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Drosophila Models of Human Disease

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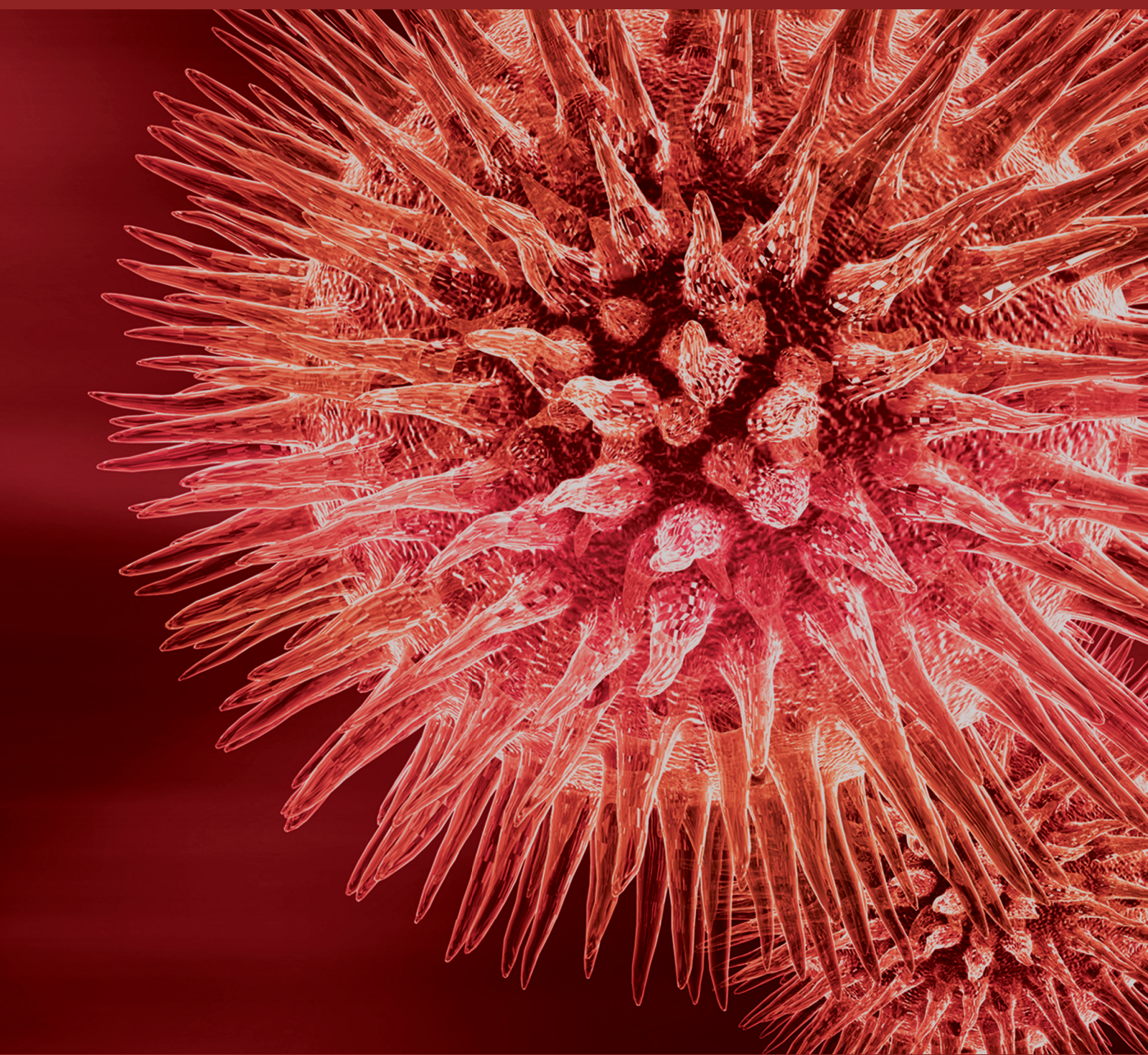

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Editorial
Drosophila Models of Human Disease

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This special edition was assembled around the theme of how the fruit fly *Drosophila* is used as a disease model. *Drosophila* has been used productively as a model organism for over a century to study a range of diverse biological processes, including genetics and inheritance, embryonic development, organ regeneration, learning, behaviour, and aging. As most of the fundamental biological mechanisms and signalling pathways that control development and survival are conserved throughout evolution, there are many precedents that demonstrate the utility of using *Drosophila* as a model system. Mechanistic details of genetic and molecular regulation of cellular processes can be thus established in the fruit fly and then transferred to other organisms.

Drosophila research has produced numerous seminal discoveries for more than a century, which have translated into beneficial health outcomes, starting with Morgan's landmark discovery that genes are carried on chromosomes, which has underpinned modern genetics. The striking observation that around 75% of the genes responsible for human diseases are evolutionarily conserved across animal species, including *Drosophil*a, has meant that the study of this organism has facilitated the understanding of multiple aspects of an increasing number of human diseases. Furthermore, the ability to perform sophisticated genetics with large progeny numbers and fast-generation time has allowed scientists to define the molecular mechanisms of gene function at a level of resolution and rapid pace not achievable with other animal models. We have selected several areas to cover in this issue, including recent advances in the understanding of the mechanisms controlling organ regeneration, neurodegeneration, cancer, and metabolic diseases.

Many neurodegenerative diseases are caused by different defects in posttranslational modification or build-up of aberrant proteins. We have included two reviews and three studies which report and discuss the use of *Drosophila* to model neurodegenerative diseases and possible therapeutic approaches. D. Denton and L. O'Keefe highlight how autophagy and defects in lysosome-mediated degradative pathways contribute to the etiology of Alzheimer's Disease and how this can be modelled using *Drosophila*. M. D. Moltó and J. V. Llorens focus on how *Drosophila* models have gained new insights into the involvement of lipid metabolism and glial cells in Friedreich's ataxia, caused by a deficit in the mitochondrial protein Frataxin. U. Mayor et al. present how the ubiquitination state of neural proteins and ubiquitin carriers can play important roles during neuronal development and in disease setting, and M. Jafari et al. show how some natural compounds may help contrast a number of neurodegenerative traits in Huntington's and Alzheimer's diseases.

Cancer is driven by complex genetic and cellular mechanisms. The use of *Drosophila* as a model to study cancer has proven essential to elucidate several mechanisms that are fundamental to cancer development. Here, we have included three reviews on how *Drosophila* is utilised to dissect the mechanisms underlying tumourigenesis and cancer progression. L.-A. Baena-López et al. present an overview on the function of caspases, independent of their traditional role in cell death, in contexts such as proliferation, differentiation, and migration, all of fundamental importance in tumourigenesis. J. B. Cordero et al. use the *Drosophila* midgut to interrogate cell autonomous, niche-derived signals which

coordinately regulate stem cell proliferation in response to tumourigenesis. Finally, H. E. Richardson and M. Portela give a comprehensive update on cooperative oncogenesis, where multiple mutations/genetic alterations cooperate to drive tumourigenesis, which can be beautifully recapitulated in *Drosophila*.

Regeneration is an amazing ability displayed in different forms by most metazoans, including humans, which allows organisms to repair or totally replace damaged organs. Given the potential therapeutic applications of understanding the mechanisms that control this capacity, in recent years a lot of effort has been focused on analysing the genetic and molecular basis of this intriguing phenomenon. In this issue, A. Baonza and S. Ahmed-de-Prado review the contribution of *Drosophila* to identify the signalling networks involved in regulating the variety of cellular responses required for regeneration.

C. Gamberi et al. and M. Mink et al. present novel *Drosophila* models of human kidney disease and explain how studies using the malpighian tubules can shed light on cyst formation in kidney disease and the importance of some basement membrane components in the context of nephropathy. Finally, J. R. Riesgo-Escovar et al. present an overview on how *Drosophila* is being increasingly appreciated as a model for human metabolic diseases. Although the physiology of insects is different from that of mammals, the genes and signalling pathways involved in growth control are highly conserved. *Drosophila* with diabetic and obese phenotypes can be generated by manipulations of pathways that cause parallel clinical manifestations in humans, further validating *Drosophila* as a model for studying human physiology and metabolism. In this sense, P. Bellosta et al. propose a *Drosophila* model of Adipose Tissue Macrophage (ATM) infiltration to study the protective role of anthocyanins in chronic tissue inflammation and related metabolic diseases.

Taken together, these reviews and studies highlight the continuous innovation in the use of *Drosophila* for the study of disease mechanisms, which has been revealing many insights in regard to human disease onset, phenotypes, and progression.

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Research Article

Cinnamaldehyde Improves Lifespan and Healthspan in *Drosophila melanogaster* Models for Alzheimer's Disease

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Cinnamon extract has been reported to have positive effects in fruit fly and mouse models for Alzheimer's disease (AD). However, cinnamon contains numerous potential active compounds that have not been individually evaluated. The main objective of this study was to evaluate the impact of cinnamaldehyde, a known putative active compound in cinnamon, on the lifespan and healthspan of *Drosophila melanogaster* models for Alzheimer's disease, which overexpress Aβ_{42} and MAPT (Tau). We found that cinnamaldehyde significantly improved the lifespan of both AD and non-AD flies. Cinnamaldehyde also improved the healthspan of AD flies overexpressing the Tau protein by improving climbing ability, evaluated by rapid iterative negative geotaxis (RING), and improving short-term memory, evaluated by a courtship conditioning assay. Cinnamaldehyde had no positive impact on the healthspan of AD flies overexpressing the Aβ_{42} protein.

1. Introduction

Alzheimer's disease (AD) results in neuronal dysfunction and locomotor impairment that would eventually lead to the loss of ability to carry out simple motor functions such as walking, talking, and holding objects [1, 2]. Other symptoms of AD include memory loss, confusion, and behavioral changes [1]. The exact etiology for AD is poorly understood, but it is generally accepted that the underlying pathology of AD is due to abnormal levels of beta-amyloid in the brain resulting in the accumulation of plaques between neurons and hyperphosphorylated Tau proteins resulting in the formation of tangles in neuronal cells [3]. Alleles of the apolipoprotein E (ApoE) gene and certain unhealthy lifestyle choices have also been proposed as possible contributors to AD by elevating Aβ and Tau in the brain [4]. For example, the ApoE4 allele has been associated with an increased risk of developing Alzheimer's disease, whereas ApoE3 has a neutral relationship and ApoE2 is negatively associated with the disease [5]. In the case of lifestyle changes, the occurrence of Alzheimer's disease has been linked to diet, social interaction, physical exercise, and mental activity [5].

Drosophila melanogaster models for AD that are based on overexpressing Aβ or Tau proteins have been used to evaluate not only the pathology of AD, but also the impact of potential interventions on the pathology and symptoms of AD [3, 6, 7]. These genetically modified models display similar neuronal dysfunction as seen in humans with AD such as neurodegeneration, neurotoxicity, and locomotion defects [3]. The overexpression of a Tau protein compromises the associative olfactory learning and memory as well as neurodegeneration of the fly [3]. The overexpression of Aβ results in the formation of diffuse Aβ deposits, gradual locomotor dysfunction, neurodegeneration, premature death, and learning defects [8]. Alzheimer's disease has also been associated with reduced gait speed, loss of muscle strength and bulk, and reduced balanced and dexterity [9]. However, these neurological manifestations are far less severe in AD than those found in either Huntington's or Parkinson's disease. While overexpression of different species of beta-amyloid, Aβ_{40} and Aβ_{42} can cause neuronal dysfunction and memory defects, only Aβ_{42} species cause neurodegeneration with amyloid deposits making the aggregation of Aβ_{42} and Tau proteins the pathological hallmark in AD brains [8]. Though

it may be appropriate to use fly models overexpressing either Aβ or Tau protein to evaluate how compounds affect the AD pathology, a faithful model of AD should feature the overexpression of both Aβ and Tau, as accumulating evidence suggests that Aβ_{42} plays a central role in the pathogenesis of AD, and Tau acts downstream of Aβ_{42} as a modulator of the disease progression [10].

Drosophila melanogaster models for AD, AD flies, have also been used to investigate the impact of pharmaceuticals and natural products, such as cinnamon, on the pathology of AD. Cinnamon is widely used by humans, both as a spice and as a traditional medicine with reported therapeutic properties such as glucose lowering as well as antioxidant and antimicrobial effects [11]. The therapeutic benefits of cinnamon are often contributed to the activities of its putative active compounds such as cinnamaldehyde, eugenol, cinnamyl acetate, and cinnamyl alcohol [12]. With respect to AD, in a comprehensive review of the pharmaceutical and phytochemical applications of the cinnamon, the extract was reported to have an inhibitory effect on Tau aggregation in AD animal models with positive effects on lifespan and motility [13]. This review did not report which specific compounds in the extract were responsible for these biological activities. Since cinnamaldehyde is considered to be the most abundant compound in cinnamon, approximately 45-62% by weight [12], we hypothesized that cinnamaldehyde should have positive impacts on the lifespan and healthspan of AD flies.

It has been reported that 40 mM of cinnamaldehyde did not extend the lifespan of normal flies (non-AD flies) [11]. For this work, we tested a dosing range of 16 to 400 mM of cinnamaldehyde on fly lifespan to identify the optimal dose. Cinnamaldehyde lifespan studies were performed as described in Schriner et al. [11]. The effects of cinnamaldehyde on both the lifespan and healthspan of *Drosophila melanogaster* model for Alzheimer's disease were examined in this study. We evaluated healthspan phenotypes in this study by two validated tests: rapid iterative negative geotaxis (RING) and a courtship conditioning assay to examine short-term memory changes [11, 14, 15].

2. Materials and Methods

2.1. Cinnamaldehyde. Cinnamaldehyde was obtained from Sigma-Aldrich in liquid form. The certificate of analysis showed a purity of 99%. We performed a dose finding study and tested the impact of 16 to 400 mM of cinnamaldehyde on fly lifespan to identify the optimal dose. As described in Schriner et al. [11], cinnamaldehyde was fed to flies by adding it to the yeast paste that was placed on the top of standard banana food.

2.2. Fly Crossing. Parental control flies of APP.Abeta$_{42}$, and MAPT (Tau), were crossed with the *Gal4*$^{elav\text{-}C155}$ *UAS* driver. This resulted in first-generation (F1) offspring expressing Aβ_{42} or MAPT, which served as our *Drosophila melanogaster* models for Alzheimer's disease (AD flies). Stock cages with 3% yeasted banana food plates were used to house the flies. After two days, the offspring eggs were collected and transferred to standard food to mature and hatch for 10 days. All flies in the assays in this work were maintained at 25°C and 55% humidity.

2.3. Survival Assay. As described by Schriner et al., the survival assay is a standard tool to evaluate the effect of genotype, interventions, or environmental conditions on the *Drosophila* lifespan [11]. This assay was performed to evaluate the lifespan of *Gal4*$^{elav\text{-}C155}$ lines that express toxic proteins in their central nervous system. The number of dead flies among cohort produced by the *Gal4*$^{elav\text{-}C155}$ crossings was recorded every other day. For this assay, 240 male and 240 female flies were transferred to fresh vials with standard food every two days until all flies had died. A total of 12 flies, 6 males and 6 females, were housed per vial.

2.4. Rapid Iterative Negative Geotaxis (RING) Assay. As described by Schriner et al., the RING assay was conducted to evaluate the impact of cinnamaldehyde on fly directionality and climbing ability [11]. This assay was used to measure locomotor ability. A total of 6 trials were tested for each group. In each trial, 20 flies supplemented with either cinnamaldehyde or control diet were placed in an empty vial and covered with a sponge. The camera was positioned to view and record the vials vertically. Flies were then tapped down to the bottom of their vials. A snapshot of the location of flies after 4 seconds was taken and the average travelled distance of 20 flies was calculated.

2.5. Courtship Conditioning. As described by Koemans et al., courtship conditioning was performed in 2 separate periods: a training period where learning occurs and a testing period to evaluate memory ability [15]. Five- to seven-day-old wild-type Oregon-R strain females, used for training and testing, were premated before the training period. To prepare premated female flies, a virgin Oregon-R female was paired with an Oregon-R male for 24 hours. To train the virgin male AD flies, flies were introduced to premated Oregon-R female flies for one hour and were allowed to initiate and learn a courtship condition that paired mating rejection with the pheromone excreted by the premated female as described in Koemans et al. [15]. One hour after the training period, the trained male flies were separated into new vials and left undisturbed to rest for one hour. The one-hour resting period was used to evaluate memory performance and memory retention from the training as well as increasing their sensitivity [15]. After this one-hour resting period, each trained male was paired with a new premated Oregon-R female fly in a new food vial. The testing period was initiated and the flies were observed and recorded for 10 minutes. The courtship behavior time of individual male flies was recorded and the courtship index (CI) was determined by dividing the total time of male courtship behavior by 10 minutes. The CI was calculated for both naïve and trained male flies [15]. The memory index (MI) was then determined by subtracting the courtship index (CI) of trained male flies from the CI of naïve male flies, then dividing this by the CI of the naïve

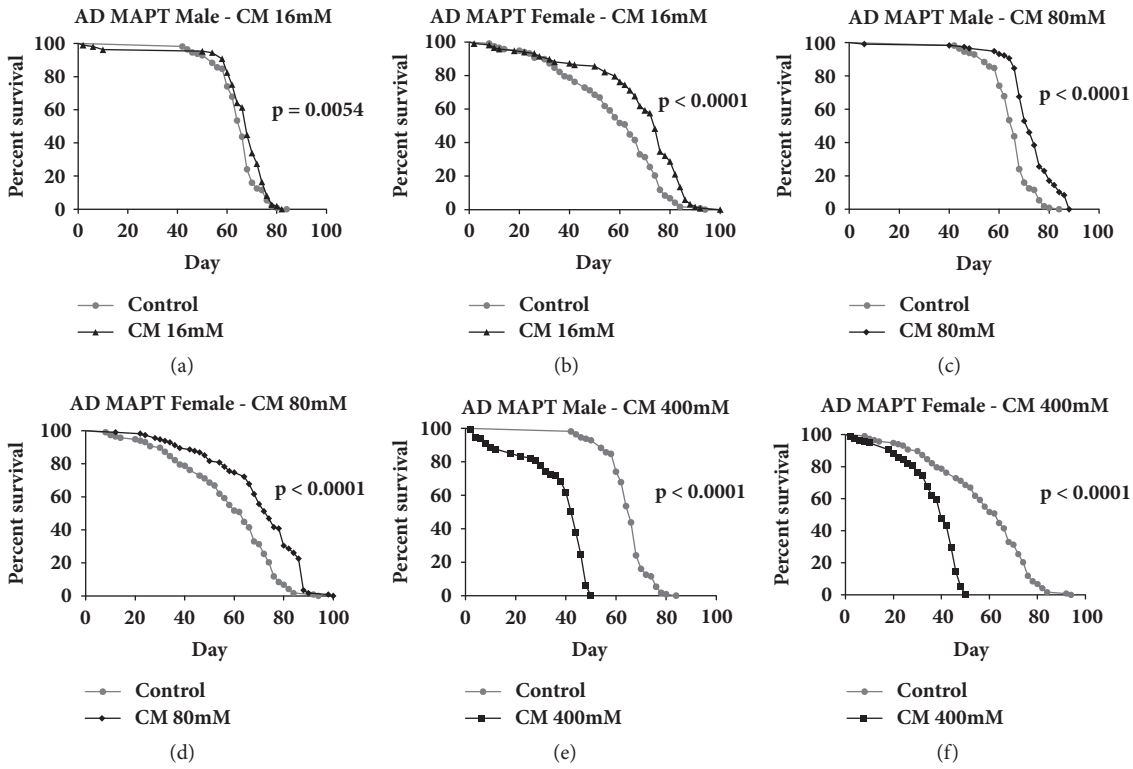

Figure 1: **The effect of cinnamaldehyde on the lifespan of Alzheimer's disease (AD) flies overexpressing MAPT (Tau).** (a, b) 16 mM cinnamaldehyde (CM) significantly increased the lifespan of AD flies overexpressing the Tau protein in males by 2% and females by 17.5% (P=0.0054, male control n = 112, male CM n = 109, $P < 0.0001$, female control n = 118, and female CM n = 118). (c, d) 80 mM cinnamaldehyde significantly increased the lifespan of AD flies overexpressing the Tau protein in males by 11.7% and in females by 20.7% (male control n = 112, male CM n = 117, female control n = 118, and female CM n = 115, $P < 0.0001$). (e, f) 400 mM cinnamaldehyde was toxic to AD flies overexpressing the Tau protein in both males and females (male control n = 112, male CM n = 113, female control n = 118, and female CM n = 118, $P < 0.0001$).

flies [15]. All male AD (APP.Abeta$_{42,}$ MAPT (Tau)) flies were maintained at 25°C and transferred to vials with fresh food every other day for 20 days before the assays were conducted [15]. Treated flies were fed 80 mM of cinnamaldehyde for 20 days after eclosion. Training and testing were performed on 20-day-old male flies.

2.6. Statistical Analysis. Data analysis was done using GraphPad Prism. Student's t-test was performed when comparing between means of two groups. A P value of <0.05 was considered significant.

3. Results

3.1. Cinnamaldehyde Increased the Lifespan of AD, Non-AD, and Parental Control Flies. The impact of three different cinnamaldehyde doses (16 mM, 80 mM, and 400 mM) on lifespan was evaluated. Cinnamaldehyde at 16 mM had no significant effect on the lifespan of AD flies overexpressing Aβ_{42} but at 400 mM, it had toxic effects on the lifespans of both male and female AD flies expressing Aβ_{42}. Cinnamaldehyde at 80 mM increased the lifespan of Alzheimer's disease fly models that overexpress the Tau protein by 11% in males ($P < 0.0001$) and by 20.7% in females ($P < 0.0001$) (Figure 1). Cinnamaldehyde at 80 mM increased the lifespan of Alzheimer's disease fly models that overexpress Aβ_{42} protein by 5.3% in males only ($P < 0.0001$) (Figure 2). In addition, cinnamaldehyde significantly increased the lifespan of conventional Gal4$^{\text{elav-C155}}$ flies for both sexes and the non-AD parental control flies that had no abnormal expression of the male Aβ_{42} and female Tau proteins (Figure 3). There was no significant improvement on the lifespan of the parental control male Tau and female Aβ_{42}. The differences in the male and female fly's physiology and the biological engineered gene could explain these sex-specific observations where they could affect antiaging mechanism differently in the male and female of each transgenic fly strain.

3.2. Cinnamaldehyde Improved Locomotion. The healthspan of AD flies was examined through the rapid iterative negative geotaxis (RING) assay that evaluates locomotion. Cinnamaldehyde significantly improved the climbing ability of male AD flies overexpressing Tau protein (Figure 4). This effect was specific to AD flies, as there was no significant improvement in climbing ability of wild-type (Oregon-R) flies treated with cinnamaldehyde (Figure 5).

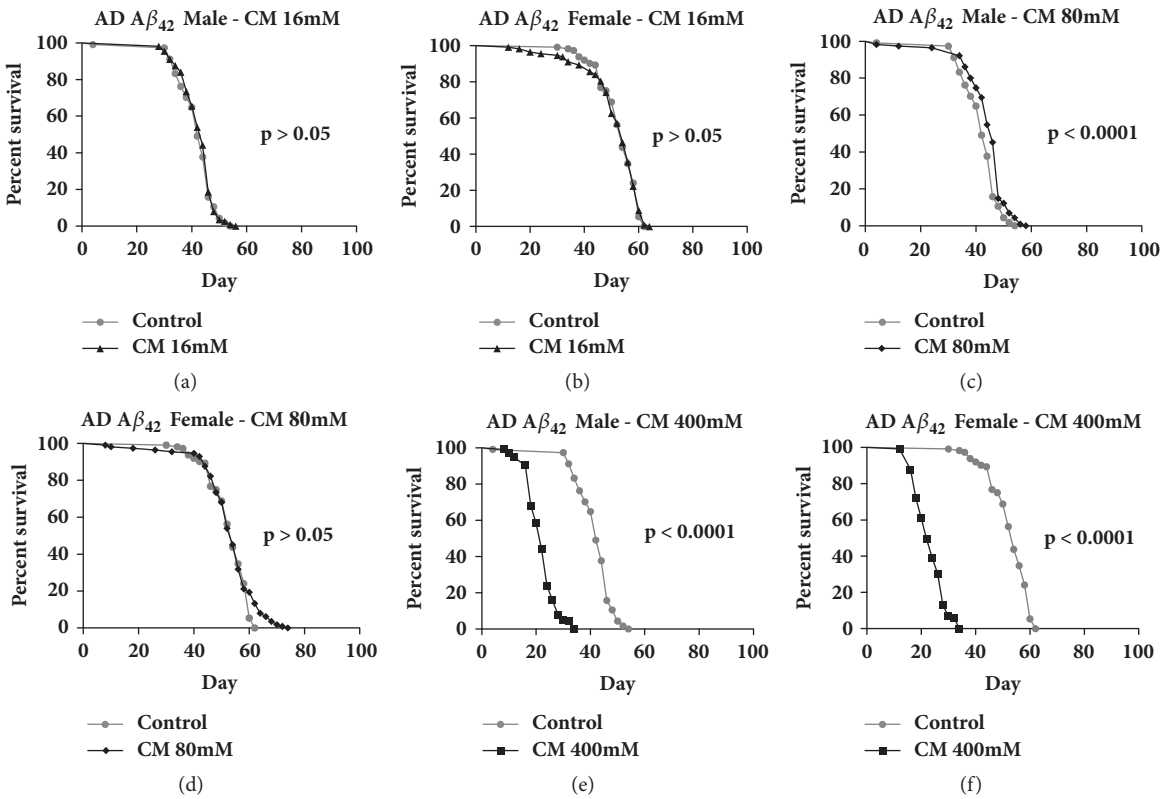

FIGURE 2: **The effect of cinnamaldehyde on the lifespan of Alzheimer's disease (AD) flies overexpressing Aβ_{42}.** (a, b) 16 mM of cinnamaldehyde (CM) had no significant effect on the lifespan of AD flies overexpressing the Aβ_{42} protein (male control n = 114, male CM n = 113, female control n = 112, and female CM n = 112, $P > 0.05$). (c, d) 80 mM of cinnamaldehyde significantly increased the lifespan of male AD fly model overexpressing the Aβ_{42} protein by 5.7% (male control n = 114, male CM n = 115, $P < 0.0001$) and it had no significant impact on the lifespan of females (female control n = 112, female CM n = 113, $P > 0.05$). (e, f) 400 mM of cinnamaldehyde was toxic to AD flies overexpressing the Aβ_{42} protein in both males and females (male control n = 114, male CM n = 113, female control n = 112, and female CM n = 115, $P < 0.001$).

3.3. Cinnamaldehyde Improved Short-Term Memory. The effect of cinnamaldehyde on short-term memory through courtship and mating in the *Drosophila melanogaster* models for AD was also examined using a validated conditioned courtship assay as described in Koemans et al. [15]. The courtship index was first examined to determine whether training was conducted successfully. Here, the courtship index was significantly decreased after training and flies were fed either control diet or 80 mM cinnamaldehyde in both fly models of AD (Figure 6). The observed decrease in courtship index demonstrated successful training as the trained male flies spent less time performing courtship with the new premated female than the naïve (untrained) male flies. We also observed that cinnamaldehyde supplementation led to a significant improvement in short-term memory for the AD flies overexpressing the Tau protein, but not AD flies overexpressing Aβ_{42} (Figure 7).

4. Discussion

Cinnamon extract has been reported to improve the lifespan and healthspan of *Drosophila melanogaster* models for Alzheimer's disease (AD) [13]. In this study, we evaluated the impact of cinnamaldehyde, the predominant putative active compound in cinnamon, on the lifespan and healthspan of *Drosophila melanogaster* models for AD. While the exact etiology of later-life AD is still far from clear, the pathology of AD is postulated to be due to the accumulation of extracellular plaques between the neurons from abnormal levels of beta-amyloid and the formation of neurofibrillary tangles from the hyperphosphorylation of Tau proteins. Thus, the majority of interventional studies in AD focus on the inhibition of the aggregation of Aβ and the tangles of the Tau protein.

We had previously reported that cinnamon increased lifespan in both males and females of two different strains of flies, w^{1118} and *JIV*, and that two putative active compounds in cinnamon, cinnamaldehyde (40 mM) and coumarin (35 mM), not only failed to increase the lifespan of these flies but they also appeared to have sex-specific toxicity [11]. We were not able to draw any conclusions on the impact of lower doses of these compounds on lifespan since we did not perform a dose finding assay. Since cinnamaldehyde is considered the most abundant compound in cinnamon, approximately 45-62% [12], we hypothesized that the lifespan extension that we observed with cinnamon could be due to cinnamaldehyde

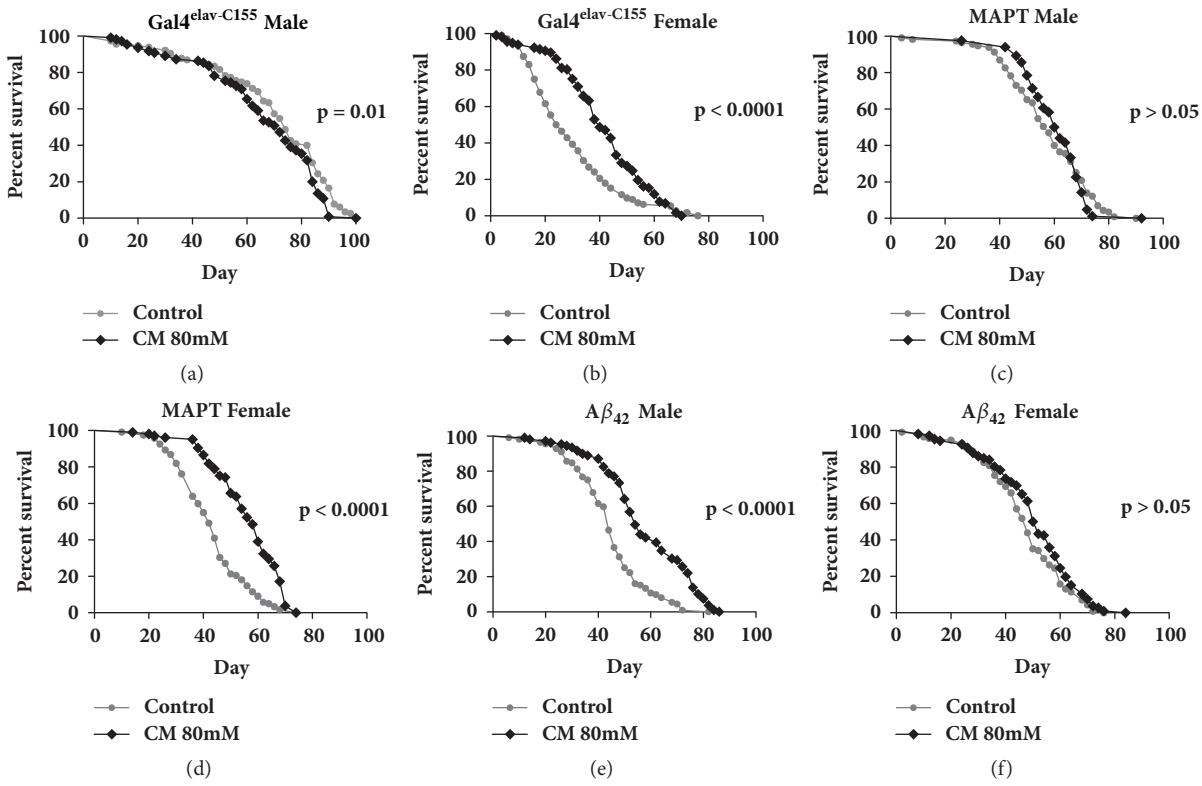

Figure 3: **The effect of 80 mM of cinnamaldehyde on the lifespan of non-Alzheimer's disease (AD) parental control flies.** (a, b) Cinnamaldehyde significantly increased the lifespan of male Gal4$^{elav\text{-}C155}$ parental control flies by 5.4% (male control n = 115, male CM n = 110, $P = 0.01$) and females by 39.9% (female control n = 109, female CM n = 111, $P < 0.0001$). (c) Cinnamaldehyde had no effect on the lifespan of non-AD MAPT parental control males (male control n = 113, male CM n = 84, $P > 0.05$). (d) Cinnamaldehyde increased the lifespan of non-AD MAPT parental control females by 30.9% (female control n = 121, female CM n = 104, $P < 0.0001$). (e) Cinnamaldehyde increased the lifespan of non-AD Aβ_{42} parental control males (male control n = 110, male CM n = 109, $P < 0.0001$). (f) Cinnamaldehyde had no effect on the lifespan of non-AD MAPT parental control females (female control n = 110, female CM n = 104, $P > 0.05$).

and that this compound might have positive impacts on healthspan for AD fly models. In order to determine the optimal dose of cinnamaldehyde that could effectively rescue the fly models that express MAPT (Tau) and Aβ_{42} a dose finding assay was performed and the impact of three different cinnamaldehyde doses (16 mM, 80 mM, and 400 mM) on lifespan was evaluated. Since cinnamaldehyde at 80 mM significantly increased the lifespan of both MAPT (Tau) and Aβ_{42} male flies by 11.7% and 5.7%, respectively ($p<0.05$), this dose was selected as the optimal dose for other assays such as locomotion and short-term memory. We also observed that while this dose significantly increased the lifespan of female AD flies that overexpressed Tau proteins by 20.7%, there was no significant impact of that dose on the lifespan of female AD flies that overexpressed Aβ_{42} proteins. We also evaluated the impact of 80 mM of cinnamaldehyde on the lifespan of three groups of non-Alzheimer disease parental control flies (non-AD Aβ_{42}, Gal4$^{elav\text{-}C155}$, and non-AD MAPT). Cinnamaldehyde significantly increased the lifespan of these three groups, none of which had abnormal expression of Aβ_{42} and/or Tau proteins. Since cinnamaldehyde increased the lifespan of AD and non-AD flies, independent of abnormal expression of Aβ_{42} and/or Tau proteins, it appears that the compound acts to extend lifespan through a pathway that is not related to Alzheimer's disease pathology.

We also evaluated the impact of cinnamaldehyde on the climbing ability of AD flies using the rapid iterative negative geotaxis (RING) assay. Cinnamaldehyde significantly increased the climbing ability of male AD flies overexpressing the Tau protein, but it did not improve the climbing ability of wild-type (Oregon-R) flies. The compound also improved short-term memory of male AD flies overexpressing the Tau protein. Since the positive impact of cinnamaldehyde was only observed in AD flies overexpressing the Tau protein, the positive effects of this compound could be due to disrupting Tau aggregation. In a study that evaluated the impact of epigallocatechin-3-gallate (EGCG) from green tea on the clearance of pathological Tau species in a cultured cell model, EGCG enhanced the clearance of phosphorylated Tau species in primary neurons [16]. Since phosphorylated Tau isoforms form the majority of Tau species in AD pathogenesis, their clearance could potentially be therapeutic [16].

The mechanism through which cinnamaldehyde improves the phenotype of the AD MAPT flies is speculative at this point. One possible explanation is that cinnamaldehyde works in a similar manner to that shown

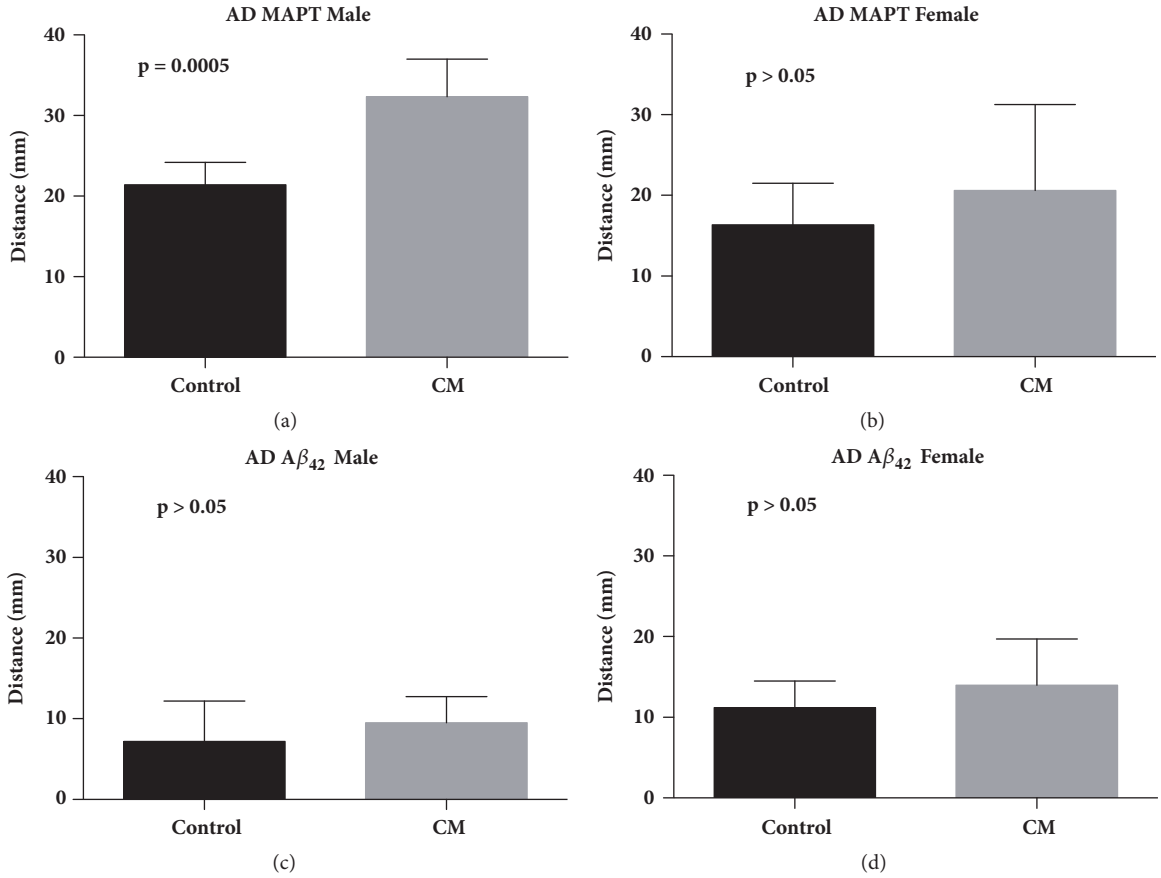

Figure 4: **The effect of 80 mM of cinnamaldehyde (CM) on climbing ability of 20-day-old *Drosophila melanogaster* model for Alzheimer's disease overexpressing MAPT (Tau) and Aβ_{42}** (a) Cinnamaldehyde significantly increased climbing ability of 20-day-old male Alzheimer's disease flies overexpressing Tau ($P = 0.0005$, control n = 123, CM n = 120). (b) Cinnamaldehyde showed no significant impact on climbing ability of 20-day-old female Alzheimer disease flies expressing Tau ($P > 0.05$, control n = 112, CM n = 115). (c) Cinnamaldehyde showed no significant impact on climbing ability of treated 20-day-old male Alzheimer disease flies expressing Aβ_{42} ($P > 0.05$, control n = 109, CM n = 121). (d) Cinnamaldehyde showed no significant impact on climbing ability of treated 20-day-old female Alzheimer disease flies expressing Aβ_{42} ($P > 0.05$ control n= 112, CM n = 121).

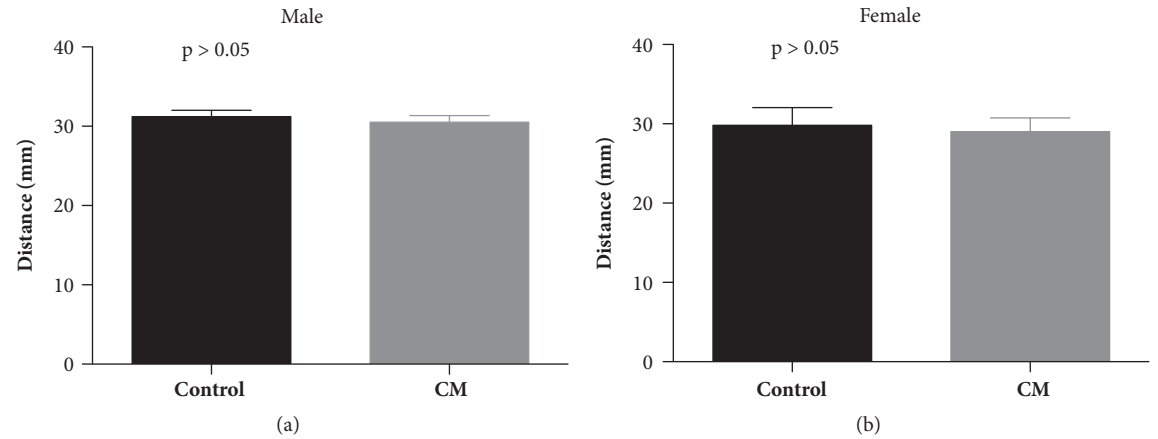

Figure 5: **The effect of 80 mM of cinnamaldehyde (CM) on climbing ability of 20-day-old wild-type Oregon-R (OR-R) *Drosophila melanogaster*.** No significant difference was observed in the climbing ability of control flies and flies treated with cinnamaldehyde in both male and female flies (male control n = 116, male CM n = 124, female control n = 116, and female CM n = 124).

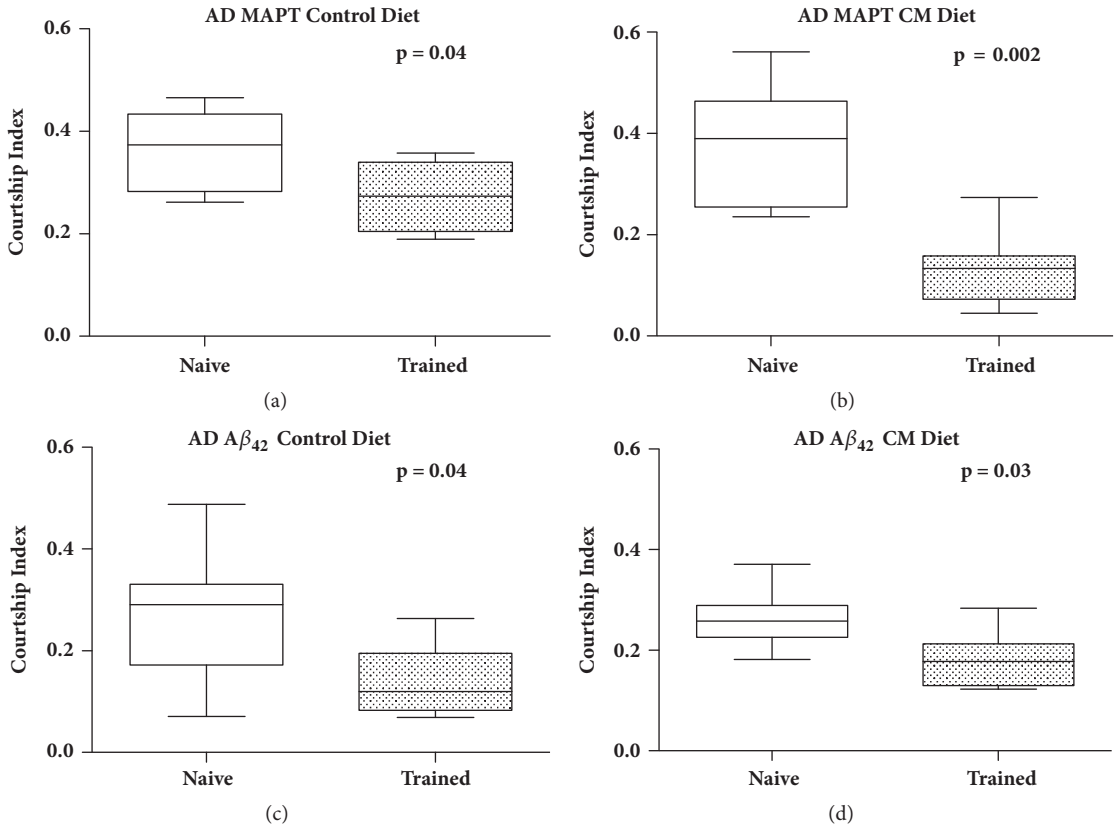

Figure 6: **The effect of training on courtship behavior in AD flies overexpressing either MAPT or Aβ_{42}.** The courtship index was significantly decreased after training in flies fed control diet ((a) and (c)), and 80 mM cinnamaldehyde ((b) and (d)) of both fly models for AD with overexpression of MAPT or Aβ_{42}. ((a). $P = 0.04$, naïve n = 46, trained n = 44; (b). $P = 0.002$; naïve n = 40, trained n = 48; (c). $P = 0.04$; naïve n = 42, trained n = 46; (d). $P = 0.03$; naïve n = 45, trained n = 44).

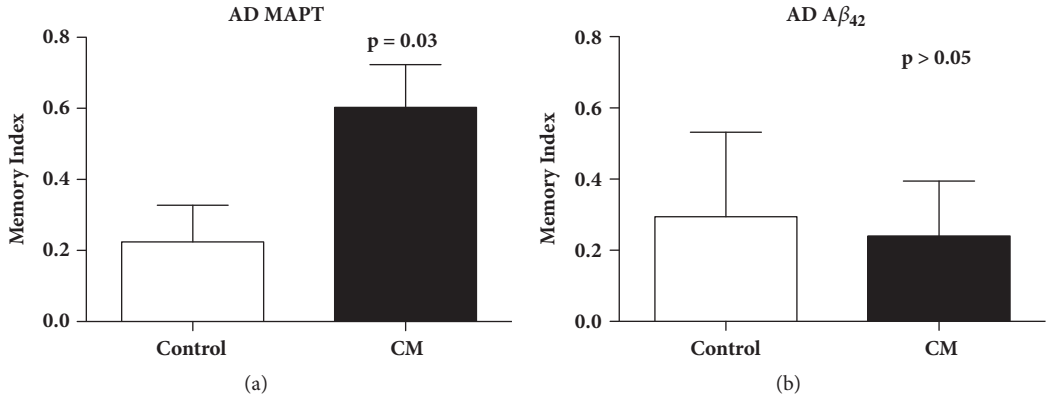

Figure 7: **The effect of 80 mM of cinnamaldehyde on short-term memory of AD flies overexpressing the MAPT (Tau) protein.** (a) Cinnamaldehyde significantly improved the memory index of AD flies overexpressing the Tau protein (P=0.03). (b) Cinnamaldehyde had no significant improvement in memory index of AD flies overexpressing Aβ_{42} protein ($P > 0.05$).

for the natural product epigallocatechin-3-gallate (EGCG), by directly disrupting Tau aggregation. Direct binding of EGCG to Tau has been reported, along with alteration of the 3D structure of Tau [17]. The effectiveness of EGCG has also been demonstrated by its ability to enhance Tau clearance in neuronal cultures and its ability to decrease the levels of soluble phosphorylated Tau in transgenic mice [16, 18].

Alternatively, cinnamaldehyde could be working in a similar manner to that of another natural product, sulforaphane, which enhances Tau degradation through the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) [16, 19]. Oxidative stress, which activates Nrf2, is thought to be one of the underlying factors driving AD. Once activated, Nrf2 binds antioxidant response elements (ARE) to induce

gene expression of downstream protective enzymes. One of these proteins is the autophagy adaptor protein NDP52, which, when expressed, can induce autophagy and clear Tau aggregates from the neurons [19].

In this study, the positive phenotypic impacts of cinnamaldehyde were only observed in AD flies overexpressing the Tau protein. Future experiments will be needed to precisely determine the mechanism through which cinnamaldehyde may impact Tau aggregation in fly models for AD.

5. Conclusion

The etiology of the complex neurodegenerative Alzheimer's disease is still far from being clear. It is speculated that the pathology of AD is due to abnormal levels of beta-amyloid in the brain that causes the accumulation of plaques between neurons and hyperphosphorylation of the Tau proteins that causes the formation of tangles within neuronal cells. It has been reported that cinnamon has an inhibitory effect on Tau aggregation of mouse and fruit fly models for AD and it has also been reported to have positive effects on lifespan and locomotion of these model species [13]. In this work, we found that cinnamaldehyde, the most abundant putative active compound of cinnamon, significantly improved lifespan and healthspan of male AD flies overexpressing the Tau proteins. Although the compound had no effect on flies overexpressing the $A\beta_{42}$ proteins, due to its safety profile, it may still be considered as a potential therapy to alleviate a known underlying cause of AD pathology. Further studies in mammalian model systems are needed to evaluate the safety and efficacy of cinnamaldehyde as a potential therapy for AD.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Rhodiola rosea Improves Lifespan, Locomotion, and Neurodegeneration in a *Drosophila melanogaster* Model of Huntington's Disease

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Huntington's disease (HD) is a dominant, late-onset disease characterized by choreiform movements, cognitive decline, and personality disturbance. It is caused by a polyglutamine repeat expansion in the Huntington's disease gene encoding for the Huntingtin protein (Htt) which functions as a scaffold for selective macroautophagy. Mutant Htt (mHtt) disrupts vesicle trafficking and prevents autophagosome fusion with lysosomes, thus deregulating autophagy in neuronal cells, leading to cell death. Autophagy has been described as a therapeutic target for HD, owing to the key role Htt plays in the cellular process. *Rhodiola rosea*, a plant extract used in traditional medicine in Europe and Asia, has been shown to attenuate aging in the fly and other model species. It has also been shown to inhibit the mTOR pathway and induce autophagy in bladder cancer cell lines. We hypothesized that *R. rosea*, by inducing autophagy, may improve the phenotype of a Huntington's disease model of the fly. Flies expressing HttQ93 which exhibit decreased lifespan, impaired locomotion, and increased neurodegeneration were supplemented with *R. rosea* extract, and assays testing lifespan, locomotion, and pseudopupil degeneration provided quantitative measures of improvement. Based on our observations, *R. rosea* may be further evaluated as a potential therapy for Huntington's disease.

1. Introduction

Huntington's disease (HD) is a dominant, late-onset disease characterized by choreiform movements, cognitive decline, and personality disturbance [1]. There is no known cure for HD which affects about 30,000 Americans. It is considered an age-related disease, with the average age of onset between 30 and 50 years [2]. This is caused by a polyglutamine repeat expansion in the HD gene, with expansions greater than 39 glutamine repeats leading to the development of the disease. The mutation of the HD gene, which encodes for the Huntingtin protein (Htt), results in striatal neuron degeneration by causing the dysregulation of autophagic cell processes that facilitate protein clearance [3].

Drosophila melanogaster, also known as the fruit fly, is useful for modeling organisms such as humans. Seventy-five percent of human disease genes have a fly ortholog [4], and one of these genes is known to encode for the Huntingtin protein. *Drosophila melanogaster* is considered an optimal model system to study neurodegenerative diseases because of its fully functional nervous system [5]. Fly models of Alzheimer's disease (AD), Parkinson's disease (PD), and spinocerebellar ataxia 3 (SCA3) have been instrumental in the discovery of the molecular basis of these neurodegenerative diseases [6–8]. Alzheimer's disease models of the fly demonstrate adult onset, progressive neurodegeneration, and enhanced mutant tau neurotoxicity [6]. Parkinson's disease fly models which are homozygous for the loss-of-function mutations in the parkin gene were important in discovering the mechanism underlying autosomal recessive juvenile parkinsonism (AR-JP) [7].

Several *Drosophila* models of HD differing in polyglutamine repeat lengths are available to conduct intervention and mechanistic studies. Models varying in the lengths of

the polyglutamine repeats have been generated and flies expressing the extended polyQ repeats mimic HD in terms of decreased lifespan, decreased locomotion, and increased photoreceptor degeneration [7–10]. For example, Q75, Q93, and Q120 transgenic flies have polyglutamine lengths of 75, 93, and 120, respectively. It has been established that there is an inverse correlation between the length of the polyQ repeat and the age of onset [9]. The onset of age-related neurodegenerative symptoms in HD is a feature of the HD fly models such as Q75, Q93, and Q120 [10].

The fruit fly has also been extensively studied in aging and anti-aging research, and a number of conserved genes and pathways modulating aging and lifespan have been discovered from these studies [11]. Such genes have been linked to the regulation of metabolic functions, mitochondrial activities, nutrient sensing, and protein synthesis [12, 13]. Since the molecular events surrounding aging and longevity have been well-characterized in the fly, this model can potentially be used to study interventions that delay the progression of age-related diseases in fly models that display age-related phenotypes such as the HD fly model.

One possible approach to alleviating the symptoms of HD is to use the root extract of *Rhodiola rosea*. This plant has been used in traditional and integrative medical practices in Europe and Asia where it is prescribed to improve mood and physical and mental stamina and to enhance protection against high altitude sickness [14]. The putative active compounds of *R. rosea*, rosavins and salidroside, are used to characterize the extract. The extract has also been shown to attenuate aging in *C. elegans, D. melanogaster, and S. cerevisiae*. In the case of *D. melanogaster*, the extract increased lifespan up to 24% through a mechanism independent of dietary restriction, a well-established intervention to increase lifespan and healthspan of various organisms [15, 16]. *Rhodiola rosea* and its putative active compound, salidroside, has been shown to inhibit the mTOR pathway and induce autophagy in bladder cancer cell lines [17]. Since the lifespan extension properties of *R. rosea* appear to be conserved among various species, *D. melanogaster*, *C. elegans*, and *S. cerevisiae* [18, 19], this plant may be a viable intervention to attenuate the symptoms of age-related diseases in humans such as HD.

2. Materials and Methods

2.1. Rhodiola rosea Extract. The *R. rosea* extract used for this study, SHR-5, was obtained from the Swedish Herbal Institute. It contains 3.5% rosavins and 1.4% salidroside (HPLC data on file).

2.2. Drosophila melanogaster Stocks. The polyQ-expressing transgenic line, 4F1, was a gift from J. Lawrence Marsh from UC Irvine. This line, containing 93 polyglutamine repeats in exon 1 of the Huntington gene, will be referred to as UAS-HttQ93 [20, 21]. The Gal4 driver used was the pan-neuronal elav driver, elavC155 from the Bloomington Drosophila Stock Center at Indiana University. Male elav-Gal4 were mated with female UAS-HttQ93 to produce offspring with females expressing the extended polyQ repeat (elav-Gal4>UAS-HttQ93) in all nerve cells. These flies will be referred to as "HD flies." The expression of the repeats was lethal to males in the late larval stage, so only females were used for assays performed on adult flies. Assays using larvae required a cross between female elav-Gal4 and male UAS-HttQ93, resulting in all offspring expressing the 93 polyQ repeats.

Table 1: The four treatment regimens used in this study.

Group	Feeding Treatment
Control	0 mg/mL *R. rosea* as larvae + 0 mg/mL *R. rosea* as adults
Larvae Feeding (LF)	25 mg/mL *R. rosea* as larvae + 0 mg/mL *R. rosea* as adults
Adult Feeding (AF)	0 mg/mL *R. rosea* as larvae + 25 mg/mL *R. rosea* as adults
Larvae Feeding and Adult Feeding (LF + AF)	25 mg/mL *R. rosea* as larvae + 25 mg/mL *R. rosea* as adults

2.3. Larval Feeding. Two days after HD flies laid eggs (Day 0), eggs (elav-Gal4>UAS-HttQ93) were transferred into food with *R. rosea* extract mixed in. To make the *R. rosea* food, the banana food was cooked as detailed in Schriner et al. [15]. After cooling the food to 48°C, 500 mL of the food was transferred into a blender along with 12.5 g of *R. rosea* extract (500 mL x 25 mg/mL). The mixture was blended until homogenous, then a pipet-aid w/a 10 mL serological pipet was used to measure out 5.5 mL of the *R. rosea* food into vials. The fly eggs were reared in this food until they reached the pupal stage.

2.4. Adult Feeding. As described in Schriner et al. [15, 22], a 3% yeast solution with 25 mg/mL of *R. rosea* extract was made, and 75 μL of the solution was added into each vial and allowed to dry to create a layer of yeast. Flies were transferred to new vials with food every other day.

2.5. Feeding Treatments. The four treatment regimens are summarized in Table 1.

2.6. Lifespan. Lifespan assays were performed as described in Schriner et al. [15]. 40 vials per group with 12 female HD flies in each vial were set up. Flies were transferred to new food every other day, with the deaths counted on transfer days.

2.7. Rapid Iterative Negative Geotaxis (RING) Assay. Sixty vials with 12 female HD flies in each were set up for this assay. Flies were transferred to new food every other day. To perform the RING assay, the 12 flies from each feeding treatment were transferred into empty vials and loaded onto the RING assay apparatus [23]. Six trials were done, using a total of 72 flies per group. As a video of the setup was being taken, three rapid strikes were applied to the apparatus. Video recording was stopped after 6 seconds. Using ImageJ, the climbing heights of the flies were measured 4 seconds after the last tap.

2.8. Pseudopupil Assay. Sixty vials with 12 female HD flies in each were set up for this assay. After 7 days of feeding with *R. rosea*, fly heads were decapitated and mounted on a slide using clear nail polish and were observed using a Zeiss Scope.A1 microscope with an N-ACHROPLAN 100x/1,25 oil

lens. Eight eyes from eight different flies were randomly selected from each feeding treatment. A minimum of 50 ommatidia were counted per eye, with the observer blinded to the identity of each group. Rhabdomeres were counted based on visibility, not on shape, size, or brightness [21, 24].

2.9. Larval Crawling Assay. Two days after eggs were laid, eggs were transferred into food with *R. rosea* (Day 0). On Day 5, larvae were collected by adding a 20% sucrose solution into the vial and waiting 20 minutes until the larvae floated to the top. Ten larvae from each group were collected using a 200 μL pipet with the tip cut off and loaded onto the center of the plate with 2% agarose placed on top of graphing paper. The larvae were allowed to crawl from the center of the plate for one minute, after which a picture of the plate was taken [25]. Crawling distances were measured using ImageJ.

2.10. Eclosion Assay. Ten vials containing 25 mg/mL of *R. rosea* and 10 vials with control food were set up with 5 males and 5 females in each vial. The vials were left in the incubator at 25°C for 24 hours. After 24 hours, the vials were cleared of flies and eggs were left in the vials. On Day 11, the number of eclosed flies were counted every 3 hours [25].

2.11. Statistical Analysis. Data analysis for all the assays was performed using GraphPad Prism 7. For the lifespan assays, Log-Rank (Mantel-Cox) Test and Tukey's Multiple Comparison Test were used. Mean increases in lifespan were analyzed using ANOVA multiple comparisons test. This test was also used for the RING, pseudopupil, larval crawling, and pseudopupil assay.

3. Results

3.1. Rhodiola rosea Extends Lifespan of Adult-Fed Female HD, elav-Gal4, and UAS-HttQ93 Flies. A lifespan assay was performed to measure the effects of the extract on the mean lifespan of the short-lived HD flies and control flies to observe if the effects were conserved among different fly strains. It has already been observed that flies expressing 93 polyQ repeats have reduced lifespan [26], but the lifespan differences between these flies and the parents used to make the cross were yet to be studied. *Rhodiola rosea* increased the mean lifespan of the parent strains, elav-Gal4 and UAS-HttQ93, and the HD flies by 25%, 21%, and 17%, respectively. The increase in lifespan was found to be significant for each strain (Figure 1).

3.2. Rhodiola rosea Extends the Lifespan of Larvae-Fed Female HD Flies. Figure 1 shows that *R. rosea* improves lifespan of HD flies when fed as adults, but the effect of the extract on fly lifespan when fed to the HD flies as larvae was yet unexplored. Lifespan assays were performed to observe the impact of feeding *R. rosea* during larval and adult stages of HD flies. This was done to elucidate the impact of the extract on lifespan when fed to the flies at varying stages in life. All feeding treatments increased the mean lifespan with feeding during both larval and adult stages having the largest increase in lifespan (Figure 2). The differences between the feedings of *R. rosea*, however, were less pronounced. The only significant difference we found was between the lifespans of the flies fed as larvae and those fed as both adult and larvae.

3.3. Rhodiola rosea Improves the Locomotion of Larvae-Fed Adult HD, elav-Gal4, and UAS-HttQ93 Flies. To observe the effects of the extract on the locomotion of the HD flies, a RING (Rapid Iterative Negative Geotaxis) assay was performed. This assay exploits the innate response of *Drosophila* to escape by ascending up the walls of a vial after being tapped to the bottom of the vial [23]. This response decreases as flies age so the RING assay can be used to evaluate the impact of interventions such as *R. rosea* on age-related decline in locomotion. *Rhodiola rosea* increased the mean climbing heights of adult HD flies when supplemented during the larval stage, as adults, or both. Of note, when *R. rosea* was supplemented to both larva and adults, the positive effect was more significant (Figure 3).

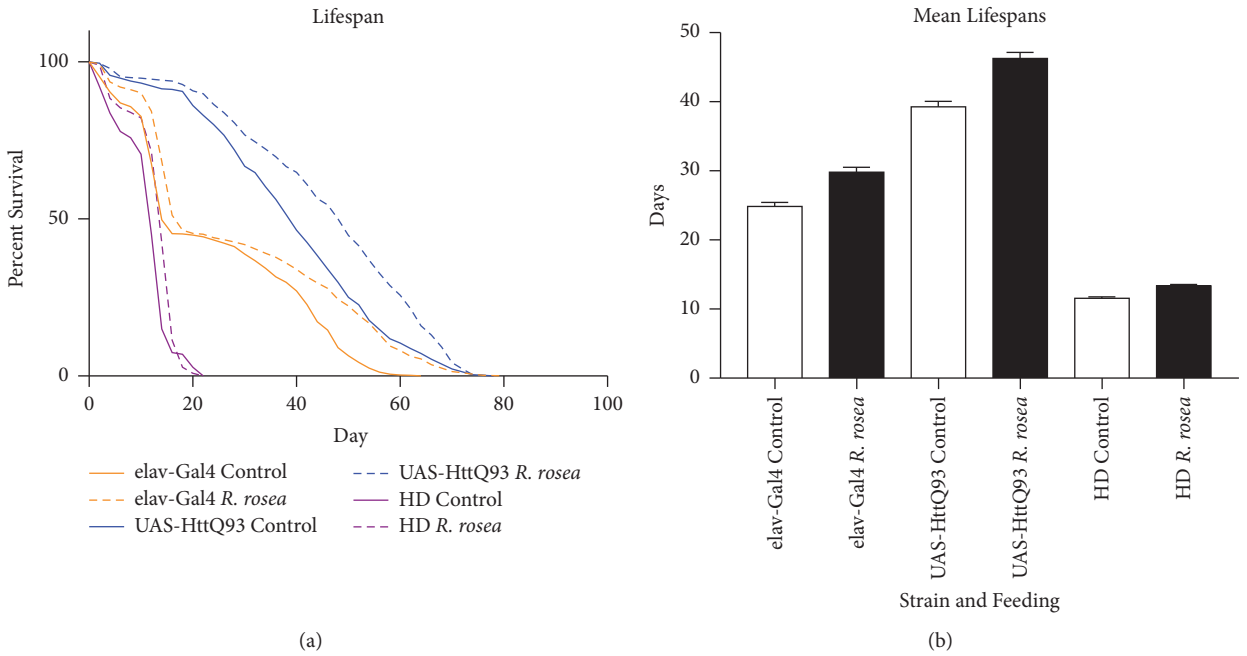
3.4. Rhodiola rosea Increases the Mean Rhabdomere Count in HD Flies. The HD flies used in this experiment had 93 polyglutamine repeats and demonstrated neurodegeneration which can be observed by counting rhabdomeres within the compound eye. The pseudopupil assay is fast and sensitive, which allows for the quantification of the degree of neurodegeneration in an *in vivo* model [21]. The supplementation of *R. rosea* at any age, larvae and adult, increased the mean rhabdomere count in the HD flies (Figure 4).

3.5. Rhodiola rosea Attenuates the Crawling Distance of HD and UAS-HttQ93 Larvae. The larval crawling assays were performed to observe the possible effects of *R. rosea* on HD fly development. This assay is a developmental assay used to study the possible toxic effects of compounds on larvae locomotion [25]. Larvae studies were performed on HD flies and control flies to observe if the effects of the extract were conserved among the strains. *Rhodiola rosea* induced a decline in mean larval crawling distance in HD and UAS-HttQ93 flies (Figure 5).

3.6. Rhodiola rosea Decreases the Percent Eclosion and Eclosion Rate of HD, elav-Gal4, and UAS-HttQ93 Flies. Another developmental assay used to observe the effects of *R. rosea* on the HD larvae was the eclosion assay which is used to measure the effect of toxins on *Drosophila* throughout the larvae-pupal stage [27]. The number of flies emerging from the pupal stage was counted to show the effects of *R. rosea* on the eclosion rate and percentage of the emerged adult flies. *Rhodiola rosea* decreased the percentage of flies transitioning from the pupal stage to the adult stage and the eclosion rates of HD, elav-Gal4, and UAS-HttQ93 flies (Figure 6).

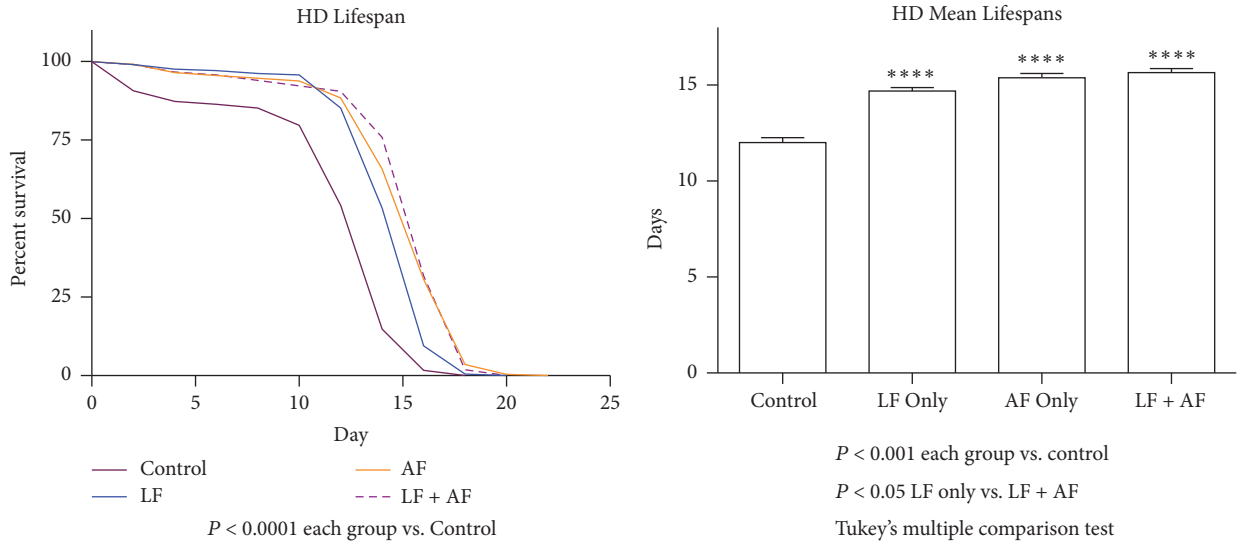
4. Discussion

Huntington's disease (HD) is a dominant, late-onset disease characterized by choreiform movements, cognitive decline, and personality disturbances [1]. Since it is considered an



(a) (b)

Figure 1: The effect of *R. rosea* on female *D. melanogaster* lifespan. (a) Compared to control-fed flies, HD, elav-Gal4, and UAS-HttQ93 flies showed a mean lifespan increase of 25%, 21%, and 17%, respectively. n=480, p<0.0001 for each strain, Log-Rank (Mantel-Cox) Test. (b) Analysis of the mean lifespan increases shows significant differences between the two treatments for elav-Gal4 and UAS-HttQ93 but not for HD flies. ****p<0.0001, ANOVA multiple comparisons test.



(a) (b)

Figure 2: The effect of *R. rosea* on the lifespan of HD flies. Relative to HD control flies (a) HD LF, AF, and LF + AF flies exhibited the following lifespan increases: 22%, 28%, and 31%, respectively. P<0.0001 (each group versus control). (b) All three groups supplements with *R. rosea* resulted in increased mean lifespan. P<0.001 (each group versus control); P<0.05 (LF only versus LF+AF), Tukey's multiple comparison test. n=240.

age-related disease, treatments that slow aging may slow the progression of HD or alleviate its symptoms. One such potential treatment may be the root extract of *Rhodiola rosea*. In this work, we evaluated the impact of *R. rosea* on a fly model of HD. We found that *R. rosea* could prevent neurodegeneration, improve locomotion, and increase lifespan. Given that the positive impacts of *R. rosea* on lifespan appear to be conserved among various species such as *S. cerevisiae*,

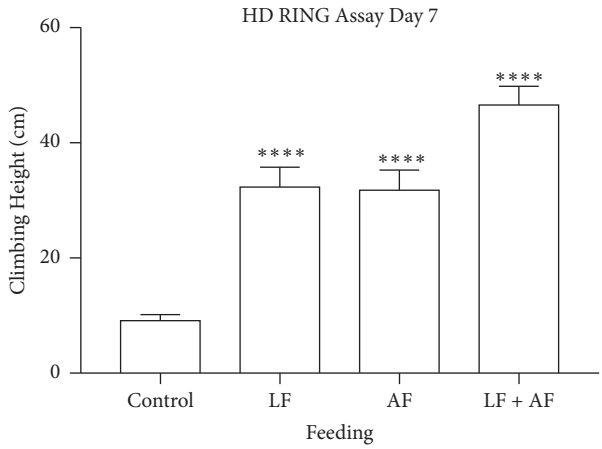


FIGURE 3: The effect of *R. rosea* on the climbing heights of adult HD flies. Flies fed *R. rosea* displayed significantly increased climbing heights compared to the control group. n~75, ****p<0.0001, ANOVA multiple comparisons test.

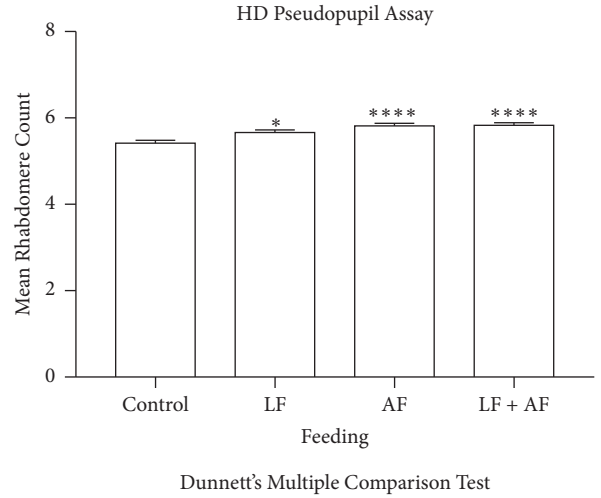


FIGURE 4: The effect of *R. rosea* on the neurodegeneration of HD fly rhabdomeres. HD flies fed *R. rosea* exhibited significant rhabdomere count increases compared to control. Data analysis was performed using an analysis of variance (ANOVA) *p<0.05, ****p<0.0001, n=50.

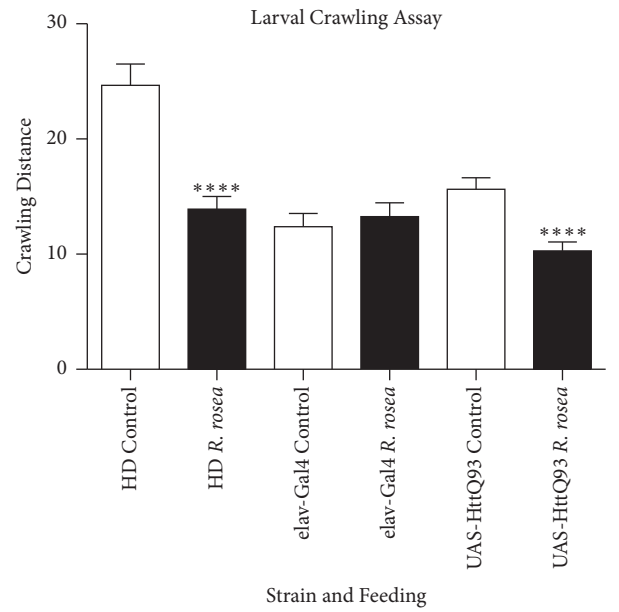


FIGURE 5: The effect of *R. rosea* on the crawling distances of larvae. Significant decreases in larvae crawling distances were observed in HD and the UAS-HttQ93 strains that were fed *R. rosea*. n =10, ANOVA multiple comparisons test.

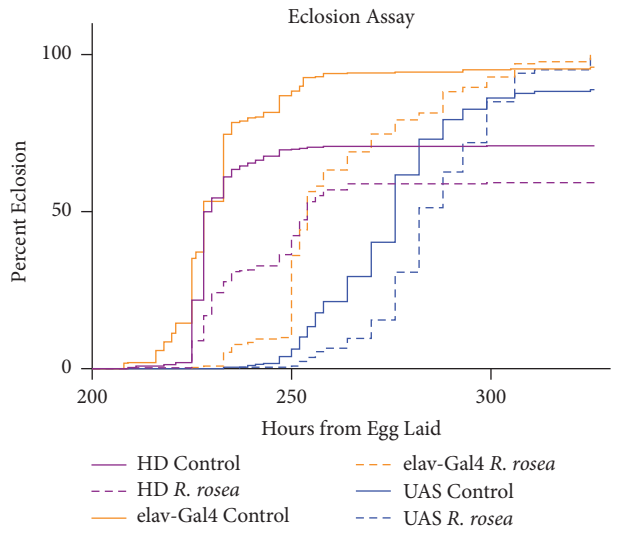


FIGURE 6: The effect of *R. rosea* on eclosion rates and percent eclosion. HD and UAS-HttQ93 flies demonstrated decreases in the percentage of flies transitioning from the pupal stage to the adult stage. HD, elav-Gal4, and UAS-HttQ93 flies showed significant decreases in eclosion rates by 5%, 13%, and 5%, respectively. The p values for the eclosion rate decreases were all significant (p<0.0001). Log-Rank (Mantel-Cox) Test.

C. elegans, and *D. melanogaster* [18, 19] and that the plant has demonstrated many therapeutic effects in clinical studies [28–31], this extract may be a viable treatment for symptoms associated with HD in humans.

The Huntingtin (Htt) protein has been implicated in the regulation of selective macroautophagy, particularly in its role in vesicle trafficking and autophagosome formation. The mutant form of the protein, mHtt, impairs the retrograde transport of vesicles to the neuron cell body, cargo loading, and the fusion of autophagosomes and lysosomes, thus leading to an abundance of empty autophagosomes and an accumulation of toxic materials in the cytoplasm [3]. Fly models of Huntington's disease (HD) have shown downregulated levels of autophagy due to the inability of the cell to degrade damaged organelles or aggregated proteins [3]. The mutant Huntingtin protein (mHtt) has also been implicated in caspase activation leading to increased toxicity and cell death [32]. The downregulation of autophagy in animal models has been linked to neurodegeneration [33], thus positing autophagy as a therapeutic target for diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease [34].

It appears that alleviating the symptoms and pathology of HD involves increasing the rate of autophagy. Researchers have observed improvements in HD disease phenotypes in fly and mouse models using mTOR inhibitors [34] and HDAC inhibitors [35]. Rapamycin, an inhibitor of mTOR, was shown to prevent the accumulation of mutant Htt (mHtt) proteins leading to cell death in cell models of HD and also conferred neuroprotective effects in a fly model [36]. Although rapamycin was found to be effective in inducing autophagy and delaying symptoms of aging in various animal models [37], adverse effects such as immunosuppression and glucose intolerance have been observed when the compound was used in humans [38], making it unsuitable for long-term prophylactic use.

Here we suggest that a standardized root extract of *Rhodiola rosea* (SHR-5) may be considered as a possible therapy to alleviate the symptoms of HD in humans. By supplementing an HD fly model with *R. rosea*, we observed improvements in the HD phenotype in terms of lifespan, locomotion, and neurodegeneration. The extract has also been shown to inhibit the mTOR pathway and induce autophagy in bladder cancer cell lines [19]. Although *R. rosea* was shown to act independently of the mTOR pathway in the fly model [15], the effects of the extract on rates of autophagy of *D. melanogaster* have not yet been observed. Currently, there are no reported adverse effects or drug interactions for *R. rosea* [29–31], making it a promising preventive treatment for Huntington's disease in humans.

The HD fly model that was used in this work expresses 93 polyQ repeats. A key characteristic of these HD flies is the rapid degeneration of the compound eye. By visually analyzing the rhabdomeres contained within the ommatidia of the fly eye, photoreceptor loss was quantitatively measured. The HD flies demonstrated decreased photoreceptor loss when supplemented with *R. rosea* as larvae only, adults only, and both adults and larvae. This suggests that the effects of the plant extract on neuronal loss were not dependent on the life stage and that feeding during only the larval or adult stage yields equally positive results as feeding during both life stages. Although the anti-aging mechanism of *R. rosea* is not known, we and others have found that *R. rosea* can induce autophagy [17, 33, 39]. Thus, one possibility is that *R. rosea*, through the induction of autophagy, can directly counteract the negative effects of the polyQ repeats. While being certainly an attractive hypothesis, it seems unlikely because of the marginal effects of *R. rosea* on the rhabdomere number relative to the marked effects that it has on locomotion. A more plausible explanation could be an indirect effect of *R. rosea*. Interestingly, *R. rosea* still resulted in a positive effect in adults when fed to larvae only. This suggests that the mechanism of action of *R. rosea* could be related to alteration in gene expression.

Adult HD flies also display impaired locomotion in the form of decreased climbing heights compared to the parental strains. We found that *R. rosea* increased locomotion in adult HD flies. However, surprisingly, the extract improved locomotion in the adult stage to equivalent degrees whether fed during adult or larval stages. The effect on locomotion during the adult stage was even greater when fed to both. The magnitude of effect and the benefit of larval feeding are both striking. Again, we can only speculate on the mechanism, but it appears that feeding *R. rosea* during either development or adult stages resulted in improved locomotion. Since the polyQ repeats in the HD fly model are expressed during the larval stages [21], we can speculate that *R. rosea* prevents polyQ repeats from aggregating before a significant problem can occur. If this were the case, why then is the effect on rhabdomere formation relatively marginal when the extract is fed to larvae? It may also be that *R. rosea* protects different cell lineages to differing degrees considering that the extract protected motor neurons more significantly than rhabdomeres. In such a case, *R. rosea* may have a more positive impact on physical performance in humans with HD, as suggested by its positive impact on locomotion in HD flies, as opposed to a marginal positive impact on the pathology of HD in humans, as suggested by the effects seen in the rhabdomere assay in HD flies.

With respect to adult lifespan, we observed that *R. rosea* had a positive effect in all of the three fly strains used in this study, the disease model, and the two parental controls. This suggests that, with respect to lifespan alone, *R. rosea* may not directly be benefiting only HD flies. It is more likely that *R. rosea* confers an overall strain independent increase in lifespan. This may not necessarily be an issue, as the extract did provide a modest decrease in pathology, but a marked increase in physical performance. Thus, an improved lifespan coupled with improved health can only be seen as a benefit. As for the locomotion assay, *R. rosea* increased lifespan whether fed to adults, larvae, or both. This clearly demonstrates that *R. rosea* mediates changes during development which affect the adult animal. The effect on lifespan was somewhat different from what was seen for locomotion in that there appeared not to be an additive effect between larval and adult feeding. Thus, for lifespan it did not seem to matter when *R. rosea* was added; the effect is the same.

To evaluate whether *R. rosea* had any harmful effects on the larvae of HD flies, we examined its impact on the percent eclosion, rate of Eclosion, and larval crawling. In all these cases, *R. rosea* was found to have a negative effect; it slowed larval crawling assay, decreased percent Eclosion, and delayed the time of eclosion. These types of assays typically indicate a toxic effect of a given agent. It may be that the extract slowed development, giving the larval cells additional time to clear the polyQ repeats before they can cause much damage. The larvae then moved on to the next stages of development possessing lesser amounts of the polyQ repeats. Such clearance may have conferred improved locomotor ability and longer lifespan. The negative effects of the extract on the larvae may have also been due to a nonoptimal dose. Further studies are warranted.

In summary, *R. rosea* seemed to provide beneficial effects in a fly model of Huntington's disease. These beneficial effects were observed when *R. rosea* was supplemented to either larvae alone, adults alone, or both. While the mechanism is not clear, it may be that *R. rosea* activates autophagy which counteracts the negative effects of the mutant Huntingtin proteins. Despite not knowing how *R. rosea* might work, due to its positive effects on other organisms and its overall

excellent safety profile, *R. rosea* may be further studied as a potential new and natural therapy to alleviate the symptoms of Huntington's disease.

5. Conclusion

In conclusion, our results show that the supplementation of *R. rosea* to the Q93 adult model of Huntington's disease improves its phenotype in terms of lifespan, locomotion, and neurodegeneration. Although the extract appeared to have a toxic effect on the larval crawling and eclosion rate, it still seemed to have a positive impact on the phenotype of adults that were fed as larvae. More studies need to be performed to evaluate the mechanism of action of the toxicity observed during the larval stage. In summary, *R. rosea* extract may be further studied as a possible therapy to alleviate the phenotype and symptoms of Huntington's disease.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Review Article

Modeling Renal Disease "On the Fly"

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Detoxification is a fundamental function for all living organisms that need to excrete catabolites and toxins to maintain homeostasis. Kidneys are major organs of detoxification that maintain water and electrolyte balance to preserve physiological functions of vertebrates. In insects, the renal function is carried out by Malpighian tubules and nephrocytes. Due to differences in their circulation, the renal systems of mammalians and insects differ in their functional modalities, yet carry out similar biochemical and physiological functions and share extensive genetic and molecular similarities. Evolutionary conservation can be leveraged to model specific aspects of the complex mammalian kidney function in the genetic powerhouse *Drosophila melanogaster* to study how genes interact in diseased states. Here, we compare the human and *Drosophila* renal systems and present selected fly disease models.

1. Introduction

Defective kidney function can lead to potentially lethal end-stage renal disease (ESRD) and chronic kidney disease (CKD), for which therapeutic options are limited. ESRD and CKD may be remedied by dialysis and renal replacement therapy (renal transplant), which are both costly [1] and greatly affect the quality of life of both patients and their families. The complexity of the human kidney has posed a formidable challenge to experimental probing for many pathologies. In many cases disease progression is well described; however, the underlying mechanisms at the molecular and cellular levels are incompletely understood, which affects our capacity to design remedial therapeutics. Animal model research on kidney disease has traditionally used rodents for their mammalian-type kidney similar to the human one. However, rodent and human kidneys also share the same complexity, which challenges experimentation. Zebrafish, featuring a streamlined pronephros, has also been used to model renal disease, albeit less frequently, in part because of its adaptation to an aquatic environment. With an open circulatory system, the fly's renal system is aglomerular and urine formation is based on active transport rather than selective readsorption [2]. However, *Drosophila* is a clear evolutionary intermediate towards the glomerular kidney, with recognizable cell types responsible for fulfilling the kidney's main functions: detoxification, filtration, and endocytosis [3, 4]. The small body size and the fastest filtration rate known [5] allow flies to have separate compartments for renal function: the Malpighian Tubules (MTs), which are analogous to the renal tubules [6], and two clusters of nephrocytes within the body cavity, which are analogous to podocytes in the glomerular kidney. Because of extensive functional similarities, the fly has been successfully used to model aspects of mammalian renal function. We will compare the human and *Drosophila* renal systems and discuss the strategic use of fly modeling of human renal disease.

2. The Human Renal Filtration System

The human kidneys, found in the mid to lower back of the trunk on each side of the spine, are bean-shaped organs roughly the size of a person's own fist. Composed of two main layers, the cortex and the medulla, they play a leading role in blood filtration, solute reabsorption, and metabolic waste excretion, which result in urine production. The kidney medulla contains so-called renal pyramids, conical regions collectively holding about one million functional units, which

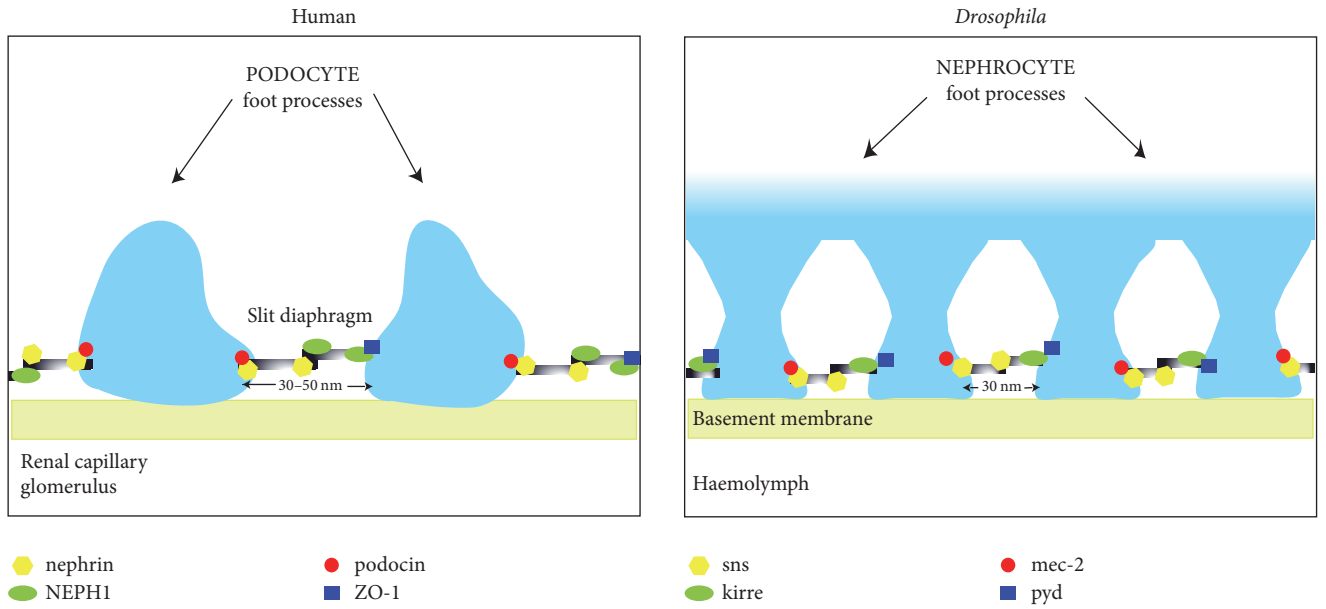

Figure 1: *Comparison of the human podocyte and the Drosophila nephrocyte slit diaphragms.* Selected evolutionary conserved proteins are indicated. The same symbols indicate orthologous proteins between human and *Drosophila*. In the fly, Duf was found to directly interact with Pyd and Sns with podocin ortholog Mec-2 [6].

are called nephrons. The nephrons span both cortex and medulla, starting and ending in the former, with the latter containing variable lengths of the central portion of the tubule. Nephrons modify the filtered fluid and produce urine, which drains into collecting tubules (also called collecting ducts) that in turn fuse into larger ducts that empty into the minor calyx, the ureter and, eventually, the bladder.

Each nephron consists of a tubule closed at one end and enlarged into the cup-like Bowman's capsule, which surrounds a tuft of capillaries called glomerulus. Together, the Bowman's capsule and glomerulus are referred to as renal (or Malpighian) corpuscle. The renal corpuscle filters blood via specialized cells that respond to physiological cues. Glomerular capillaries are fenestrated, that is have pores which allow fluids and small molecules such as ions and sugars to leave the blood and, instead, retain cells and proteins exceeding pore size, complexes of carrier proteins and lipids, as well as calcium ions (Ca^{2+}). Wrapped around the capillaries and with characteristic protrusions called foot processes which contact the capillary's basement membrane are podocytes, specialized epithelial cells integral to the filtration barrier [6–8]. Adjacent foot processes are separated by slit diaphragms about 14 nm wide with 30–50 nm wide pores carrying out filtration [6] (Figure 1). Major components of the slit diaphragm include members of the nephrin protein superfamily and NEPH1, which are coexpressed and form the diaphragm via homotypical and heterotypical interactions [6]. Together, the basement membrane, slit diaphragm, and podocyte processes form a barrier between plasma and filtrate which is essential to glomerular function. Its disruption can lead to kidney disease [9]. Differences in pressure between the glomerulus and Bowman's capsule determine the glomerular filtration rate (GFR, the amount of filtrate produced per minute), which is used to measure kidney function. Because ion and fluid balance depend on flow efficiency, glomerular filtration rate is subject to multiple regulatory mechanisms. The glomerular filtrate is first collected in the Bowman's capsule and directed through the nephron, flowing through the proximal convoluted tubule and the descending and ascending branches of the Loop of Henle, rising through the distal convoluted tubule while being modified, and, finally, arriving to the collecting duct as urine (Figure 2).

2.1. Human Nephron Development. Mammalian nephrons form during the late embryonic and early postnatal stages and display limited cell turnover, resulting in low regeneration rate in the adult [10]. Interactions between two mesoderm-derived tissues, the ureteric bud (UB), and the adjacent metanephric mesenchyme (MM) initiate nephrogenesis [10]. In part driven by developmental regulators Ret, Gfra1, Wnt11, Wnt6, and Pax2 in the UB and Bmp4, Gdnf, Pax2, and Wt1 in the MM, the UB invades the adjacent MM, generating the collecting duct [10–16]. The UB then branches to form a T shape within the MM. The two ends of the T structure then induce formation of the cap mesenchyme, which contains nephron stem cells and progenitors, as well as stromal cells that support kidney ontogenesis by producing signaling molecules, for example retinoic acid, promoting expression of Ret, ERK, MAPK, PI3K, PLC, and WNT [14, 17–20] in the UB. The iteration of this process produces both the branched structure of the ducts and nephron multiplicity. The MM cells then aggregate and, responding to Wnt signaling [21], undergo mesenchymal-to-epithelial transition to produce the renal vesicle or nephron progenitor [10–13]. Vesicle cells polarize, first establishing a proximal-to-distal axis [22] followed by an apical-basal one [23, 24]. After polarization,

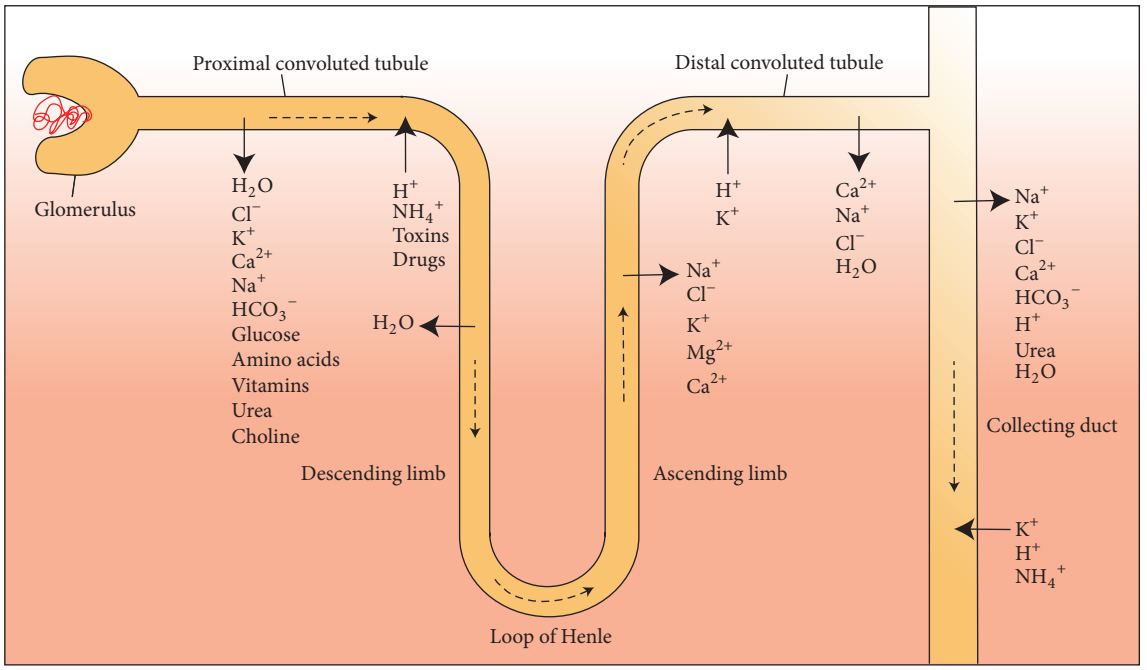

Figure 2: *Schematic features of a generalized human nephron*. The glomerulus, the different regions of the nephron, and corresponding ion and solute transport are indicated. The dashed arrows depict direction of the fluid flow.

the renal vesicle reshapes in the form of a comma, then that of an S, and fuses with the UB, while cells differentiate morphologically [10, 25]. The S-shaped body gives rise to the glomerulus, Bowman's capsule, and both proximal and distal tubules. The intermediate region of the S-shaped body, instead, yields the loop of Henle [26]. Mesangial cells, integral to the glomerulus, derive from the stromal cells [15, 27]. Finally, the vasculature is formed by mesodermal cells that migrate into the developing kidney [28].

3. The *Drosophila* Renal System

In the fly, filtration is carried out by specialized cells called nephrocytes, which display remarkable similarities to the human podocytes [6]. Nephrocytes were originally discovered in the 1800s and found to uptake and store multiple compounds, including silver nitrate, albumin, and dyes [29–34]. Nephrocytes are found in two clusters: one, called pericardial, near the tubular heart and the other, called garland, harboring two nuclei, close to the esophagus [6, 32–36]. *Drosophila* nephrocytes display characteristic in-folding of the plasma membrane which form channels flanked by foot processes [6]. Ultrastructural studies have revealed that nephrocytes form a three-layered filter morphologically similar to that formed by the vertebrate podocytes [6, 32–34, 36, 37]. Like human podocytes, pericardial nephrocytes filter and reabsorb solutes from the *Drosophila* circulating fluid, called haemolymph, via channels regulated by 30 nm wide slit diaphragms [38–40]. Slit diaphragms feature two filaments composed of proteins encoded by genes *sticks and stones* (*sns*) and *dumbfounded* (*duf*, also called *kirre*), a NEPH1 ortholog [6]. Other protein components of the nephrocyte slit diaphragm include the products of genes *mec-2*, a podocin ortholog, and *pyd*, a ZO-1 ortholog [6]. Alike human podocytes, a basement membrane enwraps each nephrocyte. Together, the nephrocyte diaphragm and the basement membrane form the filtration barrier in *Drosophila*, the integrity of which is maintained by protein-protein interactions between orthologs of the human slit diaphragm proteins (Figure 1). The filtrate is actively endocytosed from the sides of the channels, retained in cell vacuoles and either broken down (proteins) or stored (toxins, silver nitrate) [41]. The recent findings that nephrocytes may be apicobasally and basolaterally polarized reinforced their similarity with human podocytes [42, 43]. Moreover, nephrocytes and podocytes appear to respond similarly to pharmacological treatment. Administration of puromycin [44] and protamine sulfate [42] was found to disrupt the filtration barrier. Because of their extensive functional overlap with the necessary podocytes, the discovery that nephrocytes are, instead, dispensable in the adult fly, yet necessary for larval survival [45], was surprising and future studies are being targeted to understand this apparent paradox.

Nephrocytes have been used to model human nephrotic syndromes, in which podocyte processes are effaced as a consequence of mutations in the genes encoding for slit diaphragm proteins. Because these models have been recently reviewed, we refer interested readers to [46].

3.1. Malpighian Tubule Morphology. In *Drosophila* two pairs of MTs are made of a single-layered epithelium and depart from the interface between mid- and hind-gut (Figure 3). With one tubule residing more anteriorly and the other more posteriorly within the abdominal cavity, the MTs are folded

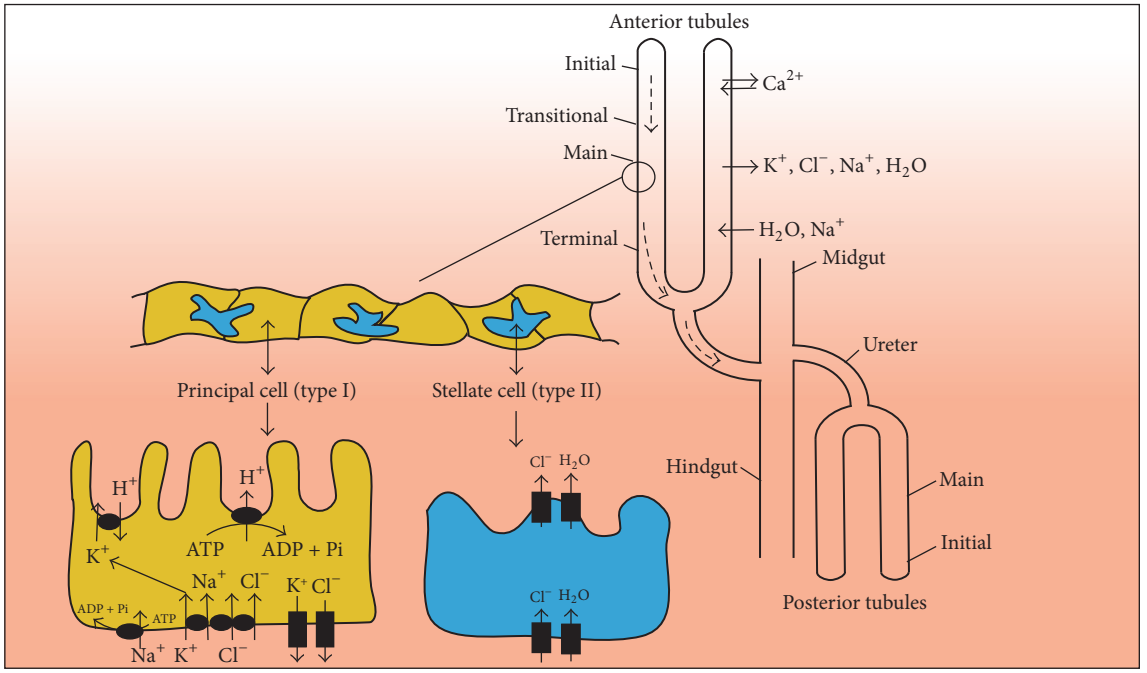

Figure 3: *Schematic features of the Drosophila Malpighian tubules*. The anterior and posterior tubules with relative functional segments and ion and water transport are indicated. Dashed arrows depict the direction of the fluid flow. Features and functions of principal and stellate cells are shown (below).

in a stereotypical way, which is thought to ensure efficient metabolic waste removal and osmoregulation in the open circulatory system typical of insects. Unlike the mammalian closed circulatory system in which the circulating fluid is subject to glomerular ultrafiltration, *Drosophila* haemolymph is, instead, filtered. Also different from the mammalian nephrons that are embedded in organ tissues, the two MTs are free inside the fly's body cavity and can be cleanly microdissected. Anterior and posterior MTs can be distinguished both functionally and morphologically, because of their distinct transcriptomes and the anterior tubule being longer [47].

The anterior tubule pair can be divided into four sections: initial, transitional, main, and terminal (Figure 3). These regions contain type I cells, known as principal cells, and type II cells, known as stellate cells (Figure 3). Principal cells arise from a key interaction between the midgut and the hindgut, constitute about ~80% of all tubule cells, and are responsible for the transport of cations and organic solutes [48]. Stellate cells are scattered around the principal cells and are responsible for water and chloride ion (Cl^-) flow [48, 49]. Reducing tubular expression of the vacuolar- (V-) ATPase by using fruit flies heterozygous for a lethal insertion in the gene encoding for the V-ATPase beta subunit revealed that cation transport may solely be performed by principal cells [50]. Stellate cells were only found in secretory regions and were absent from reabsorptive regions, suggesting that they may have secretory roles [50]. The cells in the MT initial segment are thinner and may excrete specifically Ca^{2+} ions at high rates [51]. Cells of the terminal segment appear to regulate ion and water balance via selective readsorption from and secretion into primary urine and by removing nitrogen-containing catabolites from the haemolymph via active transport of uric acid to the tubule lumen and passive diffusion of other molecules through the intercellular spaces. As the primary urine is transported along the tubule, it is sequentially transformed, a process that requires both apicobasal cell polarization of the tubular epithelium and planar cell polarity. Tubular cells were found to be functionally differentiated in a proximal-to-distal fashion and the processed urine is eventually secreted by the more distal cells. Because of their fundamental role in detoxification, the normal development of MTs is essential in the fruit fly.

Just like human nephrons, *Drosophila* MTs exhibit internal marked asymmetry which corresponds to distinct spatial domains of gene expression [50, 51]. Analyses of enhancer trap expression in the MTs revealed that the initial, transitional, and main segment of the anterior tubules and the sole main segment in the posterior tubules correspond to different cell types and distinct physiological functions [50]. The main segment was found to secrete potassium chloride (KCl) and water at high rates [52] and the lower third of the tubule carried out reabsorption [50]. The lower regions of the MTs appeared to modify the travelling fluid from the main segment by reabsorbing potassium ions (K^+) [53] but not water [54], contrary to what was previously reported [50]. Moreover, the lower tubules were found to acidify the fluid and transport Ca^{2+} into the lumen (Figure 3) [53]. Remarkably, in just about 15 seconds, the cells located in the main segment of the *Drosophila* MTs were found to secrete fluid in amounts equal to their own volume, making the MTs the fastest known filtering system [52, 54]. While the observed functional complexity of the MTs was initially

found surprising and in apparent contradiction with its reputation as a simplified epithelial developmental model [50], this same functional complexity turned out to be an asset for modeling human renal disease in combination with the available genetic and technological tools for probing ion transport [52].

3.2. Ion Transport and Fluid Secretion in the Malpighian Tubules. In the MTs, multiple ion transporters regulate ion balance in different sections. An apical V-ATPase generates a primary proton gradient that fuels the activity of sodium/proton (Na^+/H^+) and K^+/H^+ exchangers, also apically localized, which release Na^+ and K^+, respectively, in the lumen [54]. K^+/Cl^- cotransporters localized at the basolateral membrane [55] decrease K^+ concentration in the secreted fluid as it passes through the lower tubule [53]. Channels found in stellate cells transport Cl^- from the haemolymph to the lumen and are under control of the leucokinin peptide-hormone family [49, 54]. Leucokinins are synthesized in response to increased intracellular Ca^{2+} and promote both fluid secretion and epithelial permeability to Cl^- [56].

Cardioacceleratory neuropeptide CAP2b was found to stimulate fluid secretion specifically via cyclic GMP (cGMP) and to activate the nitric oxide (NO) signaling pathway [52, 57] that regulates salt and water balance in the fly MTs [58]. Early studies tested if increased concentration of intracellular Ca^{2+} could stimulate CAP2b and activate the NO/cGMP pathway in different cell types [59]. Producing the first Ca^{2+} reporter system in *Drosophila* MTs, Rosay and collaborators expressed aequorin, a Ca^{2+}-sensitive luminescent protein, in principal cells in the tubule main segment via the GAL4/UAS binary expression system [59, 60]. As aequorin was produced in the tubules in vivo, luminescence indicated both the amount of aequorin and Ca^{2+} amounts. Stimulation of CAP2b-dependent physiological responses caused rapid Ca^{2+} release from internal stores [59]. Because in this system no CAP2b stimulation was observed in stellate cells, principal and stellate cells of the main segment are unlikely to be connected through gap junctions [59].

3.3. Malpighian Tubule Development. The MTs start forming as four primordia derived from the hindgut primordium and visceral mesoderm in the six-hour embryo [4, 61] in a process requiring the gap gene product Krüppel (Kr) and the transcription factor Cut [48, 62, 63]. The specification of future tubule cells is determined via Kr [48] and, similar to mammalian kidney development, the Wnt pathway [10, 48].

Each tubule primordium contains a unique tip cell specified by lateral inhibition via the Notch pathway [4, 48, 64]. The tip cell segregates and activates the Epidermal Growth Factor receptor homolog DER [65] which promotes cell proliferation, tubular growth, and development of the MTs excretory system [64, 65]. As the MTs grow closer to the caudal mesoderm they induce mesenchymal-to-epithelial transition in nearby cells that will insert themselves in the tubules and become stellate cells [4]. The ectoderm-derived tubular epithelium is formed of principal cells [4] and the ureter of ectodermal cells [4]. Cells divide a definite number of times to give rise to 146 principal and 33 stellate cells in each anterior tubule and 105 principal and 22 stellate cells in each posterior tubule in *Drosophila* [50, 66]. Most of tubule ontogenesis is completed during embryogenesis, and the MTs are not histolysed during metamorphosis. Using positively-marked mosaic lineage with GFP-labeled proliferating cells enabled the discovery of multipotent adult stem cells in the lower tubule and ureter [4]. Such cells require JAK-STAT signaling for self-renewal and are analogous to stem cells activated during repair of kidney ischemic injury [4].

3.4. Immune Function of the Malpighian Tubules. The MT epithelium is part of the fly's defenses against pathogens. The MTs display innate immunity with both humoral and cellular responses and no adaptive response, as is typical for insects [67, 68]. Remarkably, studies in *Drosophila* first revealed the immune function of Toll-receptor signaling [69]. In fact, the *Toll* gene, originally identified for its function in embryonic polarity [70], was later found to function in immunity [71] and to have a few homologs in the fly, including *"18-wheeler"* [72] and multiple vertebrate ones dubbed Toll-Like Receptors (TLRs). Unlike other fly organs involved in immunity, the MTs display constitutive production of antimicrobial peptides (AMPs) [73]. Upon sensing infection, the MTs activate distinct pathways when triggered by specific pathogens. The Toll pathway was found to respond to fungal and Gram-positive bacterial infections and the immune deficiency (IMD) pathway to respond to Gram-negative bacterial infections [74, 75]. MTs may also initiate a Toll-independent humoral response [76]. All these eventually trigger release of seven groups of AMPs, either directly from the MTs [73] or indirectly from the fat-body, the latter being the fly liver-equivalent [68, 69, 77]. The groups of AMPs, Drosomycin, Metchnikowin, Defensin, Attacin, Cecropin, Drosocin, and Diptericin, appear to inhibit growth of haemolymph-invading microorganisms [77]. Both the IMD and Toll pathways were found to sense superficial peptidoglycan on the bacterial cell wall via signaling by peptidoglycan-recognition proteins (PGRP) in the MTs principal cells and gut [69, 78, 79]. PGRP function has mainly been studied in the gut [80], yet the pathway appears to function similarly in the MTs. The Toll proteins display homology to the cytoplasmic domain of the vertebrate interleukin 1 receptor and participate into similar intracellular signaling cascades [81]. The IMD pathway is considered to be equivalent to the vertebrate TNF pathway [75]. Both Toll and IMD pathways result in activation of NF-κB-like transcription factor Relish and induce transcriptional changes [82].

The steroid hormone ecdysone that regulates principal and stellate cell fluid secretion [68] also affects MT-dependent immunity. Ecdysone may promote haemocyte proliferation and fast pathogen encapsulation [83]. In S2 cells, ecdysone was also found to induce transcription of the *PGRP-LC* gene encoding the peptidoglycan receptor and, independently, of a subset of AMPs [84]. Ecdysone also triggers histolysis during metamorphosis [83]. However, the MTs are resistant to this process, possibly due to their fundamental role in immunity. Diap2, an antiapoptotic protein, was also found to contribute

to the innate response in the IMD pathway, possibly via regulation of MT ion channels [85]. Diap2 levels increased in the MTs when there was an immune threat; conversely, decreased Diap2 made flies more prone to infections [85].

Finally, upon septic infection, the MT-dependent immune response may alternatively be activated via the NO pathway, which in turn initiates the IMD pathway and leads to increased NO Synthase (dNOS) and improved fly survival [67, 68].

With growing appreciation for the importance of the MT immune function, the ongoing mechanistic studies of gut-mediated immunity will provide resources and paradigms to better define the role of the MTs in the defense from pathogens.

4. Malpighian Tubules to Model Disease

MTs have been utilized to study the physiology of fluid transport because of their anatomical accessibility, streamlined anatomy, and one-cell-thick epithelium. In MTs, the proliferation of the founder cells (anlage), their spatial organization, patterning, and differentiation occur in sequence, rather than concurrently as in other epithelia, enabling studies of separate stages in time course experiments. MTs and mammalian nephrons share functionally distinct regions (Figures 2 and 3), analogous functions, and display remarkable transcriptome conservation [47, 86]. For example, similar to mammalian renal tubules, MTs carry out detoxification thanks to high levels of cytochrome P450 and glutathione transferase [87]. Likewise, mutations in evolutionarily conserved V-ATPase subunits were initially discovered in *Drosophila* because of their renal phenotypes [88, 89]. Three years later, equivalent mutations in the human *ATP6B1* V-ATPase were reported to cause similar defects in patients [90]. As the interest in modeling renal function in the fly continues to grow, we review some of the successful examples below.

4.1. Nephrolithiasis. *Drosophila* has been used to model the most common kind of human kidney stones, namely, calcium oxalate nephrolithiasis [91]. Nephrolithiasis refers to the formation and movement of kidney stones in the urinary tract [91]. There are multiple types of kidney stones that are distinguished for their different composition and origin. Largely dependent on diet and metabolism, kidney stones in the urinary tract are most commonly composed of calcium oxalate (CaOx) and, in lesser quantities, calcium maleate or phosphate. Also dependent on diet and metabolism, cysts composed of uric acid develop when urine is too acidic, for example in severe dehydration, gout, or following chemotherapy. Struvite cysts are caused by kidney infections and may result in urinary obstruction [91]. Finally, cystine stones form as crystals of leaked cystine in rare cystinuria patients. While rats had been the model of choice for CaOx stone formation [92], prohibitive costs of breeding and caring inspired Chen and colleagues [92] to model nephrolithiasis in the fly. Similar to rodents, flies appeared to respond to oral administration of lithogenic agents ethylene glycol, hydroxyl-L-proline, and sodium oxalate, by inducing formation of CaOx crystals in the MTs between two and three weeks after ingestion. Importantly, response severity was dose-dependent [92].

Recently, RNAi-mediated knockdown of the enzyme xanthine dehydrogenase *(Xdh)* in the fly was shown to induce ectopic calcification and accumulation of crystals and stones in the MTs [93]. Well-fed *Xdh*-knockdown flies only survived three days, as opposed to 60 days of the wild type control. Chemical analysis of the stones by micro X-ray fluorescence revealed significant amounts of Ca^{2+} and zinc (Zn). Because the latter had never been involved in kidney stone formation before, genetic confirmation was obtained by RNAi-mediated inhibition of Zn transporters in the fly, which was found to decrease stone formation [93]. Dietary and pharmacologic modulation of Zn levels in the fly and analyses of human kidney stones further confirmed Zn as a bona fide component [93]. In this case, the *Drosophila* model enabled the discovery of a new contributor to nephrolithiasis and indicated Zn-metabolic enzymes as potential therapeutic targets [93]. One issue to be clarified is that dietary Zn intake has been linked to increased risk of kidney stones in the adult (yet not in adolescent) individuals, while inhibiting Zn excretion was found to reduce cyst formation in the fly. One of the possible ways to interpret these apparently contradicting results posits that Zn may promote formation of different crystals depending on concentration [94] and indicates the need to probe additional physiological parameters in future studies to capture the complexity of kidney stone formation.

Flies have been used to study the processes leading to formation of uric acid stones because of their high levels of urate crystals normally accumulating in the tubule. Systematic analyses of the 33 genes encoding for subunits of the V-ATPase, some of which with multiple isoforms, revealed that mutants in the genes encoding core V-ATPase subunits displayed transparent MTs as a result of urine acidification, which decreased uric acid crystallization [89]. Notably, similar acidification defects were also found in patients with mutations in two V-ATPase subunits, which suggests a certain degree of functional conservation [90, 95].

4.2. Polycystic Kidney Disease. Polycystic kidney disease (PKD) is a genetic disease affecting at least 12.5 million people world-wide, regardless of ethnicity [96]. Two forms of PKD exist, one autosomal dominant (AD) and one, rarer and more severe, which is autosomal recessive (AR) [96] and will not be discussed here. ADPKD causes the development and progressive enlargement of fluid-filled cysts in the nephron, that consequently increase kidney size and cause interstitial fibrosis and chronic kidney disease by age 55 [96]. In half of the patients the severe damage results in kidney failure, making dialysis or renal transplant the only treatments [96]. The lack of a cure and dialysis costs that can surpass 150,000$ per patient per year [1] make PKD a global priority.

Genetic Underpinning of PKD. More than 85% of ADPKD patients carry mutations in the *PKD1* gene, which encodes polycystin-1, a G-protein coupled receptor (GPCR) [97]. Complete mutational inactivation of both alleles is rare and lethal pre- or peri-natally [96]; however, incompletely

penetrant *PKD1* alleles have been found in homozygosis [98]. Mutations in another gene, *PKD2*, are found in about 10% of ADPKD cases [97]. *PKD2* encodes polycystin-2, a transmembrane calcium channel of the TRPP family which was found to physically interact with polycystin-1 [99, 100]. The remaining ~5% of ADPKD patients carry unknown mutations other than *PKD1* or *PKD2* [101]. Because of their clear implication in PKD etiology, *PKD1* and *PKD2* genes and corresponding polycystin-1 and polycystin-2 gene products are being studied in much detail. Polycystin-1 and polycystin-2 complexes were found to mediate cell-matrix and cell-cell interactions, planar cell polarity, signal transduction, and cilia-mediated mechanosensation [96]. We have recently reported that cystic tissue from ADPKD patients carrying a *PKD1* mutation exhibited significant reduction of the *Bicaudal C (BICC1)* gene expression [102]. Similarly, $Pkd1^{-/-}$ mice displayed reduced Bicc1 protein specifically in the kidneys [102], placing *BICC1* genetically downstream of *PKD1*. Mutations in the *BicC* gene of many vertebrates, including humans, cause the development of renal cysts [103–110]. *BicC* was originally discovered in the fly during a screen for embryonic polarity determinants in the germline [111].

Cyst formation is complex and unfolds over time. ADPKD patients carrying *PKD1* or *PKD2* mutations already display small renal cysts at birth [96] yet remain asymptomatic until middle age because the renal capacity is in vast excess (in fact, donation of one kidney is compatible with life). After then, kidney function declines rapidly. Because polycystin-1 and polycystin-2 are part of multiple protein complexes with wide cellular distribution, dysregulation of either in the renal tubule affects many pathways, including apicobasal and planar cell polarity, cell proliferation, cell metabolism, fluid secretion, and the extracellular matrix [112–115]. In PKD cysts form in the renal tubular epithelium where some cells reactivate normally quiescent proliferation pathways and begin to divide. In parallel, epithelial polarization is progressively lost, impacting secretion. Fluid accumulation in the cysts, in turn, stimulates further cell division, possibly in response to increased tensional stretch in the tissue [116, 117]. It is currently unclear what triggers cyst formation. As cysts expand, tubular cells display activation of various signal transduction pathways mediated by Ca^{2+} and cAMP, e.g., Raf-MEK-ERK [118, 119], the mammalian Target of Rapamycin (mTOR), PI3-kinase-Akt, JAK-STAT, NF-kB, Wnt, Hippo, and G-proteins [115, 118–130]. In spite of the enumeration of these pathways, the mechanisms of cyst initiation and progressive cystic degeneration remain largely unknown, likely because of the anatomical complexity of the vertebrate kidney and slow disease onset, which hinder experimental probing. The *BicC* fly provided the first account of renal cysts in *Drosophila* [102]. Modeling PKD in the fly may enable biochemical characterization of the cystic tubule and define the genetics of cyst formation and progression due to low genetic redundancy, and may advance our understanding of the core cystic processes. *BicC* encodes a conserved cytoplasmic RNA-binding protein with orthologs in many species [104, 105, 109, 131–133]. BicC can bind to multiple mRNA targets and appeared to reduce their expression posttranscriptionally [134]. The resulting target upregulation in the oocytes from heterozygote *BicC* female flies was found to disrupt anterior-posterior embryonic polarity [7, 115–135], while *BicC* homozygotes displayed oogenesis arrest at stage 10 [136]. Similar to ADPKD patients, *BicC* mutant flies featured fluid-filled cysts in the MTs already at hatching; over time the cysts enlarged and became more numerous [102]. Compared to wild type, *BicC* flies were short-lived, possibly a consequence of their defective renal function [102]. *BicC* MTs also displayed extra branches, indicating underlying developmental and polarity defects. Oocytes from *BicC* mutant flies exhibited abnormal actin structures which prevented secretion of the dorsal fate determinant Gurken [137–141]. Similarly, the BicC protein was required for epithelial polarization via cadherin-mediated cell adhesion in the IMCD murine kidney cell line [142]. Initial molecular analyses of the *BicC* MTs identified the activation of the *myc* and TOR pathways, two hallmarks of vertebrate PKD [102]. Like ADPKD patients, postrenal transplantation (in which diseased kidneys are left in place) and administration of the immune suppressant and TOR inhibitor rapamycin could transiently rescue the *BicC* flies and reduce cysts, relative to untreated controls [102, 126]. Murine PKD models also exhibited mTOR cascade stimulation [143–148] and responded to rapamycin by delaying cystic onset [126, 145, 149]. In sum, the *BicC* cystic flies appeared to recapitulate many of the diseased features of PKD, displayed pharmacological response to rapamycin [102], and may be a valid model to advance our understanding of the molecular bases of renal cyst formation and the formation of extra tubular branches. One interesting aspect is that ciliary (dys)function appears prominent in vertebrate PKD [150]. The absence of ciliated epithelia in *Drosophila* raises the intriguing question of how cysts form and develop in *BicC* MTs versus human nephrons. Considering that other ciliary pathways, e.g., *hedgehog*, were originally discovered in the fly, the striking biochemical similarities between PKD-type cysts and the *BicC*-dependent cysts in the fly may not be as surprising and may suggest new hypotheses on the evolution of ciliary function.

With proper consideration of the differences between flies and humans and of the hierarchical relationship between the *BicC* and *PKD1* genes, the *BicC* cystic fly may offer opportunity to chart conserved pathways that are altered in *BicC* mutation, are relevant for cyst formation and/or progression, may allow to form new hypotheses on *BicC* function and disease mechanism, and contribute to our understanding of the larger functional context of human PKD. Considering that BicC was also found in a protein complex linked to human nephronophthisis, another cystic kidney disease [110, 133], future studies will reveal if *BicC* function may affect multiple pathways of renal cystogenesis.

5. Conclusion

The remarkable conservation of renal functions between fruit flies and humans is suggestive of the presence of strong evolutionary constraints imposed on the detoxification process of all organisms. Emerging evidence of the interplay between renal and immune functions suggests additional

requirements for the renal system. Multiple diseases causing progressive degeneration and loss of function of the kidney result in organ damage that may only be remedied by renal replacement therapy or dialysis, which are costly socially for the health care system and personally to the patients and their families, due to their negative impact on quality of life. Studies aiming at understanding the mechanisms of renal disease have been hindered by the anatomical complexity of the mammalian kidney. *Drosophila* possesses an evolutionary intermediate between glomerular and non-glomerular renal system, consisting of anatomically separated renal tubules and nephrocytes that, together, fulfill the renal functions. Similar developmental origin of the fly MTs and nephrocytes with their human counterparts, the nephron and the glomerular podocytes, respectively, is accompanied by conserved cellular pathways. Making the fruit fly a useful model to study the mechanisms of disease, the structurally streamlined, anatomically isolated, renal structures can be easily microdissected and studied biochemically; moreover, they can be probed genetically utilizing the vast array of *Drosophila* genetic tools. In multiple cases in which human renal disease has been modeled in *Drosophila*, including nephrolithiasis and PKD, the conservation seemed to extend to pharmacological responses, echoing similar examples in other fly disease models. Considering that many drug binding sites were found to be conserved in the fly [151], development of proper pharmacological screen protocols in the fly may in future provide a rapid and effective alternative strategy for drug discovery.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Cassandra Millet-Boureima contributed sections on MT and nephron function, development and disease modeling, made the figures, and edited the manuscript. Jessica Porras Marroquin wrote text on nephrocytes and podocytes and conducted a literature search that contributed to the manuscript in its current format. Chiara Gamberi contributed sections on PKD, MT, nephron function and development, planned contents, and edited the manuscript.

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Review Article

Using *Drosophila* Models of Amyloid Toxicity to Study Autophagy in the Pathogenesis of Alzheimer's Disease

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Autophagy is a conserved catabolic pathway that involves the engulfment of cytoplasmic components such as large protein aggregates and organelles that are delivered to the lysosome for degradation. This process is important in maintaining neuronal function and raises the possibility of a role for autophagy in neurodegenerative diseases. Alzheimer's disease (AD) is the most prevalent form of these diseases and is characterized by the accumulation of amyloid plaques in the brain which arise due to the misfolding and aggregation of toxic peptides, including amyloid beta (Aβ). There is substantial evidence from both AD patients and animal models that autophagy is dysregulated in this disease. However, it remains to be determined whether this is protective or pathogenic as there is evidence that autophagy can act to promote the degradation as well as function in the generation of toxic Aβ peptides. Understanding the molecular details of the extensive crosstalk that occurs between the autophagic and endolysosomal cellular pathways is essential for identifying the molecular details of amyloid toxicity. *Drosophila* models that express the toxic proteins that aggregate in AD have been generated and have been shown to recapitulate hallmarks of the disease. Here we focus on what is known about the role of autophagy in amyloid toxicity in AD from mammalian models and how *Drosophila* models can be used to further investigate AD pathogenesis.

1. Introduction

Alzheimer's disease (AD) is the most prevalent form of neurodegenerative disease characterized by deficiency in memory and cognitive functions. The predominant pathological changes of AD are the development of amyloid beta (Aβ) plaque deposits in specific brain areas and neurofibrillary tangles (NFTs) within neuronal cells, leading to the progressive loss of synapses, neuronal death, and cognitive decline [1–3]. The extracellular Aβ plaques are derived from cleavage of the amyloid precursor protein (APP). The NFTs consist of intracellular aggregates of the hyperphosphorylated microtubule-associated protein tau, mutant forms of which are also found in other neurodegenerative diseases termed tauopathies. This review will focus on the role of APP and the products arising from its proteolysis (which includes Aβ42) in AD.

While the primary mechanisms responsible for AD pathology remain to be established, there is increasing evidence for a role of the autophagy pathway in AD. Macroautophagy (referred to here as autophagy) is a conserved catabolic pathway that sequesters cytoplasmic material in a double-membrane vesicle (of nonlysosomal/vacuolar origin), the autophagosome, for delivery to the lysosome. Autophagy is induced in response to cellular stress and protects cells by eliminating dysfunctional organelles and toxic protein aggregates. Aberrant regulation of autophagy has significant adverse consequences to normal cellular functions and is associated with numerous human pathologies, including neurodegenerative diseases [4]. This review will describe the pathogenesis of AD, the conservation of components of the autophagy machinery, and their known roles in neurodegeneration. We will discuss evidence of autophagy

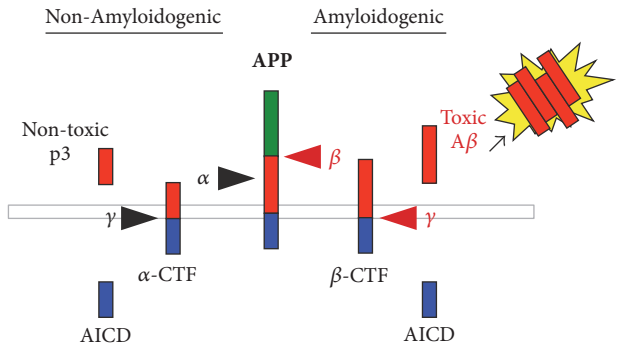

FIGURE 1: *Proteolytic processing of amyloid precursor protein*. In the nonamyloidogenic pathway, transmembrane APP is cleaved by α-secretase followed by γ-secretase generating a nontoxic P3 fragment and α C-terminal fragments (α-CTFs), thus preventing the generation of toxic Aβ. Alternatively, the amyloidogenic pathway involves sequential cleavages of APP by β-secretase followed by γ-secretase complex, thus generating toxic Aβ peptides in addition to the β-CTFs and amyloid precursor protein intracellular domain (AICD). The accumulation of Aβ peptides promotes oligomerisation and formation of insoluble plaques.

perturbation in AD and focus on *Drosophila* as an ideal model for understanding the molecular mechanisms by which autophagy contributes to AD.

2. The Genetics of Alzheimer's Disease (AD)

There are two types of AD based on genetic inheritance and age of onset. Familial AD is rare, affecting approximately 1–5% of individuals that are under 65 years of age. Autosomal dominant mutations have been identified in *amyloid precursor protein* (APP) as well as *presenilin-1 (PS1)* and *presenilin-2 (PS2)* genes that encode the catalytic subunit of γ-secretase complex that cleaves APP to promote the generation of Aβ peptides as causative agents for familial AD [13, 14]. Sporadic, late-onset AD accounts for more than 95% of cases with both genetic and environmental factors contributing to the pathogenesis. While the genetic contribution in these patients is not fully defined, genome-wide association studies have identified several loci associated with increased AD risk in genes involved in various biological pathways including cholesterol/sterol metabolism (APOE-ε4), innate immunity (CR1, CD33, and TREM2), and endolysosomal and autophagy pathways (BIN1, PICALM, and CD2AP) [15, 16]. In addition, recent studies in mammalian cells further support the role of abnormal trafficking in the endolysosomal and autophagy pathways contributing to AD [17, 18].

3. Proteolysis of Amyloid Precursor Protein

APP is a transmembrane protein that undergoes sequential cleavage by one of two pathways (Figure 1). The initial proteolytic cleavage of APP by either α-secretase (nonamyloidogenic processing) or β-secretase (amyloidogenic processing) produces APP-carboxy-terminal fragments (CTFs) as well as secreted APP peptides. In the nonamyloidogenic pathway, α-secretase (ADAM10) cleavage occurs within the Aβ region generating α-carboxy-terminal fragments (α-CTFs) and thus prevents the formation of toxic Aβ [19, 20]. The α-CTF is further cleaved by γ-secretase complex to release P3 peptide as well as an APP intracellular domain (AICD) [21]. In the amyloidogenic pathway, APP is initially cleaved by β-secretase 1 (beta-site amyloid precursor protein cleaving enzyme 1, BACE1) to produce β-carboxy-terminal fragments (β-CTFs). Subsequent cleavage of β-CTF by γ-secretase complex releases toxic amyloid-β (Aβ) peptides (Figure 1). While the processing of APP by α-secretase is predominantly localized to the cell surface, amyloidogenic cleavage occurs in endosomes, lysosomes, and autophagic vacuoles [22–24].

The amyloidogenic processing of APP increases the generation of Aβ that is susceptible to aggregation with other Aβ peptides accumulating into fibrils. This is commonly found in amyloid plaques in the brain (where Aβ42 aggregates are considered to be toxic) and is one of the hallmarks of AD. In addition to Aβ toxicity, the β-CTFs may also contribute to the pathogenesis of AD through multiple pathways [25, 26]. The AICDs of both cleavage pathways can translocate to the nucleus and induce nuclear signalling [27–29]. However, the principle physiological functions of APP remain largely undetermined. The proposed role for APP acting as a cell surface receptor or as a ligand, such as transcriptional regulation and/or synaptic functioning, requires further *in vivo* characterization [30]. While the generation of extracellular Aβ plaques is central to the hypothesis of amyloid as the causative agent in AD [31], additional factors have been identified which may contribute to the onset and/or progression of AD with dysregulation of autophagy thought to be an early event. Despite advances in the understanding of AD pathogenesis, further studies are required to understand the molecular mechanism by which autophagy contributes to disease pathogenesis. In addition, the consequence of Aβ as well as other products from APP processing on other cellular processes including autophagy needs further investigation.

4. Autophagy Molecular Machinery

Autophagy is a highly conserved catabolic pathway that degrades/recycles cytoplasmic material such as large protein aggregates and organelles. The cytoplasmic components are engulfed by a double-membrane vesicle, the autophagosome, for delivery to the lysosomes for degradation (Figure 2). Autophagy has essential functions in normal development, cell growth, metabolism, cell death, infection, and immunity [32–34]. It also acts to protect cells by eliminating toxic protein aggregates, unwanted cellular contents, dysfunctional organelles, and invading pathogens. Under growth-promoting conditions, low basal rates of autophagy are required to maintain cellular homeostasis. In response to extracellular and intracellular stresses, such as nutrient limitation, intracellular metabolic stress, organelle damage, and infection, high levels of autophagy are induced to recycle cytoplasmic material to maintain vital cellular processes [35]. The tightly coordinated multistep process of autophagy is regulated by a number of distinct autophagy-related (ATG)

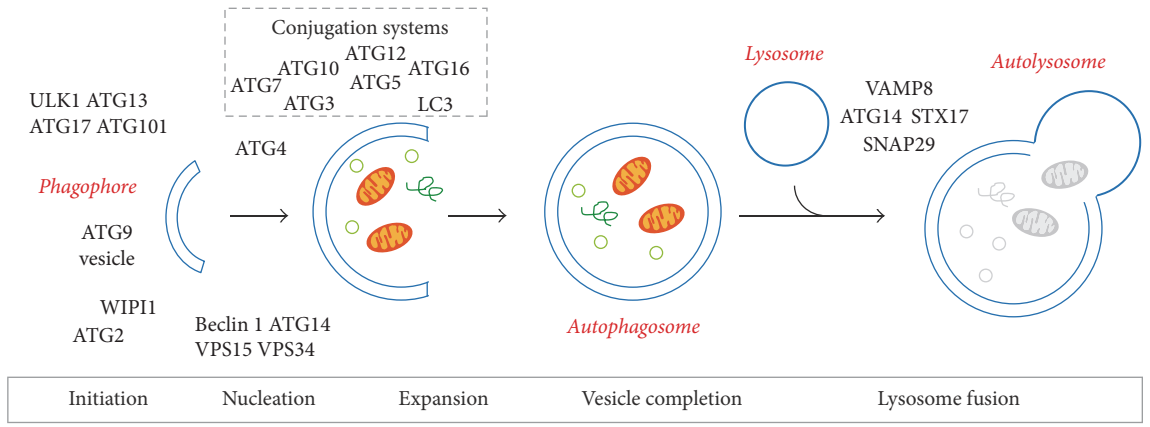

Figure 2: *Schematic representation of the autophagy pathway and the regulatory machinery*. The multiple steps of autophagy can be divided into initiation, nucleation, expansion, vesicle completion, and lysosome fusion. Several ATG proteins form distinct complexes that function in different stages of autophagy. The ULK1/ATG1 complex (consisting of ULK1, ATG13, ATG17, and ATG101) is responsible for the initiation of autophagy. The class III phosphatidylinositol 3-kinase (PI3K) complex (BECN1, VPS34, VPS15, and ATG14), ATG9, and ATG2-WIPI complex nucleate and assemble the membrane to form the double-membrane phagophore. The LC3 and ATG12 conjugation systems can be involved the formation of the autophagosome. Once completed, the autophagosome fuses with the lysosome where the enclosed components are degraded by lysosomal enzymes.

gene products that assemble into specific complexes [36]. Many of these components are evolutionarily conserved from yeast to mammals, including in *Drosophila* (Table 1). The autophagy process/complexes can be functionally divided into (i) initiation, (ii) autophagosome nucleation, (iii) expansion and vesicle completion, and (iv) lysosome fusion [37–40] (Figure 2).

4.1. Initiation and Nucleation. Autophagy is initiated by the formation of a double-membrane structure called the phagophore (also called the isolation membrane) that further expands to encapsulate part of the cytoplasm into the autophagosome [41]. A key early step in autophagy induction requires the activity of the ULK1 (Atg1 in *Drosophila*) kinase complex, comprising ULK1/Atg1, ATG13, FIP200/ATG17, and ATG101. The activity of this complex is regulated in response to stress signals [42]. Nucleation from the phagophore (isolation membrane) requires active ULK1 kinase complexes for the recruitment of class III phosphatidylinositol 3-kinase (PI3K) complex. This complex consists of VPS34, VPS15, ATG14, and Beclin 1 (Atg6 in *Drosophila*) to generate phosphatidylinositol 3-phosphate (PI3P) required for vesicle nucleation.

4.2. Expansion and Vesicle Completion. The expansion and completion to form the autophagosome requires two ubiquitin-like conjugation systems: the Atg8/LC3-lipid phosphatidylethanolamine (PE) and the ATG12-ATG5 systems [37, 43, 44]. There are six Atg8 family members in mammals, including LC3A, LC3B, LC3C, and GABARAP proteins [45] and two in *Drosophila* with Atg8a shown to be essential for autophagy [46]. Prior to lipidation, LC3/Atg8 is cleaved to produce a C-terminal glycine residue (LC3-I form) by the cysteine protease, ATG4. This enables the conjugation of LC3 to PE mediated by ATG7 (E1-like enzyme) and ATG3 (E2-like enzyme). In the ATG12-ATG5 conjugation system, ATG7 and ATG10 (E1- and E2-like enzymes, respectively) mediate the conjugation of ATG12 to ATG5, which associates with ATG16. To enable phagophore expansion a supply of lipid bilayers is required and is thought to involve the transmembrane protein ATG9; however its exact function remains unclear. Membrane closure is thought to involve ATG2, in combination with WIPI1/Atg18, to regulate autophagosome formation [47].

4.3. Lysosomal Fusion. The final step in autophagy is fusion of the autophagosome with the lysosome to form an autolysosome [37]. Lysosomes are specialized organelles that function to break down extracellular materials and recycle cellular components from the secretory, endocytic, autophagic, and phagocytic pathways [48]. The lysosome contains hydrolytic enzymes required for degradation, including cathepsin proteases that are activated by the acidic pH within the lysosome generated by proton-pumping vacuolar H+ ATPase (v-ATPase) [49]. The fusion between autophagosome and lysosome requires the soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) complex consisting of syntaxin 17 (STX17) and synaptosomal-associated protein 29 (SNAP29). This complex forms on autophagosomes to promote tethering with vesicle-associated membrane protein 8 (VAMP8) on lysosomes, resulting in fusion to form autolysosomes [50].

4.4. Amphisome Formation. Instead of fusing with a lysosome, an autophagosome can also fuse with a late endosome/multivesicular body to form an amphisome [51]. Amphisomes contain markers of both autophagosomes (lipidated LC3) and endosomes (RAB5, RAB7, and RAB11) [52, 53]. Members of the endosomal sorting complex required for transport (ESCRT) complex are required during endocytosis as well as for later endosomal maturation and amphisome formation during autophagy [54].

Table 1: Conserved *Autophagy-related* genes in *Drosophila*, neurodegenerative phenotypes, and/or modification of AD models.

	Human gene	*Drosophila* gene	Neurodegenerative phenotype in *Drosophila*	Modification of AD model in *Drosophila*
Initiation	ULK1/ATG1	Atg1	Decreased lifespan and climbing defect [6]	Deficiency line with decreased *Atg1* reduced lifespan of Aβ42 expressing flies [5]
	ULK2			
	ATG13	Atg13		
	FIP200/RB1CC1	Atg17		
	ATG101	Atg101		
Nucleation	BECN1	Atg6		
	ATG14	Atg14		
	PIK3R4/VPS15	Vps15/ird1		
	PIK3C3/VPS34	Vps34/Pi3K59F		
Conjugation systems	ATG3	Atg3/Aut1		
Conjugation systems	ATG4A	Atg4a		
	ATG4B			
	ATG4C	Atg4b		
	ATG4D			
Conjugation systems	ATG5	Atg5	Climbing defect [7]	Decreased *Atg5* reduced Aβ42 accumulation [8]
Conjugation systems	ATG7	Atg7	Decreased lifespan and climbing defect [9]	
Conjugation systems	MAP1LC3A	Atg8a	Reduced lifespan [10]	
	MAP1LC3B			
	MAP1LC3C			
	GABARAP			
	GABARAPL1	Atg8b		
	GABARAPL2			
Conjugation systems	ATG10	Atg10		
Conjugation systems	ATG12	Atg12		Decreased *Atg12* reduced Aβ42 accumulation [8]
Conjugation systems	ATG16L1	Atg16	Decreased lifespan and climbing defect [11]	
ATG9 trafficking system	ATG9A	Atg9		
	ATG9B			
ATG9 trafficking system	ATG2A	Atg2		
	ATG2B			
ATG9 trafficking system	WIPI1	Atg18a		Deficiency line with decreased *Atg18a* reduced lifespan of Aβ42 expressing flies [5]
	WIPI2			
	WDR45B/			
	WIPI3	Atg18b		
	WDR45/WIPI4			

4.5. Selective Autophagy. The targeting of cytoplasmic material to the autophagosome can also occur in a specific manner, by recognizing selective substrates. These can include, for example, damaged mitochondria (mitophagy), excess peroxisomes (pexophagy), and aggregate-prone proteins, including those causing many neurodegenerative conditions (aggrephagy) [38]. The selection of autophagic cargo can be determined by cargo receptors that interact with LC3 family member proteins on the membrane [55]. The multifunctional scaffold protein SQSTM1/p62 (known as Ref(2)P in

Drosophila) binds ubiquitinated proteins and acts as a cargo receptor by binding LC3/Atg8 targeting ubiquitinated proteins for degradation by autophagy [56]. The type of ubiquitin linkages on the substrate can lead to different functional outcomes. The most common ubiquitin linkage tags proteins for degradation of the ubiquitin-proteasome system, whereas other linkages can direct nonproteasomal fates. There are a growing number of identified cargo receptors that bind specific substrates that are tagged with ubiquitin chains. Thus, the receptors serve as a link between ubiquitinated cargo and the autophagy pathway to enable the selective incorporation of the cargo into autophagosomes.

5. Role for Autophagy in Maintaining Neuronal Homeostasis

Multiple upstream signalling pathways regulate autophagy induction with nutrient deprivation, one of the most well characterized signals. The target of rapamycin (TOR) kinase is a central mediator in regulating the response to nutrients and growth signalling and forms a multisubunit complex, mTORC1 [57]. In the presence of growth signals, mTORC1 is activated, thus preventing autophagy by inhibiting ULK1/Atg1 kinase. Under growth-limiting conditions, mTORC1 is no longer active in enabling autophagy induction by activation of ULK1/Atg1 [58, 59]. Numerous studies link alterations of mTOR pathway to age-dependent cognitive decline and to pathogenesis of Alzheimer disease (AD) [60], highlighting the importance of maintaining physiological levels of autophagy to promote neuronal health.

Several nervous system-specific conditional knockout mouse models of autophagy pathway genes have highlighted the importance of autophagy in maintaining the normal functions and homeostasis of the nervous system. The conditional deletion of *Atg5* and *Atg7* in neuronal precursor cells results in autophagy deficiency, accompanied by the accumulation of intraneuronal aggregates in neurons resulting in neuronal loss and neurodegeneration [61–63]. The accumulation of these aggregates in otherwise normal mice suggests that autophagy plays a key role in removing aggregate-prone proteins. Other mouse models of autophagy deficiency, including conditional knockout for *FIP200* and *Wipi4*, as well as *Ulk1*/2 double knockout [64–66], similarly show reduced survival and early-onset, progressive neurodegeneration across broad areas of the brain. However, each model presents variations in the pathology observed which may be due to the specific stage of autophagy that is disrupted, as well as any potential autophagy independent gene functions.

Reduced function of conserved autophagy genes in *Drosophila* also results in neurodegenerative phenotypes (Table 1). Initiation of autophagy requires *Atg17/Fip200*, and reduced expression in adult flies resulted in a climbing defect as well as decreased survival [6]. *Atg5* null flies displayed mobility defects [7], and decreased *Atg16* resulted in climbing defects and decreased survival [11]. *Atg7* mutants show a shortened lifespan as well as accumulation of aggregated ubiquitin-positive lesions in neuronal cells [9] while *Atg8a* mutants that are viable show decreased lifespan [10]. Taken together, these studies demonstrate the critical and conserved role of autophagy in neuronal homeostasis with the impaired clearance by autophagy likely to be a key factor in the accumulation of toxic peptides in the neurons.

6. Autophagy in Alzheimer's Disease

A hallmark of AD and other neurodegenerative diseases is the accumulation of large protein aggregates/inclusions and defective organelles. Autophagy is an essential degradation pathway involved in the clearance of abnormal protein aggregates as well as maintaining protein homeostasis in neuronal cells [67]. There is substantial evidence from both AD patients as well as animal models for the dysregulation of autophagy in this disease. Current findings suggest that impairment of the autophagy pathway leads to defects in the clearance of protein aggregates which is likely to occur early in the pathogenic process, before plaque formation or NFTs deposition [68–70]. However the role of autophagy in AD (in particular which stage is affected) and its alteration during disease progression in neurons is complex and remains largely unclear. Alterations to autophagy have also been identified in other neurodegenerative diseases, including Parkinson's disease and Huntington's disease [71]. There is also evidence for mitophagy in these diseases; however, that is outside the focus of this review and has been reviewed elsewhere [72]. In healthy neurons, autophagy is constitutively active and highly efficient, with low levels of autophagosomes detected [73]. Early observations revealed the accumulation of abnormal subcellular vesicles in the dystrophic or swollen neurites in AD patient brains [74]. Further evidence for disruption to autophagy flux in AD was revealed by the identification of autophagosomes and other immature autophagic vesicles that accumulated in dystrophic neurites in AD brains [68]. While clinical data has identified defects in autophagosomal biogenesis, whether this is pathogenic or a consequence of earlier defects is still controversial. Also, there is evidence that autophagy may not only act to promote the degradation of Aβ but may also be involved in its generation [8, 75].

To aid in understanding the role of autophagy in AD, animal models have provided a tool for *in vivo* studies. A number of transgenic mouse models have been generated based on the genetic pathways disrupted in AD [76]. In an APP/PS1 transgenic mouse model that contains human transgenes for APP and PS1, both of which are carrying human disease mutations, neuronal autophagy is detected in the brain before the appearance of Aβ plaques [22]. Consequently, autophagosomes and late autophagic vacuoles/intracellular trafficking vesicles accumulate in dystrophic dendrites, suggesting impaired maturation of autophagosomes to lysosomes [22]. Similarly, in another study young (4- to 6-month-old) APP/PS1 mice accumulated abnormal immature autophagosomes in axons of hippocampus neurons before neuronal loss [77]. The localization of both APP and PS1 to autophagic vacuoles suggests that Aβ may be generated during autophagy [22, 78]. This indicates that accumulation of autophagic vacuoles/intracellular trafficking vesicles may be a source of Aβ production contributing to AD progression.

Altering the level of autophagy has also been examined in AD models. APP transgenic mice with *Atg7* deletion showed a reduction in Aβ extracellular secretion and plaque formation [79, 80]. This block in Aβ secretion resulted in an accumulation of intracellular Aβ and enhanced neurodegeneration was observed. An increase in the level of autophagy by rapamycin inhibition of mTOR in APP transgenic mice reduced Aβ levels and prevented AD-like cognitive deficits [81]. These findings suggest that autophagy may function in either degradation or secretion in Aβ and supports a role for autophagy in limiting the accumulation of toxic Aβ.

There is further evidence from animal models that basal autophagy is beneficial for decreasing the pathology in AD. In the APP mouse model of AD, heterozygous deletion of *BECN1* decreases neuronal autophagy and increases the accumulation of both intraneuronal and extracellular Aβ deposits followed by neurodegeneration [82]. In support of this, reduced levels of *Beclin 1/BECN1* have been detected in the brains of patients with severe AD [82]. Consistent with this, a mouse knockin of a Beclin 1 gain of function mutation resulted in constitutively active autophagy and, when combined with an AD mouse model, showed reduced Aβ accumulation, prevented cognitive decline, and restored survival [83]. This suggests that in AD, BECN1 induced autophagy contributes to reduction in levels of Aβ peptides/aggregates. In an alternative approach, aged (7-month-old) APP/PS1 transgenic mice were transfected with miR-124 lentiviral vector that downregulates BACE1 [84]. These mice also showed increased Beclin 1 with alleviation of AD pathology but surprisingly they had decreased expression of other autophagy markers. This suggests that Beclin 1 may not be acting via the autophagic pathway in this system and may have other roles such as in the PtdIns 3-kinase complex (Rubicon-UVRAG-Beclin 1-hVps34-hVps15) that localizes to the late endosome/lysosome and inhibits autophagy [85]. Together these data highlight the need for comprehensive *in vivo* analyses to dissect the role of individual autophagy genes in AD pathogenesis.

7. Crosstalk between Autophagy and the Endolysosomal System in AD

The subcellular distribution of APP plays a key role in Aβ production and occurs within the autophagy and endolysosomal systems [86, 87]. The early endosome is the site of colocalization of APP and BACE1 promoting the proteolytic cleavage of APP [88, 89]. Indeed, endosomal pathology is one of the earliest defects observed in AD [90, 91]. Altered levels of the endosomal small GTPase, Rab5, precede Aβ deposition [91], and Aβ colocalizes in Rab5 endosomes in neurons from AD brain [23]. More recently, expression of a dominant negative Rab5 mutant was shown to reduce APP-induced axonal blockages in both cultured neurons and an *in vivo Drosophila* model [92]. Genome-wide association studies identified mutations in endosomal genes including *BIN1*, *CD2AP*, and *PICALM*, which supports the involvement of the endosomal network in processing and trafficking of APP proteolytic fragments [15]. *Drosophila* homologues of these genes show interactions with increased tau expression [17, 93, 94] but they have not been tested with respect to amyloid pathology.

The metabolism of APP in endolysosomal and autophagy networks is consistent with crosstalk between these pathways. Autophagic and lysosomal genes are coordinately regulated by a complex transcriptional program mediated by Transcription Factor EB (TFEB) [95]. TFEB levels have been found to be decreased in brains of Alzheimer's patients [96] while an increase in TFEB expression has been shown to be protective for Aβ-induced pathogenesis [97]. Similarly, in an APP/PS1 mouse model, the overexpression of TFEB increases lysosome biogenesis and reduces Aβ levels [98]. In *Drosophila* there is a single TFEB orthologue, *Mitf*, which has been shown to have a role in regulation of the v-ATPase proton pump as well as other components of the lysosomal-autophagic pathway to promote clearance of protein aggregates [99, 100].

As both endocytic and autophagic pathways lead to the lysosome, it is not surprising that aberrant lysosomal function contributes to AD pathogenesis. Defective lysosomal membrane integrity has been detected in AD patients suggesting dysfunction [101]. Increased expression levels of lysosomal proteases in the early phase of AD patients have also been reported [102]; it is likely that this increased lysosomal function is in response to increased pathogenic load. The AD-associated risk factor gene Apolipoprotein E4 (ApoE4) also affects lysosomal function. Transgenic mice that overexpress ApoE4 accumulate Aβ42 in lysosomes and there is death of neurons in the hippocampus [103]. Also in Neuro-2a cells, ApoE4 can affect lysosomal membrane permeabilization causing the release of proteolytic enzymes that can mediate cell death [104]. Further support for the function of lysosomes in AD was highlighted by the role of PS1 in the assembly of the v-ATPase pump in the lysosomal membrane, thus promoting acidification and contributing to autophagy degradation in a γ-secretase-independent way [105]. An alternative report suggested that the lysosome dysfunction resulting from loss of PS1 could be attributed to alterations in lysosomal calcium storage [106]. Increased or sustained activation of Glycogen synthase kinase-3 also affects lysosome acidification and has been shown to affect the autophagic degradation of APP [107, 108]. In addition, consideration needs to be given to the physiology of neuronal cells where retrograde transport of distally located autophagic vacuoles (mostly amphisomes) is required before any fusion can occur with lysosomes that are located in the soma [109].

These findings and others, including cell culture studies not described here, clearly establish autophagic and endolysosomal dysfunction in AD. Using model organisms to gain an understanding of the exact contribution of these pathways to the pathogenesis of AD will be a priority to enable the development of specific therapeutic interventions that do not affect other essential cellular processes.

Table 2: The human genes that function in APP proteolysis and their *Drosophila* orthologues.

Human gene	*Drosophila* gene	Functions
Amyloid precursor protein (APP)	Appl	APP is an integral membrane protein containing an Aβ-like region that is cleaved by BACE1.
Amyloid precursor-like proteins (APLP1 and APLP2)		Sequence divergence at the internal Aβ site of APLP1 and APLP2 prevents cleavage by BACE1. The principal functions of APLP1 and APLP2 remain unknown.
Presenilin 1	Presenilin	The catalytic subunit of the γ-secretase enzyme complex, also required for lysosomal acidification.
Presenilin 2		Component of γ-secretase complex.
ADAM 10	Kuzbanian	A neuronal α-secretase that cleaves APP at the plasma membrane via nonamyloidogenic processing.
BACE1	Bace	β-secretase enzyme activity cleaves APP in early endosome and promotes amyloidogenic processing with Aβ production.
BACE2		β-secretase related to BACE1 that is thought to contribute to Alzheimer's disease.

8. Advantages of Using *Drosophila* to Model Amyloid Pathology

More than 77% of human disease genes listed on the OMIM database have an orthologue in *Drosophila*, confirming their utility as a model for human genetic diseases [110]. In addition, it is possible to avoid complications that could arise from redundancy as there is often a single gene in *Drosophila* compared with multiple genes in mammalian systems as is the case for APP (Table 2). Knockdown and ectopic expression constructs are readily available in *Drosophila* for most genes and the genetic toolkit available for analyses is constantly being developed and refined [111]. Ectopic expression via the GAL4/UAS system is used most frequently where various tissue-specific "drivers" (i.e., gene-specific promoter regions upstream of a GAL4 transcriptional activation domain) give particular patterns of expression. Driver lines most useful for studies of molecular mechanisms of AD include the endogenous APPL promoter (appl-GAL4), the eye driver (gmr-GAL4), the neuronal driver (elav-GAL4), and ones that express specifically in cholinergic neurons (cha-gal4), glial cells (repo-GAL4), or ubiquitously (da-GAL4 or actin5C-GAL4) (Figure 3(a)) [12]. Inducible expression systems are also available (e.g., GeneSwitch) which allow for studies where the timing of transgene expression can be regulated more precisely [112].

Ectopic expression of human sequences encoding full length APP (with or without BACE1) or Aβ1–42 peptides (wild-type or mutant) in transgenes under UAS control gives rise to neuronal dysfunction which can be measured as retinal degeneration, locomotor defects, decreased longevity, learning and memory defects, and alterations to various cell biological markers [113] (Figure 3). These have been used as the basis for genetic and/or pharmacological screening [114–117]. Other novel approaches to ameliorating AD symptoms in *Drosophila* models include immunotherapy and photodynamics [118, 119]. In addition, the contribution of nonneuronal cell types to AD disease progression is well established. Glial cells have been shown to clear neurotoxic Aβ peptides in the adult *Drosophila* brain through a Draper/STAT92E/JNK cascade that may be coupled to protein clearance pathways such as autophagy [120]. The genetic systems available in *Drosophila* also allow for elegant approaches for understanding the complex interactions that occur between neurons and glia that could contribute to AD [121].

9. *Drosophila* Models for Amyloid Toxicity

Components of APP proteolysis are conserved in *Drosophila* (see Table 2). Although there is limited sequence conservation across the Aβ42 region, it has been shown that neuronal dBACE like enzyme activity can lead to cleavage of the APP-like (APPL) protein in *Drosophila* where the resultant peptide gives rise to neurodegenerative phenotypes that are accompanied by Aβ-like deposits [122]. Processing of APPL gives rise to the same types of cleavage fragments shown for human APP in Figure 1 including small membrane bound intracellular CTFs and neurotoxic Aβ-like peptides, and these have been shown to be expressed throughout the nervous system during development [123]. Given that APPL is conserved throughout evolution suggests that it does have important functions, some of which have been uncovered including its role in neuronal outgrowth and synapse formation, regulation of the circadian clock, and providing neuroprotection in models for AD as well as other neurodegenerative diseases [123–125].

Despite the conservation of endogenous APP processing and function in *Drosophila*, disease models have predominantly been generated based on ectopic expression of human counterparts based on the mutations identified in genetic pathways involved in AD (Table 2) (Figure 3). Various model systems have been developed whereby the human gene products are ectopically expressed in *Drosophila*. Many studies have determined the effects of expressing the Aβ42 toxic peptide directly and it has been shown to give rise to age-dependent neurodegenerative phenotypes that are accompanied by significant disruption to the correct functioning of the autophagic-lysosomal system [126]. It was shown that Aβ42 carrying the "Arctic" APP human disease mutation (E22G) has more severe effects as it is thought to increase the rate of Aβ42 aggregation [127, 128]. However these

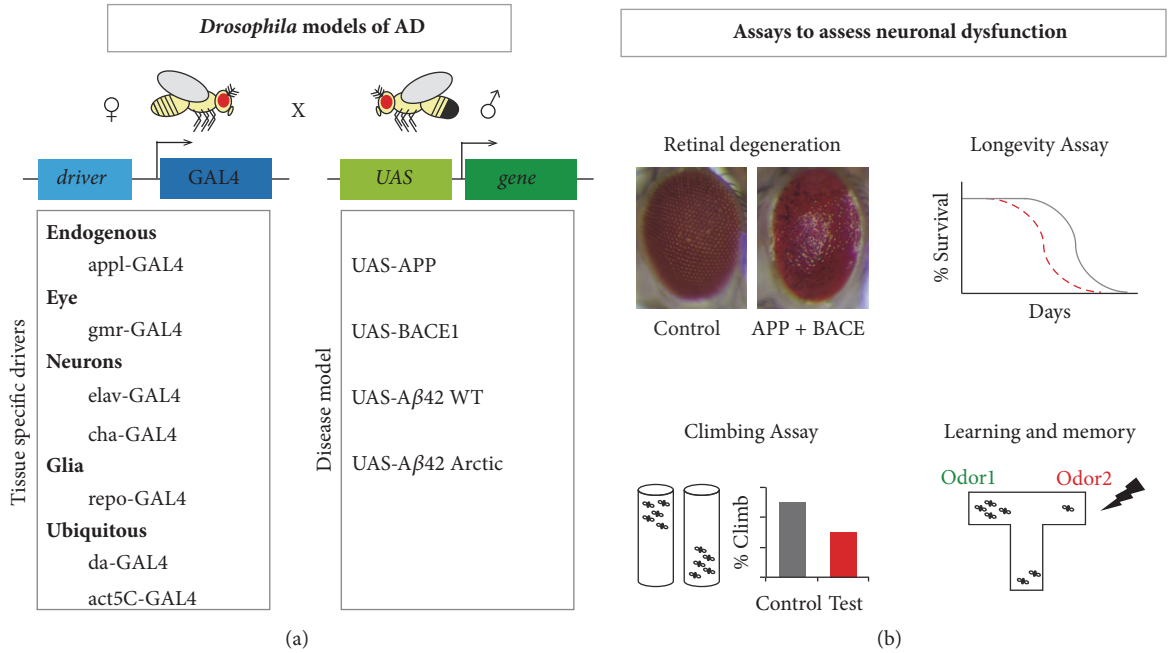

Figure 3: *Drosophila models of AD and assays for neurodegeneration*. (a) *Drosophila* models of AD. The GAL4/UAS system is routinely used in *Drosophila* to drive expression of a gene of interest [12]. There are ubiquitous or tissue-specific enhancers that drive expression of GAL4. By crossing lines containing the driver-GAL4 to the UAS-gene of interest, the progeny will result in ectopic expression. Eye, neuronal, glial, or ubiquitous drivers are used to express Aβ or APP and BACE1 transgenes resulting in specific phenotypes. These can be assessed for neural degeneration and dysfunction. (b) Assays to assess neuronal dysfunction. Using the eye-specific driver GMR-GAL4, APP + BACE1 can be expressed during eye development and the adult eye disruption can be observed. The degenerative eye shows disruption of ommatidial structure, reduced size, and loss of pigmentation. This is a useful system to screen for modifiers of APP + BACE1 toxicity. Lifespan analyses can be performed using neuronal, glial, or ubiquitous cell type driver lines and the effect of genetic modifiers on the longevity of APP + BACE1 flies can also be monitored. Climbing assays can be used to examine locomotor deficits that are known to degenerate with age. Flies are tapped to the bottom of a measuring cylinder and the number of flies that can climb above a certain height is recorded. Also relevant for studies in AD are assays for learning and memory such as odour preference teamed with an electrical shock treatment.

Aβ42 expression constructs require the inclusion of signal sequences from unrelated genes to ensure their secretion and it has been shown that, at least in some cases, these can give alternative effects [129]. Nonetheless, Aβ42 is localized within endosomes and has been proposed to be the cellular source of pathogenic Aβ42 [8]. The presence of Aβ40 was also observed but found not to correlate with toxicity. Similarly comparison of Aβ40 with Aβ42 by others has also shown differential effects in memory testing [130]. In addition ectopic expression of Aβ43 was tested separately and found to be neurotoxic, potentially by acting to prime the formation of amyloid aggregates [131].

Ectopic expression of the full length (695 amino acid) APP is also used in *Drosophila* models for AD where again both wild-type and disease associated mutations have been investigated. Wild-type human APP expressed in combination with ectopic human BACE1 enzyme gives effective processing of APP and leads to neuropathology [132]. Synaptic abnormalities have also been reported when APP and BACE1 are coexpressed specifically in neuronal cells [133]. Interestingly, it has been shown that equivalent amounts of Aβ42 peptide produced from processing of APP (when it is expressed together with BACE1) give stronger effects *in vivo* than Aβ42 peptide expressed directly as the secreted form [134]. This suggests that incorporating the findings from APP and BACE1 ectopic expression models will contribute significantly to the understanding of the molecular pathogenic mechanisms of the proteolytic products of APP.

10. Role for Autophagy in *Drosophila* Models for AD

Similar to mammalian systems there is accumulating evidence for a role of autophagy in the pathogenesis of *Drosophila* models for AD. Amyloid toxicity models tested to date have concentrated on those ectopically expressing the Aβ42 peptide (Table 2). Increased basal autophagy by various methods in these models suppresses ectopic Aβ42 induced phenotypes [135, 136]. Specific components of the autophagy pathway have also been investigated by genetic modification analyses in these Aβ42 models. Decreased expression of *Atg1* or *Atg18* was found to enhance the neurotoxic effect in flies expressing Aβ42, also supporting a protective role for autophagy [5]. However, contrary to this, the knockdown of *Atg5* or *Atg12* was shown to decrease accumulation of Aβ42 [8]. These findings suggest that there is a complex role for components of the autophagic pathway in AD which may be attributed to the particular stage of the process and/or

correlate with timing of disease progression. Together they highlight the need for a comprehensive genetic dissection of the autophagy pathway to determine its contribution to AD.

11. Ageing, Autophagy, and AD

Age is the most prominent risk factor in the development of AD. Age-related dysfunction of autophagy may play a causative role in the onset and progression of AD. It has been suggested that the neuronal autophagy-lysosomal system may shift from a functional and protective state to a pathological and deleterious state either during brain ageing or via Aβ42 neurotoxicity [137]. In support of this there is also an age-related decline in clearance of Aβ42 via the X-box protein 1 [138]. An aged onset model has been developed in *Drosophila* where human APP and human BACE1 are expressed at low levels during development followed by increased expression throughout adulthood [139]. This type of model will enable *in vivo* studies in *Drosophila* to more closely represent disease progression as it occurs in humans. *Drosophila* is also an excellent model to dissect the molecular mechanisms of ageing that are relevant for AD related neuronal dysfunction [140].

12. Conclusions and Future Directions

The contribution of autophagy to AD has been controversial. In particular, it remains to be determined whether autophagy plays a causative or a protective role in AD or whether autophagy defects are a consequence of disease progression. The detection of aberrant autophagy alone is not sufficient to support a causative role, and further detailed molecular analysis is required. However, there is clear evidence to suggest that autophagy is involved in AD pathophysiology. With therapeutic intervention based on modulating autophagy, it will be critical to understand the role of autophagy in the different stages of the disease as well as defining the molecular mechanisms underlying autophagy dysfunction in AD. While the strongest evidence for the contribution of dysfunctional autophagy to AD comes from *in vivo* studies, *in vitro* cell studies have contributed to the understanding of autophagy defects in AD.

Disruption to autophagy could occur at different steps in the pathway from initiation, elongation, cargo selection, lysosomal fusion, and degradation. This may result in altered autophagic flux, with accumulation of autophagosomes, autolysosomes and/or amphisomes, and lysosomal defects that may present as different pathological outcomes. In addition, there is a tissue-specific requirement for distinct components of the autophagic machinery as well as autophagy independent functions of a number of *Atg* genes [46, 141]. Given the controversy as to the protective and/or pathogenic role of autophagy in AD, using *Drosophila* models to dissect out the contribution of the different steps will provide important information about the origin of dysfunctional autophagic processes in AD.

Alzheimer's disease pathology is remarkably complex and human genetic mutations have highlighted alterations to amyloid processing as a primary event that gives rise to neuronal toxicity. Autophagy as part of a cellular clearance mechanism has been shown to play a prominent role in disease progression but its functional contribution to neurotoxicity and/or neuroprotection has not been fully defined. In addition some clues have emerged as to the role of nonneuronal cells, in particular glial cells and their interactions with neuronal cells that can affect neuronal function. Using the genetic platform provided by *Drosophila*, these pathways can be fully dissected and cellular mechanisms of neuronal dysfunction identified. This could include a multigenic approach where more than one candidate can be tested for their effects on APP processing and disease progression. In addition, given that ageing is the most prominent risk factor in AD, the time-frame that would be required for determining efficacies of drugs in humans is not feasible. With the development of technology that can detect amyloid in the blood as an early biomarker for Alzheimer's disease [142, 143], this now provides the opportunity for early intervention and there is a pressing need for identifying new therapeutic compounds. By understanding the role of autophagy in progression/prognosis, this will provide potential novel ways to treat AD and/or provide prognostic biomarkers of disease. Again *Drosophila* presents as an ideal system where specific autophagic mechanisms could be targeted for the development of novel therapies for early intervention in AD.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Review Article

Learning on the Fly: The Interplay between Caspases and Cancer

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The ease of genetic manipulation, as well as the evolutionary conservation of gene function, has placed *Drosophila melanogaster* as one of the leading model organisms used to understand the implication of many proteins with disease development, including caspases and their relation to cancer. The family of proteases referred to as caspases have been studied over the years as the major regulators of apoptosis: the most common cellular mechanism involved in eliminating unwanted or defective cells, such as cancerous cells. Indeed, the evasion of the apoptotic programme resulting from caspase downregulation is considered one of the hallmarks of cancer. Recent investigations have also shown an instrumental role for caspases in non-lethal biological processes, such as cell proliferation, cell differentiation, intercellular communication, and cell migration. Importantly, malfunction of these essential biological tasks can deeply impact the initiation and progression of cancer. Here, we provide an extensive review of the literature surrounding caspase biology and its interplay with many aspects of cancer, emphasising some of the key findings obtained from *Drosophila* studies. We also briefly describe the therapeutic potential of caspase modulation in relation to cancer, highlighting shortcomings and hopeful promises.

1. Introduction

As the second leading cause of death worldwide, cancer claimed the lives of nearly 9 million individuals in 2015 (http://www.who.int). Consequently, a great deal of effort has been expended towards understanding all aspects of tumorigenesis and potential treatments. As part of these efforts, recent investigations have linked some of the defining traits in carcinogenesis, or "hallmarks of cancer," with the deregulated activity of cysteine-aspartic proteases known as caspases [1–11]. In particular, it has been shown that caspase malfunctions could be crucial for explaining tumour cells' ability to evade cell death mechanisms [6, 7], to promote tumour-enabling inflammation and avoid immune destruction [3, 4, 11], to maintain high rates of cell proliferation without entering into the cell differentiation program [2, 10, 12, 13], and to metastasize [5, 14, 15]. However, the molecular basis linking the activity of caspases with these tumorigenic properties is not fully understood. Here, we review studies connecting the activity of these enzymes with different aspects of carcinogenesis, dedicating special attention to some of the key findings obtained from different *Drosophila* models.

For over a century, the fruit fly has proven to be an effective model organism to study a wide range of biological phenomena and carcinogenesis (Figure 1) [16, 17]. Beyond the practical advantages for maintaining this insect in laboratory conditions (e.g., low cost, short life cycle, and high breeding rate), several other reasons posit this model organism at the forefront of genetic research. *Drosophila* contain a simpler and less redundant genome compared to humans, while preserving 77% of genes relevant for human disease [18, 19]. They also possess an extremely versatile set of genetic tools for manipulating gene expression with spatiotemporal control (Gal80/Gal4/UAS, QS/QF/QUAST, and Gal80/LexA/LexOP systems), accurate systems for generating genetic mosaics (FLP/FRT, CRE/LoxP systems), readily available methods for incorporating stable genetic elements into the genome (P-element random transformation, specific integration using attP/attB recombination sites), and genome editing techniques with base-pair precision (CRISP/Cas9

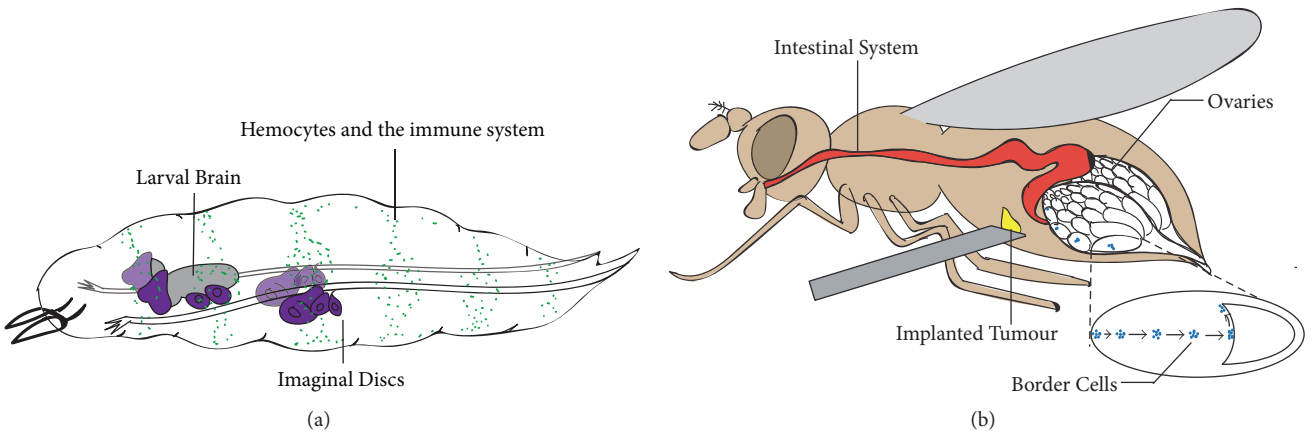


Figure 1: Schematic diagram showing a *Drosophila* larva (a) and an adult fly (b). (a) The larval brain (grey in (a)) and the imaginal discs (purple in (a)) have often been genetically manipulated to induce metastatic tumours with physiological relevance in humans. The immune system (green dots in (a)) represent the macrophage-like *Drosophila* cells, hemocytes. Hemocytes have been used to study immune responses and tumour associated inflammation (a). (b) Recent studies have exploited systems in the adult fly to investigate metastatic and tumorigenic properties. Adult ovaries (white in (b)) are often used for testing the invasive ability of implanted tumours (originating from imaginal discs or the larval brain) in the abdomen (yellow in (b)). The natural migratory ability of ovarian border cells (blue in (b)) has been used to decipher the molecular mechanisms of cell migration during development. The *Drosophila* intestinal system (red in (b)) is a well-established system for modelling many aspects of tumorigenesis related to colon carcinomas.

and homologous recombination) [20, 21]. These advantages have enabled the identification of many oncogenes, tumour suppressors, and signalling components using *Drosophila* cellular models [17]. Similarly, fly research has provided key insights about caspase biology.

Caspases were first discovered in *Caenorhabditis elegans* as regulators of cell death and, later, were implicated in the regulation of inflammation [22–24]. Caspase-mediated apoptosis is an essential process in multicellular organisms that helps to control organ size, shape, and tissue homeostasis, through the elimination of unnecessary or unhealthy cells [25]. All members of this protein family are synthesized as inactive zymogens (procaspases), and only after several steps of proteolytic processing do they become fully active [26]. Structurally, caspases contain two subunits that form the catalytically active pocket. In addition, some members contain N-terminal protein recruitment domains (DEDs or CARDs), which facilitate the formation of large protein complexes (e.g., apoptosome, inflammasome, and PIDDosome) essential for their efficient activation [26, 27]. Caspases can be subdivided into two categories depending on their temporal activation during the process of apoptosis. Initiator/apical caspases are activated at early stages of apoptosis and, immediately after, trigger the enzymatic activation of effector/executioner members [9]. During apoptosis, high levels of caspase activation can enzymatically cleave a plethora of protein substrates throughout all subcellular compartments, thus leading to the stereotyped disassembly of organelles and subsequent shutdown of all essential cellular tasks [27]. In *Drosophila* the apical caspases are encoded by the genes *death regulator Nedd2-like caspase (dronc)*, *death related ced-3/Nedd2-like caspase (dredd)*, *and Ser/Thr-rich caspase (strica)*, while the executioner members are *death related ICE-like caspase (drice)*, *death-caspase-1 (dcp-1)*, *death executioner caspase related to Apopain/Yama (decay)*, and *death associated molecule related to Mch2 caspase (damm)* [28]. As suggested by their nomenclature, caspases are tightly regulated to prevent the inadvertent activation of apoptosis. This regulation does not rely exclusively on enzymatic processing, but often demands different post-translational modifications (e.g., phosphorylation, ubiquitination) [29, 30], as well as transient interactions with regulatory protein partners: inhibitors of apoptosis proteins (IAPs), or pro-apoptotic factors *head involution defective (hid), reaper (rpr), grim (grim)*, and *sickle (skl)* [28]. Beyond their apoptotic role, caspases have recently been implicated in a broad range of non-lethal activities, including the regulation of the immune response [3, 4, 7, 31], stem cell properties [10, 12], cell differentiation [13], cell migration [5, 32], and intercellular communication [12, 14, 33–35], though little is known about these novel non-apoptotic functions. Therefore, if deregulated, caspase activity can contribute to almost every step of tumorigenesis (overproliferation, evasion of cell death and immune destruction, tumour-promoting inflammation, and metastatic invasion). This manuscript aims to provide key examples of what we have learned from *Drosophila* models about the interplay between caspases and cancer.

2. Caspase-Aided Survival and Proliferation of Tumoural Cells

Fundamental to the pathological progression of cancer is the capacity of tumorigenic cells to excessively proliferate while escaping apoptotic death [1]. Therefore, it is not surprising that insufficient caspase activation is one of the defining features of cancerous cells [6–8, 36–38]. Indeed, the evasion

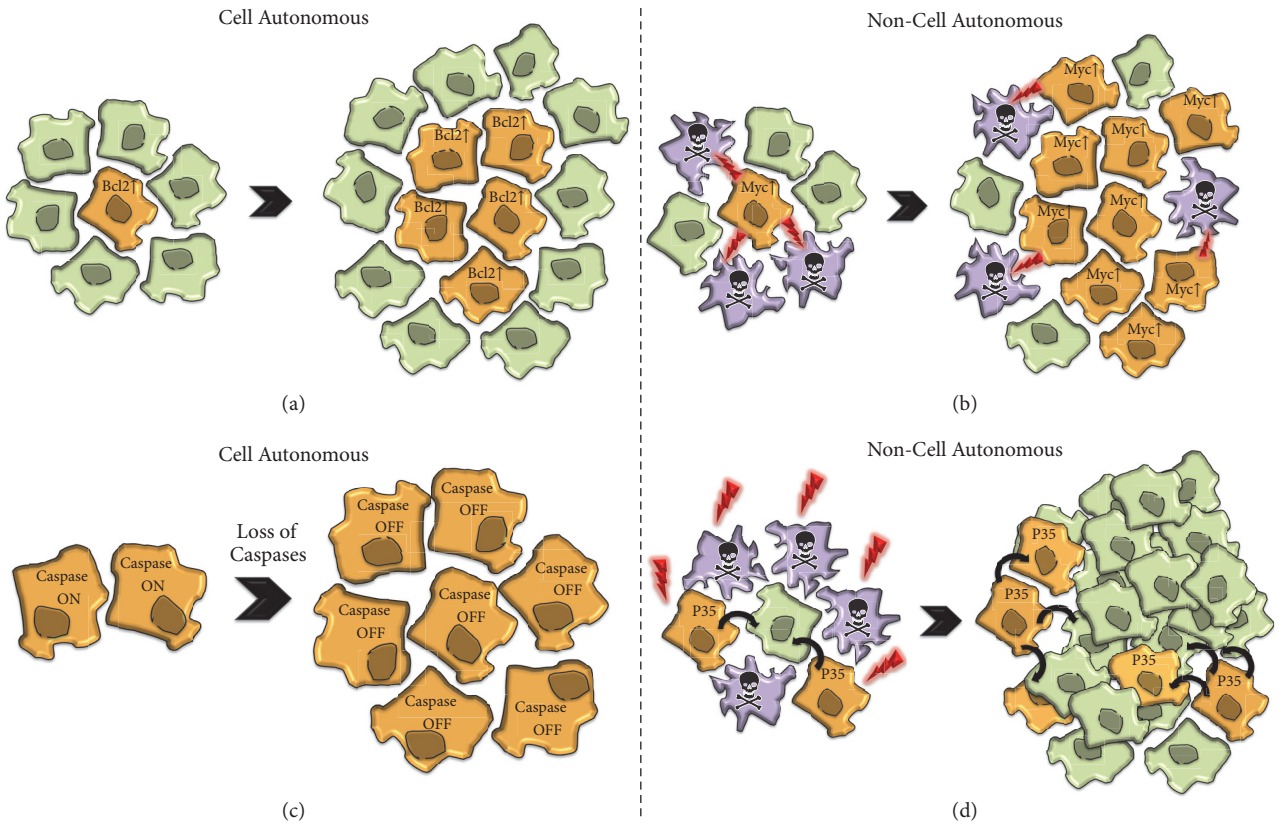


Figure 2: Examples of cellular phenomena that contribute to the clonal expansion of tumour cells. (a) Cancerous cells evade apoptosis through the upregulation of prosurvival proteins such as BCL-2, thus facilitating their clonal expansion. (b) The complex phenomenon of cell competition enables the elimination of slow-proliferating cells (purple), if confronted with fast-proliferating Myc-expressing cells (orange). Lightning symbols indicate the lethal effect (skull symbol) of Myc-expressing cells (orange) on surrounding neighbours (b). (c) Caspase activation defects in the *Drosophila* proneural clusters promote an excess of sensory organ precursor cells. The non-apoptotic activation of the caspase cascade via Drice leads to cleaved Shaggy, thus modulating the number of sensory organ precursors (c). (d) Drawing showing a non-cell autonomous caspase-mediated phenomenon that facilitates tumorigenesis. Following ablation of cells though irradiation (red lightning symbol) most of cells die (d). If apoptosis is impeded in such a scenario, by ectopic expression of P35, the so-called undead cells (in orange) release pro-proliferative signals (black arrows) into surrounding neighbours (in green), thus instigating tumour formation (d). The dashed line separates examples in which caspases have cell autonomous versus non-cell autonomous effects.

of cell death has been identified as a major risk factor during tumorigenesis, providing faulty cells the autonomy to undertake uncontrolled proliferation [36–38]. However, the recent descriptions of non-lethal functions associated with caspases [10–12, 14, 33, 35, 39–41] suggest a more complex intersection between these enzymes and tumorigenesis. Some of the newly identified caspase functions alter the tumorigenic cells' ability to grow and differentiate, while others can influence the cellular microenvironment non-cell autonomously, thus facilitating the cellular selection and proliferation of transformed cells. This section of the manuscript describes selected examples regarding key phenomena regulated by caspases that directly or indirectly enable the clonal expansion of tumorigenic cells.

2.1. Evasion of Cell Death. As popularized by the "hallmarks of cancer" paradigm, a fundamental aspect of cancer initiation and progression is the avoidance of cell death [1]. The literature encompassing this topic is extensive and far beyond the scope of this review. However, it is clear that transformed cells are often resistant to apoptosis due to defects in caspase activation, mainly from the upregulation of prosurvival genes or downregulation of pro-apoptotic factors [6, 7, 42–47]. Members of the anti-apoptotic family of BCL-2 such as Mcl-1 and BCL-XL are commonly overexpressed in cancer, thus resulting in enhanced tumour progression and poor patient prognosis (Figure 2(a)) [6, 7, 42, 43]. Conversely, downregulation of pro-apoptotic proteins such as BAX is often inactivated in colon carcinomas and specific subtypes of breast cancer [44–47]. Different examples obtained from *Drosophila* studies have not only confirmed these theories, but also provided key molecular details towards our understanding of how some types of tumours prevent the apoptotic programme.

The tumour-suppressor signalling cascade referred to as Hippo pathway was delineated in *Drosophila* [48]. However, some years before its formal description, a link had already been described between one of the key members of the

pathway *(Mst-1)* and caspases. In particular, it was observed that the caspase-3-mediated cleavage of Mst-1 had pro-apoptotic effects [49] and facilitated chromatin condensation [50]. On the other hand, it was described that the same biochemical events had a pro-differentiating role in skeletal muscle progenitor cells [51]. In *Drosophila*, the activation of the Hippo pathway normally prevents the translocation of the transcriptional activator Yorkie (Yki) into the nucleus and the subsequent activation of target genes. Whereas some of the Yki target genes promote cell division (e.g., Cyclin-E and Myc) [48, 52–57], others are potent inhibitors of apoptosis (e.g., the *Drosophila* inhibitor of Apoptosis 1 (Diap-1) and the *bantam* microRNA) [55, 58, 59]. The regulatory regions of the *diap-1* locus contain binding sites for the Yorkie-Scalloped (Yki-Sd) complexes, which potently stimulate the transcription of the gene upon binding [52, 55–57]. In turn, *bantam* can post-transcriptionally bind to the mRNA of the pro-apoptotic factor Hid, triggering its degradation [60]. Furthermore, the Hippo complex can also limit the activity of the caspase-2/9 ortholog in flies, Dronc [61]. These effects collectively facilitate the survival and rapid clonal expansion of Yki-activating cells. A further example illustrating the mechanisms of cell death evasion present in tumour cells was obtained investigating the ectopic activation of the Epidermal Growth Factor (EGF) signalling pathway. EGF signalling deregulation often correlates with tumour overgrowth and metastasis [62, 63]. Different studies have shown that, upon EGF activation, pro-apoptotic genes such as Hid are transcriptionally repressed [64]. Furthermore, post-translational inhibitory phosphorylation events also prevent the function of Hid [65]. As previously described, these effects promote cell survival and, ultimately, proliferation of EGF-activating cells. Importantly, most of the signalling pathways deregulated in tumours often crosstalk between themselves in a context-dependent manner (e.g., EGFR signalling regulates the Hippo pathway in mammals by phosphorylating the Yki-like protein YAP) [66]. In tumorigenic situations, this complicates the interpretation of their biological effects, in terms of survival and proliferation.

2.2. Caspases as Key Regulators of Cell Competition. The phenomenon of cell competition was first described in *Drosophila* around 40 years ago through the detailed analysis of wild-type genetic mosaics in heterozygous flies for the *Minute* genes [67–70]. The *Minute* genes encode for several ribosomal proteins that impede protein biosynthesis in mutant conditions. Although *Minute* heterozygous flies are phenotypically normal [68], heterozygous cells proliferate at a slow rate and are selectively eliminated if surrounded by wild-type cells [68, 70]. Importantly, without changing the final size of organs, this process facilitates the clonal expansion of faster-proliferating cells (winner cells) and the simultaneous elimination of slower-proliferating cells (loser cells) via apoptosis [70, 71]. Loser cells can be readily identified at the final stages of the elimination process by the activation of cell death markers such as cleaved caspase-3 and the apoptosis assay TUNEL [72]. Furthermore, recent work by Levayer and coauthors also indicates that caspase activation could precede the delamination of loser cells from tissues [73]. Notably, the suppression of caspase activation can strongly suppress the phenomenon and ultimately the tissue colonization of faster-dividing cells [74]. Considering the scope of this review, a key finding was the discovery that the upregulation of the growth factor Myc (commonly found to be deregulated in cancers) [75] and other tumorigenic-promoting conditions (e.g., combined upregulation of EGFR pathway and loss of cell polarity, or the Hippo pathway) are able to exploit this phenomenon for unrestrained clonal expansion (Figure 2(b)) [76–78]. In recent years, a vast amount of literature has emerged demonstrating the evolutionary conservation of the phenomenon from worms to mammals and some of the molecular pathways implicated in the process [70, 79, 80]. Cell competition has thus been hypothesised to partake in the selection of cancerous cells in tumorigenesis [70, 81–88]. Reciprocally, it has been suggested that cell competition could act as a tumour suppressing mechanism when wild-type cells have the ability to outcompete potentially dangerous cells [70, 89]. Since a fundamental component of cell competition is caspase activation in loser cells, it is conceivable that tumours can take advantage of this biological phenomenon to grow, through blocking caspase activation autonomously, or abnormally triggering it in the wild-type surrounding neighbours.

2.3. Cell Autonomous Caspase-Mediated Regulation of Cell Proliferation. Beyond affecting cell death, caspase deregulation could compromise the activity of key signalling pathways (e.g., Hippo, Notch, TGF-β, and JAK-STAT) and cell cycle regulators (e.g., p21, p27, and cyclin-D2) promoting tumour cell proliferation in many organisms [90–93]. In *Drosophila*, caspase-3-like activation (mediated by the Hippo pathway) has been demonstrated to cleave the chromatin remodelling protein, Brahma, reducing intestinal cell proliferation [94]. Therefore, in this cellular context, caspase defects are associated with the clonal expansion of intestinal precursor cells upon damage [94, 95]. Moreover, in the *Drosophila* brain, protein-protein interactions between Dronc and the Notch signalling regulator, Numb, block the activity of the latter, preventing unrestrained cell proliferation [96]. The caspase-mediated regulation of cell proliferation appears to be conserved from *Drosophila* to mammals. Kennedy and collaborators demonstrated a decrease in the proliferation of human T-cells following application of caspase inhibitors [97]. These defects were also correlated with flaws in the regulation of the cell cycle proteins p21, p27, and cyclin-D2 [93, 98]. Paradoxically, current literature also suggests that caspases could limit proliferation in tumorigenic scenarios, inducing the expression of cell proliferation inhibitors; caspase-7 reduces proliferation in breast cancers through the downregulation of the cell cycle regulator $p21^{cip}$ [92]. The explanation for these opposing roles, and how this discrepancy occurs, is still unknown.

2.4. Regulation of Caspase-Dependent Stem Cell Function and Differentiation. The proliferative potential of cells can also be maintained though the regulation of cell differentiation. Indeed, the act of differentiation itself could be considered a powerful mechanism for limiting tumour growth.

Importantly, caspases are emerging as potent controllers of stem cell properties, as well as differentiation factors [12, 13]. In the *Drosophila* proneural clusters, the sequential activation of the different members of the caspase cascade (Dark > Dronc > Drice) leads to a cleaved form of the fly homolog of GSK-3, *shaggy46 (sgg)*. This caspase-dependent event limits the number of sensory organ progenitor cells without affecting their cell viability [99]. Accordingly, loss-of-function mutations in either the aforementioned caspases or *sgg* generate an excess of sensory organ precursors and neurogenic defects (Figure 2(c)) [100]. Further highlighting the relationship between differentiation and the apoptotic program, it has been reported that the expression of the transcription factor Cut simultaneously promotes differentiation and inhibits apoptosis [101]. The authors suggested that this regulation prevents the expansion of cancer cells through the removal of uncommitted precursors *in statu nascendi* [101]. Interestingly, the cell death regulatory role of Cut is conserved in vertebrates, and Cux1 human cancer cells show apoptotic defects. Many examples have been identified supporting the implication of caspases in the regulation of embryonic and adult stem cell properties [12, 13, 51, 99, 102, 103]. Conversely, it has also been shown that caspases can revert the differentiation state of specific cell types to generate induced-pluripotent stem cells (e.g., generation of induced-pluripotent stem cells from differentiated fibroblast [104]).

Taking into account all the evidence, it is conceivable that caspase deregulation may partake in the aberrant differentiation of cancerous cells. Indeed, direct examples of such exist. Downregulation of caspase-9 results in poorly differentiated colon malignancies, whereas its upregulation results in highly differentiated tumours with decreased proliferation and increased apoptosis [105]. Furthermore, expression of cleaved caspase-3 is a common feature of advanced cancer stages associated with aberrant differentiation of the cancerous cells [106]. More controversial is the role of caspase-14 in cancer pathology [107, 108]. Despite the tentative correlation between caspase-regulated differentiation and cancer pathologies, the biochemical interactors orchestrating these tumorigenic phenotypes are largely unknown.

2.5. Remote Caspase Effects Facilitating Tumorigenesis. In addition to the cell autonomous caspase-regulated effects, these enzymes can also contribute to tumoural transformation through non-cell autonomous mechanisms. Recent investigations have uncovered the phenomenon of apoptosis-induced cell proliferation (AiP) [109]. This phenomenon encompasses all forms of induced proliferation facilitated by the activation of caspases and is crucial for ensuring homeostatic cell numbers within organs and the regenerative process [10, 11, 74, 109–113]. Seminal studies in *Drosophila* demonstrated that high doses of ionizing irradiation during larval stages could eliminate more than 50% of the prospective imaginal epithelial cells; however, healthy full-size adult flies emerged [111, 114]. Interestingly, the artificial suppression of effector caspase activity upon triggering the caspase pathway (e.g., irradiation) generates large hyperplastic phenotypes (Figure 2(d)) [10, 115, 116]. Importantly, the hyperplasia and the regeneration process are severely compromised upon blocking the upstream component of the caspase cascade, Dronc [115, 117, 118]. These observations suggested that caspase-activating cells were releasing mitogenic signals in order to promote tissue regeneration, which can lead to tumour formation if these cells are not effectively eliminated [10, 11, 74, 110, 111, 113, 116]. Although the biological nature of these mitogenic signals is not fully understood and likely context dependent, it is becoming apparent that pro-inflammatory molecules and the production of reactive oxygen species could participate in this process (see Section 4). It is unknown whether caspase-9 in mammals shares a comparable ability to induce apoptosis-induced proliferation like its fly counterpart, Dronc [116]. However, caspase-3 is commonly downregulated in particular cancers [119]; if correlated with the activation of upstream caspase components, this may lead to the promotion of abnormal growth in the wild-type surrounding cells.

3. Caspase-Aided Cell Migration and Metastasis

While overproliferation and the evasion of cell death are some of the most fundamental traits of cancer cells [1], the spreading of transformed cells from the primary tumour to other sites of the body (metastasis) is one of cancer's most deadly attributes. Indeed, the vast majority of deaths related to cancer result from the appearance of secondary tumours called metastases [120]. Because of this, a great deal of effort has been expended towards understanding the invasion mechanisms and the metastatic process. The invasion and colonization in metastasis require the detachment of cells from neighbours through the disruption of cell-cell contacts, degradation of the surrounding extracellular matrix (ECM), and extensive remodelling of the cytoskeleton [121, 122]. Under normal conditions, these cellular tasks are tightly regulated; however, in cancer cells such regulation is commonly perturbed [123–129]. This section of the manuscript compiles some of the key findings relating the activity of caspases with cell migration and metastasis of transformed cells (Figure 3).

3.1. Drosophila Models Linking Caspases, Migration, and Metastasis. During apoptosis, dying cells undergo major cytoskeletal reorganization that demands caspase-mediated pathways (Figure 3) [130]. Additionally, caspases are known to directly modify intercellular attachments by modulating the turnover of cell adhesion molecules (Figure 3) [129, 131, 132]. They can also indirectly affect the secretion of inflammatory factors and matrix metalloproteinases (MMPs) to degrade the ECM (Figure 3) [133–135]. Collectively, this supports the hypothesis that caspases play a key role in regulating the cellular motility in normal and metastatic cells [32, 135]. Support for this hypothesis has been obtained from different organisms, including flies.

A *Drosophila* model describes how the simultaneous activation of caspases and the inhibition of cell death through the effector caspase inhibitor P35 facilitate cell extrusion and spreading of wing imaginal cells [134]. This work attributes the invasive ability of the genetically modified cells to the

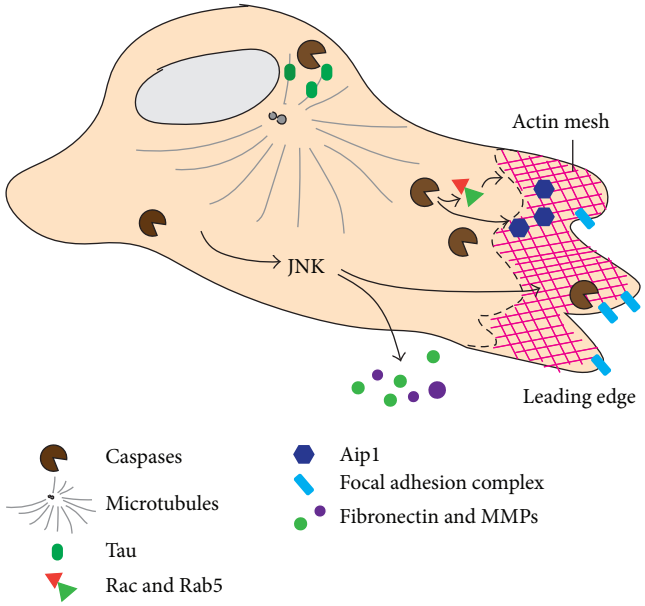


Figure 3: Graphic summary of caspase effects in different subcellular locations during cell migration and metastasis. Caspases are known to regulate the cytoskeleton remodelling elements crucial for migration (such as the microtubule-stabilizing protein tau and actin regulators Aip1, Rab5, and Rac), as well as modulating the stability of focal adhesion complexes. They also modulate the secretion of factors into the ECM that facilitate invasion and migration (MMPs and fibronectin).

non-apoptotic activity of Dronc and the downstream activation of the c-Jun N-terminal kinase (JNK) signalling pathway. Importantly, these factors induce the expression of the matrix metalloproteinase-1 (MMP-1), which ultimately degrades the ECM and basement membrane [134]. MMP production is also observed in *Drosophila* transplantation models of metastasis, in which larval metastatic brain tumours are transferred into the abdomen of host adult flies. Once transplanted, tumours in the abdominal cavity of the host can metastasize into other tissues, such as the ovary [136, 137]. This is a particularly powerful assay that can be used to highlight the differences in metastatic potential arising from different tumour-inducing mutations [137].

Another *Drosophila* model conventionally used to study cell migration and metastasis relies on the ovaries and a collection of follicle cells within the egg chamber, called the border cells, which show invasive and migratory properties [138]. Border cells rearrange their cytoskeleton, cell polarity, and adhesive properties to detach from the epithelium and migrate towards the namesake border of the developing oocyte [138]. Many of the pathways governing this migratory process share strong similarities with the metastatic behaviour of many human cancer cells [138–140]. Importantly, this model also began to shed light on the role of caspases during metastasis, when it was reported that the overexpression of Diap-1 rescued the migration defects caused by a dominant negative mutant for the GTPase Rac [139]. Evidence also indicated that Diap-1 could directly interact with Rac and profilin to regulate actin dynamics. Simultaneously, it was reported that low levels of Dronc activation could have an inhibitory effect on the migration of border cells [139].

3.2. Caspase Implication during Physiological Cell Migration and Metastasis in Mammalian Models. As in *Drosophila*, there is solid evidence suggesting the prominent role of caspases in physiological cell migration and the metastatic behaviour of mammalian cells. In physiological conditions, caspase-11 has been shown to interact with the actin-interacting-protein-1 (Aip1) to promote actin depolymerisation and cell migration [141]. Correlated with this observation, caspase-11-deficient macrophages show reduced motility [141]. Caspase-8 is also heavily implicated in cell migration and metastasis [142], and Caspase-8 knockout mouse embryonic fibroblasts (MEFs) are unable to form actin-based lamellipodia, leading to defective integrin-mediated cell motility [143]. Additionally, caspase-8 has been observed to be recruited and localized to leading lamellae in endothelial cells [144], as well as the leading edge of actin-based lamellae at focal adhesion complexes in neuroblastoma cells [145]. Interestingly, this promotes cell migration through a mechanism independent of its protease activity on effector caspases [144, 145]. This is not surprising, since many of the caspase-8 pro-migratory effects could be mediated by the modulation of actin-dynamics regulators such as Rac and Rab5 [143, 146, 147]. However, the lack of caspase-8 may also promote migratory behaviour. Loss of caspase-8 activity is known to have a major role in activating anoikis, a form of programmed cell death activated by the detachment of epithelial cells from the ECM, in a variety of cancer types [148, 149]. Since the development of anoikis resistance is critical for tumour metastasis [150, 151] and loss of caspase-8 in cancers compromises the apoptosis triggered during anoikis [151], it could be interpreted that caspase-8 pro-migratory effects during metastasis are an indirect consequence of aiding cell survival. Supporting this hypothesis, it has been shown that caspase-8 deficiency also promotes the dissemination of implanted cancerous cells in the embryonic chick due to a lack of cell death [152]. However, caspase-8 deficiency in a mouse neuroblastoma model led to a significant increase in metastases, due to ECM structural changes and production of inflammatory cytokines such as TGF-β [153]. These findings collectively indicate a complex and context-dependent intersection between caspase-8 and cell migration/metastasis.

Caspase-3 has also been linked to the process of cell migration in physiological and metastatic scenarios. It has been shown that the neuronal microtubule-stabilizing protein Tau is cleaved by caspase-3 in PC12 cells [154]. Caspase-mediated cleavage of Tau then enables the dispersion of these cells, suggesting that caspase-3 activity may regulate the cytoskeleton disassembly required for neuronal precursors to migrate towards their destinations [154]. Procaspase-3 was also found to have an inhibitory role in fibronectin secretion, and MEFs deficient for caspase-3 show increased adhesion to substrates and decreased migration velocity in wound-healing assays [155]. Interestingly, these regulatory capabilities were independent of caspase-3's catalytic activity,

as the decreased migration velocity and increased adhesion of caspase-3 deficient MEFs were rescued following introduction of a catalytically dead version of the protein [155]. These results suggest a promigratory role for caspase-3 independent of its enzymatic action. In metastatic scenarios, caspase-3 has been shown to play a pro-migratory role. Whereas caspase-3 inhibition reduces glioblastoma motility and invasiveness [156], its activation promotes migration and invasion in ovarian, melanoma, and hepatoma cancer cells [157–159]. However, conflicting evidence also suggests that caspase-3 could be an inhibitory factor in stroke-induced migration and neurogenesis [160]. Altogether, the described findings illustrate that caspase roles in cell migration and metastasis are far from straightforward and highly context dependent.

4. Caspase-Aided Evasion of Immune Destruction and Tumour-Promoting Inflammation

Components of both the innate and adaptive immune system have been located in virtually every type of tumour [161], often making the tumour's environment mirror that of a physiological inflammatory response [162]. Initially, it was thought that the presence of immune cells indicated the body's attempts to eliminate the tumour; however, it is now apparent that the immune response and resulting inflammation can have a stimulating effect on tumour growth and cancer progression [1]. Exactly how cancers evade immune destruction and instead highjack specific immune responses to promote their own growth is an intense subject of research. However, it is clear from decades of work that the release of bioactive molecules from immune cells can contribute towards every step of tumorigenesis (e.g., enhanced growth, angiogenesis, and initiation of metastatic programs) [163–165]. Indeed, tumour-promoting inflammation is now considered a core enabling characteristic of cancer, and the evasion of immune destruction has joined the ranks of other cancer hallmarks [1].

Since the original association of caspase-1 with the inflammation process in mammals [166, 167], intense research efforts have been devoted to understanding the role of the so-called "inflammatory" caspases in macrophages and other immune cells [168–170]. The primary function of this subgroup of caspases appears to be regulating the maturation and release of proinflammatory cytokines responsible for the inflammatory response [3, 168, 171]. Additionally, inflammatory caspases are potentially involved in the dampening and sequestering of proinflammatory signals released by infected and tumorigenic cells [4, 172]. Despite the fact that classical inflammatory caspases have not been described outside of vertebrates [170], *Drosophila* is known to be a useful model for investigating the immune response. Signalling and transduction pathways are conserved, and analogous elements of the immune system exist [173]. While the presence of a primitive form of adaptive immunity is still under debate [174–176], the *Drosophila* innate immune system shares many similarities with ours and conserves most of the elements and signalling pathways implicated in the cellular and humoural responses (e.g., Toll/IL-R, NF-kB, and Eiger/TNF-α) [173, 177, 178]. The innate *Drosophila* immune system is also sufficient for providing immune surveillance, while producing the proinflammatory responses associated with wound healing, pathogen defence, and tumour response [179–181]. Hemocytes are the circulating immune cells in *Drosophila* analogous to the phagocytic mammalian macrophages [173]. Like their mammalian counterparts, hemocytes are responsible for a large cohort of cellular immune responses, including the clearance of apoptotic bodies in tissue damaged areas, production of signalling molecules, and encapsulation/elimination of pathogens, and are recruited to tumours [180]. Additionally, the *Drosophila* immune response is largely caspase-dependent [182–187]. In response to specific pathogens and tissue damage, the *Drosophila* caspase *dredd* is essential for triggering immune responses through the activation of the key transcriptional factor NF-kB [183–185, 188, 189]. Despite not being formally included in the group of inflammatory caspases, the mammalian homolog of *dredd*, caspase-8, has strong links to inflammatory processes through RIPK activity in normal cells and in transformed cells through the release of inflammatory exosomes [3, 190]. Additionally, the "apoptotic" caspase Dronc has also been associated with the inflammatory response [182]. These similarities between flies and mammals make a strong case for considering *Drosophila* as a viable model for investigating the interplay between caspases, the immune response, and cancer.

Along these lines, *Drosophila* investigations have correlated the expansion of genetically induced tumours with the recruitment of tumour associated hemocytes (TAHs) and their production of Eiger (TNF-α in flies) [191, 192]. Although the pioneering works were not able to identify the molecular mechanisms essential for TAH recruitment, recent data has shown the requirement of Dronc during this process [193]. In the induced-cancer cells, the upregulation of JNK signalling prompts non-apoptotic caspase activity, which ultimately stimulates reactive oxidative species (ROS) production [193]. ROS production is a potent hemocyte recruitment factor [194, 195] that attracts these immune cells towards areas with transformed cells [193]. Hemocytes can then interact with the tumour cells and produce Eiger, which further stimulates JNK activity in cancer cells [191]. All of these events close a positive feedback loop that promotes tumour growth [193]. This cancer model beautifully illustrates the interplay between caspases and the immune system (Figure 4), while confirming the power of this model organism for uncovering fundamental aspects of cancer [17].

Interestingly, inflammation and signals released from inflammatory cells, such as ROS, are able to touch upon another enabling characteristic of tumorigenesis: genome instability and mutations [1]. ROS and other chemicals released from inflammatory cells are actively mutagenic, quickening the genetic evolution of cancer cells towards malignancy through DNA damage [164]. Similarly, DNA damage caused by sublethal levels of caspase activity has been shown to promote genome instability and carcinogenesis, through the activation of endonucleases such as endonuclease G (EndoG) and caspase-activated DNase (CAD) [196–198]. Paradoxically, CAD-induced DNA damage can also

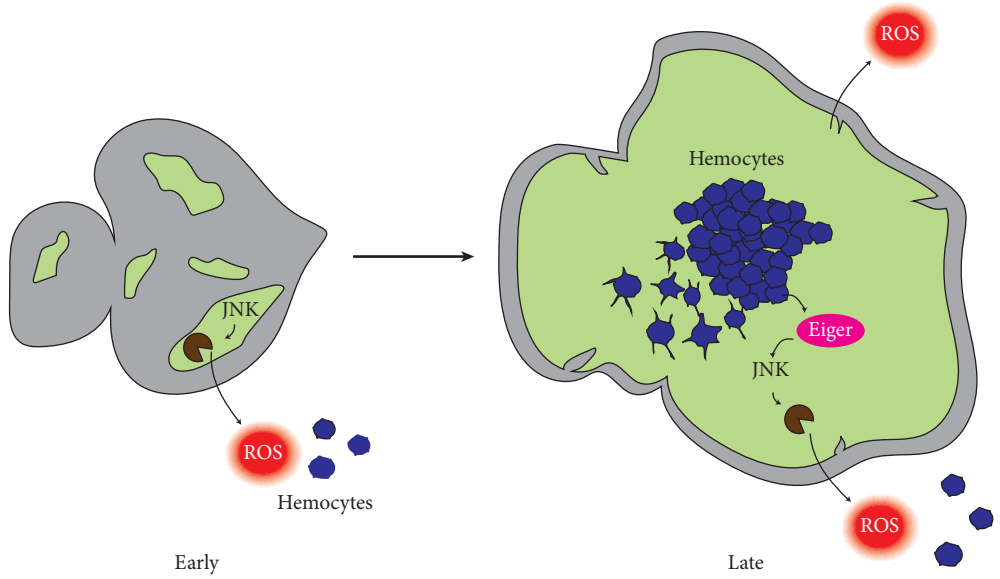


Figure 4: Schematic diagram showing a recently developed model of clonally induced tumours in *Drosophila* eye imaginal discs. During early stages of tumorigenesis, cancerous cells (in green) activate JNK signalling. This induces the production of ROS (in red) and the attraction of hemocytes (in blue) into the transformed areas (green). Upon interaction with the tumour, hemocytes become activated, releasing the TNF ligand Eiger (in magenta). Eiger goes on to stimulate further JNK activity, creating a positive feedback loop that promotes tumour growth and inflammation.

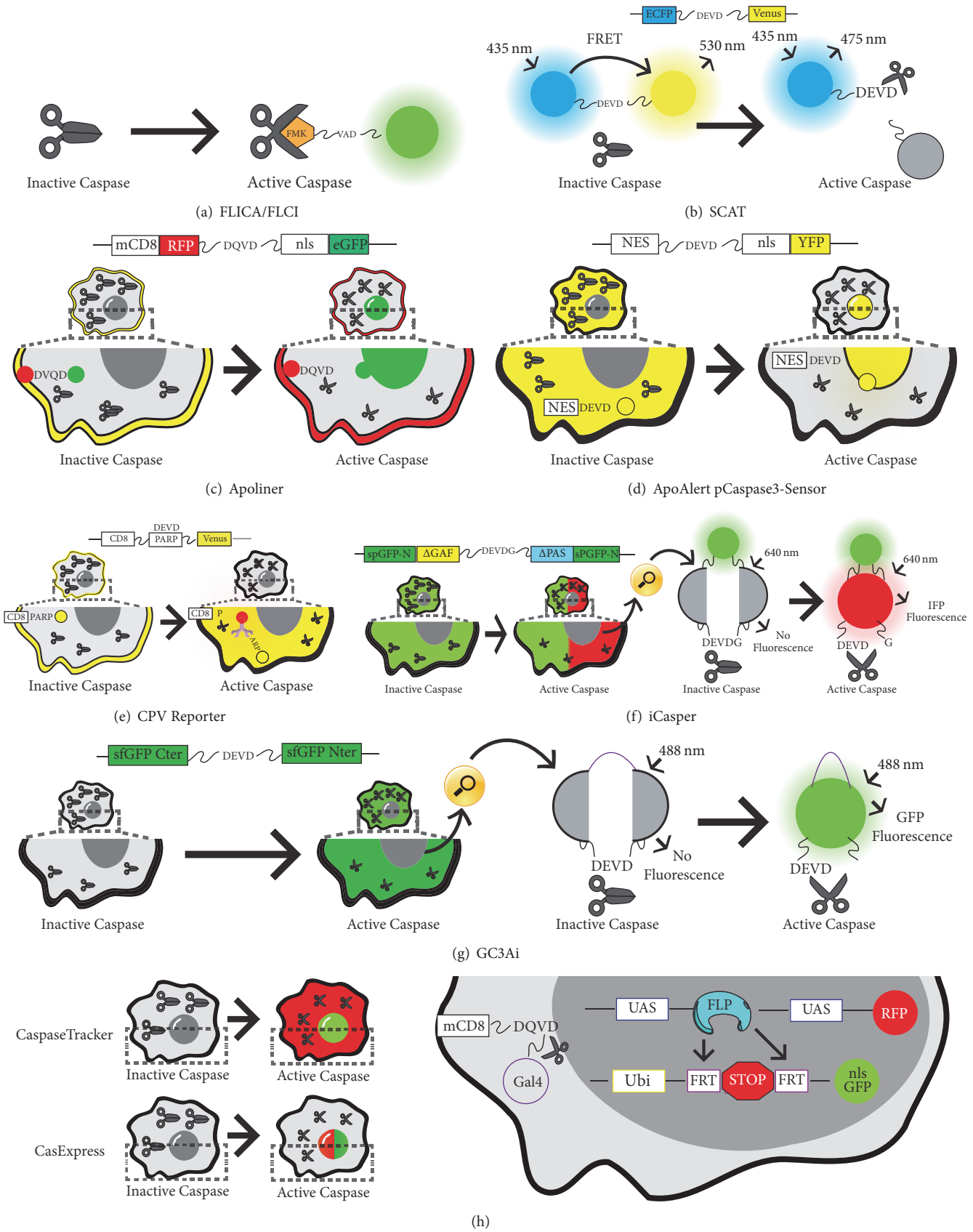
regulate the differentiation of myoblasts in physiological conditions [199]. Although there is no direct evidence connecting the activity of DNases with tumorigenesis in *Drosophila* models, the evolutionary conservation of these proteins [200] suggests that *Drosophila* could be used to investigate the role of caspase-induced DNA damage in carcinogenic processes.

Inflammatory caspases in mammals have also been demonstrated to partake in inducing cell proliferation in normal and cancer cells. The literature is vast on this subject and outside of the scope of this review [113, 163–165]; however, here we provide a few selected examples. Colonic epithelial cells show increased proliferation and reduced apoptosis when deficient for caspase-1 [201]. Caspase-11 has been also implicated in promoting intestinal epithelial cell proliferation through the inflammasome-meditated cleavage of the proinflammatory cytokine IL-18 [202]. Importantly, defective signalling from the inflammasome has been shown to contribute to colitis, but also colorectal tumorigenesis, through loss of intestinal barriers and aberrant proliferation [203]. These studies collectively described the complex intersection between caspase signalling and the immune response, while highlighting its decisive role in the appearance and clonal expansion of cancerous cells.

5. Therapeutic Potential of Caspase Modulation and Drosophila as a Vehicle for Drug Discovery

The enzymatic nature of caspases and their ability to regulate the process of apoptosis has attracted the interest of pharmaceutical companies to discover compounds with caspase-modulating activity. Indeed, there are a substantial number of apoptotic-regulatory compounds in preclinical or phase trials for treating specific diseases [34, 204, 205]. However, several factors have traditionally hampered the transition of such molecules from the bench to bedside. From the therapeutic perspective, the desired adjustment to caspase-kinetics appears dependent upon the underlying pathology and is not always easy to attain both *in vitro* and *in vivo*. Whereas studies by Akpan and collaborators demonstrated that inhibition of caspase-9 was neuroprotective after stroke [206], other studies have conversely demonstrated the efficacy of promoting a pro-apoptotic response during cancer therapy [204] to facilitate the elimination of cell death resistant cancerous cells. Several concerning side effects have also been detected upon treatment with pro-apoptotic agents. Recent studies have reported an increased risk of bone metastasis and osteoporosis linked to these therapies, as well as undesirable side effects due to low compound specificity [207, 208]. Finally, caspase-modulating molecules can impact the inflammatory response with highly diverse consequences occurring depending on the cellular context [208]. Altogether, the evidence highlights the therapeutic potential of caspase-modulating molecules, while stressing the need to anticipate side effects through research in complex cellular models.

Drosophila melanogaster has recently emerged as an excellent model for drug discovery and the evaluation of compound pharmacodynamics [209–212]. For example, methotrexate, gemcitabine, and topotecan are all FDA approved compounds originally validated and/or developed in *Drosophila* [213–215]. Until recently, screening for caspase-modulating chemotherapeutics in *Drosophila* was problematic, owing to the absence of *in vivo* tools able to monitor


Inactive Caspase
FMK
VAD
Active Caspase
(a) FLICA/FLCI
ECFP
DEVD
Venus
435 nm
FRET
530 nm
435 nm
475 nm
DEVD
DEVD
Inactive Caspase
Active Caspase
(b) SCAT
mCD8
RFP
DQVD
nls
eGFP
DVQD
DQVD
Inactive Caspase
Active Caspase
(c) Apoliner
NES
DEVD
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Active Caspase
(d) ApoAlert pCaspase3-Sensor
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Active Caspase
mCD8
DQVD
Gal4
UAS
FLP
UAS
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Ubi
FRT
STOP
FRT
nls GFP
(h)

Figure 5: Rational design and activity of different caspase sensors. (a) Caspase visualization based on artificial fluorochrome labelled inhibitors (FLICA and FLCI). (b) Schematic diagram that shows the SCAT FRET. When caspases are inactive the Enhanced Cyan Fluorescent Protein (ECFP) through Fluorescence Resonance Energy Transfer (FRET) allows the fluorescence of a Venus fluorescent protein at 530 nm (b). Upon caspase activation cleavage of the tethering sequence occurs, resulting in FRET signal loss and fluorescence emission at 475 nm (b). (c) Schematic diagram of Apoliner. This reporter is tethered to cellular membranes through a consensus CD8 membrane anchor domain. Upon caspase activation a DQVD sequence is cleaved (c), releasing a GFP moiety that is translocated into the nucleus; however, the red fluorescent signal RFP is retained at the cellular membrane upon caspase activation (c). (d) ApoAlert pCaspase3-Sensor. The Yellow Fluorescent signal (YFP) is initially retained in the cytoplasm, but a nuclear localization signal (NLS) allows the translocation into the nucleus upon caspase activation (d). (e) CPV reporter. Caspase reporter containing a Venus fluorescent protein tethered to the intercellular membrane through a consensus CD8 sequence. Upon caspase activation the caspase-recognition linker contained in the PARP protein enables the diffusion of Venus-FP into the cytoplasm. The cleaved PARP conjugated to the Venus fluorescent protein can be recognized by an Anti-parp antibody (e). (f) Schematic diagram of iCasper reporter. This reporter consists of two segments of a split GFP protein tethered by a linking region, in addition to a separated infrared fluorescent protein containing the caspase cleavage sequence: DEVD. The presence of the DEVDG linker separates the infrared fluorescent protein (IFP) inhibiting its light emission. Caspase activation results in cleavage of the consensus sequence, allowing IFP fluorescence following excitation of 640 nm. (g) Schematic representation of iGC3 reporter. This reporter consists of two segments of a green fluorescent protein (GFP) tethered by a caspase cleavage recognition sequence, DEVD. Upon caspase activation, the DEVD sequence is cleaved allowing the interaction of both GFP fragments and subsequent fluorescent emission (f). (h) CaspaseTracker and CasExpress. A CD8 sequence tethers a DQVD caspase cleavage sequence and a Gal4 transcription factor to the intracellular membranes. Caspase activation results in cleavage of the sequence and Gal4 transport into the nucleus (h). Gal4 then can activate cell markers with variable protein perdurance upon binding to UAS sequences (e.g., RFP cytoplasmic signal) (h). Additionally, it produces a flippase recombinase that mediates the excision of a stop cassette flanked by FRT sites. Upon excision a permanent marker (nuclear GFP) is expressed under the regulation of a constitutive promoter (Ubiquitin), resulting in a permeant labelling of caspase-activating cells (h). CasExpress has the same rational design as CaspaseTracker; however the authors used a nuclear RFP for showing short-term activation of caspases, instead of a cytoplasmic marker. In all panels black scissors can represent either active or inactive caspases (open or closed, resp.).

caspase activity using a high-throughput approach. Historically, measurement of *in vivo* caspase activation was achieved through the cellular application of fluorescently tagged, small non-reversible binders of activated caspases (Figure 5(a)) [216, 217]. Despite the short half-life of these compounds, concerns were raised regarding the biological significance of these molecules in physiological conditions. Luciferase reporters were then developed; however, they suffered from similar criticisms [218]. One of the pioneering breakthroughs in the *in vivo* monitoring of caspase activation in *Drosophila* came with the publication of the SCAT reporter [219, 220]. The SCAT sensor consists of two fluorophores suitable for FRET microscopy linked via a short caspase cleavage site specifically recognized by effector caspases (ECFP-DEVD-Venus). The expression of the sensor in Hela cells and *Drosophila* tissues reliably detected caspase activation upon caspase cleavage in lethal and non-lethal scenarios (Figure 5(b)) [219, 220]. Since then, the toolkit in flies of caspase sensors has significantly been expanded. Although all subsequent sensors have maintained a core caspase-recognition site for effector caspases, multiple combinations of flanking fluorophores have conferred upon them different capabilities (Figures 5(b)–5(h)). One of the sensors described after SCAT included two fluorescent proteins that change their subcellular localization upon caspase cleavage (Apoliner [CD8-RFP-DQVD-nlsGFP]) (Figure 5(c)) [221]. ApoAlert pCaspase3-Sensor (NES-DEVD-YFP-NLS) was another reporter based upon changes in the subcellular localization of fluorescence (Figure 5(d)) [222]. Alternatively, other sensors exploited the immunoreactivity of specific epitopes upon caspase-mediated excision for detecting caspase activation (CD8-PARP-Venus) (Figure 5(e)) [223]. More advanced and recent methods have used split fluorescent proteins that only fluoresce upon caspase-mediated excision of the short linker joining the two subunits of the fluorophore (Figures 5(f) and 5(g)) [224, 225]. Highly sensitive sensors like these are able to potentially detect caspase activation with subcellular resolution in *Drosophila* tissues (Figure 5(g)) [225]. Finally, new sensors have been published with ability to provide a temporal perspective of caspase activation. The rational design of these sensors includes a transcriptional activator (Gal4) that is released from the cellular membranes upon caspase-mediated cleavage of a short caspase-recognition motif. Once in the nucleus, Gal4 can drive the expression of transient or permanent cellular markers under the regulation of Upstream-Activating-Sequences (UAS) (Figure 5(h)) [226, 227]. These sensors have proven extremely useful for detecting the presence of caspase-activating cells that do not enter the apoptotic program, while enabling their genetic manipulation. Although only some of these sensors are truly suitable for high-throughput drug screens, they promise to bring new opportunities in the coming years for uncovering the effects of caspase-modulating molecules in complex *Drosophila* settings. Furthermore, they could potentially help to anticipate obvious pharmacological complications such as tissue toxicity, compound clearance properties, and tissue targeted delivery.

6. Conclusion

In this review, we have highlighted how the roles of caspases extend far beyond their canonical functions during apoptosis, in either normal or tumorigenic scenarios. Along this line, we have discussed the latest evidence indicating the critical roles of caspases in the regulation of fundamental biological processes and how caspase malfunction contributes to almost all aspects of tumorigenesis (summarized in Figure 6). We hope to have illustrated that although there has been much

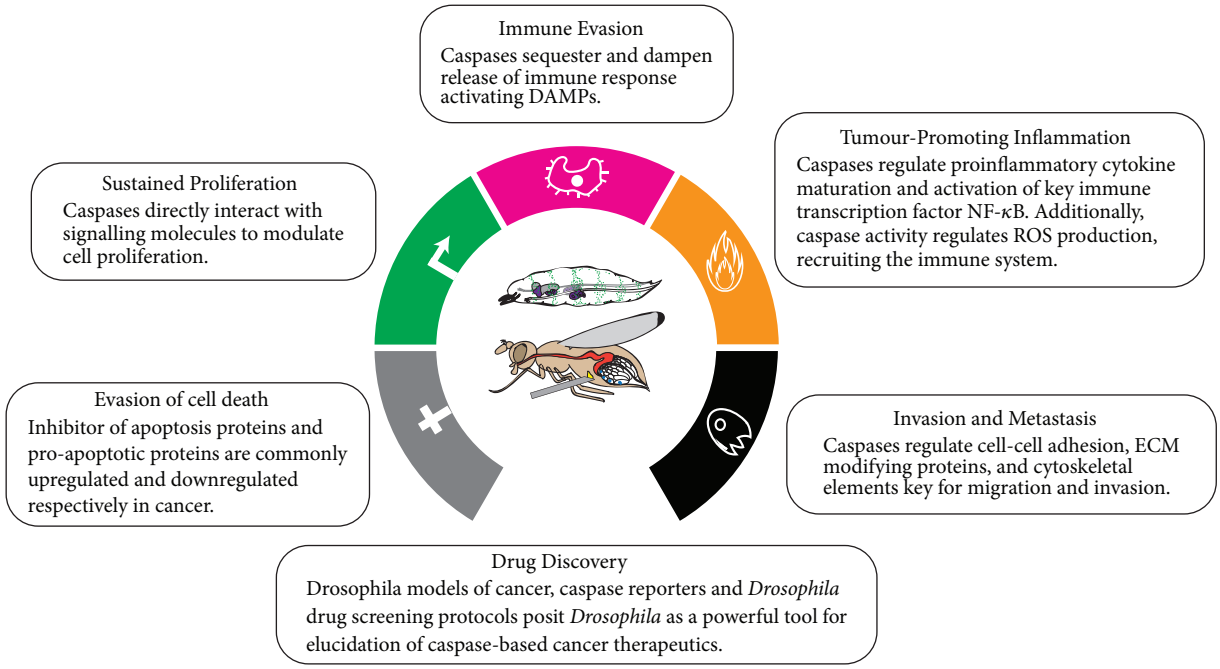

Figure 6: Schematic diagram summarizing the implication of caspases in many hallmarks of cancer. Modified from Hanahan and Weinberg, 2011.

progress, the molecular mechanisms behind these newly identified caspase roles are still largely unclear. More research should be undertaken in order to fully understand caspase biology and its connection to tumour development. Finally, we have shown that many of the findings discussed in the manuscript have emerged from research conducted in the simple but genetically powerful model organism *Drosophila melanogaster*. Indeed, given the previously stated advantages of research in flies, we consider this model organism uniquely positioned to studying the intersection between caspases and cancer, as well as uncovering novel compounds aimed at modulating caspase activity from a therapeutic perspective.

Disclosure

The authors would like to apologise in advance to the scientific community, if they have inadvertently missed any relevant literature in this review.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Lewis Arthurton and Derek Cui Xu made the original bibliographic search. All authors equally contributed to writing the original text and figure preparations. Derek Cui Xu and Lewis Arthurton are co-authors with equal contribution.

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Review Article

Drosophila melanogaster as a Model for Diabetes Type 2 Progression

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Drosophila melanogaster has been used as a very versatile and potent model in the past few years for studies in metabolism and metabolic disorders, including diabetes types 1 and 2. *Drosophila* insulin signaling, despite having seven insulin-like peptides with partially redundant functions, is very similar to the human insulin pathway and has served to study many different aspects of diabetes and the diabetic state. Yet, very few studies have addressed the chronic nature of diabetes, key for understanding the full-blown disease, which most studies normally explore. One of the advantages of having *Drosophila* mutant viable combinations at different levels of the insulin pathway, with significantly reduced insulin pathway signaling, is that the abnormal metabolic state can be studied from the onset of the life cycle and followed throughout. In this review, we look at the chronic nature of impaired insulin signaling. We also compare these results to the results gleaned from vertebrate model studies.

1. Introduction

Diabetes is a chronic metabolic malaise that affects and is forecast to affect many millions of people in the world [1]. It is a disease caused by insulin deficiency or loss of insulin action. In addition to genetic factors, certain lifestyles such as high dietary fat content and physical inactivity are risk factors for the development of diabetes [2]. It has outpaced many other diseases and is predicted to become one of the major health concerns in the future [3]. According to data cited by the World Health Organization, by 2014 incidence of diabetes had risen to 8.5% [3]. In Mexico, for example, 2017 figures show that over 15% of adults are diabetic, which is a very high incidence and concern [4]. As of now, diabetes is an incurable and incapacitating disease with a long and protracted progression. It is also a disease being diagnosed more often in younger patients [2].

In human diabetic patients where the condition has existed for some time, there are several comorbidities. It courses with macrovascular complications, leading to heart disease and stroke, and increased cardiovascular morbidity and mortality. In addition, microvascular complications lead to nephropathy, retinopathy, and neuropathy [1]. Little is known of the onset and early progression of the disease, except for familial cases, which are the minority, and the higher risk of diabetes type 2 for babies where mothers had hyperglycemia or diabetes [2, 5].

Diabetes mellitus is divided into basically two types: type 1 and type 2, a division that reflects the cause of the metabolic dysfunction. Diabetics type 1 have a reduction in insulin secretion, and as a consequence, blood glucose does not attain homeostatic levels after food ingestion and digestion. Physicians normally treat them by prescribing exogenous insulin injections on a regular basis. These diabetics represent around 10% of all diabetic patients, and in most cases, their condition is due to the death of pancreatic Langerhans islets ß-type cells, which normally secrete insulin to clear elevated glucose levels from the bloodstream, like after a meal [6].

It leads to elevated blood glucose levels, as expected, and to general body wasting.

Diabetes type 2 represents the majority of cases, ranging between 90 and 95% of all diabetic patients. It is characterized by a combination of insulin resistance and insulin secretion defects, resulting in relative insulin deficiency and hyperglycemia [6]. Diabetic type 2 patients normally represent patients that have had a long progression, initially suffering from metabolic syndrome, and/or being overweight, and/or being obese for several years. Environmental factors, like diet and level of physical exercise, also play an important role in the inception and progression of the disease, as noted above.

Finally, there is also a third type of diabetes: gestational diabetes. This form of diabetes occurs in pregnant women, leads to increased risk of diabetes for the offspring, and may lead to diabetes type 2 in the mothers after birth [2].

There are, in sum, many factors causing diabetes type 2, both genetic and environmental, and the composite picture is complex, as it may change depending on the actual combination present in populations and individual patients [2]. While all of the factors cited above are recognized contributing factors, it is not clear how they weigh in the initiation and early progression of the disease. Therefore, it is important to elucidate the precise molecular mechanisms underlying the development and progression of the disease.

In general, the diabetic state is multifactorial encompassing several origins and progressions. Studying its causes, effects, and consequences is paramount in the actual diabetes "epidemic," but it is not easy or even possible to study many of these aspects using human patients as test subjects. Scientists have developed model systems where diabetes can be controlled to a higher extent, and in which experimental setups with a high degree of rigor and reproducibility can be used, with genetic uniformity, and highly controlled environments. Principles uncovered in these systems can then be applied in a more general fashion, as the insulin pathway and glucose control is a common, evolutionarily conserved mechanism in the animal kingdom (Figure 1).

1.1. The Insulin Pathway. Insulin is an anabolic hormone in glucose homeostasis in experimentally pancreatomized dogs [7] discovered by Banting and Best, who won the Nobel Prize for this discovery [8]. In general in vertebrates, insulin is secreted from pancreatic Langerhans islets ß-type cells in response to increased glucose levels. In some teleost fish, insulin is produced in Brockmann's bodies [9]. Secreted insulin in the bloodstream binds to membrane receptors, especially in muscle cells, and initiates a transduction cascade that leads to glucose internalization and an anabolic response. In invertebrates, the insulin molecule is slightly longer, has one more disulphide bridge, and is secreted from specialized neurons (insulin-producing cells, or IPC) and glia in the brain [10]. Recently, in a striking novel use, insulin-like peptides have been identified in the venom of certain *Conus* mollusks able to bind insulin receptor molecules and induce hypoglycemia in fish prey [11, 12].

Insulin is a small polypeptide constituted by two chains linked by disulphide bonds, synthesized from the same gene [13]. Whereas vertebrates have one insulin gene, the *Drosophila* genome codes for seven several insulin-like peptides, secreted from the insulin-producing cells (IPC) of the brain. A further eight *Drosophila* insulin-like peptide, DILP8, is really a relaxin homolog, binding to a different type of receptor, and controlling corporal symmetry [14–17].

The *Drosophila* insulin-like peptides (ILPs) also have nonredundant functions [18–20]. The ILP2 peptide has the highest homology to the vertebrate insulin gene and is synthesized together with ILP1, ILP3, and ILP5 in the IPCs of the brain, and their synthesis depends on ILP3. ILP3 expression also activates the insulin pathway in the fat body [21]. ILP4, ILP5, and ILP6 are expressed in the midgut, ILp7 is expressed in the ventral nerve chord, and ILp2 is also expressed in the salivary glands and imaginal discs [22]. The *Drosophila* IPCs are the equivalent of the mammalian Langerhans' islets ß pancreatic cells [23]. ILP6 is synthesized in the fat body and can partially substitute for ILP2 and ILP5. ILP2 loss-of-function mutations lead to an increase in lifespan, while loss in ILP6 causes reduced growth [23, 24].

An insulin monomer is around 50 amino acid residues in length, but dimers form in solution. Insulin is synthesized as a single polypeptide called preproinsulin, which is processed in the endoplasmic reticulum forming proinsulin, which then undergoes maturation through the action of peptidases releasing a fragment called the C-peptide and the A and B chains, linked by disulfide bonds. Mature insulin is exocytosed into the circulation by glucose stimulation and binds to plasma membrane receptors with tyrosine kinase activity.

Insulin is a potent anabolic hormone in vertebrates [25]. It also exerts a variety of actions in flies including effects on glucose, lipid, and protein metabolism. It directly promotes growth and proliferation in tissues, rather than differentiation [26, 27]. In vertebrates, insulin stimulates glucose uptake in skeletal muscle and fat, promotes glycogen synthesis in skeletal muscle, suppresses hepatic glucose production, and inhibits lipolysis in adipocytes [28]. Although vertebrate skeletal muscle, liver, and adipose tissue are considered the main target tissues of insulin action, there is evidence that insulin has important physiological functions in other tissues such as the brain, pancreas, heart, and endothelial cells [29, 30]. Pretty much the same is true for invertebrates in equivalent tissues, where insulin action has been shown to impinge on the physiology of many tissues, including the brain [31]. In vertebrates, there are insulin-growth factor binding proteins (IGFBPs) that conform to an evolutionarily conserved superfamily, regulating insulin-growth factor function; in *Drosophila* the homolog is *ecdysone-inducible gene 2 (Imp-L2)* [32].

In vertebrates, it is thought that insulin-like growth factor binding proteins, IGFBPs, and a third protein, ALS (acid-labile subunit), form ternary complexes with IGFs to regulate IGF function, separating insulin functions from IGFs functions [33]. In flies, ILPs have both vertebrate insulin and IGF functions. The *Drosophila* genome codes for a putative IGFBP-acid-labile subunit (IGFBP-ALS) homolog, *convoluted*, that has been shown to bind in vitro by ectopic expression to ILPs and Imp-L2 forming a ternary complex [34]. However, mutations (even null mutations) in *convoluted* have mutant phenotypes that differ from insulin pathway

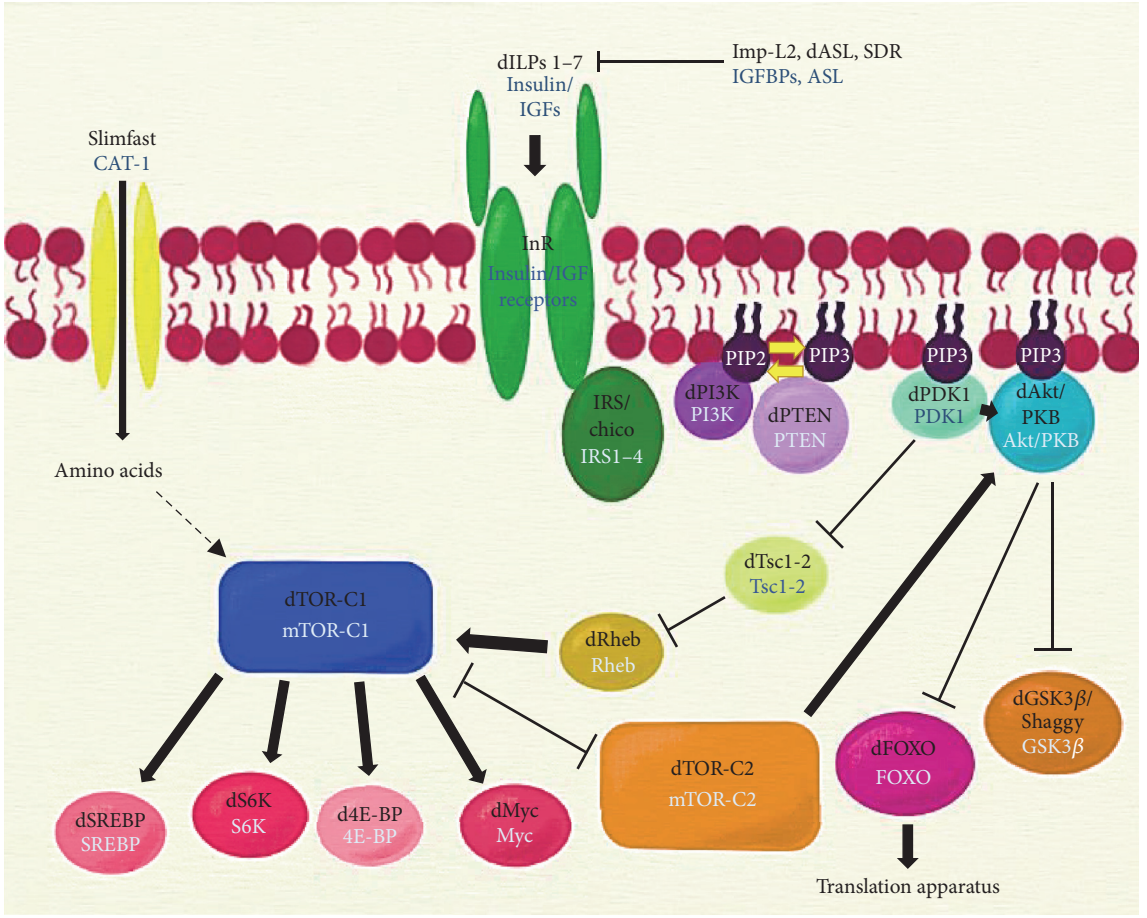

FIGURE 1: The insulin signaling pathway. The binding of insulin to its receptor initiates a phosphorylation cascade that results in the regulation of metabolism through several effectors. Names for the vertebrate counterparts of the pathway appear below their *Drosophila* names. CAT-1: cationic amino acid transporter-1; Imp-L2: ecdysone-inducible gene L2; IGFBPs: insulin-like growth factor binding proteins; ASL: acid-labile subunit; SDR: secreted decoy of InR; dILPs 1–7: insulin-like ligands 1–7; IGFs: insulin-like growth factors; InR: insulin receptor; IRS/chico: insulin receptor substrate; PI3K: phosphatidylinositol 3-kinase (two subunits: Pi3K92E is the catalytic subunit, and Pi3K21B is the regulatory subunit); PIP2: phosphatidylinositol 4,5-bisphosphate; PIP3: phosphatidylinositol 3,4,5-trisphosphate; PTEN: phosphatase and tensin homolog; dPDK1: 3-phosphoinositide dependent protein kinase-1; GSK3β: glycogen synthase kinase 3 beta; Tsc1-2: tuberous sclerosis proteins 1 and 2; Rheb: Ras homolog enriched in brain; TOR-C1: target of rapamycin complex 1 (the TOR-C1 complex consists primarily of TOR, regulatory associated protein of TOR (raptor), and lethal with Sec-13 protein 8 (LST8)); TOR-C2: target of rapamycin complex 2 (the TOR-C2 complex consists primarily of TOR, rapamycin-insensitive companion of TOR (Rictor), and stress-activated protein kinase-interacting protein 1 (Sin1)); Myc: Myc protein; SREBP: sterol regulatory element-binding protein; S6K: ribosomal protein S6 kinase beta-1; 4E-BP: eukaryotic translation initiation factor 4E-binding protein 1; FoxO: Forkhead box O transcription factor. Dashed lines indicate an indirect interaction; arrows and bar-headed lines indicate activation and inhibition, respectively.

mutants. *Convoluted* mutants are larval lethal and affect tracheal morphogenesis and motor axon guidance. In addition, *convoluted* has a higher homology to extracellular matrix proteins like Chaoptin than to vertebrate ALS [35, 36]. Taken together, all of these facts cast doubt on whether a fly ALS homolog actually exists. It seems reasonable to postulate that since ILPs are both insulin and IGFs in flies, no separation in complexes is necessary.

Insulin or insulin-like peptides bind to the insulin receptor (IR), a heterotetrameric protein that consists of two extracellular α-subunits and two transmembrane β-subunits connected by disulfide bridges [37–40]. Insulin binding oligomerizes the receptors, allowing for cross-phosphorylation of the receptor molecules in tyrosine (tyr) residues in the IR domain of the intracellular part of the β-subunit. Despite some differences, vertebrate and invertebrate insulin receptors are equivalent [41], as chimeric fruit fly-vertebrate insulin receptors have been shown to be activated with a similar mechanism as vertebrate insulin receptors in mammalian cells [42]. In flies, there is also a secreted decoy of the insulin receptor, *secreted decoy of InR (Sdr)*, that binds some dILPs in circulation in the hemolymph, necessary for the negative regulation of Dilp action [43].

Phosphorylation in InR tyr residues in the intracellular part of the β-subunit, and the carboxy-terminal extension in the fruit fly insulin receptor [44], leads to the generation

of protein binding sites. This leads to the subsequent recruitment, binding, and tyr phosphorylation of members of the insulin receptor substrate (IRS) family proteins [37]. In *Drosophila*, besides the carboxy-terminus extension of the insulin receptor, the IRS homologs *chico* [45] and *Lnk* [46] act as IRS type molecules. Whereas *chico* is the sole IRS homolog in flies [45], *Lnk* is the fly homolog of vertebrate SH2B adaptor proteins [47]. Lnk acts as an adaptor molecule that favors Chico and InR membrane localization [46].

The phosphorylated tyrosine residues in both the activated receptors and the IRS proteins create further binding sites for other molecules, like the catalytic subunit of phosphatidylinositol 3′ kinase (Pi3K92E), which via the regulatory subunit, Pi3K21B, can now be brought in proximity to its substrate, phosphatidylinositol (4, 5) bisphosphate [48].

The phosphorylated residues of vertebrate IRS1 (or Chico, in the fruit fly) mediate an association with the SH2 domains of the p85 regulatory subunit of phosphatidylinositol 3-kinase (Pi3K) (Pi3k21B in flies [49]) leading to activation of the p110 catalytic subunit, which then catalyzes the formation of phosphatidylinositol (3, 4, 5) trisphosphate (PI 3, 4, 5-P3) from phosphatidylinositol (4, 5) bisphosphate (PI 4, 5-P2) in the inner leaflet of the plasma membrane [50]. This then creates binding sites for proteins with pleckstrin homology domains (PH) [51], like the phosphoinositide dependent kinase (PDK1) [52] and protein kinase B (PKB, also known as Akt) [53]. Both proteins bind, via their PH domains, the phosphatidylinositol trisphosphate generated in the inner membrane leaflet of the plasma membrane via action of Pi3K92E [54, 55]. PDK1, a serine-threonine kinase, then phosphorylates and activates Akt [56, 57].

The phosphorylating activity of Pi3K92E is counteracted by PTEN (phosphatase and tensin homolog deleted in chromosome ten), a lipid and protein phosphatase and tumor suppressor gene in vertebrates and flies [58, 59]. The lipid phosphatase activity, of phosphatidylinositol (3, 4, 5) trisphosphate to phosphatidylinositol (4, 5) bisphosphate is thought to be the main catalytic activity. It is deregulated in many tumor types in humans and in neurodegenerative diseases, like Parkinson's disease [60]. In *Drosophila*, another negative regulator of Pi3K92E is Susi, binding to the p60 regulatory subunit of PI3K, the 60 Kd molecular weight subunit [61, 62].

Akt/PKB is considered a critical node in insulin signaling. Akt/PKB acts by phosphorylating many different proteins [40]. In so doing, Akt/PKB activates different outcomes: (1) Glut 4-mediated glucose transport in vertebrates, by activating the protein Akt substrate of 160 kDa (AS160), (2) glycogen synthesis through inhibition of glycogen synthase kinase 3ß (GSKß), and hence, favoring glycogen synthase (GS) activity, (3) protein synthesis through the mammalian target of rapamycin (TOR) pathway, (4) inhibition of the Forkhead transcription factor FoxO, a major positive catabolic regulator, and (5) others targets, such as the SIK2 (salt-inducible kinase 2) [22, 63–66]. In *Drosophila,* Melted interacts with both FoxO and the TOR kinase via the tuberous sclerosis complex 2 protein (TSC2) and acts as a bridge within the insulin pathway regulating the activities of these two proteins [67].

Besides the direct effect on glucose metabolism, GSK3β also regulates cellular metabolism through the inhibitory regulation of transcription factors that globally control specific metabolic programs, and many of them are also regulated by TOR complexes: cell survival or proliferation (including c-Myc), the sterol regulatory element-binding proteins (SREBP1c), hypoxia-inducible factor 1-alpha (HIF1a), and the nuclear factor- (erythroid-derived 2-) like 2 (Nrf2) [68]. Thus, Akt signaling can stabilize these proteins by inhibiting GSK3β and by indirectly activating TOR-C1 [27]. There is evidence that the insulin pathway control of Myc is evolutionarily conserved in *Drosophila*. In biochemical experiments in tissue culture cells and in ectopic expression studies, the *Drosophila* insulin pathway, via inhibition of *shaggy,* the *Drosophila* homolog of GSK3β, regulated *Drosophila* Myc protein stability. *Drosophila myc* is coded by the gene *diminutive* [69, 70].

Perhaps the best-documented cases of downstream components activated by Akt/PKB are the target of rapamycin (TOR) kinase and the FoxO transcription factor. The ser/thr kinase TOR interacts with different proteins to form the complexes TOR-C1 and TOR-C2 [62, 71]. This kinase positively regulates cell growth, proliferation, motility, and survival. TOR-C1 appears to play a role in acute feedback inhibition of Akt, negatively regulating insulin action. Activation of TOR is not direct from Akt/PKB: in the *Drosophila* ovaries, Akt/PKB represses the proline-rich Akt substrate 40 kDa (PRAS40). There is also a PRAS40 homolog in vertebrates [72]. In the fly ovaries, PRAS40 represses TOR, decoupling reproduction from growth in the cells of this organ [73]. In other tissues, Akt/PKB phosphorylates and might repress TSC1 (in flies) and TSC2, which are normally thought to repress the GTP-binding protein and GTPase Rheb that activates TOR-C1, yet it is unclear whether indeed this is the case [74]. TOR-C1 activation leads to longer S1 phase in cells [75, 76].

TOR is another central component downstream of insulin signaling. The TOR kinase in the TOR-C1 complex phosphorylates and regulates several proteins. TOR kinase in the TOR-C1 complex phosphorylates (1) S6 kinase, to promote translation (S6 is a component of the ribosomes) [77], (2) the translation regulatory factor 4E-BP, which also promotes protein synthesis [78], (3) the transcription factor Myc [69, 79, 80], (4) SREBP [81], and (5) autophagy proteins (phosphorylation of these autophagy proteins represses them) [82]. TOR also regulates endocytosis to promote growth and repress catabolism [83]. Besides regulation by the insulin pathway, TOR-C1 is also regulated via a nutrient sensing signaling pathway, specifically via the activity of the amino acid transporter *Slimfast* [84]. *dS6K* promotes ILP2 expression in the IPCs [21]. ILP secretion by IPCs is controlled by nutritional status, and this nutritional status is conveyed to IPCs by fat body cells, which secrete the Unpaired2 cytokine in fed conditions, which regulates GABAergic neurons in the brain, releasing the GABAergic tonic inhibition they exert on the IPCs, leading to ILP secretion [85, 86].

The other Akt/PKB well-studied target is FoxO. FoxO is a transcription factor (a family in mammals) that favors catabolism, counteracts anabolism, and is phosphorylated by Akt/PKB to repress its activity [22, 87]. Activation of insulin

signaling leads to acute translocation of FoxO proteins out of the nucleus and attenuation of their transcriptional program [88, 89]. In vertebrates, the Forkhead box O (FoxO) family consists of FoxO1, FoxO3, FoxO4, and FoxO6 proteins; a distinct gene encodes each one. *Drosophila* has only one such gene [90, 91]. FoxO proteins bind to the insulin response element (IRE) to stimulate target gene expression on diverse pathways including cell metabolism, proliferation, differentiation, oxidative stress, cell survival, senescence, autophagy, and aging, counteracting insulin action [92].

FoxO repression via insulin signaling activity results in an attenuation of FoxO-dependent expression of genes like those coding for glucose 6-phosphatase or phosphoenolpyruvate carboxykinase [93, 94]. Among other genes, FoxO-regulated antioxidants include the Mn-dependent superoxide dismutase [95]. Signaling pathways that regulate stress and redox status also regulate FoxO proteins, thus, impinge on insulin signaling and the diabetic state: p38, AMP-activated protein kinase (AMPK), among others. The NAD-dependent protein deacetylase sirtuin-1 (Sirt1) directly modifies FoxO transcription factors and promotes their nuclear translocation and activation of target genes [96]. In addition, the acetylation state of histones and the FoxO coactivator PGC-1α (peroxisome proliferator-activated receptor γ-coactivator-1α) may modify the effect of a stimulus on FoxO-induced gene transcription [97]. In conclusion, the FoxO genes transcription is regulated by a variety of physiological cues and pathological stress stimuli frequently associated with increased oxidative stress.

1.2. Oxidative Stress and Insulin Signaling. Oxidative stress is considered a key factor in the development and progression of diabetes and its complications [98]. In vertebrates, Sestrins 1–3 (Sesns) form a family of conserved stress-responsive proteins [99]. The Sesns regulate the insulin pathway by regulating the AMP kinase and TOR [100, 101]. Sesn1 was identified as the product of a gene (PA26) activated by the transcription factor p53 in cells exposed to genotoxic stress. Later, it was isolated also as a FoxO responsive gene in growth factor stimulated cells [102]. Sesn 2 promotes the degradation of Kelch-like protein 1 (Keap1) leading to upregulation of Nrf2 signaling and the induction of genes for antioxidant enzymes. The adaptor protein p62 is required for the Sesn 2-dependent activation of Nrf2 [103]. Sesns block TOR-C1 activation and thereby reduce reactive oxygen species accumulation [104]. In *Drosophila*, a single Sestrin homolog has been isolated and characterized. It is activated by accumulation of reactive oxygen species and regulates insulin signaling. Mutant flies suffer from metabolic disarray, muscle wasting, and mitochondrial dysfunction [105]. The *Drosophila* Sestrin acts through two GATOR protein complexes. These GATOR complexes regulate the activity of the RagB GTPase, necessary for TOR-C1 activity. The *Drosophila* Sestrin binds to GATOR2. Bound to Sestrin, GATOR2 frees GATOR1. Free GATOR1 inhibits RagB function by activating its GTPase activity, thus, inhibiting TOR-C1 activation [106, 107]. In vertebrates, the GATOR complexes act in the same fashion; GATOR complexes are evolutionarily conserved in metazoans [108].

Reduction of energy levels in the cells causes the activation of the AMP-activated protein kinase, AMP kinase. This, in its turn, results in TSC2 phosphorylation and subsequent TOR-C1 inhibition. A hypoxic state also reduces TOR activity via the hypoxia-inducible factor-1 (Hif-1) that affects the hypoxia-induced response genes *Redd1* [109] and *Scylla* [110]. *Scylla* forms a complex with *charybdis*, negatively regulating TOR-C1 acting downstream of Akt/PKB and upstream of TSC [110].

2. Experimental Animal Models: Vertebrates

Type 1 diabetes is characterized by progressive β-cell destruction. Insulin resistance in target tissues characterizes type 2 diabetes. The majority of obese individuals do not become diabetic, although over weight or obesity are clear risk factors for diabetes. In the United States, 87.5% of adults over 18 years old were overweight (including obese and morbidly obese individuals), and an estimated 12.2% of the population is diabetic in 2017 [111], suggesting that β-pancreatic cells failure is required to cause hyperglycemia [112]. Due to its overall evolutionary conservation, animal models are used to identify mechanisms, principles, and potential drug targets, besides elucidating general underpinnings of biological metabolic significance. Many animal models of diabetes are currently available for elucidating the pathophysiology of diabetes and testing novel therapies for complications. However, since diabetes etiology is multifactorial, no single animal model may exactly replicate the human situation. Several of these animal models can be used to study chronic diabetes phenotypes.

In principle, all of the models reviewed below could be used for chronic aspects of diabetes and the accruement and evolution of the diabetic state. In spite of this opportunity, in most cases experiments are begun when the diabetic model organisms have advanced to a frank diabetic state (for example, when the resting glucose level is above 250–300 mg/dl several days/weeks after streptozotocin (STZ) injection in rats; see below). It is desirable to study the initial states, starting when the STZ injection is given, and studying the acquirement of the diabetic state, as well as its ulterior evolution. The models reviewed here could well serve or have served this purpose.

2.1. Chemical Induction of Diabetes. Alloxan and STZ treatments are the most used diabetes models for diabetic complications in vertebrates. Both chemicals are toxic glucose analogues transported into the cells via the Glut 2 transporter [113]. Both treatments lead to necrosis, importantly of insulin-producing cells, but by different mechanisms. Alloxan generates toxic free radicals, leading to cell death via necrosis. STZ is cleaved, generating free methylnitrosourea that induces DNA fragmentation and necrotic cell decay [114]. Although STZ may also have toxic effects on other organs, its effectiveness and side effects depend mainly on tissue-specific Glut 2 expression, animal age, and nutritional status [114]. STZ administration to 0–2-day-old rats induces an inadequate beta cell mass used as a type 2 diabetes model [115].

A variety of mammals, rodents, rabbits, dogs, pigs, and nonhuman primates, have been used as models of STZ- and alloxan-induced diabetes. The small size of rodents and rabbits results advantageous for maintenance costs, especially in longitudinal studies, but somewhat limits sample material available per animal. In recent years, the pig has gained importance because of its size and close similarity to human physiology. Minipigs have clear advantages over domestic pigs, and genetic modifications leading to diabetic phenotypes have been developed [116].

2.2. Genetic Vertebrate Models of Diabetes. Yet to date, rodents represent the predominant vertebrate species used in biomedical research because of traditional use and accumulated knowledge, known animal husbandry, evolutionarily conserved metabolic pathways, the capacity to conduct experiments in organs and study physiology, and, more recently, genetic manipulation possibilities. Among them, several genetic mutant strains are extensively used, depending on the diabetic aspect under study.

The Akita mice have an Ins2+/C96Y mutation, a single nucleotide substitution in the insulin 2 gene (Ins2). This mutation causes reduced insulin secretion, resulting in the development of type 1 diabetes [117]. The db/db mouse is the most popular model of type 2 diabetes. They have a deletion mutation in the leptin receptor resulting in defective receptor function for the adipocyte-derived hormone leptin. This mutation leads to the developing of obesity, insulin resistance, and diabetes [118]. Other mutants with altered metabolism such as the agouti (Ay) mouse, a polygenic model of obesity-induced diabetes, and the ApoE −/− (apoliporotein E deficient) mouse, an atherosclerosis model, are available [119–122].

The Zucker fatty (ZF) rat ports a homozygous missense mutation (fatty, fa) in the leptin receptor gene and develops obesity without diabetes, although rats develop progressive insulin resistance and glucose intolerance [123]. The Wistar fatty (WF) rat is a congenic strain of the Wistar Kyoto rat that also has a fa/fa homozygous missense mutation in the leptin receptor gene. This strain develops obesity [124, 125]. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a recognized model of type 2 diabetes. Rats show impaired glucose tolerance, observed from 8 weeks of age, hyperglycemia, and peripheral insulin resistance [126, 127]. The Goto-Kakizaki (GK) rat is a model of nonobese type 2 diabetes. This is a Wistar substrain that develops mild hyperglycemia, insulin resistance, and hiperinsulinemia [128–131]. The ZDF-Lepr *fa*/Crl rat was originated in a colony of Zucker rats, expressing type 2 diabetes, among other models [132–134].

In addition, there are also a variety of different polygenic models of obesity that include the KK-AY mice [135], New Zealand obese (NZO) mice [136], the TALLYHO/Jng mice [137], and the OLETF rats [127], besides diet-induced models of obesity [30]. These models also lead to diabetic states.

3. Invertebrate Insulin Signaling

Besides vertebrate diabetes models, two main invertebrate models have been used in experiments. These two invertebrate models are the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*.

3.1. Caenorhabditis elegans. In the nematode *Caenorhabditis elegans* some of the insulin pathway main components were first characterized, like the nematode homolog, *age-1*. In *C. elegans*, faulty insulin signaling leads to life extension, metabolism changes, disrupted growth, and stress resilience, reminiscent of some diabetic phenotypes [138, 139].

In this nematode the insulin pathway genes were discovered by virtue of their control of dauer larva formation and longevity, evidencing a relationship between aging/nutrition/lifespan [140]. Dauer larvae are formed between larval stages two and three and represent an alternative third larval stage that can survive harsh environmental conditions for up to four months. The pivotal genes in the *C. elegans* insulin pathway are evolutionarily conserved. The insulin receptor homolog is *daf-2* (from *abnormal dauer formation*), the IRS homolog is *ist-1* [141], the PI3K catalytic subunit is *age-1* (from *aging alteration*), and the regulatory subunit is *aap-1* [141], PTEN is *daf-18*, Akt is Akt-1 and Akt-2, and FoxO is *daf-16* [142]. Similar to the case in *Drosophila*, the insulin pathway is unique and required for many functions including nutritional assessment and metabolism, growth, lifecycle, longevity, and behavior. The study of dauer larvae formation in *C. elegans* has already yielded insights into metabolic/nutritional control and longevity with relevance to humans [143]. There have also been studies regarding behavior modifications, degenerative diseases, and the roles played by FoxO transcription factors in the worm and humans [144]. Learning, memory, and organismal growth are also other chronic conditions where research in *C. elegans* insulin pathway has pinpointed general functions [142].

3.2. Drosophila and Diabetes. *D. melanogaster* insulin signaling has been evolutionarily conserved, and both types 1 and 2 diabetes can be modeled. Reducing or nearly abrogating expression of the insulin-like peptides (ILP) in the fly can achieve type 1 diabetes [23]. On the other hand, several manipulations can lead to diabetes type 2: mutations in the insulin pathway components downstream from the ILPs [27, 45], dietary manipulations leading to obesity, metabolic imbalance, and hyperglycemia [145–148], or studies in other *Drosophila* species with different lifestyles/diets [149, 150]. As these different experimental protocols, applied in genetically homogeneous fly populations converge, essentially, in a reproducibly diabetic state and faulty insulin signaling, they can all be used for longitudinal studies characterizing the accruement and evolution of compromised diabetic signaling, diabetic phenotypes, and their consequences.

Drosophila is uniquely poised to study the insulin pathway and diabetes chronic aspects: it has a very well-developed genetic toolkit simply not available, or not as easily amenable, and with higher genetic background homogeneity and rigor as other models, a very highly polished sequenced genome,

a "simplified" insulin pathway, with components exhibiting far less redundancy than, for example, vertebrate models, and the availability of different species with similar sequenced genomes that represent "natural" experiments with different lifestyles and diets, among other advantages. It is particularly of note the capacity to generate different types of genetic mosaics in the whole organism, allowing study and analysis of the cell, tissue, and organismal consequences, and cell independence of mutations, and the localization of functional "foci." Another related advantage is the possibility of generating space and time limited genetic mosaics that can be used to distinguish between developmental defects versus metabolic defects, for example.

3.2.1. Different Drosophila Species Lead to Different Lifestyles. Most of the well-known *Drosophila* species are saprophytic [151]. Inside this big genus (over 2,000 species described so far), there are both generalist and specialist ones, with omnivorous or restricted diets. The environmental conditions that each population faces, together with the availability of nutrients, altitude, latitude, temperature, and so on, can impinge on differences and adaptations that have effects, whether direct or indirect, on insulin signaling. It can affect the levels of activity and, in general, the lifestyle of populations. There are studies examining the effect of varying diets in different *Drosophila* species, whether or not they support life of the organisms in a long-term basis [149, 150, 152]. Some of these changes may or may not have to do with adaptations involving the insulin pathway [153, 154]. In any case, the fact that the genomes plus many other ecological and genomic variables are already known [155–157] implies a great advantage for insulin pathway studies of these ecologically diverse species [158]. This avenue of research represents a window of opportunity as there are more and more *Drosophila* species characterized that can be cultivated in the laboratory, with their genomes sequenced, available for study [159].

Other examples of studies with different *Drosophila* species include *D. simulans*, where metabolic rate, longevity, and resistance to stress have been studied [160, 161]. *D. sechellia*, found only in the Seychelles archipelago and requiring the fruit *Morinda citrifolia* as specialized and niche nutrition, toxic for other species, has been thoroughly researched [162, 163]. A recent adaptation of a population of *D. yakuba* to the same nutritional resource as *D. sechellia* in an island population (as opposed to conspecific populations in the continent), namely, Morinda fruit, is striking. This represents a particularly interesting case of a recent adaptation to a major diet shift [164]. *D. mojavensis* requires cacti as a feeding resource and has even specialized to different host cacti in different populations [165]. Together, they may allow dissection of the mechanisms behind the differences and preferences for specific nutrients, oviposition sites, and their tolerance and metabolism. For example, *D. mojavensis* shows a better resistance to the presence of alcohol, a product of the fermentation of cacti [166]. It will be interesting to study in these examples the changes, if any, in the insulin pathway due to their specialized and restricted nutritional resources, and how might a diabetic state alter their metabolism.

3.2.2. Drosophila melanogaster and Insulin Signaling. In *D. melanogaster*, the growth of the organism is regulated by insulin signaling and the interaction of this signal with the levels of juvenile hormone and ecdysone. Additionally, there is the role played by the kinase TOR of the insulin pathway, which, as stated above, couples growth with the amount of available nutrients [167], at least in part via the amino acid transporter Slimfast [84]. Since the fruit fly is a poikilothermic organism, it is affected by ambient temperature in a direct way, presenting a larger size at lower temperatures, and an increase in size with latitude and altitude [168]. The number of ovarioles in females is also susceptible to these factors, being lower in tropical populations and it has been shown that insulin signaling activity underlies these differences [169, 170].

Besides growth hormones, diet, and temperature, gut microbiota can modulate insulin pathway activity. Between populations and lines there are differences in the microbiome, and this influences insulin signaling [171, 172]. Strains infected with the *Wolbachia* endosymbiont exhibit increased insulin signaling, whereas lack of *Wolbachia* worsens insulin mutants phenotypes, particularly the decline in fecundity and adult weight [173]. Loss of ILPs in the brain, on the other hand, extends lifespan if *Wolbachia* is present [24]. *Lactobacillus* partially rescues growth in poorly fed larvae and *Acetobacter pomorum* also modulates insulin signaling [174, 175]. All of these factors have to be taken into consideration, ideally, when longitudinal studies are performed, since many of these factors may vary with age, independent of the status of the fly.

Longevity has also been tied at times with insulin signaling [176–178]. Experimental model organism lines that were selected because of their increased lifespan, often present changes in the insulin pathway. Hoffman et al. [179] performed WGS and GWAS studies on long-lived *Drosophila* strains and found that the metabolites that decline with age are associated with glycolysis and the metabolism of glycophospholipids. Changes were observed associated with age and sex in biogenic amines, and carnitines, required for the transfer of fatty acids in the mitochondria where they pass through beta-oxidation generating acetyl-coA required for the Krebs cycle.

3.2.3. Inducing the Diabetic State through Diet. Providing *Drosophila* diets with increased or decreased nutrients provokes the deregulation of its metabolism and of insulin signaling. High-sugar as well as high-protein diets increase insulin-like peptide expression (ILPs) [148, 180]; this initial increase in ILP expression is consistent with what is observed in vertebrates in the accruement of insulin resistance, where the organism initially tries to increase its insulin production to compensate for excess nutrient input. However, in vertebrates, the eventual deterioration of beta cells leads to ultimate failure of this initial compensation [181]. Similarly, in overfed flies, the fat body secondarily reduces its insulin response to increased circulating ILPs, and this diminution decreases significantly as flies age, rendering flies completely resistant at advanced ages [147]. These results support the observation that a diet rich in fat initially increases levels of

different ILPs, rescuing at a first stage the overfed phenotype by means of hyperinsulinemia. This increase in insulin signaling, plus hyperglycemia, though, leads to an increase in free fatty acids by inappropriate lipolysis and the generation of insulin resistance, particularly in the fat body. High fat diets also contribute to heart dysfunction [145, 182].

Sugar, lipid, and protein variation in diets have led to effects in fertility, longevity, sugar and fat accumulation, weight changes, induction of insulin resistance, and aging [147, 183]. In general, the results are consistent with protein and carbohydrate balance determining lifespan. In some cases diets high in carbohydrates and low in proteins allow greater longevity, often accompanied by lower fecundity. In other studies, extra protein intake results in lean and longer lived flies, while carbohydrate intake leads to obese flies; finally, balanced, intermediate carbohydrate:protein ratios diets have also been found to lead to longer-lived flies [183–187].

Dietary restriction has been variously applied to flies, often leading to longer life-spans and insulin signaling involvement [188–190]. Dietary restriction effects are also seen in immunity via insulin signaling regulation [191]. The insulin signaling is clearly part of the equation in all these studies, although it may not be the sole determinant [192, 193]. One such type of dietary restriction is caloric restriction. Caloric restriction is defined as a reduction in caloric intake without malnutrition and has shown in several models a positive effect on calorie consumption, including yeast and *C. elegans* besides *D. melanogaster* [194–196]. Another variation of diet manipulation with nutritional and lifespan consequences is methionine availability in the diet [197]. Also, parental obesity leads to transgenerational effects [198].

Clearly, diet manipulation can be used to generate and evolve diabetic states akin to diabetes type 2 in flies, and by its very nature, it is easily amenable for longitudinal studies. Yet an important problem besieging all these studies, and one that may explain conflicting results, is that all these diet regimes are semidefined chemically, at best, so that studies that modify diets are very difficult to compare. They typically define "protein" as amount of yeast in the medium, or "carbohydrate" as unrefined sugar or molasses, for example, which are clearly broad generalizations, as yeast cells have carbohydrates, lipids, and other nutrients besides proteins, and unrefined sugar or molasses are not only composed of carbohydrates. In the future, diets should strive to be defined chemically, so that they can be comparable and used reproducibly by different laboratories. In addition, total consumption should be measured, since unconstrained flies have free access to their food source and are able to regulate their caloric intake, at least in the case of *D. melanogaster* [183]. Also, these studies are subject to environmental variations, the use of different sexes, different genetic backgrounds, different strains, different age of flies, and so on, all of which affect the outcome of the studies. And while these studies show that changes in diet have clear effects on the body and insulin signaling, lack of definition constitutes a limiting factor in these experimental approaches.

3.2.4. Diabetes in Flies by Virtue of Mutations in the Insulin Pathway. Flies homozygous mutant for genes in the insulin pathway are born diabetic. There are advantages to this approach: the nature of the defect is known, and the genetic background and environmental conditions can be controlled in a rigorous manner. The different stages of the lifecycle can also be exploited, with stages where feeding occurs (larvae, adults), and stages without food input (pupae). Faulty insulin signaling by virtue of mutations can both be used to model diabetes type 1 (ILPs loss-of-function mutations [23], and even explore genes regulating insulin secretion [199]), and also diabetes type 2, with loss-of-function mutations in the rest of the pathway, as the net effect would be insulin resistance [27, 31, 71]. Furthermore, both whole organisms can be mutant (in hypomorphic conditions, as null alleles are mostly lethal), or only in selected tissues and organs ([18, 45, 48, 77, 91, 200, 201], among other references cited throughout this review).

Drosophila has seven insulin-like peptides (ILPs). They are partially redundant, so knock-outs for a particular ILP usually have moderate effects, and it is necessary to have more than one ILP gene loss-of-function mutation to generate lethality [23, 24]. In contrast, mutations in *InR, Dp110,* and other components of the pathway are homozygous lethal, so heterozygous (as there are some dominant effects [45, 202]) or heteroallelic flies are often used. The latter have the benefit of allocating more robustly the defects observed to the studied mutation (for example, see [31]). *chico* (the insulin receptor substrate fly homolog) is a particular case. The homozygous mutant $chico^1$ allele was originally described as viable, helping establish the typical mutant phenotypes of partial loss-of-function of insulin pathway mutants [45]. Later it was found out that, when devoid of the endosymbiont *Wolbachia*, *chico* loss-of-function conditions are homozygous lethal, underscoring the close association between gut microbiota and insulin signaling [173].

The most common phenotypes caused by mutants in the insulin pathway are a decrease in fertility, decreased size of organisms, changes in longevity (decrease in normal conditions, often an increase in longevity when there is caloric restriction), defects in fat body morphology, in heart, retina, and brain physiology, increased levels of triacylglycerides, and higher amounts of circulating sugars in the hemolymph [31, 45, 71, 145, 147, 203–205].

Insulin/TOR pathway function is critical in the regulation of growth, autophagy, cell and organism survival, and anabolism (regulating lipid and carbohydrate homeostasis) [22]. Lack of nutrients or ATP impedes its function, and overfeeding can lead to loss of balance: insulin participates in the accumulation of lipids and carbohydrates, so that an excessive intake of nutrients can lead to hyperactivation of the pathway, and lipid and glycogen accumulation. TOR kinase is regulated by insulin signaling and by amino acids, acting as a central point in metabolism regulation. It has also been implicated in aging [206–208].

Besides nutritional input, there are other conditions that regulate insulin signaling: as an example, one such state is the systemic response to stress (which, of course, is activated by

lack of nutrition, among other stimuli). One such stress trigger is infection, and innate immunity activation. Activation of Toll in the fat body leads to the induction of immunity, redistribution of resources, and activation of JNK and NFK-β by inflammation. Here, attenuation of insulin signaling leads to FoxO activity, which regulates genes participating in stress response and metabolic control: blockade of gluconeogenesis, glycogenolysis, and the use of storage lipids for catabolism [209]. It may also increase longevity if FoxO is upregulated in adipose or intestinal tissue [210]. In addition, chronic intestinal activation of FoxO may lead to deregulation of lipid homeostasis [211].

3.2.5. Towards the Characterization of Chronic Diabetes in Flies. What can be studied in these fly diabetes models in a longitudinal study? Nearly every aspect of diabetes mellitus mentioned so far: from initial phenotypes, to its evolution and consequences at old age, up to death. We have discussed above metabolic imbalances and longevity as two of the most studied effects [212, 213]. Other mutant phenotypes include decreased fertility and altered physiology of various organs and systems (nervous system, heart, fat tissue, muscles, etc.), for example, showing involvement of nervous system function: electrical activity or octopamine neurotransmission [214, 215] or heart dysfunction [216].

Perturbations like chronic stress can be addressed, and the effects of external factors on the diabetic state, such as nutritional variation, light regime, and temperature, can also be addressed, for example, the effect of artificial sweeteners upon insulin pathway signaling [217] or the effect of xenobiotics on the insulin pathway [218]. Fundamentally, though, the inception and "normal" progression of the mutant condition or the diseased state can be closely followed, for example, the relationship between sleep and metabolism alterations via insulin signaling [219–221]. Unfortunately, to this day few studies have consciously addressed these chronic aspects of diabetes, although some do compare flies at different stages, like during oogenesis [222], or pupariation [223], or neurite remodeling [224], or adult stages [225].

Most of the studies to date that touch on longitudinal aspects address longevity, fertility [226], and its phenocritical period [23, 202, 227, 228], like the earlier appearance of locomotor defects [229], size and growth phenotypes [45, 200, 201, 230], and metabolism [71]. In summary, the *Drosophila* model represents a window of opportunity not only to study fundamental aspects of diabetes and the diabetic state, but also its complications, and the effect of various external stimuli and factors in the accruement and development of the disease. Future studies will, undoubtedly, target and address these issues to a much greater extent.

4. Conclusions

Notwithstanding the ubiquity and utility of vertebrate models of diabetes (especially rodent models), and when compared with other experimental models of diabetes, *Drosophila* has clear advantages. Despite sometimes ill-defined parameters in diet regimes and environment, the model strengths, namely, a robust, extensive, and highly developed genetic system, with a plethora of isolated and characterized insulin pathway and general metabolism genes, ease of manipulation and use, low cost, high fertility, and numbers, short generation time, high evolutionary conservation, and homogeneous genetic backgrounds, among other positive characteristics, make the fly a premier system for insulin pathway and metabolic studies.

Although many aspects of diabetes mellitus have been studied in the different fly models specially, there is still a dearth of longitudinal studies. In such studies, ideally exception should be taken of the differences that occur in metabolism and lifestyle normally as flies age; that is to say, these studies should always par appropriate control flies with experimental ones throughout the life cycle with the same genetic background, to effectively tease away differences due to the occurrence and evolution of the diabetic state from normal aging. This is especially true of a disease that touches many different aspects of the organisms' wellbeing, and one that by its very nature is very pleiotropic and polygenic.

Notwithstanding this, the fruit fly represents one of the more promising, rigorous, and thoroughly researched models of diabetes in which we carry out such research. Due to great evolutionary conservation, it allows for particularly detailed and controlled studies covering nearly all aspects of this chronic and fatal disease. Compared to other available models, that is, vertebrate studies, be it organismal or even cell tissue culture ones, the fly favorably compares, allowing for more holistic and encompassing approaches.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Review Article

Drosophila melanogaster Models of Friedreich's Ataxia

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Friedreich's ataxia (FRDA) is a rare inherited recessive disorder affecting the central and peripheral nervous systems and other extraneural organs such as the heart and pancreas. This incapacitating condition usually manifests in childhood or adolescence, exhibits an irreversible progression that confines the patient to a wheelchair, and leads to early death. FRDA is caused by a reduced level of the nuclear-encoded mitochondrial protein frataxin due to an abnormal GAA triplet repeat expansion in the first intron of the human *FXN* gene. *FXN* is evolutionarily conserved, with orthologs in essentially all eukaryotes and some prokaryotes, leading to the development of experimental models of this disease in different organisms. These FRDA models have contributed substantially to our current knowledge of frataxin function and the pathogenesis of the disease, as well as to explorations of suitable treatments. *Drosophila melanogaster*, an organism that is easy to manipulate genetically, has also become important in FRDA research. This review describes the substantial contribution of *Drosophila* to FRDA research since the characterization of the fly frataxin ortholog more than 15 years ago. Fly models have provided a comprehensive characterization of the defects associated with frataxin deficiency and have revealed genetic modifiers of disease phenotypes. In addition, these models are now being used in the search for potential therapeutic compounds for the treatment of this severe and still incurable disease.

1. Introduction

Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disorder and the most common form of hereditary ataxia among populations of European origin (2–4/100,000) [1]. This disabling condition typically manifests before age 25, with progressive neurodegeneration of the dorsal root ganglia, sensory peripheral nerves, corticospinal tracts, and dentate nuclei of the cerebellum. A large proportion of patients develop hypertrophic cardiomyopathy, which is the major cause of reduced life expectancy in this disease. Diabetes mellitus and impaired glucose tolerance are also seen in a significant number of FRDA patients (reviewed in [2]).

FRDA is caused by loss-of-function mutations in the *FXN* gene, which encodes the frataxin protein [3]. Frataxin is a small protein encoded in the nucleus, expressed as a precursor polypeptide in the cytoplasm and imported into mitochondria [4–6]. The majority of FRDA patients are homozygous for an abnormally expanded GAA repeat in intron 1 of *FXN*, resulting in strongly reduced frataxin protein expression (from 5% to 30% of the normal level) [7]. The remaining FRDA patients are compound heterozygotes, carrying the GAA repeat expansion on one *FXN* allele and another pathogenic mutation on the other allele, including point mutations and insertion and/or deletion mutations [8].

A lack of available patients and the inherent limitations of cellular models often hinder the discovery and detailed analyses of genes and pathways relevant to the pathology of rare human disorders such as FRDA. Fortunately, the high evolutionary conservation of frataxin (Figure 1) has enabled the development of disease models in several organisms, from bacteria to mice, that have significantly contributed to the

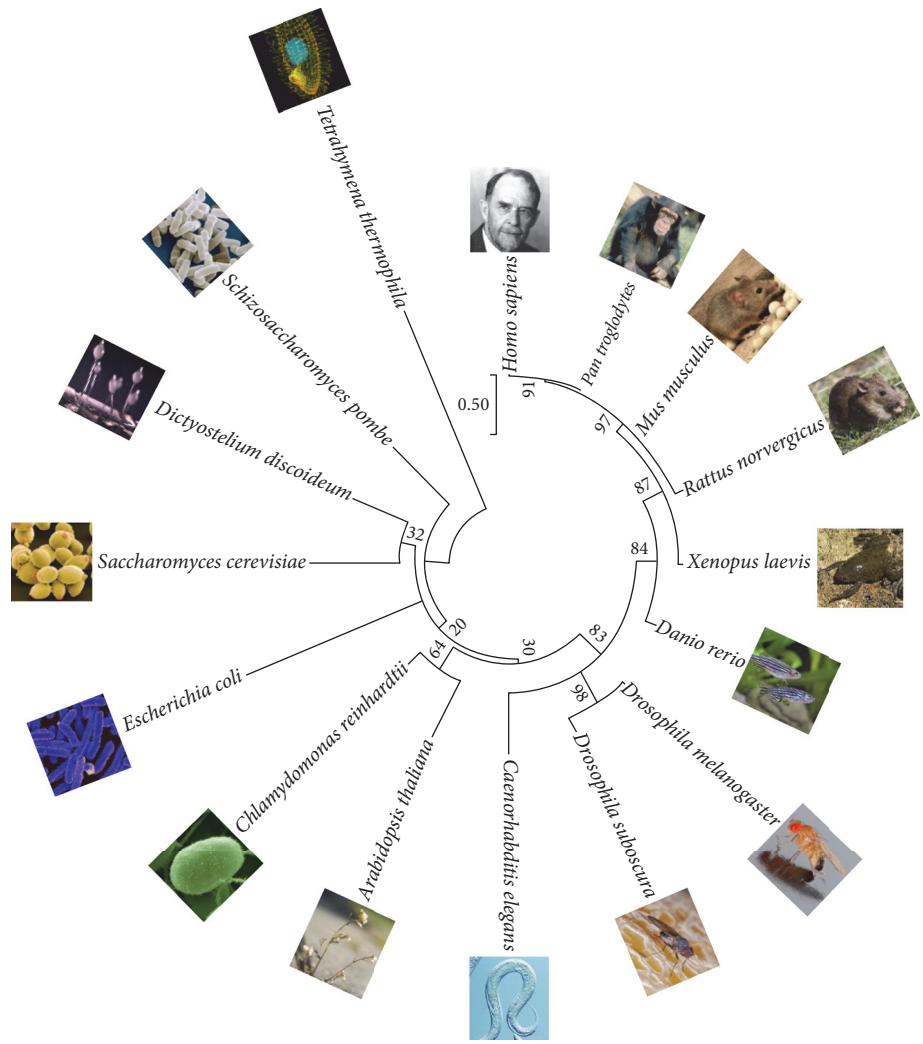


Figure 1: Molecular phylogenetic analysis of frataxin sequences from different species. The picture of Thomas Hunt Morgan was chosen to represent *Homo sapiens* because, as a result of his work, *D. melanogaster* became a major model organism in genetics. Methods: evolutionary history was inferred with the maximum likelihood method based on Le and Gascuel model [9]. The tree with the highest log likelihood (−2026.7976) is shown. Initial trees for the heuristic search were obtained automatically by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with the superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 2.4842)). The tree is drawn to scale, with branch lengths representing the number of substitutions per site. The analysis involved 16 amino acid sequences. All positions containing gaps and missing data were eliminated. A total of 90 positions were present in the final dataset. Evolutionary analyses were conducted in MEGA7 [10].

understanding of frataxin function. The development of these disease models is an essential step in elucidating underlying pathological mechanisms and identifying efficient treatments in FRDA.

Seminal findings reported by key studies in model organisms (reviewed in [14–23]) have suggested potential roles for frataxin in iron homeostasis and cellular defense against reactive oxygen species (ROS), as an activator of the mitochondrial respiratory chain, as a mitochondrial chaperone, and as a regulator of Fe-S cluster (ISC) assembly. Although frataxin function is not yet fully characterized, its role in ISC biogenesis is generally accepted [24–26]. Major alterations associated with frataxin deficiency include mitochondrial iron accumulation, oxidative stress hypersensitivity, impaired ISC biogenesis, and aconitase and respiratory chain dysfunction (reviewed in [27–29]).

Although the arthropod lineage diverged from the vertebrate lineage more than 600 MYA, genome sequencing projects have revealed a large number of biological processes that are conserved between flies and vertebrates. Most of the genes implicated in familial forms of disease have at least one *Drosophila* ortholog [30, 31]. This species offers many different genetic tools that can be applied to investigate basic biological questions in a multicellular organism, with the advantages of easy manipulation and culture.

2. The *Drosophila* Ortholog of the *FXN* Gene

The *D. melanogaster* frataxin ortholog was cloned and characterized in our laboratory in the early 2000s. It was named *dfh (Drosophila frataxin homolog)* [32]. This gene is referred to as *fh (frataxin homolog)* in FlyBase (CG8971, FBgn0030092), and this name will be used throughout this review. We isolated *fh* by screening a genomic library from *D. subobscura* using human *FXN* probes. Database searches employing the sequence of *D. subobscura* positive clones led to the identification of the *D. melanogaster* STS 125a12, mapped to the 8CD region on the X chromosome and cloned in cosmid 125a12. Further characterization of this cosmid showed an open reading frame (ORF) encoding a frataxin-like protein. Screening of an adult cDNA library from *D. melanogaster,* using the genomic frataxin ORF, revealed two transcripts with two different polyadenylation signals. We confirmed that this gene is located in the 8CD region by in situ hybridization analysis of polytene chromosomes of *D. melanogaster* using *fh* cDNA as a probe.

The genomic organization of *fh* is much simpler than that of the human gene (Figure 2(a)) [32]. *fh* is approximately 1 kb and is composed of two exons of 340 bp and 282 bp, separated by an intron of 69 bp. RNA in situ hybridization in whole embryos showed ubiquitous expression of *fh* in all developmental stages examined (from 2 to 16 h). ~1 kb major transcript was identified by Northern blot analysis, in agreement with the predicted size of one of the two mRNA sequences detected by cDNA library screening. This transcript was found in embryonic, larval, pupal, and adult stages [32]. Accordingly, the protein was present in all developmental stages at varying levels, reaching its highest level in late embryos [33].

The encoded fly protein was predicted to have 190 amino acids, with a molecular weight of ~21 kDa. A sequence comparison of frataxin proteins from different species showed better alignment in the central and the C-terminal regions (Figure 2(b)), whereas no alignment was found in the N-terminal region of the protein. Importantly, this region of fly frataxin (FH) also showed typical frataxin features, such as a mitochondrial signal peptide and a putative α-helix with abundant positively charged amino acids and few negatively charged residues [32]. Colocalization experiments using an FH-enhanced green fluorescent fusion protein (EGFP) and a mitochondrial marker confirmed the localization of FH in mitochondria [34]. The mature form of FH has a molecular weight of ~15 kDa [33]. The secondary structure of FH matches the α-β sandwich motif characteristic of other frataxin proteins encoded by orthologous genes [32]. Predictions of the 3D structure generated using the Phyre 2 [11] and Chimera 1.12 [12] software show that FH has an organization similar to that of the human protein (Figure 2(c)). The biophysical properties of FH indicate that its thermal and chemical stabilities closely resemble those of human frataxin [35]. Unlike other eukaryotic frataxin proteins, FH shows enhanced stability *in vitro*, making it a more attractive candidate for evaluation of metal binding and delivery properties. In these experimental conditions, FH can bind and deliver Fe(II), which is required for ISC biosynthesis [35], and, as previously described for human frataxin [36], it interacts with Isu (the Fe cofactor assembly platform for ISC cellular production) in an iron-dependent manner [35]. Recently, some authors have provided experimental evidence that the initial complex of the mitochondrial ISC biosynthetic machinery is conserved in *Drosophila* [37, 38]. These results, along with those reported in mouse (reviewed in [39]), suggest an evolutionarily conserved role for frataxin in ISC biosynthesis.

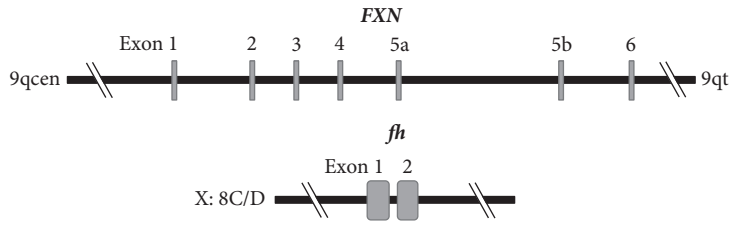
3. Modeling FRDA in Flies

Several models of FRDA have been developed in *D. melanogaster*, mainly taking advantage of GAL4/UAS transgene-based RNA interference (RNAi) methodology. RNAi allows the posttranscriptional silencing of a gene via the expression of transgenic double-stranded RNAs [40]. The GAL4/UAS system [13] has been incredibly successful in *D. melanogaster* and can induce the expression of a transgene under the control of UAS (Upstream Activating Sequences) and the transcriptional activator protein GAL4 (Figure 3). This experimental strategy has been used to induce tissue-specific and ubiquitous knockdown of *fh* (Table 1). Therefore, this strategy allows the phenotypes of FRDA patients to be mimicked by reducing rather than completely eliminating FH.

The first UAS-transgene construct for RNAi-mediated silencing of *fh* expression was reported by Anderson et al. [33]. This construct consisted of inverted repeats containing the first 391 nucleotides of the *fh* coding region, which were subcloned into the pUAST vector. Fly transformants were crossed to the da^{G32} GAL4-driver line (which exhibits widespread GAL4 protein expression throughout development and in most tissues under the control of regulatory sequences of *daughterless*) to examine *fh* silencing. Three transgenic lines (UDIR1, UDIR2, and UDIR3) were selected in which the GAL4-regulated transgene substantially reduced the FH protein level [33, 41]. Similarly, Llorens et al. [34] generated another UAS-transgene construct (named UAS-*fh*IR) containing two copies of the *fh* coding region in opposite orientations, separated by a GFP fragment as a spacer. A transgenic line (*fh*RNAi line) was selected showing milder effect than the GAL4-regulated transgene in UDIR1/2/3 when crossed with the da^{G32} GAL4 line (Table 1).

The RNAi lines from John Phillips's laboratory [33] have also been combined with a ligand-inducible GAL4/UAS system to deplete frataxin in the *Drosophila* heart [42]. This system is based on a steroid-activated chimeric GAL4 protein, specifically the GAL4-progesterone-receptor fusion protein that is activated by RU486 (mifepristone) [43, 44]. Transgene expression is induced by supplementing the fly food with RU486, and the level of expression is controlled by changing the dosage of the steroid ligand [43].

More recently, Chen et al. [45] identified the first mutant allele of *fh* (fh^1) in an unbiased genetic screen of the X chromosome designed to isolate mutations that cause neurodegenerative phenotypes. The mutant allele consisted of an ethyl-methanesulfonate-induced missense mutation (S136R) located in a highly conserved region (S157 in the human protein) required for the binding of human frataxin to the



(a)

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Homo sapiens                MWTLGRRAVAGLLASPSPAQAQTLTRVPRPAELAPLCGRRGLRTDIDATCTPRRA
Mus musculus                MWAFGGRAAVGLLPR-TASRASAWVGNPRWREPIVTCGRRGLHVTVNAGAT-RHA
Drosophila melanogaster     -MFAGRLMVRSIVGRACLATMGRWSKPQAHASQVILPSTPAI-AAVAIQCE-EFT
Caenorhabditis elegans      ----------------MLSTIL------------------------------RNN
Saccharomyces cerevisiae    ------------MIKRSLASLVRVSSVMGRRYMIAAAG--GERARFCPAVTNKKN

                                                                  *       * *
H. sapiens                  SSNQRGLNQIWNVKKQSVYLMNLRKSGTLGHPGSLDETTYERLAEETLDSLAEFF
M. musculus                 HLNLHYL-QILNIKKQSVCVVHLRNLGTLDMPSSLDETAYERLAEETLDSLAEFF
D. melanogaster             ANRRLFSSQI-------------------ETESTLDGATYERVCSDTLDALCDYF
C. elegans                  FVRRSFSSRI------------------------FSQNEYETAADSTLERLSDYF
S. cerevisiae               HTVNTFQKRFVESSTDGQVV--------PQEVLNLPLEKYHEEADDYLDHLLDSL

                                  **    ** *       ****** * ******* ** *** *
H. sapiens                  EDLADKPYTFEDYDVSFGSGVLTVKLGGDLGTYVINKQTPNKQIWLSSPSSGPKR
M. musculus                 EDLADKPYTLEDYDVSFGDGVLTIKLGGDLGTYVINKQTPNKQIWLSSPSSGPKR
D. melanogaster             EELTENASELQGTDVAYSDGVLTVNLGGQHGTYVINRQTPNKQIWLSSPTSGPKR
C. elegans                  DQIADSFPVSEQFDVSHAMGVLTVNVSKSVGTYVINKQSPNKQIWLSSPMSGPKR
S. cerevisiae               EELSEAHPDCIP-DVELSHGVMTLEIPAF-GTYVINKQPPNKQIWLASPLSGPNR

                             *         *     * *   *  *
H. sapiens                  YDWTG----KNWVYSHDGVSLHELLAAELTKAL-KTKLDLSSLAYSGKDA
M. musculus                 YDWTG----KNWVYSHDGVSLHELLARELTKAL-NTKLDLSSLAYSGKGT
D. melanogaster             YDFVGTVAAGRWIYKHSGQSLHELLQQEIPGILKSQSVDFLRLPYCS---
C. elegans                  YDLEE---EGKWTYAHDGEQLDSLLNREFRKILADDRIDFSRHV------
S. cerevisiae               FDLLN----GEWVSLRNGTKLTDILTEEVEKAISKSQ-------------
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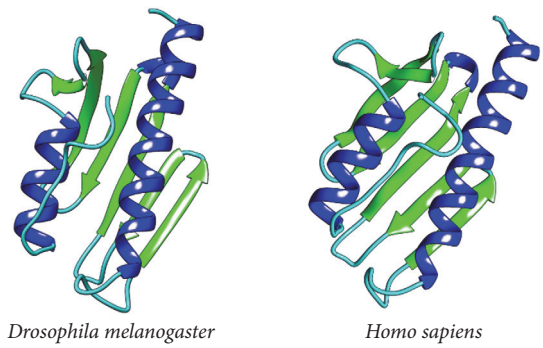
(b)



(c)

Figure 2: The *Drosophila* frataxin ortholog. (a) Genomic organization of the human *(FXN)* and the fly *(fh)* genes encoding frataxin. *FXN* is located in 9q21.11 and contains seven exons. *fh* is located in chromosome X: 8C14 and has two exons. (b) Multiple alignment of the frataxin protein sequences of *Homo sapiens*, *Mus musculus*, *D. melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*. The letters indicate the amino acid in each position, and the colors classify the amino acids according to their biochemical properties, as described in the MEGA7 program [10]. Invariant amino acids are marked with an asterisk. (c) The 3D structure prediction of the frataxin protein using the Phyre 2 [11] and Chimera 1.12 software [12]; α-helixes appear in blue and β-sheets in green.

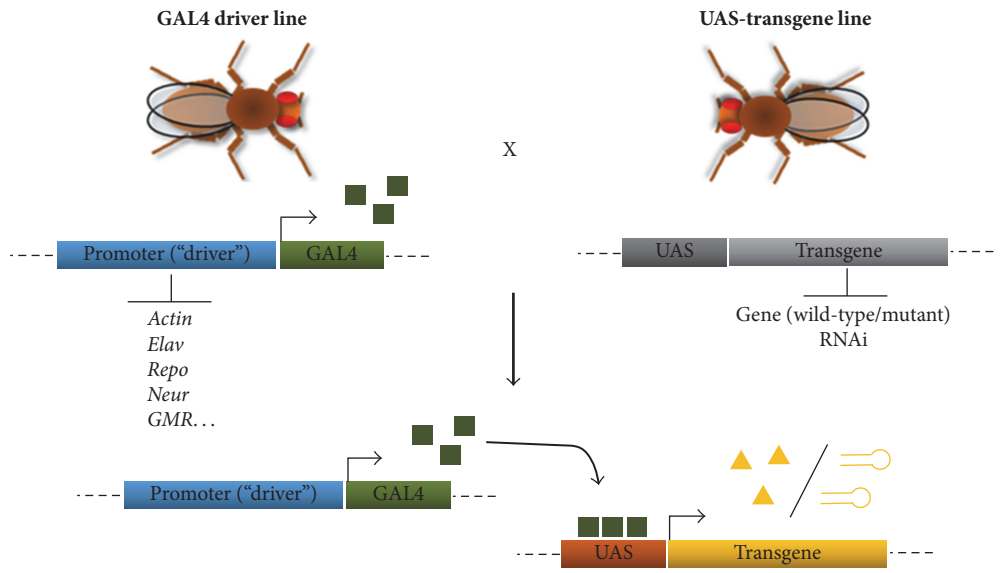


Figure 3: The GAL4/UAS system, adapted from yeast, involves the use of two transgenic lines in *Drosophila* [13]. One line carries the GAL4 transcription factor under the control of a promoter of known expression pattern (the driver line), and the other line contains the transgene of interest downstream of UAS (the responder line). Many GAL4 driver lines are available, carrying the promoters of genes such as *actin* (ubiquitous), *elav* (pan-neuronal), *repo* (glial cells), *neur* (sensory organs), and *GMR* (eye). This system is very versatile and allows the expression of specific genes or gene constructs to be induced or suppressed. Triangles indicate a wild-type or mutant protein; the hairpins represent double-stranded RNA molecules that mediate RNAi.

ISC assembly complex [45, 46]. The authors also generated mosaic *fh* mutant mitotic clones of adult photoreceptor neurons using the eyeless-FLP/FRT system to bypass the lethality associated with the fh^1 mutation [45].

These *Drosophila* models of FRDA have been employed to study frataxin function, analyze conserved pathological mechanisms, and search for genetic modifiers and potential therapies. The main results of such studies are described in the following sections.

4. Phenotypes of Frataxin Deficiency in *Drosophila*

The loss of *fh* function in *Drosophila* recapitulates important biochemical, cellular, and physiological phenotypes of FRDA. In addition, some phenotypes have been described for the first time in this organism, revealing new key players in FRDA pathogenesis. All these phenotypes have been obtained using the *fh* constructs and alleles that were described above. Table 1 details these features as well as the temperature of the crosses when available, because the GAL4/UAS system is sensitive to this parameter.

Near-complete frataxin depletion in *Drosophila* seriously affects viability, similar to observations in the FRDA mouse model [47] and most likely in humans, since no patients carrying a pathogenic point mutation or deletion or insertion mutations in both *FXN* alleles have been reported. Ubiquitous *fh* suppression affects larva and pupa development, and individuals do not reach the adult phase [33, 34]. In agreement with these results, individuals that are hemizygous for the fh^1 mutant, carrying the missense S136R mutation, show lethality from the instar 3 larva to pupa stages [45]. Silencing of *fh* in developing muscle and heart tissue (using the *24B* and *Dot* driver lines) is also lethal in pupal stages, while reduction of *fh* expression in subsets of neurons (C96, *Ddc*, *D42*, *c698a*, and *neur*) allows the development of viable adults. Importantly, when *fh* expression is specifically reduced in the peripheral nervous system (PNS), using the *C96* and *neur* GAL4 lines, the adult flies show a shortened lifespan and reduced climbing ability [33, 34]. These results indicated that, in *Drosophila*, as in humans, frataxin is an essential protein and that different tissues have distinct sensitivity to frataxin deficiency.

Tricoire et al. [42] obtained the first fly *in vivo* heart images after heart-specific depletion of frataxin using the UDIR2 line and the RU486-inducible Geneswitch driver HandGS. They observed major cardiac dysfunction including impaired systolic function and substantial heart dilatation, resembling the phenotypes observed in FRDA patients. The cellular neuropathology of frataxin deficiency was examined in larval motor neurons using the UDIR1 line [48]. Loss of mitochondrial membrane potential was detected in the cell bodies, axons, and neuromuscular junction of segmental nerves from second to late third instar larvae. These effects were followed by defects in mitochondrial retrograde transport in the distal axons, leading to a concomitant dying-back neuropathy. A dying-back mechanism has also been described in sensory neurons and the spinocerebellar and corticospinal motor tract in patients (reviewed in [29]).

To more closely mimic the patient situation, viable adults with ubiquitous reduction of FH were obtained by Llorens et al. [34] by crossing the *fh*RNAi line with the actin-GAL4 driver at 25°C. Under these experimental conditions, the *fh* mRNA level was reduced to one-third compared with

Table 1: *Drosophila* models of frataxin deficiency. The *fh* construct or allele and the GAL4 driver used to obtain the different phenotypes of frataxin reduction are specified.

RNAi/mutant allele	GAL4 driver line	Phenotypes
UDIR1–3 [33] frataxin reduction to undetectable levels (25°C)*	da^{G32} Ubiquitous	(i) Prolonged larval stages, reduced larvae viability, and inability to pupate [33, 88] (ii) When raised at 18°C, survivor adults exhibit high initial mortality, with some escapers that survive up to 40 days [33, 83] (iii) Reduction of activity of aconitase and respiratory complexes II, III, and IV in larvae and adults [33] (iv) Increase in free fatty acid content in larvae [83]
	C96 Adult peripheral nervous system	(i) Viable adults with a shortened lifespan and increased sensitivity to H_2O_2 [33, 41]
	D42 Motor neurons and interneurons in L3. Adult motor neurons	(i) Normal development and longevity [33] (ii) Loss of mitochondrial membrane potential and reduced mitochondrial transport in the distal axons. Distal axonal degeneration and cell body loss in the ventral ganglion in late L3 [48] (iii) Normal ROS levels [48]
	Repo Pan-glial	(i) Viable adults accompanied by some preadult lethality [83] (ii) Reduction of lifespan, increased sensitivity to hyperoxia (99.5% O_2), and impaired climbing capability [66, 83] (iii) Lipid droplet accumulation in glial cells and brain vacuolization [66, 83]
	HandGS Heart-specific RU486-inducible Geneswitch driver	(i) Induction starting at L3. Viable adults that display heart dilatation and impaired systolic function [42, 88]
	GMR Developing eye	(i) Mild rough eye phenotype [82]

Table 1: Continued.

RNAi/mutant allele	GAL4 driver line	Phenotypes
	actin and da^{G32} Ubiquitous	(i) Lethal at the mature pupa stage at 29°C [34] (ii) Viable adults that exhibit shortened lifespan, sensitivity to oxidative stress, and reduced climbing ability [34, 49, 66, 81, 82] (iii) Exposure to hyperoxia causes a substantial reduction in aconitase activity and oxygen consumption [34, 81, 83] (iv) Increased levels of lipid peroxides [81–83] (v) Increased mitochondrial iron content [49] (vi) Sensitive to increased iron content in diet [66] (vii) Complete ablation of iron-dependent ferritin accumulation, reduction of *IRP-1A* expression, and enhanced expression levels of *mfrn (mitoferrin)* [66] (viii) Increased levels of Fe, Zn, Cu, Mn, and Al [82]
UAS-fhIR [34]: Up to 70% frataxin reduction (25°C)*	*neur* Sensory organs and their precursors	(i) Viable adults at 29°C [34] (ii) Reduced lifespan and climbing capability at 25 and 29°C [34, 49]
	Nervous system: *D42*, motor neurons *Ddc*, aminergic neurons and *c698a*, brain	(i) Viable adults at 29°C [34] (ii) Lifespan and climbing capability unaffected at 29°C [34]
	Repo Pan-glial.	(i) Viable adults [83] (ii) Reduction of lifespan, increased sensitivity to hyperoxia (99.5% O_2), and impaired climbing capability [66, 83]
	Other tissues: *Dot*, heart and *24B*, mesoderm	(i) Lethal at the mature pupa stage at 29°C [34]
fh^1 [45]: Ethyl-methanesulfonate-induced missense mutation (S136R). Severe loss of *fh* function Mosaic *fh* mutant clones of adult photoreceptor neurons by the eyeless-FLP/FRT system		(i) Hemizygous fh^1 mutants are lethal from L3 to pupa stage [45] (ii) Removal of maternal *fh* mRNA or protein in the egg causes embryonic lethality [45] (iii) Age-dependent degeneration of photoreceptors [45] (iv) Abnormal mitochondrial cristae morphology, reduced ETC CI activity, and impaired ATP production [45] (v) No increase in ROS [45] (vi) Accumulation of Fe^{2+} and/or Fe^{3+} and iron-dependent stimulation of sphingolipid synthesis and activation of the Pdkl/Mef2 pathway [45]

*The most used temperature in the different experiments.

the normal level. As in humans [7], the remaining frataxin (approximately 30% of the normal level) allowed normal embryonic development but resulted in decreased lifespan and impaired motor performance in adulthood. Specifically, survival analysis showed a decrease of 60% and 32% in the mean and maximum lifespan, respectively, compared with controls. The FRDA flies showed limited climbing ability in negative geotaxis assays, with 5-day-old adults exhibiting a 45% decline compared with control flies.

Frataxin deficiency in flies also triggers iron accumulation [45, 49] restricted to mitochondria [49], consistent with findings in other model organisms and FRDA patients. Importantly, the role of iron in the pathophysiology of FRDA has not yet been completely established and is still a matter of debate. The discovery of iron deposits in the hearts of FRDA patients in the late seventies [50, 51] was the first indication of an association between frataxin and this transition metal. This relationship became more important after the discovery that the loss-of-function of the yeast frataxin ortholog results in mitochondrial iron accumulation [52]. Since then, iron-enriched granules have been further confirmed in patient hearts [53–55] and in several other patient tissues [56, 57]. Surprisingly, analyses of iron levels in neuronal tissues have shown inconsistent results, even in tissues with high frataxin expression. On the one hand, histological and imaging approaches have detected alterations in the expression of iron-related proteins that support the hypothesis that iron redistribution rather than iron accumulation is the key defect underlying frataxin deficiency in the nervous system [58, 59]. On the other hand, increased iron content has been reported in critical brain areas of FRDA patients [60, 61]. In *Drosophila,* Chen et al. showed that iron accumulates in the nervous system in fh^1 mutants [45]. These authors also found increased levels of iron in the nervous system in an FRDA mouse model that exhibits less than 40% of the normal level of frataxin mRNA in this tissue [62]. By contrast, no iron deposits have been reported in the nervous system in other mouse models of FRDA [47, 63–65]. In line with the proposed iron toxicity in FRDA, all *Drosophila* models share an enhanced sensitivity to increased iron content in food [33, 45, 66].

The analysis of the iron-frataxin relationship in several FRDA models has provided experimental evidence supporting a role for frataxin in iron homeostasis (storage, redistribution, chaperone, and ISC biosynthesis, reviewed in [23, 24]). Supporting a role for frataxin in ISC assembly, loss of FH expression is associated with impaired activity of Fe-S containing enzymes, including proteins involved in the mitochondrial electron transport chain (ETC) and aconitase [33, 34]. This effect causes problems in ATP production, which is reduced in *Drosophila* models independently of the levels of functional frataxin [33, 34, 45], as well as in FRDA patients [67, 68]. In addition, the biochemical and biophysical characterization of FH is consistent with its expected role as an iron chaperone acting as a regulator during ISC biosynthesis [35]. In line with this role for frataxin, its suppression in the prothoracic gland impairs the ability of larvae to initiate pupariation [69]. This organ produces ecdysteroid hormones, such as 20-hydroxyecdysone, that mediate developmental transitions. Interestingly, some Fe-S-containing enzymes such as Neverland (converts cholesterol into 7-dehydrocholesterol) and the fly ferredoxins Fdxh and Fdxh2 participate in the metabolism of ecdysone, and their activities are likely impaired in frataxin-deficient larvae. In agreement with this hypothesis, 20-hydroxyecdysone supplementation improves the defective transitions associated with frataxin deficiency in the prothoracic gland [69]. An ecdysone deficiency would explain the giant, long-lived larvae phenotype reported by Anderson et al. in their fly model using the UDIR2 line and da^{G32} GAL4 driver [33]. Interestingly, *Drosophila* models have also revealed that iron deregulation occurs before the decrease in the activity of mitochondrial enzymes [49, 66]. This is in agreement with results from an inducible yeast model in which the iron regulon was activated long before decreased aconitase activity was observed [70].

It has been suggested that ROS are generated by iron accumulation through Fenton's reaction, damaging the mitochondrial ETC and mediating the pathophysiology of FRDA (reviewed in [20, 71]). However, the role of oxidative stress in the disease is still questioned, and controversial results have also been reported in *Drosophila*. Overexpression of ROS-scavenging enzymes such as catalase (CAT), superoxide dismutase 1 (SOD1), or SOD2 could not rescue the pupae lethality caused by ubiquitous UDIR1 and UDIR2 expression [33] or the photoreceptor neurodegeneration in fh^1 mutant clones [45]. CAT overexpression and treatment with EUK8 (a synthetic superoxide dismutase and catalase mimetic) also failed to improve cardiac function in frataxin-depleted hearts [42]. Shidara and Hollenbeck [48] did not detect increased ROS levels in frataxin-deficient motor neurons, but these neurons responded to the complex III inhibitor antimycin A with a larger increase in ROS than control neurons.

However, increasing evidence from different FRDA models and patient samples suggests that oxidative stress is a major player in FRDA [34, 41, 65, 72–80]. In *Drosophila*, increased levels of malondialdehyde (MDA, a lipoperoxidation product) have been reported in flies with ubiquitous FH suppression using the *fh*RNAi line and the *actin* GAL4-driver line [81, 82]. These flies and flies with tissue-specific frataxin deficiency in the PNS *(C96)* or glial cells *(repo)* showed increased sensitivity to external oxidative insults (see Table 1) such as hyperoxia or H_2O_2 treatment [41, 81, 83]. Hyperoxia induces enhanced aconitase inactivation in the frataxin knockdown flies [34, 83], which compromises the entire respiratory process. In fact, hyperoxia leads to reduced oxygen consumption rates in mitochondrial extracts of the frataxin-depleted flies [34]. Overexpression of the H_2O_2-scavenging enzymes CAT, mitoCAT (using a synthetic transgene that targets CAT to the mitochondria), or mitochondrial peroxiredoxin (mTPx) rescues the shortened lifespan and increased sensitivity to H_2O_2 in flies with reduced frataxin expression in the PNS *(C96)* [41]. These scavengers also restore aconitase activity in flies with systemic reduction of FH using the UDIR1 line and the da^{G32} GAL4 driver [41], supporting the role of oxidative stress in aconitase inactivation. In addition, scavengers of lipid peroxides have

been shown to improve frataxin-deficient phenotypes [83, 84].

Recently, Hugo Bellen's laboratory identified a new mechanism for neuronal degeneration in FRDA, in which iron toxicity is not associated with ROS damage [45]. These authors showed in their *fh* mutant that iron accumulation induces sphingolipid synthesis and activates the expression of the genes *3-phosphoinositide dependent protein kinase-1 (Pdk1)* and *myocyte enhancer factor-2 (Mef2)* and their downstream targets, causing loss of photoreceptors in fly ommatidia. In agreement with these results, inhibition of sphingolipid synthesis by downregulating the expression of the rate-limiting enzyme lace (the fly ortholog of serine palmitoyltransferase) or feeding the mutant flies Myriocin (a compound that inhibits serine palmitoyltransferase) was sufficient to partially revert the cellular degeneration [45]. Similarly, silencing *Pdk1* or *Mef2* expression also suppressed the neurodegenerative phenotype. Remarkably, the authors found that loss of frataxin in the nervous system in mice and in heart tissue from patients also activates the same pathway, suggesting a conserved mechanism [62]. These results highlight, once more, the relevance of *Drosophila* in the study of human disorders such as FRDA. In addition, they strongly suggest that iron plays an instrumental role in *Drosophila* frataxin biology.

Similarly, *Drosophila* has also been a pioneer model organism in highlighting the role of frataxin in lipid homeostasis [83]. Ubiquitous frataxin knockdown or targeted frataxin downregulation in glia cells triggered lipid accumulation. Increased amounts of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1), and linoleic acid (C18:2) were found. These results suggested that loss of mitochondrial function also affects fatty acid beta-oxidation, leading to the accumulation of the most abundant lipid species [83]. The presence of lipid droplets had already been characterized in mouse models [63], and the fly findings indicated the content of these droplets and their likely association with the disease pathophysiology. These findings were followed by assessments of lipid deregulation in other models [85] and in patient samples [86]. The association between frataxin and lipid metabolism has been extensively reviewed elsewhere [87].

5. Frataxin Overexpression Phenotypes

Although frataxin overexpression does not model the disease, it is an excellent complementary tool to further describe the cellular roles of frataxin. In this regard, *Drosophila* models have shown that some increase in frataxin expression is beneficial, whereas its excess beyond certain thresholds is clearly detrimental. Table 2 summarizes the phenotypes reported for frataxin overexpression in flies using several GAL4 lines that drive ubiquitous or tissue-specific *fh* expression.

Flies with ubiquitous *fh* expression at a level approximately fourfold higher than the physiological level show increased longevity, antioxidant defense responses, and resistance to treatment with paraquat (a chemical known to specifically affect mitochondrial complex I and to generate free radicals), H_2O_2, and dietary iron [89]. Similarly, it has been reported that frataxin overexpression in mice [90, 91] or in cultured cells [92–94] is innocuous or has a positive effect, stimulating ATP production or inducing antioxidant defense responses.

A systemic 9-fold increase in *fh* mRNA expression impairs muscle, heart, and PNS development in fly embryos, leading to lethality from larva to pupa stages [34]. Frataxin overexpression restricted to developing heart and muscle tissue (*Dot*, *24B*; Table 2) also has deleterious effects [34]. In contrast, overexpressing FH pan-neuronally *(Appl, elav)*, in sensory organs *(neur)*, motor neurons *(D42)*, and glial cells *(repo)* produces viable adults, but they show a reduced lifespan and decreased locomotor performance [34, 95]. The effect of human frataxin expression has also been tested in *Drosophila*. FXN is correctly expressed and targeted to mitochondria in flies and can rescue the aconitase activity of UDIR2-knockdown flies [95]. These results provide *in vivo* evidence that human and fly frataxins have conserved functions, which was further confirmed by Tricoire et al. [42] and Chen et al. [45]. As expected, FXN overexpression in flies produces similar but slightly stronger phenotypes at biochemical, physiological, and developmental levels than those observed in flies overexpressing FH [95]. Initially, it was proposed that frataxin overexpression might act as a dominant negative mutation and that its toxic effect might be mediated by oxidative stress [95]. The mechanism underlying frataxin overexpression has recently been further investigated [96]. In this study, the authors reported that frataxin overexpression increases oxidative phosphorylation and modifies iron homeostasis. Such an increase of mitochondrial activity alters mitochondrial morphology and sensitizes cells to oxidative damage leading to neurodegeneration and cell death. Importantly, authors found that iron was a pivotal factor in the neurodegeneration [96].

These results in *Drosophila* show that frataxin requires an optimal balance in expression to function properly and that control of its expression is important in treatments that aim to increase its protein level.

6. Genetic Modifiers of FRDA

Drosophila models are important because they offer the ability to carry out genetic screens for mutations that affect a particular biological process. This powerful tool provides a way to identify genetic modifiers of human diseases (Figures 4(a) and 4(c)). Our group has collaborated with Juan Botas's laboratory in two studies using this methodology in *Drosophila* models of FRDA. These studies followed a biased candidate approach, selecting genes related to disease pathophysiology [81, 82]. We set out to test whether genetic modification of key pathways would improve FRDA phenotypes in flies. Candidate genes were selected from pathways involved in metal homeostasis, the response to oxidative stress, apoptosis, and autophagy. Approximately 300 lines were analyzed, including RNAi lines from the Vienna *Drosophila* Resource Center and loss-of-function and overexpression lines from the Bloomington Stock Center (Indiana University). The external eye morphology and motor performance of adult flies were used

Table 2: Frataxin overexpression in *Drosophila*. The *fh* construct and the GAL4 driver used to obtain the different phenotypes are indicated.

Overexpression line	GAL4 driver line	Phenotypes
UAS-dfh^{1} and *UAS-dfh*^{2} [89]: fourfold increase in *fh* mRNA expression (25°C)*	*Actin* Ubiquitous	(i) Viable adults [89] (ii) Increased lifespan [89] (iii) Significant increase in tolerance to iron-induced stress ($FeCl_3$), paraquat, and H_2O_2 (measuring survival) [89] (iv) Significant increase in total antioxidant activity (bathocuproine dye) [89]
UAS-fh [34]: 9-fold increase in *fh* mRNA expression and a strong increase in protein levels (29°C)*	*Actin* and *da*^{G32} Ubiquitous	(i) Lethal at early pupae or 3rd instar larvae at 29°C [34] (ii) Defects in developing muscles, axonal tracks, and axonal pathfinding (1D4 staining) and an increase in the number of sensory ventral neurons. No abnormalities detected in the CNS [34] (iii) At 25°C, viable adults that are sensitive to oxidative stress and iron [34, 96]. Young individuals have higher catalase and aconitase activities and ATP production than controls but are hypersensitive to hyperoxia [96]
	Appl and *elav* Pan-neural	(i) Viable at 29°C and 25°C (ii) Reduced lifespan and climbing capability [95, 96]. Locomotor defects are rescued by mitochondrial catalase expression and *mfrn* silencing [96]. (iii) Reduced ferritin and mitoferrin levels [96] (iv) Brain vacuolization [96]
	Other neuronal drivers	
	neur Sensory organs and their precursors	(i) Viable adults at 29°C and 25°C [34] (ii) Reduced climbing capability and lifespan at both temperatures *(neur/D42)* [34, 95]. (iii) Lifespan is recovered by mitochondrial catalase *(neur)* [95]
	D42 Motor neurons	
	Ddc Aminergic neurons	(iv) *Ddc*, *TH*, and *c698a*: lifespan and climbing capability unaffected at 29°C or 25°C [34, 96]
	TH Dopaminergic neurons	(v) Strong promotion of mitochondrial fusion and ROS-mediated cell death of dopaminergic neurons *(TH)* [96]
	c698a Brain	
	Repo Pan-glial	(i) Reduced lifespan and climbing capability [95] (ii) Expression of mitochondrial catalase increases lifespan and climbing capability [95]
	Other tissues: *Dot*, heart and *24B*, mesoderm	(i) Lethal from the early pupa stage to adult eclosion from the puparium at 29°C and 25°C [34, 95] (ii) Lack of some pericardial cells along the tubular structure of the developing heart (ECII staining) in embryos at 29°C [34]

Table 2: Continued.

Overexpression line	GAL4 driver line	Phenotypes
UAS-FXN[#] [95]: Expression of human frataxin. Stronger phenotypes than UAS-*fh* (25°C)[*]	*Actin* and da^{G32} Ubiquitous	(i) Lethal in pupae [95] (ii) Reduced aconitase activity in larvae [95] (iii) Reduced NDUFS3 protein levels in larvae [95]
	Appl Pan-neural	(i) Viable adults, lethal at 29°C [95]
	neur Sensory organs and their precursors	(i) Reduced lifespan and climbing capability and increased sensitivity to oxidative insult [95] (ii) Expression of mitochondrial catalase increases lifespan [95]
	Repo Pan-glial	(i) Morphological disruption of glial cells and formation of lipid droplets [95] (ii) Expression of mitochondrial catalase increases lifespan and improves climbing capability [95]
	24B Mesoderm	(i) Lethal during pupariation [95]

[*]The most used temperature in the experiments. [#]UAS-*FXN* triggers the same defects as UAS-*fh*. To avoid repetition, only new phenotypes have been included; CNS: Central Nervous System.

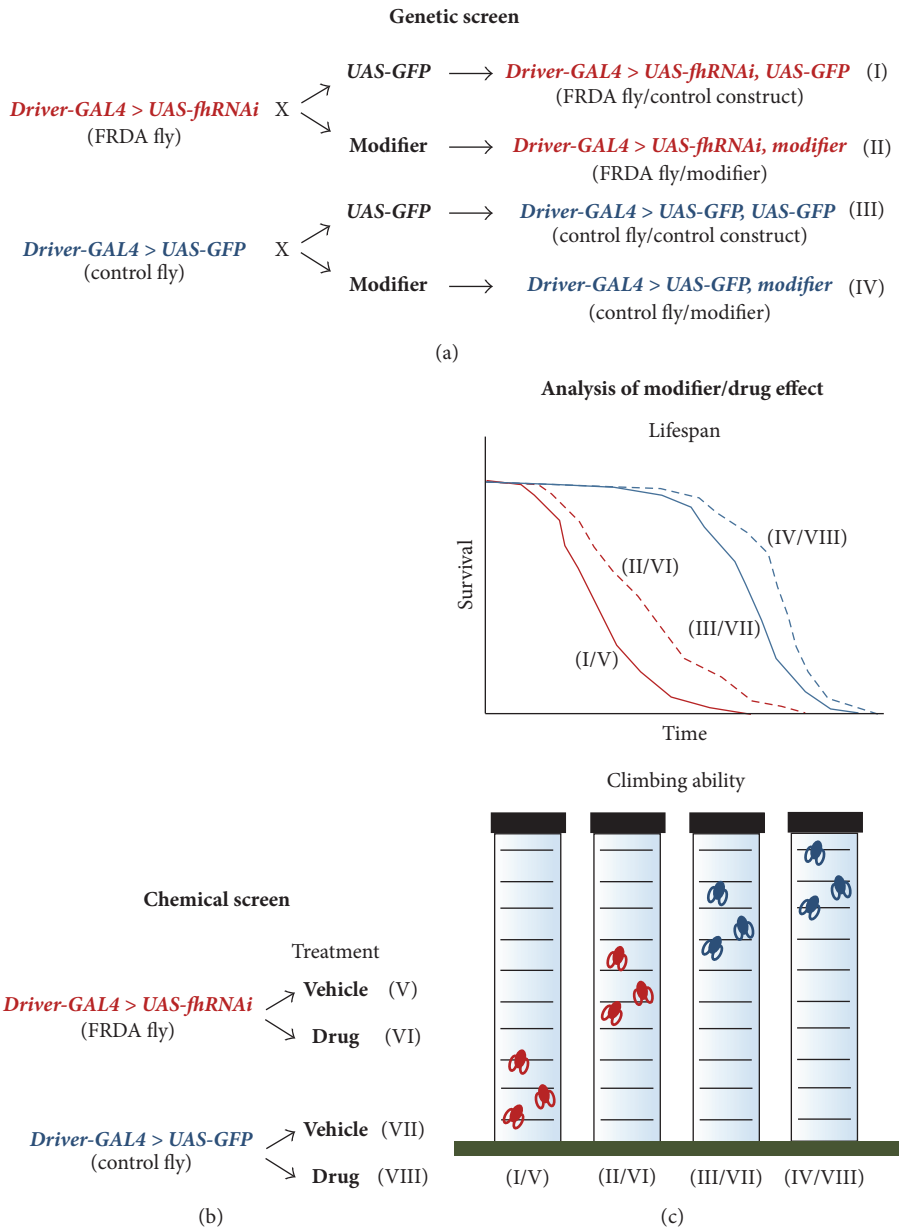


FIGURE 4: Schematic design of a genetic (a) or chemical (b) screen to identify genetic modifiers or potential therapeutic compounds in FRDA using *Drosophila* as a model organism. The effect of a genetic modifier or drug is evaluated by monitoring the lifespan and climbing ability of FRDA flies. (c) A UAS-GFP construct is included in this strategy as an internal control to determine whether the drug can interfere with the GAL4/UAS system and the potential dilution of the GAL4 protein due to the presence of two UAS construct. In parallel, the effect of the modifier or drug treatment is analyzed in control flies to identify frataxin interactors. GFP: green fluorescent protein. Vehicle: DMSO/H_2O depending on the drug solubility.

as screening phenotypes. The UDIR2 line [33] (with a 90% reduction in FH expression when expressed ubiquitously) produces a mild rough eye phenotype when expressed in the developing eye [82]. The *fh*RNAi line [34] (with a 70% reduction in FH expression that is compatible with normal development) impairs motor performance when expressed ubiquitously. We applied a tiered strategy to examine the effect of metal-related genes on eye morphology, followed by the effect of eye modifiers on motor performance [82]. In Calap-Quintana et al. [81], we reported the effect of the remaining candidate genes on the motor performance of the *fh*RNAi line.

Five suppressors of both the eye and motor performance phenotypes were identified: the iron regulatory proteins encoded by the genes *Irp-1A* and *Irp-1B*, their target Transferrin (*Tsf1* and *Tsf3*), and *Malvolio (Mvl)*, the *Drosophila* ortholog of the mammalian gene *Divalent metal transporter-1 (DMT1)*. The suppression of these FRDA phenotypes was mediated by reducing the iron abundance associated with frataxin deficiency [82]. On the one hand, reduced expression

of *Mvl*, *Tsf1*, and *Tsf3* decreases cellular iron uptake, which in turn reduces mitochondrial iron accumulation. On the other hand, downregulation of *Irp-1A* and *Irp-1B* reduces IRP activity, as suggested in [33, 66], and thus recovers ferritin expression and normal cellular iron distribution. In agreement with these findings, *Irp1* knockout reduces mitochondrial iron accumulation in frataxin-depleted mouse livers [97].

Another iron player that can suppress FRDA phenotypes in flies was identified by Navarro et al. [66]. It is a member of the mitochondrial solute carrier family named mitoferrin (Mfrn), which is located in the inner mitochondrial membrane, and its function is to translocate iron into mitochondria [98–100]. Downregulation of *mfrn* was sufficient to improve iron metabolism in frataxin-deficient flies and to ameliorate neurodegeneration triggered by targeted frataxin silencing in glia cells [66]. In this study, overexpression of ferritin subunits was unable to counteract neurodegeneration, whereas another study reported that ferritin overexpression had a positive effect in *fh* mutant clones of fly photoreceptors [45]. It is likely that the different metabolic requirements of each cell type might be reflected in the factors that can exert protective roles.

Knockdown of zinc transporters and copper chaperones also ameliorates FRDA phenotypes in flies [82]. Members of the two conserved gene families of zinc transporters (the ZnT and Zip families) improve the eye and motor performance phenotypes by normalizing iron levels in some cases. It has been previously reported that several members of the Zip family can also transport iron in addition to zinc [101–103]. Genetic reduction of *Atox1*, which encodes a chaperone that delivers copper to ATP7 transporters located in the trans-Golgi network [104], and *dCutC,* encoding a protein involved in the uptake, storage, delivery, and efflux of copper [105], suppressed both FRDA phenotypes. We also found that the *Metal-Responsive Transcription Factor-1* Gene *(MTF-1)* is a modifier of the motor impairment phenotype, acting as a suppressor when overexpressed and as an enhancer when downregulated. Overexpression of *MTF-1* in *Drosophila* also reduces the toxicity associated with oxidative stress [106], human Aβ42 peptide expression [107], and a parkin null mutation [108]. Under stress conditions, such as metal overload and oxidative stress, MTF-1 is translocated to the nucleus and binds to metal response elements (MREs) in the regulatory regions of its target genes, such as metal-sequestering metallothioneins (Mtns). Mtns are small cysteine-rich proteins that maintain low levels of intracellular free metal due to their ability to bind metals with high affinity. Contrary to what was expected, Mtn knockdown suppressed FRDA phenotypes [82], which could be explained by the role of Mtns as prooxidants under oxidative stress conditions [109–111]. Therefore, the beneficial effect of *MTF-1* overexpression may not be mediated by Mtns but rather by reduced iron accumulation, because the iron level is normalized in *fh*RNAi flies with *MTF-1* overexpression [82]. These results demonstrate that metal dysregulation in FRDA affects other metals in addition to iron. Importantly, zinc and copper redistribution have been reported in the dentate nucleus of the cerebellum in FRDA patients [112].

The genetic screen conducted in Calap-Quintana et al. [81] revealed four modifiers of the motor performance phenotype in FRDA flies. These genes encode tuberous sclerosis complex protein 1 *(Tsc1),* ribosomal protein S6 kinase *(S6k)*, eukaryotic translation initiation factor 4E *(eIF-4F)*, and leucine-rich repeat kinase *(Lrrk)*. These proteins are involved in the TORC1 signaling pathway, which regulates many major cellular functions such as protein synthesis, lipid biogenesis, and autophagy. We found that genetic reduction in TORC1 signaling activity is beneficial, while its genetic activation produces a detrimental effect in frataxin knockdown flies by inducing semilethality. Table 3 shows these genetic mediators of frataxin deficiency as well as other modifiers individually identified in other studies.

7. Potential Therapeutic Compounds for FRDA Treatment

Currently, there is no effective treatment for FRDA, although different therapeutic strategies are being developed or tested in clinical trials (http://www.curefa.org/pipeline). These strategies include lowering oxidative damage, reducing iron-mediated toxicity, increasing antioxidant defense, and increasing frataxin expression and gene therapy [83, 113, 114]. *Drosophila* models are also gaining increasing significance in biomedical and pharmaceutical research as a valuable tool for testing potential treatments (Figures 4(b) and 4(c)).

Table 4 lists the compounds that have been found to improve some FRDA phenotypes in *Drosophila*. Our group has validated the utility of frataxin-depleted flies for drug screening [49]. We separately tested the effect of two compounds, the iron chelator deferiprone (DFP) and the antioxidant idebenone (IDE), that were already in use in clinical trials for this disease. DFP is a small-molecule, blood-brain-barrier-permeable drug that preferentially binds iron and prevents its reaction with ROS. IDE is a synthetic analog of coenzyme Q10 and can undergo reversible redox reactions, improving electron flux along the ETC. Each drug was administered in the fly food at two starting points: early treatment (from larva to adult stage) and adult treatment (in adult phase). Both drugs improved the lifespan and motor ability of flies expressing the *fh*-RNAi allele in a ubiquitous pattern or in the PNS *(neur)*, especially when given at the early treatment timepoint. DFP improved the FRDA phenotypes by sequestering mitochondrial iron and preventing toxicity induced by iron accumulation. IDE rescued aconitase activity in flies subjected to external oxidative stress [49].

Another compound with electron carrier properties, methylene blue (MB), has been described as a potent therapeutic drug for heart dysfunction in FRDA [42]. Cardiac defects were decreased in a dose-dependent manner in flies with heart-specific frataxin depletion treated with different concentrations of MB. The authors demonstrated that this drug was also able to reduce heart dilatation associated with deficiencies in several components of complexes I and III in mutant flies. These results indicate that respiratory chain impairment is involved in the cardiac defects associated with frataxin deficiency and that compounds showing electron

Table 3: Genetic modifiers of FRDA phenotypes in *Drosophila*.

Modifier	Pathway	Effect
Fer1HCH/Fer2LCH (Co-expression)	Iron storage	Suppressor of reduced life span [66], ERG, and photoreceptor neurodegeneration [45]
Fer3HCH (OE)	Iron storage and oxidative stress protection	Suppressor of reduced life span [66] ERG, and photoreceptor neurodegeneration [45]
Irp-1A (RNAi) *Irp-1B* (RNAi) *Irp-1B* (LOF)	Iron sensor	Suppressor of mild rough eye and impaired motor performance [82]
mfrn (RNAi)	Mitochondrial iron importer	Suppressor of reduced aconitase activity and IRP-1A and ferritin levels, impaired motor performance, and increased brain vacuolization [66]
mfrn (OE)		Enhancer of locomotor defects and brain vacuolization [66]
Mvl (RNAi)	Iron absorption	Suppressor of mild rough eye and impaired motor performance [82]
Tsf1 (LOF) *Tsf3* (RNAi)	Serum iron binding transport proteins	Suppressor of mild rough eye and impaired motor performance [82]
dZip42C.1 (RNAi) *dZip42C.2* (RNAi) *dZip88E* (RNAi)	Zinc importer	Suppressor of mild rough eye and impaired motor performance [82]
dZnT35C (RNAi)	Zinc transporter to vesicles	Suppressor of mild rough eye and impaired motor performance [82]
dZnT41F (RNAi)	Zinc homeostasis	Suppressor of mild rough eye and impaired motor performance [82]
dZnT63C (RNAi)	Zinc exporter	Suppressor of mild rough eye and impaired motor performance [82]
foi (LOF)	Zinc importer	Suppressor of impaired motor performance [82]
Atox1 (RNAi)	Copper chaperone donor	Suppressor of mild rough eye and impaired motor performance [82]
dCutC (RNAi)	Copper uptake and storage	Suppressor of mild rough eye and impaired motor performance [82]
MTF-1 (OE)	Metal responsive Transcription Factor	Suppressor of impaired motor performance [82]
MTF-1 (LOF)		Enhancer of impaired motor performance [82]
MtnA (RNAi)	Heavy metal detoxification	Suppressor of mild rough eye and impaired motor performance [82]
MtnB (RNAi) *MtnC* (RNAi)	Heavy metal detoxification	Suppressor of mild rough eye [82]
Tsc1 (RNAi)	TORC1 pathway	Enhancer of reduced survival [81]
S6K (DN)	TORC1 pathway	Suppressor of impaired motor performance [81]
S6K (CA)		Enhancer of reduced survival [81]
eIF-4E (LOF)	TORC1 pathway	Suppressor of impaired motor performance [81]
Lrrk (RNAi)	TORC1 pathway	Suppressor of impaired motor performance [81]
Cat (OE) *mCat* (OE) *mTPx* (OE)	Antioxidant (hydrogen peroxide scavengers)	Suppressor of reduced lifespan when overexpressed in the PNS [41]

Table 3: Continued.

Modifier	Pathway	Effect
dGLaz (OE)	Antioxidant defense	Suppressor of reduced life span, impaired motor performance, aconitase inactivation, and lipid peroxidation [83]
Pdk1 (RNAi)	Embryonic development (insulin receptor transduction pathway and apoptotic pathway)	Suppressor of photoreceptor neurodegeneration [45]
Mef2 (RNAi)	Muscle differentiation	Suppressor of photoreceptor neurodegeneration [45]
lace (RNAi)	Sphingosine biosynthesis pathway	Suppressor of photoreceptor neurodegeneration [45]

CA: constitutively active mutation; DN: dominant negative mutation; ERG: electroretinograms; LOF: loss-of-function mutation; OE: overexpression; RNAi: RNA interference.

Table 4: Compounds that showed beneficial effects in *Drosophila* models of FRDA.

Compound	Mechanism of action	Improved phenotype
Idebenone	Antioxidant	Motor performance and lifespan in adults [42, 49]
Methylene blue	Electron carrier	Adult heart function [42]
Toluidine blue	Electron carrier	Adult heart function [42]
Deferiprone	Iron chelator	Motor performance and lifespan in adults [49]
Deferoxamine	Iron chelator	Pupa development [88]
LPS 01-03-L-F03	Possible iron chelator	Pupa development [88]
LPS 02-25-L-E10	Possible iron chelator	Pupa development [88]
LPS 02-13-L-E04	Possible iron chelator	Pupa development [88]
LPS 01-04-L-G10	n.d.	Pupa development [88] Adult heart function [88]
LPS 02-14-L-B11	n.d.	Pupa development [88]
Rapamycin	TORC1 inhibitor	Motor performance and oxidative stress in adults [81]
Myriocin	Serine palmitoyltransferase inhibitor	Photoreceptor function [45]

n.d.: not described.

transfer properties could prevent heart dysfunction in FRDA patients.

A yeast/*Drosophila* screen to identify new compounds for FRDA treatment was carried out by Seguin et al. [88]. The authors showed the utility of using a strategy based on two complementary models, a unicellular and a multicellular organism. Accordingly, a frataxin-deleted yeast strain was used in a primary screen, and positive hits were tested in flies ubiquitously expressing the UDIR2 allele (secondary screen). Approximately 6380 compounds were evaluated from two chemical libraries (the French National Chemical Library and the Prestwick Collection) to test the ability of the drugs to improve the fitness of yeast mutants using raffinose as the main carbon source. Yeast cells with frataxin deficiency grew slowly when raffinose was provided as the carbon source [115]. A total of 12 compounds, representative of the different chemical families, were selected from the yeast-based screen and their effect was analyzed on the FRDA fly model. Six of them improved the pupariation impairment of flies, with LPS 01-04-LG10 and Deferoxamine B (DFOB) being the most promising compounds. DFOB, an iron chelator, was suggested to increase the pools of bioavailable iron and to reduce iron accumulation in mitochondria. LPS 01-04-L-G10, a cinnamic derivative, partially rescued heart dilatation in flies with heart-specific frataxin depletion [88].

The efficacy of iron chelators as potential treatments has already been assessed in FRDA patients, but unfortunately the results were not conclusive. Studies have reported improvement of the cardiac and/or neurological conditions [61, 116, 117], no significant effect [118], or even worsening of some conditions [119]. However, the *Drosophila* models of FRDA indicate that iron is an important factor in FRDA pathophysiology. Genetic or pharmacological interventions through pathways regulating iron homeostasis and the sphingolipid/Pdk1/Mef-2 pathway are new approaches that might be explored in preclinical studies. In addition, *Drosophila* has shown for the first time that alteration of genes involved in metal detoxification and metal homeostasis

(copper and zinc in addition to iron) is also a potential therapeutic strategy.

Finally, the results obtained from the genetic screen in *Drosophila* [81] also suggest that rapamycin and its analogs (rapalogs) are promising molecules for FRDA treatment. Inhibition of TORC1 signaling by rapamycin increases climbing speed, survival, and ATP levels in flies [81]. This compound enhances antioxidant defenses in both control and FRDA flies by increasing the nuclear translocation of the transcription factor encoded by the gene *cap-n-collar*, the *Drosophila* ortholog of *Nrf2*. As a result, it induces the expression of a battery of antioxidant genes. In addition, rapamycin protects against external oxidative stress by inducing autophagy. Rapamycin is a well-described drug approved for human uses. There is a large amount of data regarding the safety, tolerability, and side effects of this drug and rapalogs, which could facilitate their potential use in FRDA.

8. Conclusions

D. melanogaster is one of the most studied organisms in biological research. The conservation of many cellular and organismal processes between humans and flies and the constant increase in the number of genetic tools for *Drosophila* have made this organism one of the best choices for studying human genetic diseases. Following the identification of Friedreich's ataxia gene by positional cloning, model organisms have played a decisive role in the investigation of the function of frataxin and consequently the underlying pathophysiological mechanisms of FRDA. Here, we have presented the main contributions of *Drosophila* in this area of research. Frataxin-depleted flies recapitulate important biochemical, cellular, and physiological hallmarks of FRDA. In addition, the model flies exhibit new phenotypes that reveal, for the first time, other key players in FRDA pathogenesis. These models have allowed the identification of genetic and pharmacological factors capable of modifying some FRDA phenotypes, revealing new and promising ways to find effective treatments. Nevertheless, there are still many other questions that can be addressed by taking advantage of *Drosophila* models. Additional models of FRDA in flies are expected to help us understand the transcriptional silencing of *FXN* mediated by the GAA repeat expansion. These new models will advance our knowledge of the molecular bases of this disease and facilitate the development of new drugs for FRDA.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Drosophila as a Model System to Study Cell Signaling in Organ Regeneration

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Regeneration is a fascinating phenomenon that allows organisms to replace or repair damaged organs or tissues. This ability occurs to varying extents among metazoans. The rebuilding of the damaged structure depends on regenerative proliferation that must be accompanied by proper cell fate respecification and patterning. These cellular processes are regulated by the action of different signaling pathways that are activated in response to the damage. The imaginal discs of *Drosophila melanogaster* have the ability to regenerate and have been extensively used as a model system to study regeneration. Drosophila provides an opportunity to use powerful genetic tools to address fundamental problems about the genetic mechanisms involved in organ regeneration. Different studies in Drosophila have helped to elucidate the genes and signaling pathways that initiate regeneration, promote regenerative growth, and induce cell fate respecification. Here we review the signaling networks involved in regulating the variety of cellular responses that are required for discs regeneration.

1. Introduction

Regeneration is the ability that presents some organisms and allows them to partially or fully replace missing or damaged organs. This capacity is conserved among different phyla and it involves a wide range of processes, from wound healing to the induction of regenerative growth to reconstruct a whole new organ, as in urodele amphibians [1, 2]. Studying regeneration should shed light on the mechanisms that regulate this process, paving the way for their potential therapeutic applications in regenerative medicine.

Drosophila melanogaster is a powerful model system to perform genetic analysis, and it has provided much of the information of our understanding about the genetic basis of organ morphogenesis. While the adult organs of Drosophila are incapable of regenerating, the primordia of these structures, known as imaginal discs, can undergo regenerative growth. Imaginal discs are epithelial sac-like structures that develop from the embryonic ectoderm and after a period of cell proliferation in the larval stages, they give rise to the adult cuticle.

A series of classic experiments from the mid-1940s to the 1970s laid the groundwork for our current understanding of regeneration in Drosophila imaginal discs [3–5]. The classic approach used to study regeneration in Drosophila was to grow the regenerating discs *in vivo* culture. The disc was extracted from the larva and after amputating a fragment of it, it was transplanted into the abdomen of an adult host, where the cells of the discs could proliferate and the disc would regenerate [3, 6]. After regeneration the disc was then extracted from the adult host and examined [5, 7].

One of the problems of these studies is that regeneration did not occur under physiological conditions, since the disc regenerates in an adult host. Moreover, the process of disc extraction and transplantation will cause some stress to the cells of the discs, provoking apoptosis and halting cell proliferation in the first few hours after transplantation [8]. An alternative approach based on the transient induction of cell death in specific regions of the discs has resolved some of these problems. This method implies the use of the *Gal4/UAS* binary system in combination with $Gal80^{ts}$ to express a cell-death inducer, which makes it possible to genetically ablate a

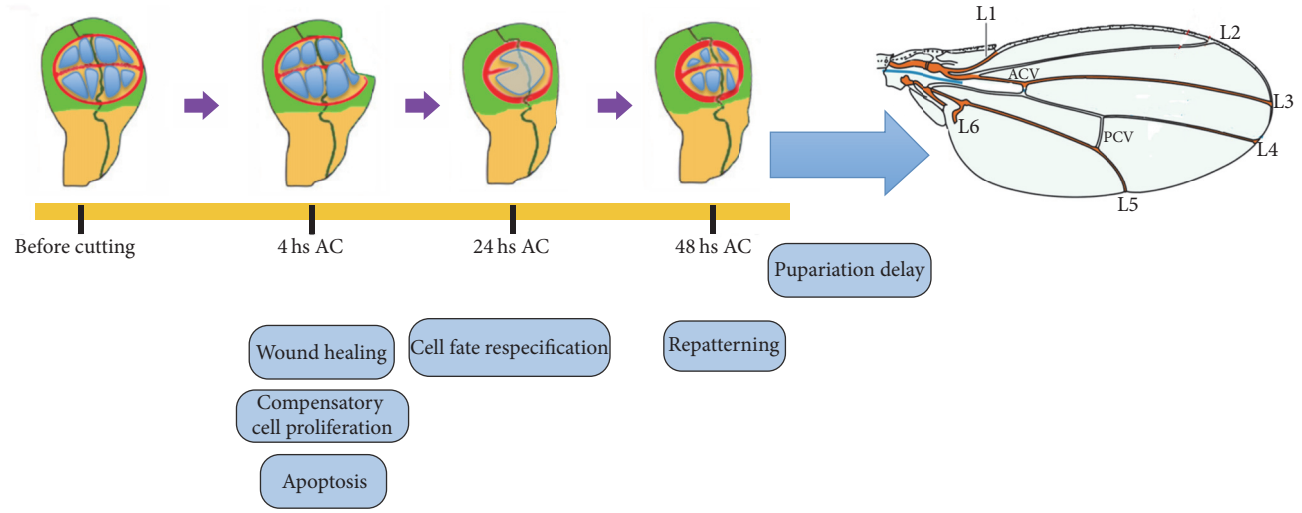


Figure 1: *Drosophila* wing imaginal discs regeneration process. After a cut or genetic ablation in the third instar wing imaginal discs various cellular processes occur. First, four hours after cut (AC), the wound heals restoring the epithelial continuity. Around 24 hours AC, some cells lose expression of markers of cell fate commitment. Finally, the pattern is restored and the discs give rise to a normal pattern and sized adult wings. However, there is a delay in pupariation to allow the tissue to regenerate.

region of the disc *in vivo* for a predetermined period of time, after which the disc recovers [9, 10]. Although this technique mimics some aspects of surgical amputation, there are important differences between these two approaches. For instance, unlike amputation the overexpression of a cell-death inducer may not be sufficient to eliminate all the cells in the region targeted, and thus dying cells will coexist with living cells during the period of recovery. A further method has been developed to study disc regeneration in its normal developmental context. This system consists of removing a section of the disc "in situ" inside the larvae without extracting the discs from the larvae [11, 12]. The results obtained using these different approaches revealed that the initial stages of disc regeneration involve different processes. Thus, after wound healing a zone with a high rate of cell proliferation appears at the edges of the wound, similar to the blastema that originates during limb regeneration in amphibians and teleost fish. In addition, there is a temporary loss of markers of cell fate commitment and repatterning (Figure 1) [5, 7, 10, 12, 13]. All these cellular processes are triggered and regulated by the action of different signaling pathways. In this article we will focus on the signaling networks that regulate the various cell responses required to control the early stages of imaginal disc regeneration.

2. Reactive Oxygen Species (Ros) Are Induced in Response to Disc Damage

One of the first reactions of the imaginal disc in response to damage is the production of reactive oxygen species (Ros). Ros have been increasingly implicated in the physiological regulation of many developmental processes, including the emergence of stem cells, inflammatory cell recruitment, or the differentiation of embryonic cardiomyocytes. Ros act at many distinct levels in biological processes, affecting gene expression, protein translation, and protein-protein interactions [14–17]. Ros are the by-products of aerobic metabolism and they include superoxide (O_2^-) and hydrogen peroxide (H_2O_2). There are numerous potential sources of Ros within the cells and various organelles within the cell can produce Ros. One important generator of oxidant is a family of NADPH-dependent oxidases (Nox/Duox). These transmembrane proteins regulate the generation of Ros and an increase in their activity is produced in response to different stimuli after damage, such as the liberation of Ca^{2+}. Indeed, wounding in Drosophila embryos provokes the binding of Ca^{2+} to Duox and its activation [18].

The specific effects of Ros are largely modulated by the reversible oxidation and reduction of reactive Cys residues, which in turn provokes the reversible modification of enzymatic activity of redox-sensitive targets, such as Tyrosine phosphatases or kinases [16, 17].

There is increasing evidence indicating that, during regeneration in vertebrates, the response to damage involves oxidative stress and, consequently, the stimulation of stress-activated protein kinases [19–21]. Ros have been also proposed to play a key role in disc regeneration [22–27]. Physical injury or genetic ablation of part of the imaginal disc provokes Ros production in cells at the wound site [22–25]. The initial burst of Ros acts as a chemoattractant for macrophages and it is necessary for the activation of different signaling pathways [22, 25, 27] (reviewed in [26]) (Figure 2). It is not clear how the first burst of Ros is generated, although different mechanisms have been identified that contribute to the increase in Ros levels in response to damage (see below). Shortly after trauma, Ros is also detected in cells adjacent to the injured region, although at a lower concentration [22].

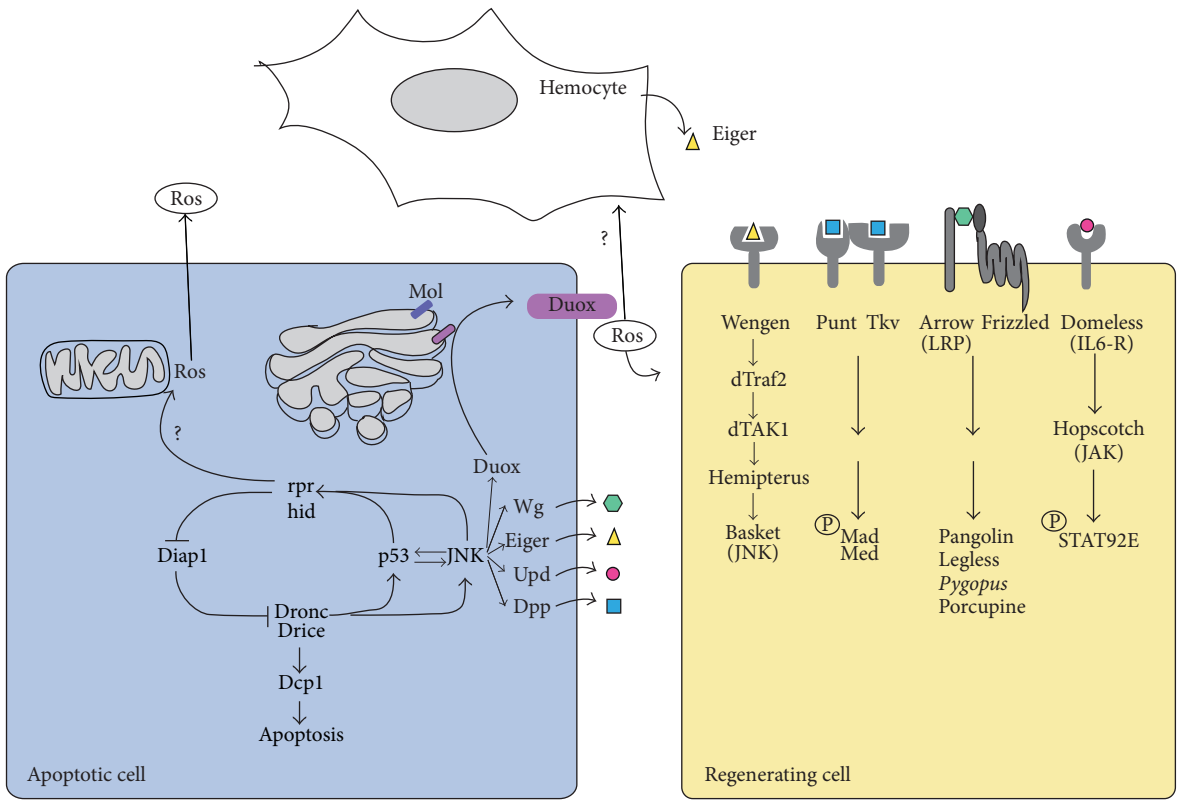


Figure 2: Signaling network promoting apoptosis and Ros production after discs damage. Damage causes the activation of different signals that lead to apoptosis and high levels of Ros accumulation in dying cells. The function of Duox leads to extracellular Ros production that is responsible for hemocyte recruitment and promotes the activation of different signaling pathways in surrounding cells. Hemocytes secrete Eiger, which activates JNK pathway in the adjacent cells. Apoptotic cells produce also different signals that influence surrounding cells.

3. Activation of JNK Signaling Promotes Ros Production and Triggers Multiple Responses

JNK signaling is initially triggered at the wound site in response to Ros, and probably by other cellular stress signals [28]. Ros has been proposed to regulate the activity of MAP3K (MEKK1) and apoptosis signal-regulating kinase 1 (ASK1 or MAP3K5), kinases that reside upstream of JNK [16, 17, 29–31]. In addition, Ros can also block the function of the MAP kinase phosphatases that inhibit JNK signaling [30, 32]. JNK signaling promotes the activation of proapoptotic genes like *reaper (rpr), head involution defective (hid),* and *grim*. The proteins encoded by these genes bind to and inhibit the activity of Drosophila IAP-1 (Diap1), which in turn blocks the initiator caspase-9 orthologue Dronc (Drosophila NEDD2-like caspase). Dronc activates the effector caspases Dcp1 and Drice (Drosophila interleukin converting enzyme), inducing apoptosis. It has been shown that *rpr* and *hid* alter cytochrome Cytochrome C in the mitochondria, leading to mitochondrial disruption [33–36]. While the origin of Ros after damage remains unclear, the action of *rpr* and *hid* on mitochondria could favor Ros production by apoptotic cells (Figure 2).

The tumor suppressor Dp53 is another factor activated by JNK signaling and that is necessary to trigger apoptosis, playing a fundamental role in the elimination of cells that cannot complete DNA repair [37]. Both dp53 and JNK can activate each other in a Dronc-independent manner, and they establish a feedback loop that amplifies the initial apoptotic signals [38]. This loop is very important to promote cell death in response to the activation of the apoptotic pathway (Figure 2) [38]. Dp53 is required to induce compensatory proliferation and to reestablish the patterning of the damaged discs. Interestingly, it has been proposed that these functions are not dependent on apoptosis [39, 40].

JNK signaling also increases the levels of Ros by transcriptionally activating the gene *moladietz (mol)* [23]. This gene encodes the Duox-maturation factor NIP that is required for the production of Ros. Therefore, the activation of *mol* favors the production of Ros and it promotes a positive feedback signal that ensures the prolonged JNK activation necessary for regenerative growth (Figure 2) [23, 25]. Ros are also involved in the activation of *Cap-n-collar (cnc)*. The transcriptional targets of *cnc* constrain Ros levels within a range in which regeneration is most efficient [24]. The cells with the highest levels of JNK signaling die and produce high levels of Ros. Indeed, at the wound edge there are apoptotic cells that have high levels of Ros in conjunction with high levels of JNK [22]. Ros propagate from dying or dead cells to the nearby surviving cells, and they activate multiple factors

and signaling pathways. Indeed, Ros can propagate from cell to cell through aquaporins [41] or gap junctions [42].

Like other signals generated by apoptotic cells, Ros can affect surrounding cells. Various studies have shown that apoptotic cells can influence the proliferation and survival of nearby surviving cells. Indeed, apoptotic cells have been proposed to send signals that induce surrounding cells to divide or to die; events are known as apoptosis-induced proliferation (AiP) or apoptosis-induced apoptosis (AiA) [43]. It has been suggested that apoptotic cells can release mitogenic factors, such as the Drosophila Wnt1 homologue Wingless (Wg), the bone morphogenetic protein (BMP) Decapentaplegic (Dpp), and the leptin-like (IL-6 family) cytokine ligands Unpaired proteins (Upd, Upd2, Upd3) (Figure 2) (see below). Therefore, apoptosis at the wound site might fulfill a fundamental role in regulating regenerative proliferation. However it has been shown that the partial inhibition of apoptosis does not have a major effect on disc regeneration [8, 44]. A possible explanation for this paradox might be that apoptosis was not fully suppressed in any of these analysis. Thus, the signals emitted by the few apoptotic cells that remain might be sufficient to induce proliferation of the surrounding cells. More studies will be necessary to clarify the true role of apoptotic cells during regeneration.

4. P38 and JNK Signaling Promotes JAK/STAT Activation

Ros generated during apoptosis promotes tolerable levels of JNK in nearby surviving cells. Thus, in addition to apoptotic cells at the wound site, nonapoptotic cells also appear that have nondeleterious levels of JNK and low levels of Ros [22]. Hemocytes stimulated by Ros are also involved in activating JNK in surviving cells adjacent to the wound, since hemocytes release the TNF ligand Eiger that can induce JNK signaling [25]. JNK signaling plays a key role in regulating many biological processes involved in regeneration, including wound healing, compensatory proliferation, apoptosis, and cytoskeletal rearrangement. Inhibition of JNK during disc regeneration impairs wound healing and reduces regenerative proliferation [8, 28, 45, 46]. These regenerative responses depend on the activation of several downstream pathways by JNK.

In addition to the activation of JNK signaling, Ros can regulate the P38 stress-activated MAP kinases in surviving cells. The activation of P38 signaling is independent of the JNK pathway [22] and it has been proposed that Ros may promote the P38 pathway through the oxidative modification of intracellular kinases, such as the redox-sensitive activating protein-1 ASK1 [31]. The nondeleterious activation of JNK and P38 MAP kinases by Ros may have multiple effects, among them the induction of cytokine expression [11, 22, 29, 47, 48]. *Drosophila* has three leptin-like (IL-6 family) cytokine ligands known as the Unpaired proteins (Upd, Upd2, and Upd3). After physical injury or cell death, the three *upd* genes are upregulated in the wound's edges in a manner dependent on JNK signaling [22, 47, 48]. It is unclear whether these factors are expressed exclusively in dead cells (as we mentioned before), or they are also expressed in surviving cells surrounding the damage region. In this review we have considered that both dead and surviving cells can express these ligands. The Upd ligands bind to the IL-6R type receptor *Domeless (dome)*, which activates the Janus kinase Hopscotch (Hop), and this phosphorylation cascade promotes the translocation of a Stat3-like transcription factor (Stat92E) to the nucleus (Figure 3). All these factors constitute the JAK/STAT signaling pathway, which has important roles in disc development, for instance appendage patterning [49–51] and the control of cell proliferation [49, 52–54]. The elimination of JAK/STAT components during leg disc regeneration impairs local cell proliferation [48]. Similarly, during wing disc regeneration, reduced JAK/STAT activity also partially disrupts adult wing recovery, leading to the generation of much smaller adults wings [22, 47]. As such, it was proposed that JAK/STAT signaling functions downstream of JNK/P38 signaling and that it is necessary to induce compensatory cell proliferation and to form the blastema (Figure 4) [48]. However, instead of promoting compensatory cell proliferation, it has been proposed that JAK/STAT might be necessary to restrain the excessive tissue damage caused by the activation of the JNK pathway, which would facilitate the initiation of compensatory responses [47]. JAK/STAT could act as a suppressor of JNK signaling and this repression could either be mediated by direct transcriptional effects on JNK components or indirectly, by suppressing apoptosis. This mechanism could restrain the nonautonomous activation of JNK and excessive apoptosis [47]. This function of JAK/STAT would be mediated by Zfh1 and Zfh2 (Zinc-finger homeobox) proteins. These ZEB proteins that act as transcriptional repressors [47] have been previously identified to be downstream effectors of JAK/STAT [47, 49, 55]. In the promoter region of *hid* as well as in the promoter of the gene *key*, which encodes for the AP-1 component dFos, appears multiple, highly clustered mammalian ZEB1-binding motifs [47]. Therefore, it has been proposed that Zfh1 and Zfh2 might be restraining JNK activation by repressing *kay*. In addition, ZEB proteins might be also competing with AP-1 for transcriptional repression of *hid*, thereby limiting the apoptosis induced by JNK signaling through *hid* (Figure 3) [47].

In addition to the possible role that JAK/STAT might have in controlling cell proliferation, this pathway also induces a physiological response by activating Drosophila *insulin-like peptide (dilp8)* [47, 48]. This paracrine factor is activated after damage and it regulates the timing of pupariation [56, 57]. It has been reported that Dilp8 regulates both developmental delay and growth coordination between regenerating and undamaged tissue. Dilp8 inhibits the production of the neuropeptide prothoracicotropic hormone (PTTH), causing developmental delay [56, 57]. Moreover Dilp8 activates Nitric oxide synthase (NOS) in the prothoracic gland. NOS limits the growth of undamaged tissues by reducing ecdysone biosynthesis [58, 59]. The function of Dilp8 is mediated by the Orphan leucine-rich G-protein coupled receptor Lgr3. Lgr3 activity is necessary in the Central nervous system (CNS), as well as in the prothoracic gland, for NOS activation following damage [60–62].

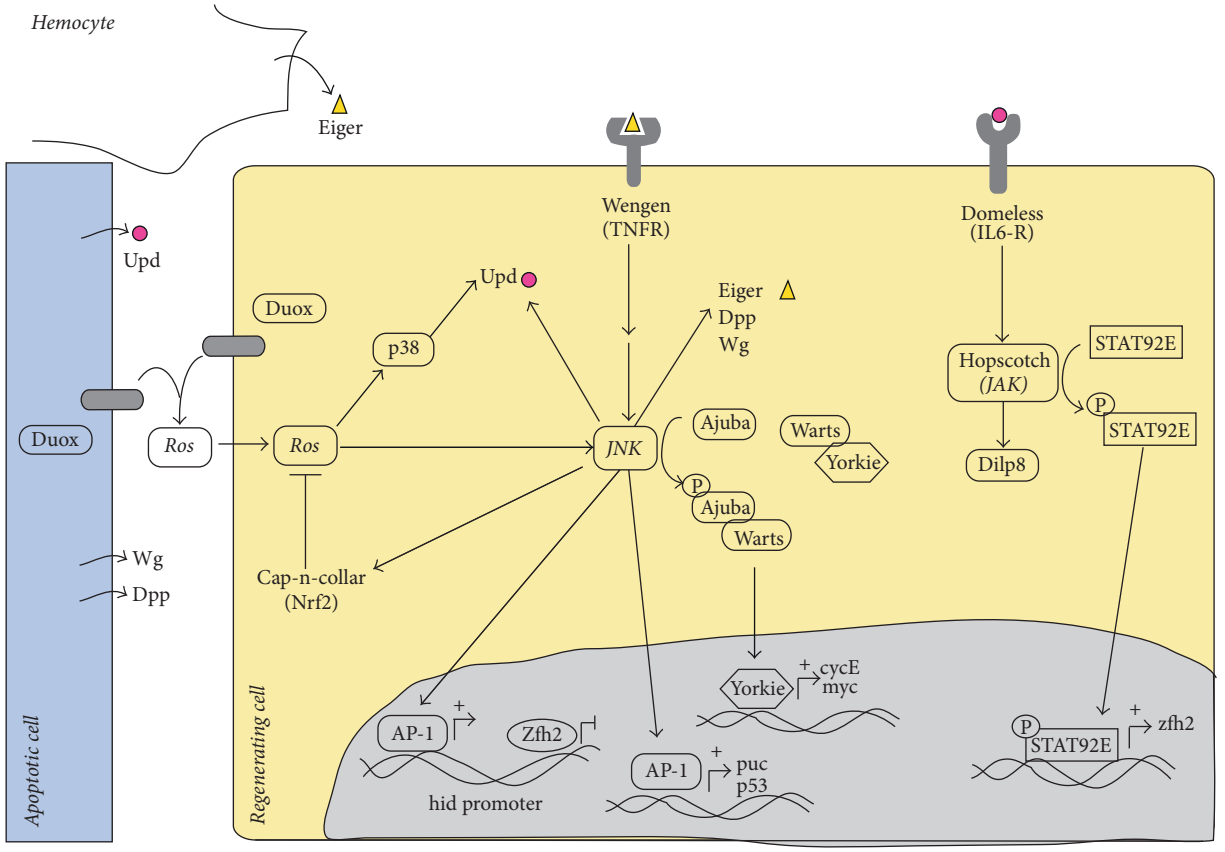


FIGURE 3: Schematic of regulatory interactions between components of signaling pathways involved in promoting Drosophila wing disc regeneration. See text for details.

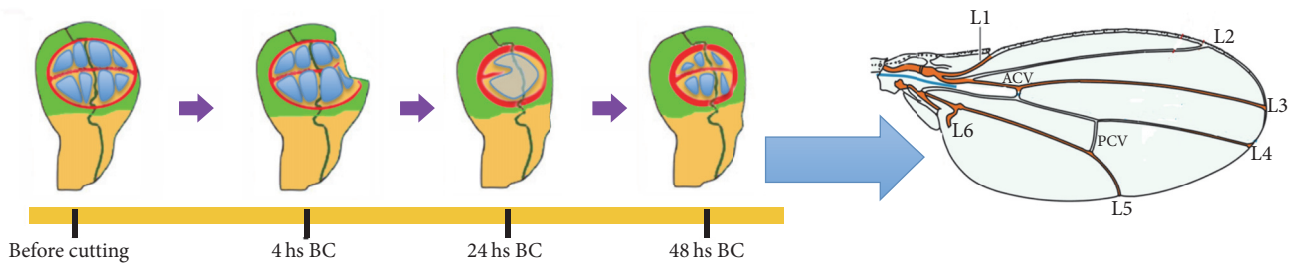
As we mentioned the activation of *dilp8* after damage depends on the JAK/STAT pathway [47, 48]; therefore, JAK/STAT signaling might favor regeneration by delaying development [47, 48, 56, 57].

5. The Role of Wg and Dpp Signaling in Disc Regeneration

The Wingless family of proteins (Wnt class) are involved in regeneration in different organisms with this capacity [2, 63, 64]. Intriguingly, different responses to *wg* expression have been observed during disc regeneration depending upon the proapoptotic gene employed or the methods of inducing the wound. Thus, *wg* (the Drosophila Wnt1 homologue) is ectopically expressed near the lesion edges before blastema formation in amputated leg and eye imaginal discs [48, 65–67]. In addition, *wg* is upregulated in the wing cells that form the blastema after genetic ablation by expressing *Eiger* or the proapoptotic gene *reaper* [9]. During these processes *wg* is activated by JNK signaling [48]. Using these experimental approaches regenerative proliferation was impaired when *wg* was reduced [9, 48]. Accordingly, it was proposed that *wg* is required for regenerative proliferation (Figure 4). This effect is at least in part due to the down regulation of Notch, which leads to Myc upregulation [9]. It has also been proposed that JAK/STAT signaling cooperates with Wg signaling to induce regenerative cell proliferation [48].

Paradoxically, when apoptosis was induced by overexpressing the proapoptotic gene *head involution defective (hid)* or when a portion of a disc is eliminated *in situ* [12], *wg* expression was not altered during disc regeneration [68]. Moreover, knocking down *wg* did not block discs regeneration after *in situ* amputation or *hid* expression [12, 68]. The basis for these differences in *wg* expression and its requirements are not yet clear. They might in part reflect differences in the efficiency of genetic ablation, or that different methods of inducing a wound elicit different responses in terms of gene expression. Alternatively, *wg* function might be redundant with the activity of other genes of Wnt family present in Drosophila, such as *wnt6*. In fact, *wnt6* and *wg* share the same regenerative enhacer (see below). Therefore, more work is needed to define the role of *wg* signaling in the regenerative response.

The bone morphogenetic protein (BMP) Decapentaplegic (Dpp) activates a signaling pathway that plays an important role in inducing growth and patterning during imaginal disc development [69–72]. Therefore, it was suggested that the Dpp pathway might be redeployed to control regenerative growth. However, the contribution of the Dpp signal to this process remains unclear. Thus, although *dpp* is transcriptionally activated in response to genetic ablation in wing



Ca2+	JAK/STAT	Downregulation of Polycomb genes
Duox	Yorkie	Trithorax
Ros	Myc	Taranis
Mol (Cap-n-collar)	Wg	
P38	P53	
JNK		

Figure 4: Schematic representation of the different factors and signaling pathways involved in the regulation of the cellular processes that occur during wing disc regeneration; see text for details.

discs [9], this is not the case in amputated wing discs [46]. Moreover, while Dpp is required for the hyperplastic growth caused by "undead" cells, when apoptotic cells are protected with P35 [73, 74], this factor is dispensable for compensatory cell proliferation when P35 is not ectopically expressed in apoptotic cells, even though Dpp is expressed in apoptotic cells [74, 75]. As yet, the basis for these differences remains unclear.

It has been proposed that both *wg* and *dpp* are activated in apoptotic cells and diffuse to surrounding cell to promote proliferation [73–75]. However, as JNK signaling pathway is active, although at low levels, in surviving regenerating cells [22], and *wg* and *Dpp* are targets of JNK signal, we do not exclude the possibility that these factors might be also expressed in some regenerating cells (Figure 3).

6. The Hippo Pathway Is Necessary for Regenerative Growth

Hippo signaling is a conserved pathway that regulates growth during development and regeneration, and its deregulation is associated with oncogenesis (reviewed in [76, 77]). This signaling pathway is constituted by a kinase cascade that can be activated by different stimuli. Hippo signaling is mediated by a transcriptional coactivator protein, Yorkie (Yki in *Drosophila*, YAP in vertebrates: reviewed in [77, 78]) (Figure 2). Yki remains inactive when the signaling pathway is active and it is retained in the cytoplasm due to its phosphorylation by the kinase Warts (Wts) [79]. When Wts is inactive, unphosphorylated Yki accumulates in the nucleus [77, 78] and in conjunction with different DNA-binding proteins, it promotes the transcription of downstream genes necessary to promote cell proliferation, such as Cyclin E and cMyc [77, 78] (Figures 3 and 4).

The Hippo pathway plays a key role in inducing regenerative growth after disc damage [80–82]. This pathway can be activated by multiple upstream inputs, including Fat–Dachsous signaling, sense tissue damage, and JNK signaling [82, 83]. JNK signaling can directly promote the activation of Yki by phosphorylating Ajuba family LIM proteins and enhancing their binding to Wts, thereby preventing their activation by Hippo [84]. Interestingly, the ability of JNK to activate YAP is conserved in mammalian cells [83, 84]. Thus, JNK increases Yki activity after wounding, a process essential to induce compensatory cell proliferation and regeneration.

7. The Control of Cell Plasticity during Imaginal Disc Regeneration by the Polycomb Group (PcG)

One of the processes associated with organ regeneration is the repatterning of the regenerating tissue, which implies genetic reprogramming of cells in order to switch their fates (Figure 4). After damage, newly formed tissue is derived from surviving cells that lie nearby and some of these cells must change their state of determination to contribute to the lost region.

During disc regeneration several observations indicate that cell fates are respecified and that there is a process of cell reprograming. For example, there is a temporary loss of markers of cell fate commitment after genetic ablation or disc amputation [9, 12, 85]. It has also been reported that after genetic ablation in the wing pouch, the cells of the hinge generate cells that become part of the pouch [68, 86]. Moreover, cell fate changes between compartments have been reported after surgical excision [66] or genetic ablation [87]. Indeed, cells near the anterior/posterior or dorsal/ventral boundary can change their identities and contribute to the compartment on the other side of the boundary [87]. Finally,

during regeneration the cells of one disc occasionally acquire the identities of different imaginal discs, switching cell fate and generating disc-inappropriate structures, a process known as transdetermination [88].

The preservation of a specific cell fate or determination state depends on a particular genetic program, which is largely maintained through epigenetic modifications that are established during development. The polycomb group (PcG) proteins function as epigenetic modifiers and they are required to maintain cell fates by controlling the expression of developmental regulators [89]. This group of proteins forms two different types of complexes, Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2). PcG can silence large numbers of genes by establishing repressive marks like histone H3 lysine 27 trimethylation (H3K27me3). There is evidence that JNK signaling downregulates PcG genes during regeneration, thereby allowing the transcription of otherwise silenced genes [90]. This process is important for cell reprogramming during regeneration. Inappropriate or excessive downregulation of the PcG by JNK during regeneration may activate genes that induce a genetic program corresponding to a different disc, provoking transdetermination. Indeed, the frequency of transdetermination is enhanced in PcG mutant discs [90]. Interestingly, ectopic activation of *wg* can induce transdetermination, possibly because *wg* might be a direct target of the PcG [90].

The preservation of the anterior/posterior compartment identity during regeneration is mediated by *taranis (tara)*, that is, the homologue in Drosophila of the vertebrate TRIP-Br (Transcriptional Regulators Interacting with plant homeodomain (PHD) zinc fingers and/or Bromodomains) family of proteins. In mutant conditions for *tara,* regenerating wing disc undergoes posterior-to-anterior transformations late in regeneration. These changes are consequence of the misregulation of posterior selector gene *engrailed (en)*. The deregulation of *en* leads to the autoregulatory silencing of the *engrailed* locus, which requires the PRC1. The misregulation and subsequent silencing of *en* are induced by JNK signaling. It has been proposed that Tara stabilizes *engrailed* expression downstream of JNK signaling to maintain the posterior cell fate identity during regeneration [91].

Recently, a defined regulatory element was identified that is responsible for the activation of *wg* expression after damage [92]. Interestingly, this regenerative enhancer (BRV118) regulates the expression of *wg* and *wnt6*. This observation suggests that the function of different members of Wnt family might be involved during regeneration in Drosophila. It has been described that within this enhancer there is a damage-responsive module that remains active throughout the third instar stage and an adjacent silencing element that nucleates increasing levels of epigenetic silencing during development. This latter element can restrict the activity of this enhancer [92]. Therefore, the loss of the regenerative capacity of the discs as development proceeds might be explained by a blockade of the damage-responsive enhancers through the activity of the silencing elements. This mechanism might prevent gene expression in the mature organism without compromising the gene activity regulated by developmental signals [92]. Interestingly, PcG-mediated epigenetic silencing is required to regulate the activity of this enhancer. Hence, the inability of the cells in adult tissue to reactivate programs necessary to promote regenerative growth or cell fate respecification could limit regeneration in adult stages.

8. Perspectives

The urodele amphibians have been used extensively as a model system to study regeneration as they present a remarkable regenerative capacity and they can fully regenerate amputated appendages [93]. While the studies carried out on these organisms allowed multiple cellular processes involved in limb regeneration to be identified [93], much less is known about the genetic mechanisms that control them, as amphibians are not the best model organisms for genetic analyses. Moreover, most studies into regenerative biology aimed at developing biomedical applications have been carried out on stem cells cultivated *in vitro*. To better understand the processes that occur during regeneration, these phenomena must be studied *in vivo*, in the context of the complex genetic and cellular interactions that take place. Drosophila is a complex model organism in which the mechanistic details of genetic and cellular processes can be defined. In addition, Drosophila has been extensively used as a model system to carry out unbiased genetic screens to identify genes involved in different cellular processes. These features make Drosophila an excellent model to identify and characterize genes involved in all aspects of regeneration. In fact, different genetic screens and studies of the changes in gene expression during disc regeneration have identified multiple signaling pathways and genes required for different processes associated with regeneration [23, 24, 94].

The conservation between flies and vertebrates of basic signaling pathways and their regulatory elements justifies using Drosophila as a model organism to establish mechanisms and genetic processes that can be translated to vertebrates. Different studies have confirmed that most of the signaling pathways required for disc regeneration are also involved in regeneration in vertebrates; for example, the Hippo pathway appears to play a fundamental role in vertebrate limb regeneration and in skin wound healing [95, 96]. JNK is very important in mammalian liver regeneration and one of its targets, the AP-1 transcription factor subunit c-Jun, is activated during liver regeneration [97–99], the cytokines TNF-α and IL-6 also being required during this process [99]. Finally, and as in Drosophila, PcGs are downregulated during murine skin repair, which provokes the derepression of *dmyc* [100]. Moreover, it has been suggested that that loss of polycomb-mediated silencing might contribute to the induction of repair genes in mammals.

In summary, basic regenerative research carried out in Drosophila can provide insights into the genetic and cellular responses involved in mammalian regeneration. This knowledge might serve to develop new therapies in regenerative medicine.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Review Article

Drosophila as a Model System to Study Nonautonomous Mechanisms Affecting Tumour Growth and Cell Death

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The study of cancer has represented a central focus in medical research for over a century. The great complexity and constant evolution of the pathology require the use of multiple research model systems and interdisciplinary approaches. This is necessary in order to achieve a comprehensive understanding into the mechanisms driving disease initiation and progression, to aid the development of appropriate therapies. In recent decades, the fruit fly *Drosophila melanogaster* and its associated powerful genetic tools have become a very attractive model system to study tumour-intrinsic and non-tumour-derived processes that mediate tumour development *in vivo*. In this review, we will summarize recent work on *Drosophila* as a model system to study cancer biology. We will focus on the interactions between tumours and their microenvironment, including extrinsic mechanisms affecting tumour growth and how tumours impact systemic host physiology.

In loving memory of Marcos Vidal, whose work and ideas inspired this review and continue inspiring our work

1. Introduction

Despite being the most studied human disease, cancer remains a leading cause of mortality worldwide. Nearly 1 in 6 deaths in 2015 was attributable to cancer, according to the World Health Organization, with an increase of 70% of new cases projected within the next two decades [1]. The seemingly restricted success in controlling and reducing the devastating outcomes of this disease is due, to a great extent, to the high complexity and variable nature of the pathology. The current limited understanding of many aspects of cancer biology is partly imposed by limitations in conventional animal models of research.

The organismal implications and ultimate outcome of tumour burden in patients are undoubtedly determined by a combination of tumour-intrinsic mechanisms and interactions between tumours and proximal, as well as distal tissues [2–4]. While cancer research has classically focused on identifying tumour autonomous processes, there is a recent growing interest in understanding the nonautonomous mechanisms that control tumour progression [5]. Indeed, pioneering work dating back to the 19th century established the notion that distant tissues influence tumour growth and metastasis, when in 1896 Sir Beatson published a report on the treatment of inoperable cases of breast carcinomas through ovariectomy [2]. More recently, many molecular mechanisms have been identified highlighting the importance of the tumour microenvironment (TME) in cancer progression [5]. The crosstalk between tumour cells and their microenvironment often resembles normal physiological responses: for example, interactions between cancer cells and the immune system imitate various aspects of host-pathogen interaction [6]. In such a context, the body can detect cancer cells and react by mounting an immune

response, to fight abnormal cell behaviours associated with the presence of a tumour. However, tumour cells appear to evolve to turn on new or divert existing physiological programs in order to evade the action of the immune system [6, 7]. The end result of such a power struggle between cancer cells and the surrounding tissues will ultimately determine the outcome of the tumour and its host. Targeting non-tumoural tissues to counteract cancer growth is becoming a prime therapeutic strategy, which takes advantage of the higher genetic stability and lesser susceptibility of normal cells to escape drug treatments [8]. Hence, the discovery of novel non-tumour autonomous mechanisms to fight cancer progression is a promising area of research. However, the physiological complexity and limitations in the genetic accessibility of mammalian models systems render *in vivo* studies of non-tumour autonomous processes difficult to accomplish in conventional whole animal model systems.

Drosophila melanogaster remains the most powerful genetic model in research. During the last decades, the development of various tumour models, including leukaemia, neuroblastoma, glioblastoma, colorectal, and ovarian cancer, has made the fruit fly an attractive *in vivo* model system to decipher tumour intrinsic (i.e., tumour cell-autonomous) and extrinsic (i.e., non-tumour autonomous) molecular mechanisms mediating tumour growth and metastasis [9, 10]. Such studies have revealed astonishing conservation in the processes driving cancer development between flies and humans [10]. The ability to spatially and temporally regulate gene expression in tumour-bearing animals, as well as the low genetic redundancy, is particularly useful for the study of non-tumour autonomous mechanisms. Major advances in the understanding of these tumour-extrinsic mechanisms have been provided through the use of models based on loss of cell polarity, utilising mutants of the *scribble*-group of tumour suppressors genes (*scribble: scrib*, *lethal giant larvae: lgl*, and *disc large: dlg*), which encode key components of the basolateral polarity complex [11]. These mutations induce transformation of larval epithelial tissues, called imaginal discs, into "benign" neoplastic tumours. In this context, activation of proto-oncogenes, such as *Ras* or *Src,* drives tumour cell proliferation, spreading to distant tissues [12, 13]. During the years following the discovery of *scrib*-group genes as tumour suppressors in *Drosophila*, research has provided growing evidence that these models are directly relevant to human conditions. Indeed, scrib and dlg proteins are known targets of several oncogenic viruses, such as Human Papillomavirus, the main agent of cervical cancers. These viruses induce the degradation of the polarity complex proteins, comprising a key part of the process of malignant transformation in these conditions [11, 14, 15]. Loss of *scrib* has also been shown to work as a tumour suppressor in human breast, liver, skin, and lung cancers [16–19]. The loss of the human homolog of the Lgl protein has been involved in colorectal cancer [20] and hepatocarcinoma [21] and is associated with an increased risk of metastasis in endometrial cancer [22]. Moreover, similar to its *Drosophila* homolog, *scrib* also cooperates with the Ras oncogene to promote tumour cell invasion [12, 23].

Here, we discuss recent discoveries in *Drosophila* that have shed light into how extrinsic signals influence tumours, as well as mechanisms that mediate the systemic impact of tumours in the host. We focus on new findings highlighting the influence of immunity and metabolism in cancer progression and cancer-related disorders.

2. Cellular and Systemic Immunity Influence Tumour Growth and Cell Death

2.1. The Immune System: A Double-Edged Sword. Work in mammals has highlighted the immune system as a key component of the tumour microenvironment (TME), which plays a critical role in defining tumour outcome. While early studies on cancer patients support anticancer activity of the immune system [24], recent research has revealed that immunity can also promote tumour growth and metastasis [25]. However, deciphering the mechanisms of this dual immune function is a challenging task, mostly due to the complex cellular and molecular composition of the mammalian immune system [5]. For the past 15 years, the development of cancer models in *Drosophila* has allowed the discovery of molecular mechanisms mediating both pro- and antitumoural immunity. In contrast to mammals, which possess both innate and adaptive immunity, *Drosophila* only relies on innate immunity to fight against pathogens and tumours. Additionally, while mammals have numerous types of white blood cells, the cellular arm of *Drosophila* innate immunity includes only three main cells types—plasmatocytes, lamellocytes, and crystal cells—commonly called haemocytes. Only plasmatocytes have been currently reported to be associated with tumours [26]; however, a possible diversity within the haemocyte population bound to tumours cannot be excluded. Even if such macrophage-like cells were unable to infiltrate tumours as macrophages do, they could still produce a cocktail of mammalian-like cytokines leading to inflammation. While short-term inflammation can be beneficial to protect the host from challenges, such as those posed by pathogenic infection, chronic inflammation is associated with tumour initiation and metastasis in both *Drosophila* and mammals [27–29].

Tumour Necrosis Factor alpha (TNF-α) is a major proinflammatory cytokine produced within the TME, which was originally characterised for its ability to induce tumour death [30]. Consistently, TNF-α's discovery led to great expectations for its use as a therapeutic target for cancer. However, further experiments have revealed a dual role for TNF-α as both an anti- and protumour factor [31]. The molecular bases of TNF-α's antagonistic actions were poorly understood. However, recent research in *Drosophila* has highlighted some key molecular aspects underlying this dual action of the cytokine. *Drosophila* possesses a single TNF-α homolog called Eiger (Egr) [32, 33], whose role as an immune proinflammatory cytokine is conserved [34]. The importance of Egr in the TME has been highlighted in *Drosophila* tumour models through the use of mutants of the *scrib*-group of tumour suppressors genes. Egr expression is induced in tumours and tumour-associated immune cells

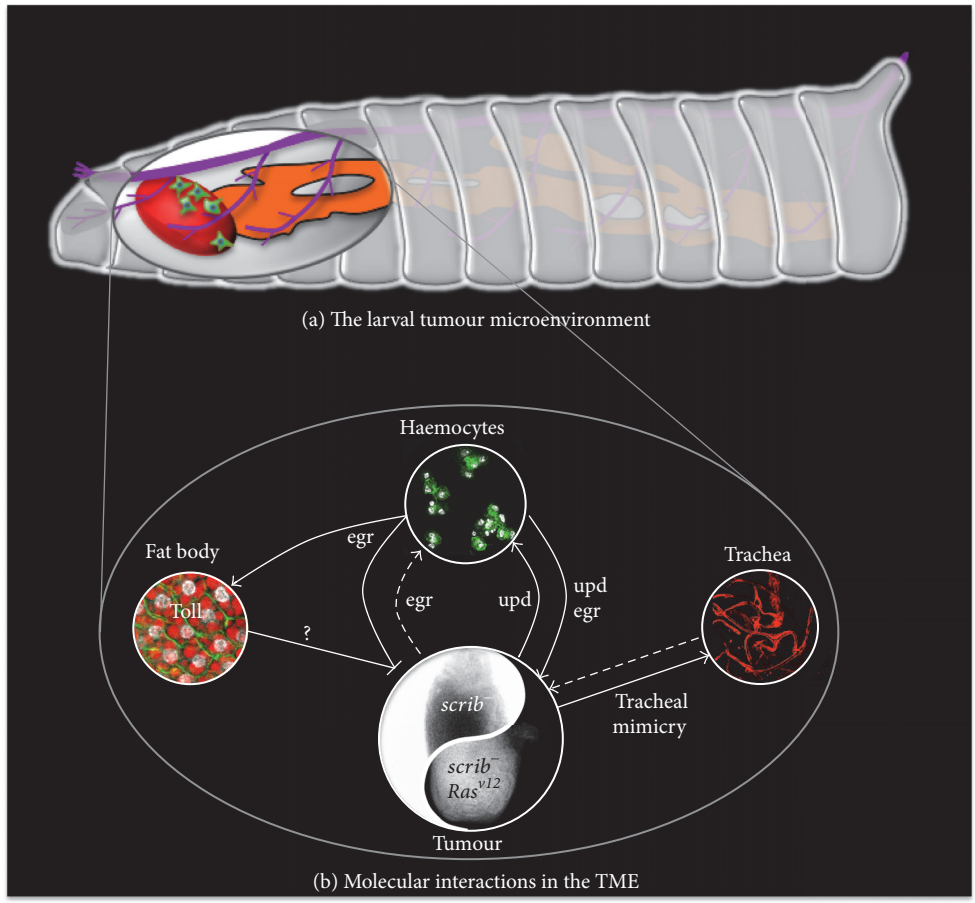

Figure 1: Immune interactions between larval tumours and their microenvironment (TME). (a) In *Drosophila* larvae, where tumours are generated in imaginal discs (tumour in red), the TME consists mostly of immune cells (in green), the fat body (in orange), and the trachea (in purple). (b) The molecular interactions within the TME are represented in this figure. Positive effects on growth and/or proliferation are highlighted by lines ending in arrowheads, while lines ending in bars show negative effects, mostly represented by increased cell death. Solid lines indicate demonstrated interactions and dashed lines potential ones. Both the immune cells and the tumour produce the fly TNF homolog Egr. It acts as a double-edge sword depending on the context of the tumour, represented as the Ying-Yang paradigm. Egr is antitumour in *scrib*-group mutant contexts, while being protumour and prometastatic when Ras^{v12} is present in the *scrib*-group mutant genetic background. The effect of tumour-derived Egr on immune cells is still an open question. Egr is required to activate the Toll pathway in the fat body, which subsequently promotes tumour cell death in combination with Egr itself, through an unknown signal (question mark). The interleukin homolog Upd3 produced by the tumour induces immune cells proliferation, while immune cell-derived Upd3 promotes tumour proliferation and invasion. While tumour can promote tracheogenesis through incorporation of tumour cells into the tracheal wall (tracheal mimicry), the effects of trachea on tumour growth and metastasis remain elusive.

[35, 36], much like mammalian TNF-α, which is detected in tumour cells as well as macrophages and T lymphocytes [31]. Given the focus of this review, we will only discuss the extrinsic role of Egr here. However, a tumour-intrinsic role of the cytokine has also been previously demonstrated [35, 37].

2.2. Cellular Arm of the Immune System and Associated Cytokines. Experimental evidence showed that immune cell-derived Egr has antitumoural activity. Patches of *scrib*, *lgl*, or *dlg* mutant cells generated in imaginal discs, delaminate, and are mostly removed from the epithelia through cell competition [12, 37–40]. However, in Egr mutant animals elimination of polarity deficient clones is abolished, and this effect can be recapitulated by knocking down *Egr* specifically within haemocytes, highlighting a conserved non-tumour autonomous anticancer function of Egr in *Drosophila* [35, 36] (Figure 1). Complementarily, loss of the TNF-α receptor Grindelwald (Grnd) in *scrib* mutant cells suppressed their removal from the epithelia [41]. In those cases, where a group of mutant cells is generated in a wild-type background, the elimination of mutant cells through cell competition relies on Egr-dependent JNK activation, which subsequently restricts cell proliferation and the survival of mutant cells [35, 38, 42]. This JNK-dependent toxic effect of TNF-α is conserved in mammals, as TNF-α induces cell death through TNFR1 and subsequent JNK signalling activation [29]. Recent discoveries of new molecules driving cell competition in *Drosophila*, including immune response proteins, may uncover new mechanisms involved in the elimination of cancer cells from a healthy tissue [43–45]. Egr has also

been shown to exert antitumoural effects independently of cell competition. Full mutants animals for *scrib*-group genes, where neoplastic tumours develop from the whole imaginal disc, also show dependency from haemocyte-derived Egr to trigger JNK activation and tumour cell death [36, 46] (Figure 1). These studies highlight the importance of the TME and demonstrate a conserved antitumoural function of TNF-α-dependent inflammation in *Drosophila* models of cancer.

In contrast to the described antitumoural functions, *Drosophila* TNF-α can also exert protumoural effects. Evidence for such a role is provided by studies on tumours where *scrib*-complex mutations are associated with a constitutively active form of Ras (Ras^{v12}). Ras is a conserved proto-oncogene mutated in many cancer types, with a 16% overall incidence rate in all analysed human tumours [47]. In *Drosophila*, clones of cells mutated for *scrib*-complex proteins and overexpressing Ras^{v12} fail to be eliminated by surrounding epithelial cells. Instead, they form neoplastic tumours that can invade distant tissues [12, 48]. While JNK is required for cell death in *scrib* mutant clones, cooperation with Ras^{v12} in these clones diverts the function of JNK pathway activation toward tumour cell proliferation and invasion [39, 49]. In this context, haemocyte-derived Egr has also been shown to promote JNK activation, as knockdown of Egr specifically in immune cells abolished JNK activation and restricted the ability of *scrib, Ras^{v12}* mutant cells to grow and invade. Strikingly, transplantation of Egr-wild-type immune cells could rescue the progression of *scrib, Ras^{v12}* tumours, as well as JNK activation, providing the final demonstration that *Drosophila* TNF shares protumour effects with its mammalian counterpart [36] (Figure 1). This is further supported by observations of high expression levels of Grnd in *scrib, Ras^{v12}* tumours and by data showing that *Grnd* knockdown in those tumours also disrupts their growth and invasive properties [41]. Interestingly, tumours display increased levels of ROS, which have been reported to promote haemocyte-dependent Egr secretion and subsequent JNK-induced proliferation in response to apoptosis, suggesting a protumoural feedback loop mechanism [50]. Further insights into the mechanisms mediating this protumorigenic role of Egr come from a recent demonstration that caspase-dependent ROS production in cancer cells is required for the recruitment of macrophages into *scrib*, Ras^{v12} tumours [51]. This work demonstrates that Ras^{v12}-driven tumour progression requires the activation of Caspases, which function as tumour promoters. This mechanism is suggested to be one of the key mediators of the switch of Egr from an antitumour to a protumour cytokine by Ras. The protumoural function of TNF-α produced by immune cells is highly reminiscent to the one described in mammalian systems. In a mouse model of skin carcinogenesis where loss of TNF-α suppresses tumour formation [52], transplantation of B-cells from TNF-α competent mice is sufficient to restore tumour formation. However, this effect appears to be indirectly mediated through TNF-α-dependent regulation of T-cell number [53]. A more direct parallel between TNF-α-dependent antitumoural responses in flies and humans comes from work on Kras-dependent intrahepatic cholangiocarcinoma. In this context, TNF-α produced by Kuppfer cells (liver-specific myeloid cells) drives preneoplastic lesions through JNK signalling pathway activation [54].

The demonstration of antagonistic actions of TNF-α in *Drosophila* and mammalian tumours suggests that the successful use of antitumoural immunity as a cancer therapy may strongly depend on, and must take into consideration, the genetic composition of the tumour. This is further supported by data showing that not all neoplastic tumours are sensitive to Egr. The neoplastic growth induced upon knockdown of *avalanche (avl)*, a Syntaxin involved in the fusion of endocytic vesicles to the early endosome, is dependent on *Grnd* but escapes the need for *Egr* [41]. Interestingly, *avl* tumours produced high levels of Wingless (Wg) protein, which is a known target of JNK pathway activation and a key driver of compensatory proliferation, which is linked to cancer progression [55, 56]. It is therefore conceivable that the genetic properties and/or tissue location of a tumour dictate its sensitivity to different signalling pathways. A recent study in *Drosophila* showed that Wg dependent tumours proliferate independently of the TME and TNF-α/Grnd [57]. Similarly, tumours bearing combined loss of Ras^{v12} and hyperactivation of the nonreceptor tyrosine kinase Src, which also feature Wg overexpression [58], are largely insensitive to Egr loss (J.B.C. personal communication). High Wg activity could therefore be one of the factors rendering tumours insensitive to TNF-α. The expression of growth factors and activation of downstream signalling pathways in epithelial tissues in general and in *Drosophila* imaginal discs in particular are usually restricted to certain tissue locations [59, 60]. Recent work in *Drosophila* identified the presence of "tumour hot-spots." Tumour hot-spots are defined as locations within tissues where neoplastic mutations are more likely to result in successful tumoural growths capable of invading normal tissues and it is a process involving differential activation of JAK/STAT signalling [61]. It is likely that additional spatially restricted factors, including graded morphogens, such as Wg, Decapentaplegic (Dpp), or Hedgehog (Hh), may influence "tumour hot-spots" and, therefore, the potential impact of TNF-α in this context.

A key phenotypic feature of *scrib*-group mutants is the loss of epithelial cell polarity. In tumours lacking *lgl*, knockdown of the JNK pathway rescues loss of cell polarity [62]. Loss of cell polarity is required for epithelial-mesenchymal transition (EMT), which drives tumour progression, including invasion [63, 64]. Given that Egr is a major driver of JNK pathway activation, the fly TNF-α may be a determinant in the loss of cell polarity in tissues carrying these neoplastic transformations. Indeed, Egr regulates asymmetric localisation of determinants of asymmetric division, Miranda and Prospero, in neuroblasts, supporting a role for Egr in cell polarity determination [65]. Interestingly, TNFα-dependent loss of cell polarity has been reported upon induction of chronic inflammation in the mouse intestine [66]. Likewise, a recent report shows that TNFα-dependent EMT increases lung cancer metastasis [67]. This possible relationship between TNF-α and cell polarity could also be the driving force for TNF-α's protumour effect

on Ras^{v12} expressing cells, as Ras hyperactivation facilitates the prosurvival function of JNK signalling.

The discovery of other immune-derived cytokines may have implications on their role in cancer progression through the TME. Haemocyte-derived Dpp, the fly homolog of Bone Morphogenetic Protein 2/4 (BMP2/4), a member of the Transforming Growth Factor beta (TGF-β) signalling family, can promote intestinal stem cell (ISC) proliferation in response to infection [68]. A similar effect has been reported in response to both septic and aseptic injuries for hemocyte-derived unpaired 2 and 3 (Upd 2/3), the *Drosophila* interleukin homologs that function as ligands of the JAK/STAT pathway [69]. Consistently, in *scrib* mutant larvae Upd 3 produced by the tumour induces JAK/STAT activation in the immune tissues (fat body and haemocytes), leading to a positive feedback loop that increases Upd 3 levels in haemocytes, which is required for JAK/STAT-induced proliferation of haemocyte and subsequent tumour suppression [26] (Figure 1). On the other hand, Upd3 can also impact JAK/STAT activation within *scrib*/Ras^{v12} tumour, where it cooperates with JNK to promote growth and metastasis [48] (Figure 1). A protumour effect of JAK/STAT signalling is also reported in fly leukaemia model, as its activation is sufficient to drive *Drosophila* blood cell neoplasia [70].

2.3. The Humoral Immune Response to Tumours. While the local immune response to tumours is receiving great interest for the design of new immunotherapies, the role of systemic immunity in mammals remains elusive. However, recent advances are highlighting the importance of systemic immunity to drive successful immunotherapy [71]. Pioneering work done in *Drosophila* has demonstrated a role of systemic or humoral innate immunity in the impairment of tumourigenesis. The main organ involved in humoral immunity in *Drosophila* is the fat body, which processes analogous functions to the mammalian liver and adipose tissues. Several conserved immune signalling pathways are activated in the fat body upon infection, including Toll, immune deficiency (Imd), and JAK/STAT signalling [72]. Activation of those pathways leads to the expression of downstream effectors (antimicrobial peptides, turandots, clotting factors, serine proteases, TEPs, serpins, and cytokines), which act by clearing the underlying infection and promoting recovery of infected tissues [73]. Interestingly, tumour-bearing animals show activation of the humoral immune response [46]. Unexpectedly, activation of the Toll signalling pathway in the fat body of tumour-bearing animals could be prevented by knocking-down the Toll ligand *Spaetzle (spz)* in haemocytes or by removing Egr from tumours, suggesting that Egr produced by the tumour promotes Spz production by haemocytes, which in turn activates the Toll pathway in the fat body [46]. Toll knockdown in the fat body leads to increased tumour size and decreased tumour cell death. Conversely, Toll overexpression is sufficient to induce tumour cell death and decrease tumour size, a process that requires haemocyte-derived Egr [46]. All together, evidence shows that TNF-α-dependent activation of systemic Toll signalling is an important component of a nonautonomous tumour suppressor program (Figure 1). The exact mechanisms of Toll activation, as well as the downstream effector(s) of the Toll pathway in tumour-bearing animals, remain elusive. Interestingly, downstream Toll targets expressed following infection include antimicrobial peptides (AMPs), which have been reported to exert antitumoural activity *in vitro* [74].

It is worth mentioning recent technical advances in flies that have provided new means to study the interactions between the tumour and the TME or more distant tissues. Tumour allografts have been a powerful technique to assess some physiological aspects of tumour growth and metastasis [57, 75–77], permitting independent genetic manipulation of tumours and non-tumour host tissues. Furthermore, it is likely that the use of new genetic tools that allow manipulation of gene expression independently from the widely used Gal4 system, such as the LexA/LexAop and QF/QS/QUAS systems [78, 79], will be extremely useful to study the influence of distant tissues on tumours. However, to this end the development of new fly lines is required, in order to establish these alternative gene-driving systems for use in large/unbiased screening of processes involved in tumourigenesis in *Drosophila*.

2.4. The Tracheal System and Its Role in Tumourigenesis. The vascular system of vertebrates is known to play a critical role in the tumour microenvironment, through interaction with the tumour and the immune system. Indeed, blood vessels deliver oxygen and nutrients, as well as immune cells, to all tissues. The fast-growing properties of cancers lead to the development of some hypoxic areas that are not vascularised. As a result, angiogenesis is required, in order to sustain the high demand for oxygen and nutrients necessary to ensure tumour growth. This therefore constitutes an attractive target for interfering with tumour development [80]. In *Drosophila,* oxygen is provided by the tracheal system that spreads throughout the animal, thus providing an analogous system to the vertebrate vasculature. Moreover, the *Drosophila* tracheal epithelium is also important in immunity, as it constitutes a physical barrier to the external milieu and is able to produce defence proteins [73]. Interestingly, a recent study showed that tracheogenesis occurs in the TME of hypoxic tumours in *Drosophila*. Strikingly, tumour cells undertake a trachea-specific developmental program and become incorporated into existing tracheal walls [81] (Figure 1). This data is reminiscent of the vascular mimicry process described in several mammalian cancer types, where tumour cells form functional blood vessel-like structures that can provide oxygen and nutrients to the tumour [82]. However, while tracheal derived Dpp is shown to influence ISC proliferation in the fly adult gut [83], the contribution of tracheogenesis to larval tumour growth and cell death and its possible contribution to antitumoural immunity remains an open question.

The studies described above highlight the importance of cellular and systemic immunity in shaping the tumour outcome. Critically, they reveal the existence of anti- and protumour mechanisms mediated by the immune system that are conserved between flies and humans and also uncover novel interactions between tumours and the immune system (Figure 1). However, even in a "simple" model system,

interactions between tumour and immune cells are extremely complex. Future work in *Drosophila* will help to better understand how the global immune response shapes the TME, and how tumours are able to influence the antitumoural immune response via interactions with their microenvironment.

3. Interactions between Host Metabolism and Tumours

3.1. Tumours Impact Systemic Metabolism. One of the striking effects of tumour burden is the alteration in host metabolism that occurs as a direct consequence of tumour development. The origins of the understanding that metabolism is altered in cancer patients can be traced back to the identification of glucose intolerance as the first systemic metabolic abnormality linked to the presence of a tumour [84]. This was followed by Warburg's discovery of the abnormal metabolism of glucose into lactate in tumours, which occurred even in the presence of oxygen [85]. Later discoveries have revealed a large panel of metabolic dysfunctions within tumours, which sustain further growth and proliferation of tumour cells. The high nutritional demand of tumours can influence nutrient availability in the TME, as demonstrated by recent work in mouse models showing that glucose restriction within the TME inhibits antitumour T-cell function [86, 87]. Furthermore, the high levels of hormone, peptides, and cytokine secretion observed during early tumour formation also affect metabolic pathways in distant tissues, leading to the hypothesis that tumours behave as "metabolic dictators" [88]. The biological complexity and limited genetic tools available in mammalian models, as well as the lack of physiological relevance of cell culture models to questions of interorgan communication, have largely hindered the investigation of altered host and tumour metabolism. As a model system, *Drosophila* has proven very relevant to the investigation of the links between tumour burden and altered systemic metabolism and the effects that this can have on both the tumour and host [89] (Figure 2).

3.2. The Effects of Diet on Tumour Burden. Obesity and type 2 diabetes are common comorbidities in modern society and are characterised by systemic insulin resistance and hyperglycaemia. These conditions are associated with an increased risk of developing cancer and are a risk factor for cancer mortality [90–94]. Insulin resistance can be modelled in *Drosophila* through the use of a high sugar diet, generating phenotypes that recapitulate the human condition [95]. In this context, small clones of noninvasive tumours cells transform into highly proliferative, metastatic tumours, due to the ability of these tumours to evade diet-induced systemic insulin resistance [58]. Tumours retain sensitivity to insulin signalling due to the overexpression of insulin receptor, as a result of elevated expression of Wg. This allows them to exploit the elevated levels of circulating glucose present in the context of the high sugar diet and peripheral tissue insulin resistance (Figure 2). It was later demonstrated [81] that activation of salt-inducible kinase in tumours from animals fed a high sugar diet functions to inhibit Hippo signalling, which facilitates the increase in Wg signalling that mediates the evasion of insulin resistance by these tumours. However, it is unclear whether nutrient availability has a universal impact on tumour growth, or whether any such dependency also relies on the genetic makeup of the tumour. An additional example of nutrient dependency can be identified in cells bearing a loss of function mutation in the tumour suppressor gene PTEN, which is commonly mutated across a broad range of cancers [96]. Under normal conditions, PTEN mutant clones in epithelial wing disc tissue show increased cell size but do not overgrow or disrupt tissue architecture. However, upon systemic nutrient restriction PTEN mutant cells display a proliferative advantage over wild-type cells, which is dependent on the function of the amino acid transporter *slimfast (slif)* [97]. Interestingly, overgrowth of PTEN mutant cells in the context of nutrient restriction was sufficient to induce systemic nonautonomous effects, decreasing the size of other tissues in the organism. PTEN mutant cells are suggested to outcompete distant wild-type cells for access to nutrients, as genetically driving growth in PTEN-competent peripheral tissues reduced the overgrowth observed in PTEN mutant cells [97]. Interestingly, the TOR pathway, a nutrient-dependent regulator of tissue growth, promotes the activity of Yki in wing discs [98], which is a known promoter of tumour growth [99–101]. This may therefore represent a possible mechanism by which increased nutrient availability promotes tumour growth in these *Drosophila* models. These findings demonstrate the drastic effect that the perturbation of host metabolism by extrinsic factors can have on tumour growth, how tumours exert systemic effects on distant tissues, and how the genetic properties of the tumour itself are critical in mediating this crosstalk.

Parallels can be drawn between the results observed in these *Drosophila* models and those found in vertebrates. Preexisting obesity and diabetes promoted tumour growth in a rat cancer model [94], while a study of over one million patients over 26 years identified diabetes as a predictor of both cancer development and cancer death [102]. *Drosophila* cancer models involving diet and obesity are therefore particularly relevant to the human condition, as the protumour effects demonstrated in the contexts of these studies appear to be conserved in higher organisms, and the mediating factors are environmental influences that are very common in developed societies. The studies discussed here highlight new aspects of tumour physiology, suggesting that tumours are direct competitors to host tissues for nutrients and are frequently able to outcompete them for access to metabolic resources through various means (Figure 2). This induces nonautonomous metabolic effects in host tissues, which are likely to be beneficial to the tumour.

3.3. Non-Tumour Autonomous Autophagy and Tumour Growth. Macroautophagy is the process of bulk degradation of cytoplasmic components, facilitating the removal of defective organelles and the recycling and remobilising of cellular resources in times of stress [103]. While intratumour autophagy has been shown to act as a tumour suppressor, Ras^{v12} tumour cells in larval wing discs activate autophagy nonautonomously in the wild-type cells of the disc,

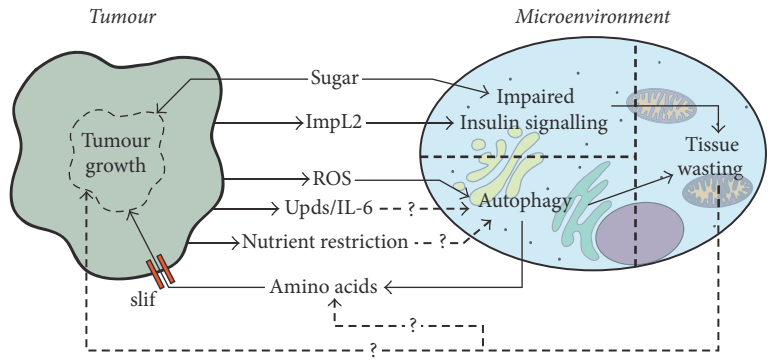

Figure 2: Metabolic interactions between tumours and their microenvironment (TME). Interactions between the tumour, the TME, and other environmental factors are represented in this figure. Solid arrows indicate demonstrated interactions, while dashed lines with question marks designate putative ones. Nonautonomous metabolic changes in the TME can affect both the TME and the tumour and are generated through various means. High levels of dietary sugar promote tumour growth and induce systemic insulin resistance in the TME. Tumours can also perturb TME insulin signalling by the secretion of an insulin-signalling antagonist, ImpL2. Autophagy in the TME promotes tumour growth through the recycling of amino acids from the TME into the tumour. Expression of the amino acid transporter *slif* in the tumour is necessary for this protumour effect. TME autophagy can be triggered by tumour-derived ROS and may also be driven by cytokine signalling or direct competition with the tumour for nutrients. Both, autophagy and impaired insulin signalling can contribute to tissue wasting and cancer cachexia. The causes of wasting in the TME and the effects of wasting in these tissues are an increasing research focus. However, the effects of TME wasting on the tumour remain an open question.

demonstrating the ability of tumours to affect the TME in this manner [104]. This was further confirmed by another study reporting systemic non-cell-autonomous autophagy in animals bearing invasive neoplastic *scrib*/*Ras*^{v12} tumours [77]. Moreover, this study demonstrated that autophagic activity in tissues both local and distal to the tumour promoted tumour growth. Inhibition of autophagy in the local TME is sufficient to significantly inhibit tumour growth and invasion, an effect that is further enhanced when autophagy is also blocked in all peripheral tissues. These results directly demonstrate that non-cell-autonomous autophagy in local and distant nontumour tissues contributes to tumour growth and invasion [77]. These data are relevant to vertebrate models, as autophagy in pancreatic stellate cells has been demonstrated to promote tumour growth in a pancreatic cancer cell line implanted into mice [105]. *Drosophila* studies have also suggested that microenvironmental autophagy fuels tumour growth through the mobilisation of nutrients from these local and peripheral nontumour tissues (Figure 2). It has been proposed that, in starvation conditions, autophagy induced by Desat1-dependent Myc activity may act in a non-cell-autonomous manner to promote tumour growth [106], while decreased amino acid transport, by the targeted knockdown of *slif* in the tumour, results in a dramatic loss of tumour growth [77]. In human cell culture models, microenvironmental autophagy has also been shown to metabolically support human pancreatic ductal adenocarcinoma in a non-cell-autonomous manner, through the provision of Alanine as a carbon source [107]. This shows that the data presented in these *Drosophila* studies is highly relevant to the vertebrate condition.

The tumour-derived factor(s) that drive the onset of microenvironmental autophagy are not yet fully defined; however, ROS signalling is an excellent candidate for further investigation (see Filomeni et al. [108] for a comprehensive review of ROS and autophagy). Starvation-induced autophagy is mediated by mitochondrially generated ROS, via the activation of the TOR pathway [109], while ROS are elevated in *scrib*/Ras^{v12} tumours, and the generation of mitochondrial ROS is sufficient to induce local autophagy in wing discs [77]. Manent et al. [104] provide evidence that ROS derived from tumour cells is sufficient to induce autophagy nonautonomously in the local microenvironment and that this also activates protumour JNK signalling in these cells. Altogether, these studies suggest that tumour-derived ROS might act as a convergent signal that triggers non-cell-autonomous microenvironmental autophagy and JNK signalling in the TME, both of which are protumour events (Figure 2). There is also some evidence in mouse models to support the idea that ROS may play an important role in TME autophagy. Fibroblasts that suffer oxidative stress induced by ROS and hypoxia in the TME undergo autophagy, which acts to degrade mitochondria. This alters the metabolism of these cells towards aerobic glycolysis, which, combined with autophagic degradation, is suggested to provide recycled nutrients from the TME to the tumour to fuel growth [110]. The transfer of energy between tumour and the TME in the form of metabolites is suggested to maintain the TME in a protumour setting [88]. Another recent work performed in cell culture and mouse models suggests that tumour-derived IL-6 may be a candidate for inducing autophagy in more tissues distal tissues from the tumour [111]. This work may represent an interesting novel target for the focus of research on the effects of peripheral tissue autophagy in *Drosophila* cancer models, as the expressions of IL-6-like Upd ligands are elevated in *Drosophila* neoplastic tumours [112]. There is little work exploring the potential interactions between ROS, autophagy, and Il-6 signalling in the context of the TME, and

given the studies discussed here, *Drosophila* may represent a suitable model for further work into the interactions between these factors and their combined impact on the tumour and the TME. The importance of *Drosophila* studies on microenvironmental autophagy is reinforced by the apparent conservation of mechanisms in human patients and other vertebrate models systems. Further work in *Drosophila* is likely to be invaluable in improving our understanding of how metabolic changes in TME may affect tumours and shape tumour-host interactions.

3.4. Cancer-Associated Cachexia. One of the best-recognised outcomes of altered host metabolism in the context of tumour burden is the condition of cancer cachexia, a paraneoplastic syndrome that results in the dramatic loss of muscle and adipose tissue [113]. Cachexia is a highly multifactorial condition with numerous metabolic aberrations implicated in the onset of the condition, including perturbed insulin signalling, systemic hypercatabolism, inflammatory and immune responses, and deregulation of muscle homeostasis [114–117]. Cachexia is a highly deleterious condition, as it decreases patient tolerance to cancer therapies, negatively affects quality of life, and increases the risk of mortality, with up to 30% of cancer patient deaths occurring as a direct result of cachexia [118, 119]. Importantly, there is no clear therapeutic gold standard for the treatment of cachectic patients, in part due to the poorly understood aetiology of the condition. Cancer cachexia represents an extreme example of the effect a tumour can have on the host, as the presence of the tumour generates such a strong alteration of the host's metabolic state that it leads to the development of a novel pathology. There are unanswered questions about the systemic effects of cachexia beyond the direct effects of the wasting itself, including whether cachexia has a functional role that affects the tumour or other tissues.

Two independent models of cancer cachexia have shown the utility of *Drosophila* in this field of research [76, 120]. Both reports demonstrated that tumours secrete high levels of *imaginal morphogenesis protein-Late 2 (ImpL2)*, a secreted insulin-signalling antagonist that functions by direct binding to Dilp2 [121]. These studies also showed that tumour-bearing flies developed systemic insulin-resistance phenotypes in tissues distal from the tumour. This insulin resistance promoted tissue wasting, a process that is also likely to occur in human patients and other animal models [122–126] (Figure 2). RNAi knockdown of *ImpL2* in the tumour was sufficient to reduce the systemic insulin resistant phenotype and thus partially rescue the wasting phenotypes observed in peripheral tissues, without impacting the growth of the tumour [76, 120]. This work provides an excellent example of the use of *Drosophila* cancer models in the field of tumour-microenvironment interactions. Research into cachexia is an emergent field, and the identification of a tumour-derived factor that mediates a systemic effect on host tissue metabolism is an important example of the ability of *Drosophila* models to recapitulate and dissect complex phenotypes. Interestingly, autophagy is one of the main mechanisms of tissue degradation during cancer cachexia [111, 127, 128]. There are direct associations between whether tumours are cachectogenic and their ability to induce autophagy [111]. Together, these studies raise an interesting open question as to the functional nature of cachexia, namely, whether the process is not just deleterious to the host, but whether it is also beneficial to the tumour, due to the mobilisation of metabolites from muscle and adipose tissues. There are also questions as to whether tumour-inherent properties drive cachexia, and thus whether genetic factors can be established that mediate cachexia. Data from human patients suggest this may be the case, as pancreatic and gastric cancers have a much higher incidence rate of cachexia when compared to other tumour types [129, 130]. The *Drosophila* models discussed here represent a good opportunity to answer some of these important open questions.

4. Concluding Remarks

The studies discussed here demonstrate that *Drosophila* is a relevant model for studying cancer and its interactions with the TME, with many parallels to orthologous vertebrate conditions. Research utilising *Drosophila* as a model system has shown that immune and metabolic processes induced in a nonautonomous manner by the presence of the tumour are sufficient to feed back to the tumour and alter its characteristics. This can be shown well in the studies of microenvironmental autophagy, which is induced in the TME by the tumour, and serves to support tumour growth and metastasis [77, 104], and in the dual role of haemocyte-derived Egr, which can promote or suppress tumour growth depending on the tumour context [36, 46] (Figures 1 and 2).

Given the effects observed in response to the tumour there are likely to be interactions between the immune system and metabolism in this context. Indeed, nutrient restriction in larvae inhibits TOR signalling in the fat body, leading to increased levels of circulating Egr. Egr binds to insulin-producing cells in the brain and suppresses the production and secretion of progrowth Dilp2 and Dilp5 [131]. As previously discussed, Egr is also a mediator of tumour-induced immunity with context-dependent pro- or antitumour function [36, 41, 46]. There is therefore the potential for crosstalk between host tissues with tumour-derived metabolic derangement and immune pathways in *Drosophila*. Work in human cell culture and mouse models has demonstrated that tumours can alter host immunity via directly influencing immune cell metabolism. Lactic acid secreted by the tumour into the TME changes macrophages metabolism, polarising them towards a tumour-promoting state [132, 133]. These macrophages produce ARG1, a metabolic enzyme that generates polyamines (metabolites essential for cell division), which promote tumour growth in this context [134]. Another example is given by the direct competition for glucose between tumours and TME T cells, which is also sufficient to alter T-cell metabolism, suppressing antitumour responses and highlighting how the Warburg effect is used to escape the immune system [86, 87].

However, there is a lack of comprehensive understanding as to how different factors such as diet and metabolism, immune responses, and tumours interact and cooperate or synergise when presented together. This is often the case in

the human condition, for example, in the case of a cancer patient with diabetes. *Drosophila* cancer models represent an excellent basis for the study of the roles these factors may play, both individually and combined together, and how nonautonomous signalling inputs might influence both tumour and host tissue responses. There are likely to be inevitable questions about the ability of simple *Drosophila* tumour models with one or two genetic drivers to fully recapitulate the complexity of tumour burden in higher animals, including human patients. However, the simplicity of these models is likely to prove advantageous when attempting to dissect the contributing roles of the multiple interacting factors that comprise tumour-TME interactions. There is also interesting work on the generation of *Drosophila* "avatars," fly lines that can generate close homologs of tumours from specific patients, including the numerous genetic aberrations that drive a particular type of tumour in humans [135]. Such avatars may represent an excellent opportunity to test principles uncovered in more simple *Drosophila* cancer models, in order to investigate whether these discoveries still hold in a more complex tumour setting, including tumour-TME responses.

The mechanisms mediating the crosstalk between tumours and local and distal tissues are still being uncovered. Improving the understanding of the signalling pathways that may link together the complex interactions between host metabolism, immunity, and tumour growth is an essential aspect towards the unravelling of such crosstalk. *Drosophila* models represent an excellent platform for the continued investigation of these complex interactions, thanks to the multiple advantages of the model system. Low genetic redundancy, powerful genetic tools, and the possibility of tightly controlling not only the genetics of the tumour but also various aspects of the tumour micro- and macroenvironment render *Drosophila* a strong paradigm for further work into these complex interactions that impact human health and disease.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Anthocyanins Function as Anti-Inflammatory Agents in a *Drosophila* Model for Adipose Tissue Macrophage Infiltration

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Epidemiological and preclinical studies have demonstrated that bioactive foods like flavonoids, polyphenolic compounds derived from fruits and vegetables, exert a protective action against obesity, cardiovascular disorders, and Adipocyte Tissue Macrophage infiltration (ATM). All these pathologies are characterized by increase in reactive oxygen species (ROS) and in proinflammatory cytokines that have been shown to favor the migration of immune cells, particularly of macrophages, in metabolically active organs like the liver and adipose tissue, that in *Drosophila* are constituted by a unique organ: the fat body. This study, using a unique *Drosophila* model that mimics human ATM, reveals the beneficial effects of flavonoids to reduce tissue inflammation. Our data show that anthocyanin-rich food reduces the number of hemocytes, *Drosophila* macrophages, infiltrating the fat cells, a process that is associated with reduced production of ROS and reduced activation of the JNK/SAPK p46 stress kinase, suggesting a fundamental function for anthocyanins as antioxidants in chronic inflammation and in metabolic diseases.

1. Introduction

Obesity is a metabolic syndrome occurring worldwide and often associated with other chronic diseases such as cardiovascular disorders, type II diabetes, and cancer [1].

The onset of obesity is the result of multifactorial elements, including a sedentary lifestyle, genetic predisposition, ethnicity, and environmental factors (such as organic pollutants) [2]; these factors with a diet rich in fats and sugars and poor in phytonutrients may result in weight gain and subsequently lead to metabolic disorders [3, 4]. Obesity is known to trigger a low-grade inflammation in metabolically active tissues and in organs such as the liver and adipose tissue [5–8]. Inflammation is the result of cellular and humoral responses with the scope to protect the organism from various insults, including infection and tissue damage, in attempt to rescue tissue homeostasis [9, 10].

In humans, the adipose tissue regulates lipid homeostasis and in normal conditions controls the storage of energy reserves in the form of triglycerides as well as functioning as an endocrine organ, producing a variety of proinflammatory cytokines such as IL-1, IL-6, and IL-8, IFNγ, and TNFα [11, 12]. In pathological conditions, such as obesity or metabolic syndrome, the adipocytes start to alter the production of these proinflammatory cytokines, which results in the activation of the innate immune system with recruitment of immune cells including macrophages leading to a state of chronic inflammation or ATM [7]. In addition, lipid accumulation and chronic inflammation in obese people are associated with a permanent increase of oxidative stress and with the production of high levels of reactive oxygen species (ROS) [13, 14], which is often associated with the activation of the c-Jun-NH_2-terminal kinase (JNK/SAPK) p46, member of a mitogen-activated protein kinases (MAPKs) downstream of JNK signaling [15]. This pathway is highly conserved in *Drosophila* and consists of a cascade of phosphorylation events starting with the activation of the JNKKK kinases, consisting of the Ask1 and Tak1, that activate MKK7, the orthologue of Hemipterous (Hep), and terminates with the activation of JNK/SAPK p46 kinase, called *basket (bsk)* in Drosophila, that is negatively regulated by *Puckered (puc),* a

phosphatase, which itself is a target of JNK/SAPK p46 kinase (see Figure 3(e)) [16].

This pathological situation influences other organs by altering their functions. Furthermore the adipose tissue from obese individuals exhibits a reduced capacity to store fat leading to an increase of circulating free fatty acids (FFAs) that promotes insulin resistance and damages the mitochondrial membrane thereby enhancing the production of ROS causing oxidative stress [17–19].

Epidemiological evidence suggests that a high intake of bioactive food is associated with a lower risk of developing chronic diseases like obesity [20]. Bioactive foods may influence the physiological and cellular activities of oxidative pathways and in recent years the attention has been focused on a class of secondary metabolites present in plant foods called flavonoids that seem to possess beneficial properties in preventing chronic diseases [21, 22]. The possible health benefits of flavonoids are linked to their potent antioxidant and free radical scavenging activities demonstrated *in vitro* and *in vivo* using different animal models [23]. Among the different classes of flavonoids, anthocyanins represent the major red, purple, and violet pigment in many plants and fruits. *In vivo* studies showed that anthocyanins added to the diet stimulate the secretion of insulin and decrease the generation of ROS [21, 24]. Preclinical studies performed on human demonstrate that dietary anthocyanins have a positive biological effect against obesity-induced inflammation and oxidative stress [24, 25], which is associated to a lower risk of type 2 diabetes. This potentially important application creates a high interest in understanding the action of these natural bioproducts in preventing metabolic diseases.

In order to study the mechanisms that control the anti-inflammatory response to flavonoids, we took advantage of a previously unrecognized conserved functional relationship between the immune cells, called hemocytes (macrophage like cells) and adipocytes (larval fat body, FB) [26]. *Drosophila* FB, a metabolic tissue with similar physiological functions to the mammalian adipose tissue and liver, acts as a functional unit to control key metabolic processes and the native immune response, in addition to storing fats and sugars [27]. In *Drosophila* the immune response is orchestrated by the hemocytes that are circulating cells in the hemolymph, present at all stages of the life cycle, and compose the fly's innate immune system [9, 28–33]. Hemocytes are essential mediators in the cell-cell communication process: they have been shown to mediate a response between the fat body and tumor cells to control their growth [34] and to promote proliferation of epithelial cells in response to the release of ROS following cell death in cells of the imaginal discs [35].

Using our model of obesity, we observed that hemocytes infiltrate the FB of obese larvae mimicking the chronic inflammation present in human obesity (manuscript in submission). In this study we report that treatment with anthocyanin-enriched food results in a significant decrease in the number of hemocytes infiltrating the FB concomitantly to a reduction in ROS and of the phosphorylation of JNK/SAPK stress kinase.

Our data demonstrate that the mechanisms driving the protective role of bioproducts like anthocyanins *in vivo* as anti-inflammatory and antioxidants are conserved in *Drosophila*. In addition, they highlight the potential use of our model to study the complex relationship between inflammation and obesity and corroborate the positive action of anthocyanins to combat chronic inflammation in humans.

2. Materials and Methods

2.1. Fly Stocks and Husbandry. Hml-RFP/CyO is a gift from Katja Brückner at UCSF. The P0206-Gal4 from [36], UAS-CG7839RNAi (BL 25992), is an RNA interference lines that reduces the expression of the CG7839 gene encoding for the orthologue of the yeast ribosomal protein Noc1; herein the CG7839-RNAi construct will be called Ni. Fly cultures and crosses were grown on standard fly food composed of yellow corn, sugar, and yeast molasses-base, at 25°C.

Feeding Experiment and Chemical Compounds. Crosses were kept in culture bottles perforated to provide adequate air circulation and eggs were collected on a grape agar plate (5%) supplemented with dry yeast every 3 hours. First instar larvae were collected after 24 hours AEL (after egg laying) and shifted into vials containing different food. First instar larvae were reared with 2 g of standard food, hereafter Normal Food (NF) and 5 ml of each flavonoid (FL) extract, one containing only flavonoids (NF + FL) and another extract containing flavonoids and 0.24 mg/ml anthocyanins (NF + FL + ACN). All these phenolic compounds were extracted from the cobs of yellow and purple corn (gift from Katia Petroni and Chiara Tonelli, University of Milan); only the purple extract is rich in anthocyanins, while the content of other flavonoids is the same in both extracts (the content of flavonoids present in the extracts are reported in [37]).

2.2. Hemocytes Quantification and Size Analysis in Larval Fat Bodies. To label *in vivo* plasmatocytes, which comprise more than 95% of the hemocytes population in the *Drosophila* larva, we used the transgene *Hml*Δ*-DsRed (Hml-RFP)* that contains the promoter for hemolectin, expressed in the hemocytes, fused with the Red Fluorescence Protein (RFP) [38]. Fat bodies from 20 larvae at 5 and 12 days AEL were dissected in phosphate-buffered saline (PBS) pH 7.4 and fixed in 4% paraformaldehyde (PFA) for 30 minutes. Hoechst 33258 (Sigma Aldrich) was added to stain DNA in a final concentration of 1 μg/ml. After washing with PBS, fat bodies were mounted onto slides with DABCO-Mowiol and images were acquired using an SP2-LEICA Lasertechnik GmbH confocal microscope. Images were analyzed with the ImageJ software. In order to analyze the cell size, the larval fat bodies were fixed in 4% PFA, permeabilized with 0.2% Triton X-100 in PBS, and rinsed in PBS 1x and membranes were stained with 1 : 100 Alexa Fluor 488 Phalloidin to visualize the cytoskeleton through the binding between Phalloidin and F-actin and Hoechst 33258 for nuclei and then mounted onto slides with DABCO-Mowiol. Photographs were taken using confocal microscopy and the area of adipose cells for each fat body was calculated with ImageJ software. In order to visualize lipids, fat bodies were stained with Nile Red (Sigma Aldrich) and with Alexa Fluor 488 Phalloidin following the protocol in [39].

2.3. In Vivo Detection of ROS Using Dihydroethidium (DHE). DHE is used to detect cytosolic superoxides and radical oxygen species (ROS). The reaction between DHE and superoxide anions generates a highly specific red fluorescent product (ethidium), which intercalates with DNA. ROS levels were detected in live tissue as described in [40]. Briefly, larval fat bodies at 5 and 12 days AEL were dissected in Schneider's insect medium (GIBCO). After incubation in 30 μM DHE (Invitrogen) for 5–7 minutes in the dark at room temperature, fat bodies were washed three times with Schneider's medium and immediately mounted with VECTASHIELD Antifade Mounting Medium.

2.4. RNA Extraction and Quantitative RT-PCR. Total RNA was extracted from 8 whole larvae using QIAGEN RNeasy Mini Kit. 1 μg total RNA from each genotype was reverse-transcribed into cDNA using SuperScript IV MILO Master Mix (Invitrogen). The obtained CDNA was used as the template for quantitative real-time PCR (qRT-PCR) using SYBR Premix Ex Taq-Tli RnaseH Plus II (TaKara), analyzed on a RT-PCR BIORAD thermocycler machine. In these experiments, gene expression levels were normalized to *actin mRNA*, used as the internal control. The following primers for qRT-PCR were used: *actin5c* 5′-CAGATCATGTTCGAGACCTTCAAC-3′ (R) and 5′-ACGACCGGAGGCGTACAG-3′ (F) and E74B 5′-GAATCCGTAGCCTCCGACTGT (R) and 5′-AGGAGGGAGAGTGGTGGTGTT (F) [39].

2.5. Protein Extractions and Immunoblotting. The larval fat bodies (10 for each genotype) were dissected in Schneider's medium serum-free and lysed in 80 μl of lysis buffer 1x (50 mM Hepes pH 7.4, 250 mM NaCl, 1 mM EDTA, 1.5% Triton X-100). Protease inhibitor cocktail (Sigma-Aldrich) was added to inhibit protease and phosphatase activities. Samples were sonicated two times for 10 seconds and then centrifuged. Protein concentration was determined by Bradford protein assay (Bio-Rad). The samples were boiled in 1x SDS and then separated on 10% SDS-polyacrylamide gels and blotted. Membrane was incubated with primary antibody anti-phospho-p46 SAPK/JNK (Cell Signaling #9521) or antiactin (Hybridoma Bank) overnight at 4°C in blocking buffer and then washed in 0.1% Tween 20 with TRIS-buffered saline (TBST). Appropriate secondary antibody was incubated for 2 hours, followed by washing. The signal was revealed with ChemiDoc Touch Imaging System (Bio-Rad Lab).

2.6. Immunostaining. Dissected fat bodies from 20 larvae were fixed in a solution of 4% PFA/PBS for 40 minutes. After permeabilization with 0.3% Triton/PBS, tissues were washed in a solution of Tween 0.04%/PBS, saturated with 1% BSA/PBS, and incubated overnight with anti-SPARC antibodies (1 : 400), a generous gift from Martinek et al. [41], and visualized using anti-Rabbit Alexa555 (Invitrogen).

2.7. Statistical Analysis. The experiments were repeated at least three times and the statistical analysis among the various genotypes was examined by Student's *t*-test. Differences were considered significant if *P values* were less than 0.05 (∗), 0.01 (∗∗), 0.001 (∗ ∗ ∗), and 0.0001 (∗ ∗ ∗∗).

3. Results

3.1. Obese Larvae Have Increased Size of Fat Cells and Increased Hemocytes in the Fat Body. In order to study the ability of hemocytes to infiltrate the fat cells, we blocked pupariation (Figure 1(a)) creating larvae *P0206-Gal4; UAS-Ni* where the reduction of the size of the prothoracic gland, the endocrine organ that produces ecdysone, resulted in reduced levels of ecdysone (Figure 1(b)), leading to animals that develop at almost normal rate and continue to feed until 3 weeks with an increased body weight (see method). *Drosophila* FB-cells function as storage for nutrients, which synthesize and release energy, and accumulate fat and sugars; in our obese animals we observed that at 12 days AEL the size of the cells from the FBs from *P0206-Gal4; UAS-Ni* larvae increased (Figure 1(c)) due also to the accumulation of fats in lipid droplets visible by Nile Red staining (Figure 1(d)). Those *P0206-Gal4; UAS-Ni* animals acquired phenotypic characteristics of obese individuals, including increased triglycerides (TAGs), glucose circulating in the hemolymph, resistance of fat cells to stimulation with insulin, and increased hemocytes in the FB (manuscript in submission).

Chronic inflammation in the adipose tissue is characterized by the infiltration of macrophages in the fat cells; we therefore analyzed if a similar event was present in the FB of our obese animals. We labeled the hemocytes *in vivo* using the *Hml-RFP* reporter line that specifically expresses Red-Fluorescence protein in hemocytes and introduced this transgene to our genetic background. *Hml-RFP* positive cells were monitored over time to visualize and quantify the number of hemocytes infiltrating the FB, from control and obese animals at 5 days AEL and at 12 days AEL in the obese larvae. These results showed that FBs from *P0206-Gal4/Hml-RFP; UAS-Ni* animals contain at 5 days AEL a small but significantly higher number of hemocytes in their FBs (5.2%, $P < 0.05$) as compared to control *P0206-Gal4/Hml-RFP* (Figure 1(e)); furthermore, at 12 days the percentage of hemocytes in *P0206-Gal4/Hml-RFP; UAS-Ni* animals was drastically increased to 17% ($P < 0.00001$). Hemocytes are characterized by the expression of high levels of the cell adhesion protein SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin or BM 40) [41]; morphological analysis of FBs from 12-day *P0206-Gal4; UAS-Ni* animals showed the presence of crown-like structures of hemocytes, positive with anti-SPARC antibodies, that surrounded the fat cells, mimicking similar structures described in the fat of obese individuals suffering from chronic inflammation (Figure 1(f)).

3.2. Obese Larvae Have Increased Phosphorylation of JNK/SAPK and of ROS Production in the FB. Chronic inflammation in obese people is often associated with high levels of reactive oxygen species (ROS) and with the activation of the c-Jun-NH_2-terminal kinase (JNK/SAPK) p46 [15].

We therefore analyzed, in our *Drosophila* model of chronic inflammation, if there was an activation of JNK signaling by looking at the levels of phosphorylation of JNK/SAPK p46. Western blot analysis using extracts from FBs of larvae from *P0206-Gal4* at 5 days AEL or *P0206-Gal4;*

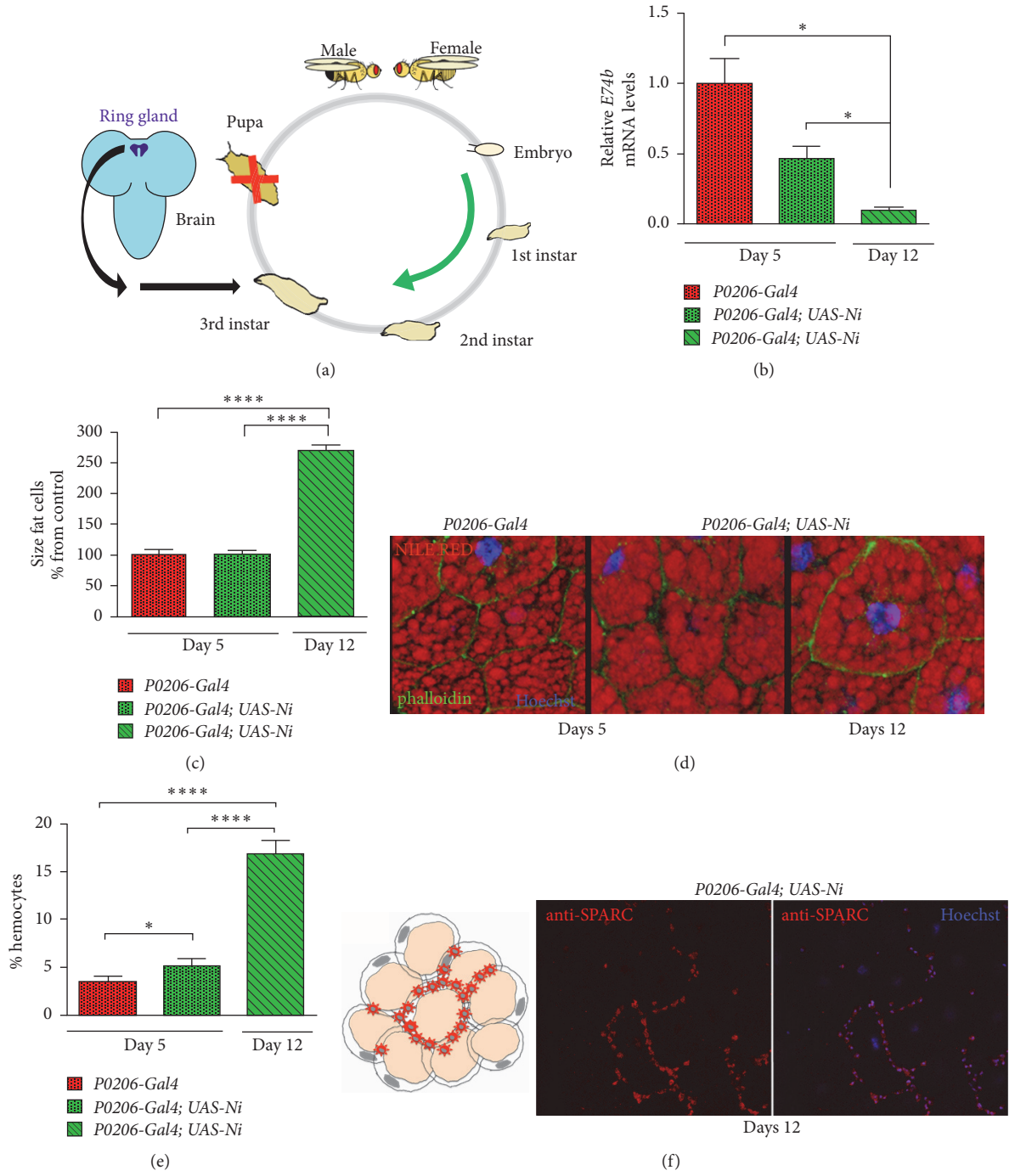

Figure 1: *Obese larvae have increased hemocytes infiltrating the fat cells*. (a) Ecdysone regulation of larval molting and metamorphosis. Reducing the size of the ring gland reduces ecdysone level in *P0206-Gal4; UAS-Ni* animals. (b) Quantitative RT-PCR in whole larvae of the indicated genotype showing the relative expression of *E74b mRNA*. *Actin5c* was used as control. (c) Relative size of cells from the FBs from animals of the indicated genotypes, at 5 and 12 days AEL. (d) Nile Red staining for lipids, Phalloidin for membranes, and Hoechst for nuclei, of FBs. (e) % of hemocytes infiltrating the FBs of animals at 5 or 12 days AEL, of the indicated genotype. Data are expressed as percentage of hemocytes in the cells of FBs. (f) Draw and confocal photographs of cell from the FB, showing hemocytes stained with anti-SPARC antibodies (red), while nuclei are visualized using Hoechst (blue). Error bars represent SEM (standard error of the mean) of three independent experiments. $^{*}P < 0{,}05$ and $^{****}P < 0{,}0001$.

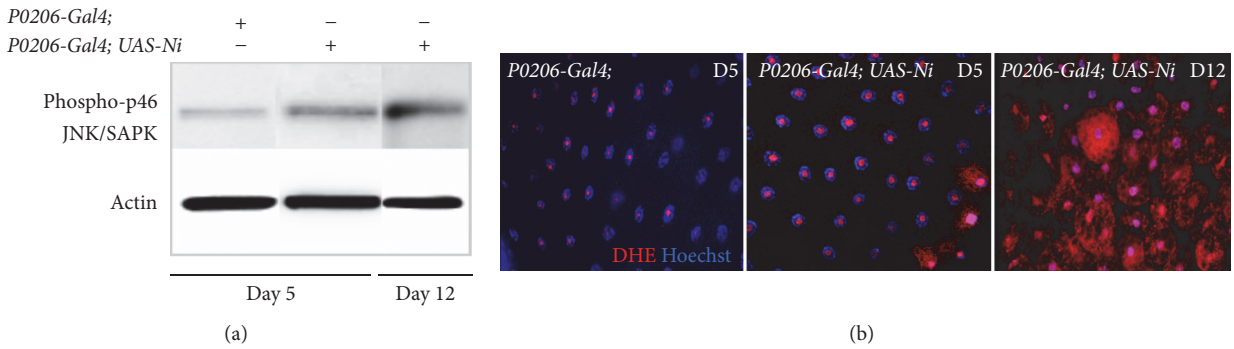

Figure 2: *Obese larvae show activation of JNK/SAPK signaling and increased ROS production*. (a) Western blot from lysates of FBs showing the level of phosphorylation of JNK/SAPN p46 kinase, in *P0206-Gal4* (control) and *P0206-Gal4; UAS-Ni* animals. Actin was used as control loading. (b) Confocal photographs (20x) of cells from FBs stained with DHE (red) for ROS and Hoechst (blue) for nuclei.

UAS-Ni at 5 and 12 days AEL shows an increase in the phosphorylation of JNK/SAPK p46 kinase (Figure 2(a)).

Since ROS are known to induce the activation of the JNK pathway we then analyzed if in the FBs from the obese larvae there was an increase in ROS signaling, using DHE as a marker. These experiments show that at 5 days AEL, DHE staining increased in FBs from *P0206-Gal4; UAS-Ni* (Figure 2(b), middle panel) animals as compared to control (Figure 2(b), left panel); moreover, DHE staining further increased at 12 days AEL (Figure 2(b), right panel) suggesting that FBs form these animals exhibit significant increase in ROS production over time.

3.3. Dietary Anthocyanins Reduce Hemocytes Infiltration in FBs and Phosphorylation of JNKSAPK p46. Flavonoids (FL) and anthocyanins (ACN) are known to have antioxidant effects against inflammation-induced oxidative stress. Therefore, we analyzed if the presence of FL or ACN in the diet of the obese animals had an effect on the chronic inflammation and stress phenotypes.

Staged first instar larvae were transferred to normal standard food (NF) or to food enriched with FL only or enriched with FL + ACN, herein called ACN (Figure 3(a) and material and methods), and their effect on the migration of hemocytes in the FBs was quantified by visualizing the number of HML-RFP positive cells on dissected FBs using a fluorescent microscope. These experiments show that after 5 days of feeding with the different diets, only food containing ACN significantly reduces from 5.7% to 3.2% the presence of hemocytes in the FBs of *P0206-Gal4; UAS-Ni* animals, while treatment with FL did not have any effect (Figure 3(b)). At 12 days instead both FL and ACN diets were able to significantly decrease the number of hemocytes (Figure 3(b)). In addition macroscopic analysis of the shape and number of hemocytes showed that at 12 days AEL both FL and ACN treatments were able to reduce the formation of crown-like structures of hemocytes surrounding the fat cells in *P0206-Gal4; UAS-Ni* animals (middle and left panel) Figure 3(c). We then analyzed the effect of FL and ACN diets on the phosphorylation of the stress-response JNK/SAPK p46; FBs from animals growing in the different diets were dissected at 5 and 12 days AEL and phosphorylation of JNK/SAPK was analyzed by western blot. These experiments showed that at 5 days AEL, feeding with ACN significantly reduced the phosphorylation of JNK/SAPK p46 in FBs from *P0206-Gal4; UAS-Ni* (Figure 3(d)), while after 12 days AEL both diets with FL and ACN were able to significantly reduce JNK/SAPK p46 phosphorylation, suggesting a potential role at later points for FL in reducing oxidative stress.

4. Discussion

Obesity and metabolic disorders are pathological conditions associated with our diet enriched of fats and sugars or with a sedentary life, but also with environmental factors that may pollute our food with chemicals that affect lipid metabolism. As consequences we have seen an increase in cardiovascular diseases, type 2 diabetes, and a chronic inflammation of the adipose tissue (ATM) induced by the persistent infiltration of macrophages into the fat cells, for which the mechanisms are not totally understood but have been associated with an oxidative stress condition present between the immune cells in the metabolic tissues. In order to study *in vivo* these relationship, we have taken advantage of the conserved relationship in *Drosophila* between the immune cells (hemocytes) and the fat body (adipose tissue) to study how bioproducts like flavonoids in particular anthocyanins that are known to act as antioxidants and that are naturally present in our food may ameliorate or counteract the migration of the hemocytes into the FB using our animal model that mimics chronic inflammation in vertebrate (ATM).

Anthocyanins are a class of flavonoids classified as bioactive food have been shown to ameliorate hyperglycemia, insulin sensitivity, and fat accumulation in obese mice fed to a high fat diet and in vertebrates studies identify a beneficial effect by anthocyanins in combating inflammation-related diseases such as diabetes, cardiovascular diseases, and obesity [25, 42, 43]. Moreover, clinical studies in humans demonstrate that higher consumption of anthocyanins is associated with weight loss in both men and women and reduces the risk of developing chronic diseases with a mechanism poorly understood [24, 44].

In this study, we are using our innovative model that mimic obesity in flies, where upon blocking growth by

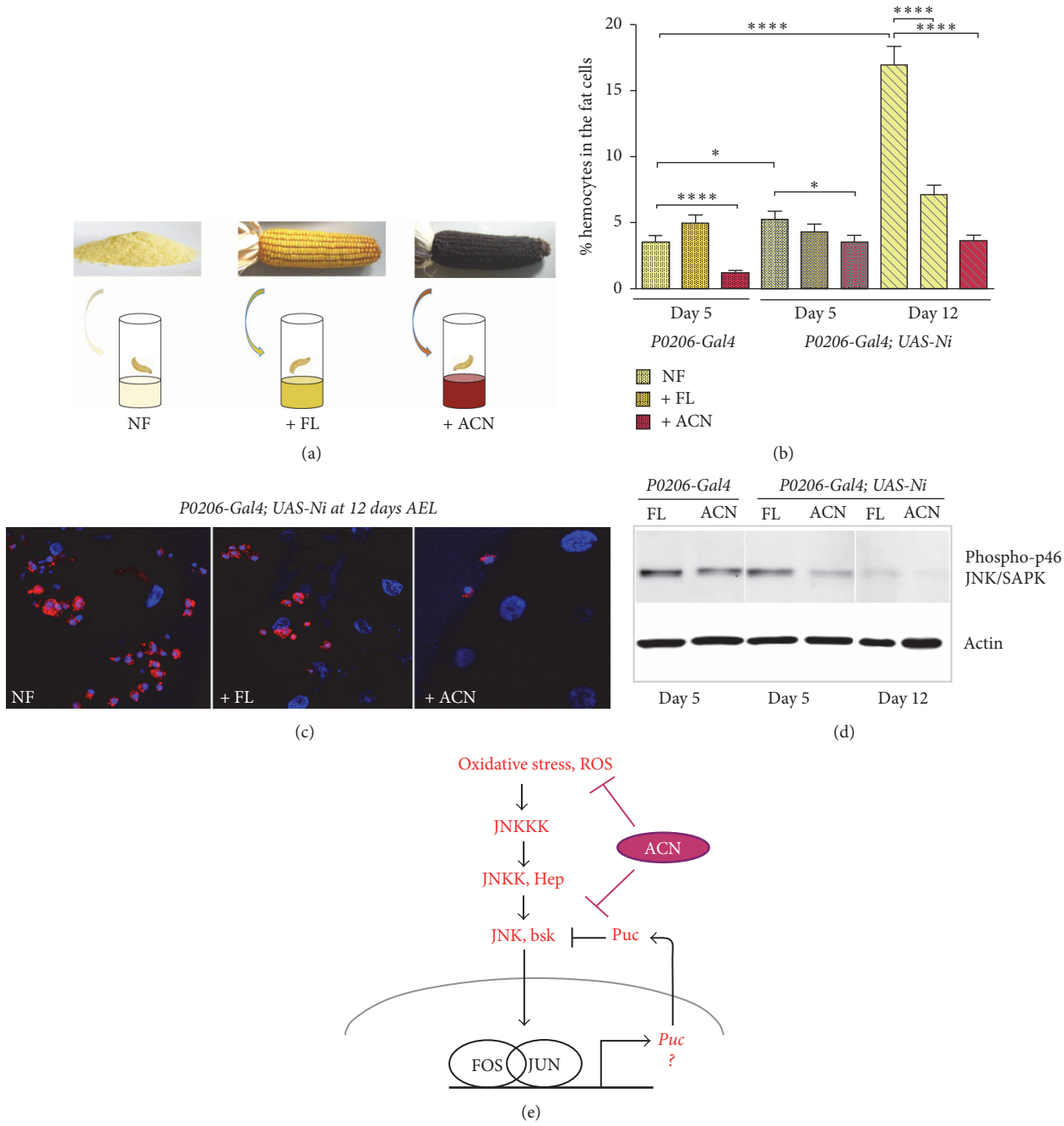

Figure 3: *Anthocyanins-rich diet reduces the infiltration of hemocytes in the FBs and the phosphorylation of JNK/SAPK*. (a) Scheme of the different diets NF (Normal Food) or enriched in FL (flavonoids) or ACN (anthocyanins). (b) % of hemocytes in the cells of the FBs from animals at the indicated genotypes and fed with the indicated diets at 5 or 12 days AEL. (c) Confocal images showing hemocytes expressing Hml-RFP (red) and nuclei stained with Hoechst (blue) from animals at 12 days AEL, upon feeding with NF, FL, or ACN enriched diets. (d) Western blot from lysates of FBs showing the level of phosphorylation of JNK/SAPK p46 kinase, in *P0206-Gal4* (control) and *P0206-Gal4*; *UAS-Ni* animals fed in FL or ACN enriched diets. FBs were taken at 5 or 12 days AEL. Actin was used as control loading. (e) Model of JNK signaling and potential action of anthocianins. Error bars represent SEM (standard error of the mean) of three independent experiments. $^{*}P < 0{,}05$ and $^{****}P < 0{,}0001$.

reducing ecdysone, the animals develop at almost normal rate but continue to feed with an increase in body weight and in the fat cell-size; these animals acquire the characteristics of obese people, with an accumulation of TAGs and insulin resistance (manuscript in preparation). Moreover, these animals present an infiltration of hemocytes (macrophage-like cells) within the cells of the FB that progressively increases until the formation of the typical "crown-like structures"

described in obese patients suffering from ATM [5]. We demonstrate that in FBs from these obese animals there is increased production of ROS, indicating the presence of an oxidative stress that may be responsible of the augmented phosphorylation of the JNK/SAPK p46 stress kinase. Because the molecular mechanisms that regulate lipid metabolism are highly conserved between humans and flies [45, 46] and hemocytes have been shown to be functionally equivalent to macrophages we can speculate that the mechanisms underlining these humoral responses are conserved also in flies. Therefore, we use our obesity model to investigate the antioxidant effect of flavonoids and anthocyanins to chronic inflammation. In our study, we find that a diet rich in anthocyanins reduces hemocytes migration in the larval FB and decreases the accumulation of TAGs in the fat cells (not shown) ameliorating several characteristics of the obese phenotypes. Moreover, we showed that anthocyanins reduce the production of ROS in cells of the FB and significantly attenuate the phosphorylation of JNK/SAPK p46 kinase providing evidence that may play a key role in regulating the JNK-mediated cellular stress responses and to control ROS signaling.

The interplay of signals that regulate the nonautonomous responses between hemocytes and the cells of the FB is coming up as a new field for important studies; indeed recently hemocytes have been shown to be responsible of mediating an humoral immune response in a model for tumor growth, where they were shown to trigger signals responsible of killing the tumor cells through a nonautonomous mechanism mediated by the activation of cytokines of the Toll and Eiger/TNFα by the fat body [34]. More recently, hemocytes were shown that upon stress conditions they are able to migrate near epithelial cells and to produce ROS to induced the release of Eiger/TNFα by the epithelial cells through the activation of the JNK signaling pathway, suggesting also in this case the presence of nonautonomous signals between the hemocytes and the cell of the epithelium necessary for tissue homeostasis [35, 47, 48]. In a similar way, we can speculate that the hemocytes in the FB from obese animals may be activated by the oxidative stress signals (ROS), present in the FB, that trigger signals to induce the production of cytokines of the Toll and Eiger/TNFα that further aggravate the oxidative stress condition that attract the hemocytes that constitutively migrate into the fat cells causing a status of chronic inflammation.

In our experiments, we show that anthocyanins are able to reduce the activation of JNK/SAPK p46 stress kinase. As mentioned before, JNK pathway is activated upstream by ROS and by cytokines including Eiger/TNFα; this pathway is inhibited by a negative regulatory feedback that induces the transcription of the phosphatase *puckered* (Figure 3(e)). In our model, we can speculate that anthocyanins may either directly block cytokines upstream of JNK signal, for example, by controlling Eiger/TNFα signaling, or they may contribute to the activation of the negative feedback that involved the activity of puckered.

Interestingly, anthocyanins were shown to act concomitantly with detoxification enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase (GST), and glutathione reductase to reduce oxidation. In *Drosophila Gst-D1* [49] and *jafrac,* an inhibitor of cell death, together with *puckered*, were shown to be transcriptional targets of *jun-fos* activity in response to the activation of JNK pathway, and these genes were shown to negatively counteract the oxidative stress response [50]. Our preliminary data however did not find any regulation in the expression of *GstD1* in the fat cells from the obese animals upon feeding with FL or ACN anthocyanins (data not shown) suggesting that probably this enzyme is not involved in the regulation of JNK signaling by anthocyanins in these cells.

In conclusion, with the present study we provide for the first time a strong evidence of the potential use of anthocyanins in the diet to control chronic inflammation and provide a link to the oxidative stress that characterize the adipose tissue in obese animals. We were able to evidence the ability of anthocyanins to decrease *in vivo* the phosphorylation of JNK/SAPK p46 stress kinase, thus providing a new insight into the mechanism of phenolic compounds in the treatment of inflammation in adipose tissues, a field of currently study since the lack of a better knowledge of the mechanisms that regulate or control ATM in pathologies such as obesity and metabolic disorders.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Review Article

Modelling Cooperative Tumorigenesis in *Drosophila*

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The development of human metastatic cancer is a multistep process, involving the acquisition of several genetic mutations, tumour heterogeneity, and interactions with the surrounding microenvironment. Due to the complexity of cancer development in mammals, simpler model organisms, such as the vinegar fly, *Drosophila melanogaster*, are being utilized to provide novel insights into the molecular mechanisms involved. In this review, we highlight recent advances in modelling tumorigenesis using the *Drosophila* model, focusing on the cooperation of oncogenes or tumour suppressors, and the interaction of mutant cells with the surrounding tissue in epithelial tumour initiation and progression.

1. Introduction: *Drosophila* as a Model for Understanding Human Cancer

For over 100 years, research utilizing the powerful genetics of the vinegar fly, *Drosophila melanogaster*, has contributed to the understanding of fundamental cellular and developmental processes relevant to the medical field (reviewed in [1, 2]). Indeed, research using the *Drosophila* model had now been granted five Nobel Prizes for Medicine or Physiology. Moreover, the *Drosophila* model has proven to be a highly suitable system for understanding cancer and in developing cancer therapies (reviewed in [3–15]). Use of *Drosophila* as a model organism for cancer research was pioneered by genetic screens, conducted in the late 1900s, which identified many *Drosophila* tumour-causing mutations (reviewed in [16, 17]). Many of these were novel tumour-suppressor genes or oncogenes, which were subsequently shown to also have tumourigenic properties in mammalian systems and to be involved in human cancer (reviewed in [8, 9, 11, 18, 19]).

The strengths of the *Drosophila* model for cancer research lie in the evolutionary conservation of genes and signalling pathways between flies and humans, its lower genetic redundancy, simpler biology, rapid life cycle, and powerful genetics (reviewed in [1, 2, 15]). Due to the sophisticated genetic tools available, cancer-causing mutations can be studied in a tissue-specific or mosaic context. In the study of tumorigenesis in *Drosophila*, the developing epithelial tissues of the *Drosophila* larval imaginal discs that generate the adult eye-antenna or wing-thorax or the epitheliums of the adult intestine are commonly used (reviewed in [7, 20–22]). Indeed, it is mosaic (clonal) analyses using these epithelial tissues that have enabled new insights into the initiation and progression of cancer. In this review, we highlight recent studies focusing primarily on *Drosophila* epithelial tissues, showing how cooperating interactions between cells, and between mutations in oncogenes or tumour-suppressor genes, drive cancer initiation and progression.

2. Cell Competition and Cooperating Interactions between Cells in Tumorigenesis

Epithelial tumours can be initiated by multiple molecular lesions, including deregulation of signalling pathways and the perturbation of cell polarity/morphology, such as those generated by loss of function of the cell polarity regulator, Scribbled (Scrib) [15, 23–25]. The clonal-analysis approach has enabled the molecular interactions between the developing epithelial tumour and the surrounding normal tissue,

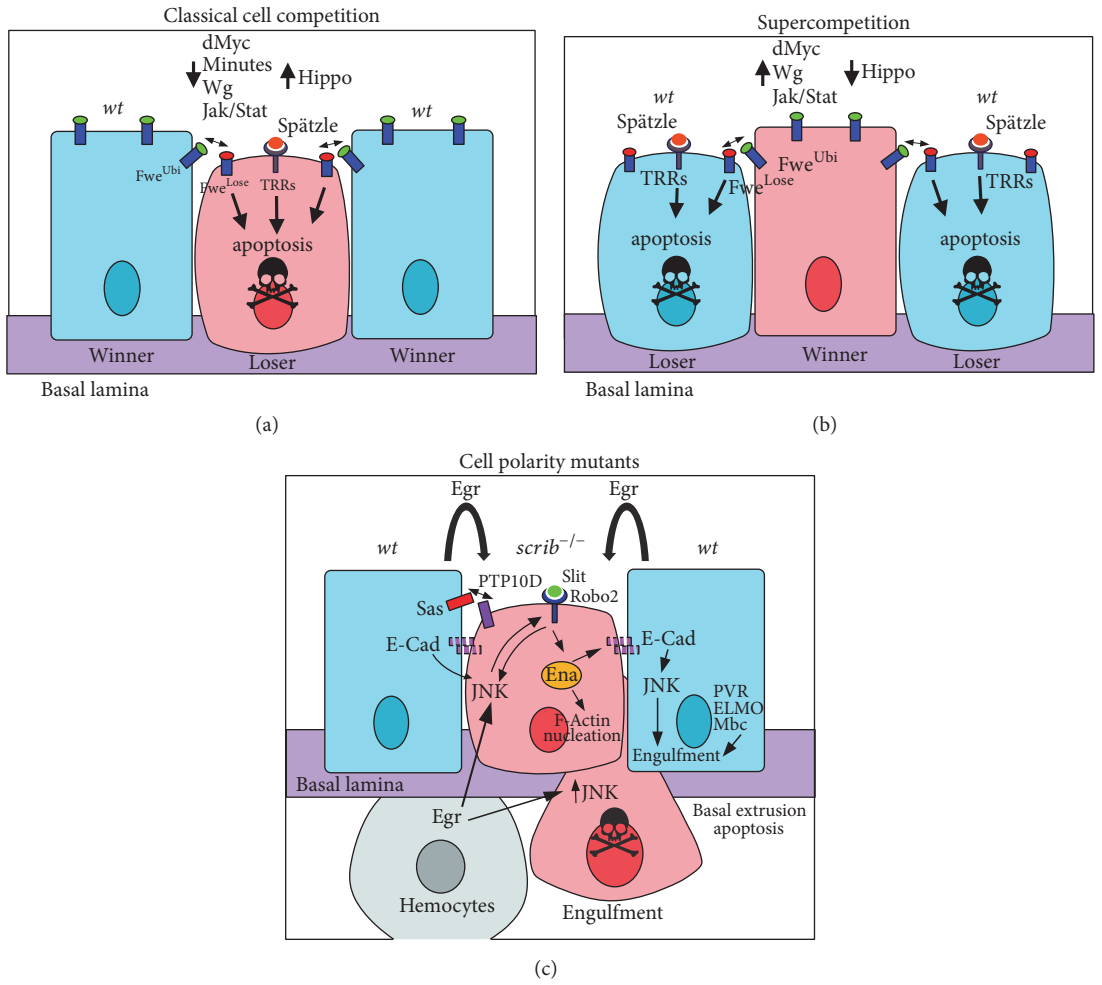

Figure 1: *Cell competition mechanisms*. The three main types of cell competition are shown. Mutant cells are in pink, *wild-type* cells are in blue, hemocytes are in grey, and the basement membrane (basal lamina) is in purple. (a) Classical cell competition: within an epithelium, cells with reduced levels of dMyc, ribosomal subunits mutants (minutes), Jak-Stat or Wg signalling, or high levels of Hippo signalling (losers) are eliminated by apoptosis, induced by the surrounding *wild-type* cells (winners). The loser cells express on their cell surface the Flower-Lose (Fwe^{Lose}) isoform (red dots), which marks them for elimination when in contact with the surrounding *wild-type* cells that express the Flower-Ubi (Fwe^{Ubi}) isoform (green dots). Additionally, signalling via the Spätzle ligand and Toll-Like Receptors (TLRs) in the loser cells triggers cell death via upregulation of cell death inducers, Rpr or Hid. Cells with upregulated Hippo signalling (or *yki* mutants) exhibit decreased dMyc levels, but cells with decreased ribosomal function, Jak-Stat, or Wg signalling undergo dMyc-independent cell competition. (b) Supercompetition: cells with high levels of dMyc, Jak-Stat, increased Wg signalling, or decreased Hippo signalling show "supercompetitor" behaviour and induce apoptosis in neighbouring *wild-type* cells. This occurs via the Flower-code or via Spätzle-TLR signalling in the loser cells. (c) Cell polarity mutant cell competition: cell polarity-impaired mutant cells are recognized by their epithelial neighbours or hemocytes (grey) and the TNFR-JNK signalling ligand, Egr (TNF), which is secreted by the *wild-type* epithelial cells or hemocytes. Mutant cells are removed by JNK-dependent and caspase-dependent apoptosis. JNK activation in neighbouring *wild-type* cells together with PVR, ELMO, and Mbc signalling is required in the *wild-type* cells for the removal of the dying cells. Hemocytes play the predominant role in engulfment and removal of the dead cells. The interaction of PTP10D in the mutant cell with SAS in the *wild-type* cell is important for "loser" cell fate of the polarity-impaired mutant cell. The Slit-Robo-Ena signalling pathway plays an important role in basal extrusion of the mutant cell, where the hemocytes are localized.

the innate immune system, or distant organs to be revealed (reviewed in [6, 26–30]). The interaction between a tumour cell and the surrounding normal cells in an epithelium is important in determining whether the tumour cell survives and proliferates or is eliminated. The phenomenon of "cell competition," a surveillance mechanism that compares the fitness of cells in an epithelium, is critical for the active elimination of cells of lower fitness (losers) by cells of greater fitness (winners) within an epithelial tissue (reviewed in [29, 31–33]) (Figure 1). Cell competition involves the interaction of cells and cell-surface molecules or a modified innate immune signalling pathway, leading to caspase-mediated apoptosis of the loser cells by the winner cells. The mechanism of cell competition depends upon the molecular lesion. Cells with low levels of the cell growth regulator, dMyc, or of ribosomal proteins, which reduce cellular growth, are recognized and eliminated

differently from those where cell polarity is impaired [34–39] (Figure 1(a)). Differentially expressed cell-surface receptor isoforms of the Flower protein [37, 38] or modified innate immune signalling involving Toll-Like Receptor-NfκB (TLR-NfκB) signalling are involved in the elimination of low dMyc or ribosomal protein expressing cells [35].

Clonal alterations in signalling pathways such as Wingless (Wg/Wnt), Jak-Stat, and the Hippo negative tissue-growth control pathways can also induce cell competition (reviewed in [33, 36, 40]). Impairment of Hippo signalling, in addition to upregulating cell cycle and cell survival genes, leads to the upregulation of dMyc and results in a supercompetitor phenotype, where the surrounding *wild-type* cells are actively eliminated [41, 42] (Figure 1(b)). However, the cell competition mechanism that occurs upon differences in the Wg or Jak-Stat signalling occur by dMyc-independent mechanisms, which are currently not well defined [43, 44].

By contrast, *scrib* mutant cell competition requires the interaction of a membrane tyrosine phosphatase, PTP10D, on the loser cell and a membrane protein, Sas, on the winner cell, which results in repression of Epidermal Growth Factor Receptor- (EGFR-) Ras small-GTPase signalling and the activation of the Jun N-terminal Kinase (JNK) signalling in the loser cell [34] (Figure 1(c)). Additionally, JNK signalling activates the Slit-Robo-Ena signalling pathway leading to downregulation of E-Cadherin (E-Cad) and the basal extrusion of *scrib* mutant cells, where they die [45, 46]. Indeed, downregulation of E-Cad appears to be important in *scrib* mutant cell extrusion and elimination, since overexpression of E-Cad in *scrib* mutant clones reduced cell extrusion and promoted clonal overgrowth [45]. JNK signalling also overrides the impaired Hippo signalling in *scrib* mutant cells in a clonal context, preventing their overgrowth [47, 48]. Furthermore, differing levels of dMyc or Jak-Stat signalling between the polarity-impaired mutant cells and the surrounding *wild-type* cells has also been implicated in the elimination of the mutant cells in particular contexts [49–51].

In addition to cell competition, the interactions between the tumour and its microenvironment are critical for whether the tumour cells will undergo apoptosis or overgrow and eliminate the normal tissue (Figures 1(c) and 2). Interactions between the surrounding *wild-type* epithelial cells, mesenchymal cells (myoblasts), or macrophage-like innate immune system cells (hemocytes) contribute to the fate of the tumour cells [52–62]. Mechanistically, the emerging picture from the study of neoplastic tumours generated in imaginal epithelial tissues (such as with mutants in the neoplastic tumour-suppressor gene (nTSG), *scrib*), is that tumour development occurs through the cooperative interaction of factors produced from surrounding epithelial cells or hemocytes and feed-forward mechanisms within the tumour cell amplifying this loop (Figure 2). Hemocytes are attracted to sites of cell competition by the secretion of fragments of the Tyrosyl-tRNA synthetase protein (dminiTyr and dEMAP), which is triggered by JNK activation and Metalloproteinase (MMP) dependent cleavage in dying loser cells [63] (Figure 2(a)). Mechanistically, dEMAP upregulates PI3K signalling in the hemocytes, which is required for hemocyte chemotaxis [64] and may be important in engulfment of the dying cells [63].

A highly important pathway in cell-cell interactions that triggers tumour cell death is the Tumour Necrosis Factor (TNF), Eiger (Egr), pathway (Figure 1(c)). Egr signals via the TNF receptor (TNFR), Grindenwald (Grnd), and leads to the activation of the JNK signalling pathway in the tumour cell, which, through the activation of caspases, results in caspase-mediated apoptosis of initiating tumour cells [143]. Egr can be produced from the adjacent *wild-type* epithelial cells, myoblasts, or the hemocytes [52, 55, 61, 89, 144]. The *wild-type* cells on the border of the mutant clone also require JNK signalling, though in a nonapoptotic role, and the induction of PVR (PDGF/VEGF receptor homolog)-ELMO (Ced-12 homolog)-Mbc (Dock180 homolog) signalling to induce engulfment of the mutant cells [54] (Figure 1(c)). Whilst there is evidence that *wild-type* epithelial cells engulf the *scrib* mutant dying cells [54], hemocytes play the predominant role in this process, as well as in cell competition due to variations in dMyc or ribosomal protein levels [145, 146]. Furthermore, in tumour development, microenvironmental "hot-spots" have been revealed where the tumour has a greater chance of progressing, which has parallels with mammalian systems [27, 147]. Molecularly, the "hot-spots" are due to endogenously higher levels of Jak-Stat signalling and the presence of a stiff basement membrane extracellular matrix, resulting in extrusion of the tumour cells apically, where they survive (Figure 2(b)). Conversely, in "cold-spots," tumour cells extrude basally from the epithelium and die, perhaps due to exposure to hemocytes (see below). Molecularly, the level of Slit-Robo-Ena signalling is important for the direction of cell extrusion and therefore dictates whether the aberrant cells will be eliminated by basal extrusion, remain in the epithelium and overgrow, or are apically extruded into the lumen and progress to invasive tumours [45, 46].

By contrast, if cell death is prevented in the mutant cells by blocking caspase activity or upregulation of a cell survival pathway, such as the EGFR-Ras signalling pathway, then the cells survive and form invasive tumours [23, 65, 66, 89–91, 144]. This occurs since TNFR-JNK signalling is repurposed to promote cell morphology changes and migratory cell behaviour (reviewed in [143]). Ras signalling prevents caspase-mediated cell death, and instead caspase activity induces the formation of reactive oxygen species (ROS) within the cell and promotes their secretion [57] (Figure 2(c)). Extracellular ROS, in turn, attracts hemocytes, which secrete TNF and amplify the JNK signalling pathway in the tumour cell [57]. Interestingly, a recent report revealed that ROS, released from the *scrib* mutant Ras^{V12}-expressing tumour cells, promotes autophagy (a catabolic process that degrades cellular macromolecules and organelles to provide energy) in the surrounding *wild-type* cells, as well as systemically in gut, muscle, and adipose tissues [60] (Figure 2(d)). The induction of autophagy may serve to provide glucose, amino acids, and other nutrients that facilitate tumour growth. In the *scrib* mutant Ras^{V12}-expressing cells, Egr-JNK-Fos (Kay) signalling together with Ras-MAPK signalling generates metabolic stress, leading to ROS production [60, 101]. JNK and impaired Hippo signalling in these tumour cells also result in the transcription of *unpaired 1–3 (upd1–3)*, which encode IL-6-related ligands for the Domeless (Dome) receptor of the

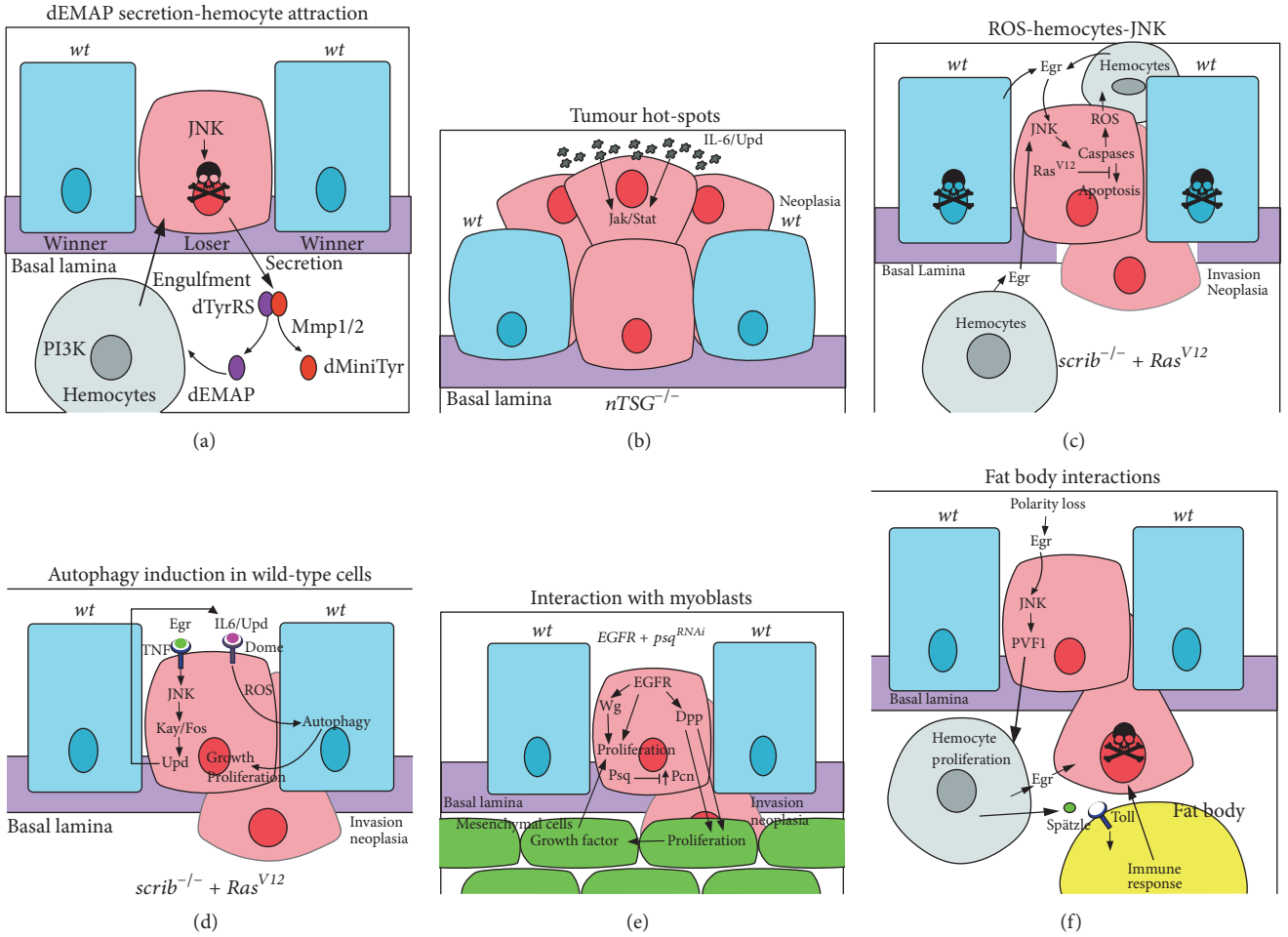

FIGURE 2: *Cooperative interactions between the tumour and surrounding cells in tumorigenesis*. Interactions between cells are shown that result in either the death of the mutant cell or cell survival, proliferation, and neoplastic transformation. Mutant cells are in pink, *wild-type* cells are in blue, hemocytes are in grey, myoblasts (mesenchymal cells) are in green, a fat body adipocyte is in yellow, and the basement membrane (basal lamina) is in purple. (a) dEMAP secretion-hemocyte attraction: JNK signalling in a cell polarity-impaired loser cell transcriptionally upregulates MMP1, which acts to cleave secreted dTyrRS to form dEMAP and dminiTyr. dEMAP attracts hemocytes to the loser cell by upregulating PI3K signalling in the hemocytes, which is required for chemotaxis and possibly engulfment of the loser cell. (b) Tumour hot-spots: neoplastic tumour-suppressor mutants (nTSGs) induce tumours more preferably, in regions where there is a stiff basal lamina and there are developmentally high levels of the Upd (IL-6) ligand to elevate Jak-Stat signalling, which promotes cell survival and proliferation of the tumour cells. (c) ROS-hemocytes-JNK: in *scrib* mutant Ras^{V12}-expressing tumour cells, a feedback loop between the hemocytes and the mutant cells promotes tumorigenesis. In the mutant cells, Ras signalling and caspase activation leads to ROS production that is released from the cells and promotes hemocytes to produce Egr (TNF). Egr signals via the TNFR-JNK pathway in the mutant cell leading to the upregulation of caspase activity, and some apoptosis, which is required for tumour overgrowth and invasion. Due to the disruption of the peripodial epithelium in large *scrib* mutant Ras^{V12}-expressing tumours, hemocytes most likely interact with the tumour on both apical and basal sides. (d) Induction of autophagy in surrounding *wild-type* cells: *scrib* mutant Ras^{V12}-expressing tumour cells are metabolically stressed, which leads to ROS production. Egr-JNK signalling leads to the transcriptional upregulation of Upd, ligands for the Dome-Jak-Stat signalling pathway, which is elevated in the mutant cells. Jak-Stat signalling and ROS production are required for the induction of autophagy in the surrounding *wild-type* cells, and also at distant sites, such as the fat body, muscle, and gut (not shown), which facilitates tumour growth and neoplastic transformation, possibly through supplying amino acids, glucose, and other nutrients to the tumour cells. (e) Interactions with myoblasts: in *EGFR*-overexpressing *psq*-knockdown tumours cooperative interactions are observed between the tumour cells and the surrounding myoblasts (mesenchymal cells). EGFR induces Wg and Dpp expression, and *psq* knockdown leads to increased levels of the extracellular matrix protein, Perlecan (Pcn). Wg acts to promote proliferation of the tumour cells, whilst Dpp, facilitated by Pcn in the basal lamina, stimulates proliferation of the myoblast cells. In turn, the myoblast cells provide unidentified growth factors that drive proliferation and neoplastic transformation of the tumour cells. Myoblasts also supply Egr (not shown), which would be expected to activate the TNFR-JNK signalling pathway in the tumour cells. (f) Interactions with the fat body: polarity-impaired tumours through Egr-JNK signalling upregulate PVF1, a ligand for the PVR receptor on hemocytes, which promotes hemocyte proliferation. Hemocytes, in turn, supply Egr to the tumour cells, and the Toll Receptor ligand, Spätzle, to the fat body, which induces innate immune system signalling in the fat body. These interactions are required to induce apoptosis of tumour cells.

Jak-Stat pathway, thereby activating this signalling pathway and promoting tumour growth [148]. Interestingly, Upd1–3 acts in an autocrine manner in the tumour cells to promote autophagy in the neighbouring *wild-type* cells, most likely by stimulating ROS production or secretion [60].

Furthermore, myoblast cells are thought to provide growth factors, which are currently unidentified, to the epithelial tumour cells to stimulate proliferation and survival [52] (Figure 2(e)). In an *EGFR*-driven *pipsqueak* knockdown neoplastic tumour model, EGFR signalling induces upregulation of Wg, which promotes epithelial tumour cell proliferation, but tumour growth is dependent on the neighbouring myoblast cells. Interestingly, a codependency occurs between the epithelial neoplastic tumour cells and the mesenchymal cells, whereby the TGFβ/Bone Morphogenetic Protein- (BMP-) family morphogen, Decapentaplegic (Dpp), produced in the epithelial cells promotes the expansion of mesenchymal cell compartment, and, in turn, the myoblast cells are required for epithelial cell tumorigenesis [52]. Recent studies have shown that the myoblast cells also produce Egr [61], which, via TNFR signalling, promotes tumorigenesis when cell death is blocked in the epithelial tumour cells. Despite studies showing the importance of Egr in inducing JNK signalling in neoplastic tumours [89], an intrinsic mechanism also exists to elevate JNK signalling in the tumour cells, involving Rho1-GTPase signalling and activation of the JNKKK, Wallenda [61, 149]. Thus, initially, impairment of cell polarity may trigger JNK activation through the Rho1-Wallenda pathway, and, subsequently, myoblasts and hemocytes in the tumour microenvironment are stimulated to produce Egr, thereby amplifying JNK activation in the tumour.

In addition to interactions between the epithelial tumour cells and their local microenvironment, there is also evidence for communication between the hemocyte and the fat body adipocytes [56] (Figure 2(f)). In polarity-impaired neoplastic tumour-bearing larvae, hemocytes supply the Toll ligand, Spätzle, to the fat body adipocytes, which leads to induction of the Toll-NFκB innate immune response signalling pathway in the adipocytes and the production of immune peptides. Egr-JNK signalling in the tumour cells also contributes to the cellular crosstalk, since it results in the transcriptional upregulation of the ligand, PVF1, which, through the PVR signalling pathway, stimulates hemocyte proliferation, thereby elevating Spätzle production from the hemocytes and innate immune signalling in the fat body. This mechanism is required to restrain tumour growth, since knockdown of Spätzle expression in the hemocytes results in reduced Toll pathway signalling in the fat body and reduced tumour cell death. However, whether the fat body-induced immune response only functions to activate the hemocytes, or also by secretion of diffusible signals, to promote tumour cell death, is presently unclear. Moreover, since a Spätzle-modified Toll signalling pathway leading to caspase activation has been observed in dMyc and ribosomal protein cell competition mechanisms [35], the hemocytes might also supply Spätzle to the tumour cells to contribute to their death. Consistent with this, crosstalk between the Toll and JNK signalling pathways in triggering cell death occurs in eye-antennal and wing epithelial tissues [150]. In these tissues, JNK signalling in the epithelial cells induces Spätzle upregulation in the surrounding peripodial membrane cells by an unknown mechanism, which, in turn, activates Toll-NfκB signalling in the epithelial cells. Thus, Spätzle production by hemocytes or peripodial membrane cells, together with Egr-JNK signalling and signals from the fat body, may all be involved in triggering tumour cell death.

To summarize, in cell competition within epithelial tissues, signals from the myoblasts, the extracellular matrix, the cellular innate immune system, and systemic responses all influence whether the tumour cells will be eliminated or survive and progress to form overgrown invasive tumours. Moreover, if cell death of the tumour cells is blocked, tumour intrinsic and cell-cell signalling pathways that are normally antitumorigenic can instead become tumour-promoting (see below). Cell competition mechanisms are conserved in mammalian systems (reviewed in [36, 39, 151, 152]), and the tumour microenvironment plays a key role in mammalian tumorigenesis (reviewed in [153–156]). Thus, the findings from these *Drosophila* studies of cellular interactions in tumorigenesis are likely to provide new insights into the understanding of human cancer initiation and progression.

3. Cooperation Interactions between Oncogenic or Tumour-Suppressor Mutations in Tumour Initiation and Progression

The development of malignant cancer requires the deregulation of many processes, including increased cell proliferation, reduced differentiation and apoptosis, increased invasion, and altered metabolism (reviewed in [157]). There are only a few tumour-causing genes that when individually knocked down or overexpressed in whole epithelial tissues or large domains, are capable of inducing all the hallmarks of cancer that can be modelled in *Drosophila* (reviewed in [3, 11, 15, 158]). Many genes, when deregulated, can cause hyperplastic tumours, characterized by increased tissue growth that are still capable of differentiating, but only a few result in neoplastic tumours, in which the tissue overgrows and shows reduced differentiation and a loss of tissue architecture (reviewed in [159]). Genes capable of conferring many hallmarks of cancer when knocked down or mutated in large domains in epithelial tissues are the junctional (cell polarity regulators, Scrib, Dlg, and Lgl) and endocytic (such as Rab5) neoplastic tumour suppressors. Moreover, recent studies have shown that *lgl* mutant tumours, in addition to possessing other cancer hallmarks, are able to induce an angiogenesis-like process in *Drosophila*, tracheogenesis, in order to obtain an increased oxygen supply [160, 161]. A gene capable of conferring neoplastic overgrowth when expressed in large epithelial tissue domains is the activated version of the receptor tyrosine kinase gene, *PVR* [159, 162, 163]. Additionally, a recent study has shown that expression of the oncogenic fusion between the KIF5B kinesin motor protein and the Ret tyrosine kinase, KIF5B-Ret, promotes many hallmarks of cancer in tracheal epithelial cells [164]. However, as cancer arises from mutations that occur in single cells surrounded by

normal tissue, it is uncommon for perturbations in any one gene to confer all the properties that are required for a normal cell to transform into a proliferative-invasive cancer within the context of a *wild-type* epithelium, since cell competition leads to the elimination of aberrant cells. Even with potent tumour-causing mutations, when generated clonally or by induction in a tissue domain, growth of the tumour beyond a certain size is required to overcome apoptosis induced by cell competition [49, 165]. Thus, the phenomenon of cell competition is one reason why at least two mutations are required for tumour progression when initiated in single cells or small patches of cells, particularly concerning mutants in cell polarity or endocytosis regulators. We will now highlight various cooperative tumorigenesis mechanisms that have been modelled in *Drosophila*, focusing primarily on epithelial tissues (summarized in Table 1), and discuss the important insights these studies have revealed. We will first cover the genes/pathways involved in cell death, caspases (cysteine proteases), and the JNK signalling pathway, since they can have context-dependent roles in tumorigenesis.

3.1. Caspases in Cooperative Tumorigenesis: Context Dependency. Blocking cell death in the mutant tissue (via blockage of effector caspase activity by overexpressing p35) can, in some cases, enable the survival of the mutant cells, thereby revealing their tumourigenic properties. Examples of caspases acting in a tumour-suppressor role occur in epithelial tissues containing *scrib, rok, mud, Sin3a, Snr1, Csk,* or *frazzled* mutant cells [125, 141, 166–168] or overexpressing a subunit of the Vacuolar ATPase (V-ATPase) complex, Vha44 [169]. However, caspases can also be oncogenic in some contexts. Indeed, activating certain caspases at low levels, insufficient to induce cell death (at least not rapidly), can promote an invasive phenotype [168, 170]. Similarly, caspase activity within the tumour is also required for growth of tumours generated by mutations of the endocytosis regulator, Rab5 [165]. Caspase activity is also observed in wing epithelial tumours generated by mutation of the cell polarity regulator gene, *lgl*, which correlates with JNK pathway activation and is important for tumour invasion [161]. Additionally, in polarity-impaired Ras^{V12} epithelial tumours, described above, reducing cell death by knocking down caspase activity reduces tumorigenesis [57]. Thus, caspase activity can be tumour promoting or tumour suppressing, depending on context. These findings have implications for cancer therapy, which is designed to induce caspase-mediated cell death, since mild-to-moderate activation of caspases may instead promote tumour growth and invasive behaviour.

3.2. The JNK Signalling Pathway in Cooperative Tumorigenesis: Context Dependency. The JNK signalling pathway can also have context-dependent roles in tumorigenesis in *Drosophila* and in mammalian systems (reviewed in [143, 171–174]). In some types of cell competition, such as that induced by polarity impairment, the JNK pathway is required to promote apoptosis and therefore is inhibitory for tumour progression (acting as a tumour suppressor) [23, 91, 136]. In these cases, when JNK signalling is blocked using a kinase-dead dominant-negative JNK transgene (bsk^{DN}), tumour cells delaminate from the epithelium, overgrow, and invade into the surrounding epithelium. This occurs in clones for cell polarity regulators, such as *scrib* or *lgl* mutants, but also occurs upon overexpression of an activated version of *aPKC* or *wild-type crb* in clones in the developing eye epithelia when bsk^{DN} is coexpressed [66, 71] (Table 1). The mechanism by which JNK-independent cell invasion occurs in these cases is unknown. Interestingly, in *lgl* mutant clones expressing bsk^{DN}, large GFP-marked tumours are observed in the eye, and clumps of GFP-marked cells occur elsewhere in the head and also in body of the pupae/pharate adult [71]. Cooperative interactions also occur upon blocking JNK and activating other signalling pathways to promote tumorigenesis. Overexpression of the Src tyrosine protein kinase gene, *Src64B*, activates JNK signalling and leads to cell death in the eye-antennal epithelium, but when bsk^{DN} is coexpressed, tumour overgrowth occurs, in a mechanism involving upregulation of the actin-cytoskeletal regulators, Rac1 and Dia, as well as Ras signalling, which inhibit the Hippo pathway, thereby promoting tumour growth [79]. Similarly, in mutants affecting endocytosis, such as *Vps4*, blocking JNK signalling promotes the formation of neoplastic tumours in epithelial tissues, by an unknown mechanism [175].

In another model of tumorigenesis in the developing eye, mutants in *frazzled* (an ortholog of mammalian Deleted in Colorectal Cancer, DCC, a regulator of axon guidance), combined with the blockage of apoptosis by expression of the effector caspase inhibitor, p35, results in elevated JNK and Rho1 activity and promotes cell invasion [125]. However, photoreceptor differentiation still occurs, leading to the migration of differentiated photoreceptor cells to distant sites. Blockage of JNK signalling in *frazzled* mutant *p35* expressing cells enhances the invasive phenotype in a Rho1-dependent manner (Table 1).

Another tumour type, where blocking JNK promotes an invasive phenotype, is the *eyeful* model [116] (Table 1). In this model, overexpression of the Notch ligand, Delta, combined with overexpression of the transcription factor genes, *lola* and *pipsqueak*, in the developing eye, promotes an invasive phenotype but does not affect differentiation, resulting in differentiated photoreceptor cells located at distant sites. This phenotype is dependent on the Polycomb group chromatin-remodelling factor, histone deacetylases, and reduced expression of Rbf1 (the *Drosophila* ortholog of the retinoblastoma tumour suppressor) [116]. Using this model, another group found that overexpression of *atonal* (a transcription factor gene, involved in eye differentiation) reduces the *eyeful* invasive phenotype, whereas knockdown of *atonal* enhances it [120] (Table 1). Atonal functions by inducing JNK activity and possibly enhances cell death and therefore blocking JNK results in restoration of the invasive phenotype [120]. Mammalian atonal, ATOH1, also acts as a tumour suppressor, which may also involve JNK activation [176]. Consistent with the involvement of JNK as a tumour suppressor in this context, in *Delta*-expressing *Drosophila* eye epithelial cells, blocking JNK activity also enhances the invasive phenotype [120]. How invasion occurs upon blocking JNK activity in *Delta*-expressing cells is unknown. Altogether, these examples indicate that blocking JNK can promote cell

Table 1: Cooperating genes in *Drosophila* tumorigenesis.

Cell-autonomous cooperative tumorigenesis		
1st mutation/mechanism	2nd mutation/mechanism	Phenotype/references
Cell polarity gene perturbations		
Loss of function in apicobasal polarity regulators Results in cell polarity loss, JNK activation, mild Hippo pathway impairment		Neoplastic overgrowth in whole tissue context and cell polarity loss and apoptosis in clonal context (reviewed in [19])
Scribble (scrib, dlg, lgl) and Par and Crb polarity module gene loss of function Scribble module loss of function phenotypes dependent on aPKC activation	Ras^{V12} overexpression Dependent on ROS production, TNF (Egr)-JNK signalling, caspase (Dronc) activity Dependent on impairment of Hippo signalling Dependent on PI3K signalling and glutamate utilization	Invasive neoplastic tumours of the larval eye-antennal epithelium [23, 48, 57, 60, 65–68]
scrib loss of function, *aPKC-CA* overexpression, *crb* overexpression	Inhibition of JNK signalling	Neoplastic tumour overgrowth in eye-antennal epithelium [66]
lgl or *scrib* loss of function Results in JNK activation	Ras^{V12} overexpression Requires Hippo pathway impairment	Neoplastic tumours in the larval wing epithelium [49]
scrib loss of function Results in JNK activation	$Notch^{intra\ (Act)}$ overexpression	Invasive neoplastic tumours in the larval eye-antennal epithelium [23, 66, 69]
scrib loss of function Results in JNK activation	Abrupt (BTB-POZ Zn finger transcription factor) overexpression Results in JNK activation Results in Hippo pathway impairment Results in downregulation of differentiation and Ecdysone response genes	Invasive neoplastic tumours in the eye-antennal and wing epithelial tissues [70]
scrib loss of function	Taiman (Ecdysone coactivator) Results in reduced differentiation	Invasive neoplastic tumours in the eye-antennal and wing epithelial tissues [70]
scrib loss of function Results in JNK activation	Slit-Robo2-Ena loss of function	Overgrown tumours in the eye-antennal epithelial tissues [45]
scrib loss of function Results in JNK activation	Slit-Robo overexpression Requires Ena Results in JNK activation and activation of a positive feedback loop	Excessive extrusion and luminal tumour overgrowth in larval eye-antennal epithelial tissues [45]
lgl loss of function Results in Hippo pathway impairment	Inhibition of JNK signalling	Invasive neoplastic tumours of the larval/pupal eye neural-epithelium [71]
lgl loss of function	Myc overexpression	Invasive neoplastic tumours of the larval wing epithelium [50]
lgl loss of function	Hippo pathway impairment	Neoplastic tumours of the larval wing epithelium [49]
lgl loss of function	$Notch^{intra\ (Act)}$ overexpression	Neoplastic tumours of the larval wing epithelium [72]
scrib, dlg or *lgl* depletion	*cno* mutants Results in activation of Ras-MAPK signalling	Enhanced neoplastic tumours of the antennal epithelium [73]
Par-1 overexpression Results in cell polarity loss and Hippo pathway impairment		Eye-antennal and wing tissue overgrowth [74]
Par-1 overexpression	$Notch^{intra\ (Act)}$ overexpression	Hyperplastic eye-antennal epithelium [75]

Table 1: Continued.

Cell-autonomous cooperative tumorigenesis		
1st mutation/mechanism	2nd mutation/mechanism	Phenotype/references
Actin cytoskeletal regulators		
Activation of Actin cytoskeletal regulators *Rac1, RhoGEF2, Pbl, Rho^{V14}, Rho1, Rok^{CAT}, sqh^{EE}* Results in activation of JNK signalling and cell morphology changes	Ras^{V12} (Raf gain-of-function) overexpression	Invasive neoplastic tumours of the larval eye-antennal epithelium [76, 77]
RhoGEF2 overexpression Results in JNK activation and cell morphology changes	Abrupt (BTB-POZ Zn finger transcription factor) overexpression Results in reduced expression of differentiation gene, Dac	Neoplastic tumours of the larval eye-antennal epithelial tissue [78]
Src64B overexpression Results in JNK activation and cell morphology changes	Blocking JNK Rac1-Dia, Ras-MAPK, Hippo pathway impairment	Eye-antennal epithelial tissue overgrowth [79]
csk loss of function (Src activation) Depends on Actin cytoskeleton regulators, JNK activation, STAT activation, Hippo pathway impairment, Wingless (Wnt) expression/signalling and insulin-PI3K signalling	Ras^{V12} overexpression Promotes cell proliferation and survival	Invasive neoplastic tumours of the larval eye-antennal epithelium [80–85]
Src42A or Src64B overexpression Results in Egr independent activation of JNK and Jak-Stat signalling	Notch$^{intra\ (Act)}$ overexpression	Neoplastic tumours of the larval eye-antennal and wing epithelium [86]
Src64B overexpression Results in cell morphology changes	Abrupt overexpression Reduces expression of differentiation gene and Dac and Dll	Neoplastic tumours of the larval eye-antennal epithelial tissue [78]
Troponin I overexpression	*Ras^{V12}* overexpression *Notch$^{intra\ (Act)}$* overexpression *lgl* mutant *Ras^{V12}* overexpression	Tumour overgrowth or neoplastic tumour overgrowth in wing epithelial tissue [87]
Signalling pathway deregulation		
Ras^{V12} overexpression Results in tissue overgrowth, which depends upon EGF-EGFR activation and Arf6 mediated Hedgehog signalling		Eye-antennal and wing epithelial tissue overgrowth [88]
	TNF-JNK signalling	Invasive neoplastic tumours in the larval eye-antennal epithelium [76, 89–91]
	Immune signalling and activation of JNK	Invasive neoplastic tumours of the adult hindgut epithelium [92]
	Ben/dUev1a E2 ubiquitin ligase overexpression Results in JNK activation (via binding Traf2)	Invasive neoplastic tumours in the larval eye-antennal epithelium [93–95]
	sds22 (PP1) loss of function Results in cell morphology/polarity loss Results in Myosin II, JNK activation	Invasive neoplastic tumours of the larval eye-antennal epithelium [96]
	PP6 phosphatase (FMT, PpV) knock down Results in Tak1-JNK activation	Invasive neoplastic tumours of the eye-antennal epithelium [97]

Table 1: Continued.

Cell-autonomous cooperative tumorigenesis		
1st mutation/mechanism	2nd mutation/mechanism	Phenotype/references
	Infection/inflammation Results in Imd-dTab2-dTak1-JNK signalling and MMP1 expression	Hindgut epithelial tumour invasion [92, 98]
	Impaired Hippo pathway signalling Results in upregulation of Ras pathway genes, Upd-Jak-Stat signalling	Eye-antennal and wing tissue overgrowth [65, 99]
	Lysosomal protein loss of function—*deep orange, carnation, vps16A*	Invasive neoplastic tumours of the larval eye-antennal epithelium [100]
	Autophagy loss of function—e.g., *Atg8a, Atg7, Atg9, Atg1, Atg13, Syx17* Requires ROS and JNK upregulation	Invasive neoplastic tumours of the larval eye-antennal epithelium [101]
	Chromosome remodelling complex mutation *polyhomeotic* Depends on ectopic Notch activation	Invasive neoplastic tumours of the larval eye-antennal epithelium [102]
	Chinmo (BTB-POZ Zn finger transcription factor) overexpression	Overgrown tumours in the eye-antennal epithelial tissues [69]
	Fruitless (BTB-POZ Zn finger transcription factor) overexpression	Overgrown tumours in the eye-antennal epithelial tissues [69]
	PTEN knockdown (Elevated PI3K signalling)	Larval-Pupal tracheal epithelial tissue invasive tumours [103]
	apc (Wingless/Wnt) signalling	Adult midgut epithelial tissue overgrowth [104, 105]
	p53, apc, pten knockdown *dSmad4, apc, pten* knockdown	Adult hindgut epithelial tissue invasive tumours [106]
	pico (MRL) overexpression *chickadee* (Profilin) overexpression *mal* (SRF cofactor gene) overexpression Requires JNK-MMP1 activity	Glial cell overgrowth and invasion [107]
EGFR activation/overexpression Depends on Ras and Hh signalling		Eye-antennal and wing epithelial tissue overgrowth [88]
	fat loss of function (Hippo pathway impairment)	Eye-antennal and wing epithelial tissue overgrowth [108]
	bantam micro-RNA expression Results in downregulation of Socs36E Leads to increased Jak-Stat signalling	Invasive overgrowth of the larval wing epithelium [109]
	miR-10 or *miR-375* Micro-RNA expression Results in downregulation of Psq transcription factor	Invasive overgrowth of the larval eye-antennal and wing epithelium [52]
	miR-8 Micro-RNA expression Results in downregulation of Peanut protein expression, cytokinesis blockage, formation of polyploid cells	Invasive overgrowth of the larval wing epithelium [110]
	PI3K pathway activation Requires Tor, Sin1, Rictor, Myc, Cyclin D-Cdk4, Rb-E2F and Cdc25 Requires RIOK1, RIOK2	Glia cell invasive brain tumours and eye neural-epithelium tumours [111–113]
Notch$^{intra\ (Act)}$/Delta overexpression Results in tissue overgrowth		Eye-antennal and wing tissue overgrowth (reviewed by [114])
Notch$^{intra\ (Act)}$ overexpression	Mef2 overexpression	Invasive neoplastic tumours of the larval eye neural-epithelium [115]

Table 1: Continued.

Cell-autonomous cooperative tumorigenesis		
1st mutation/mechanism	2nd mutation/mechanism	Phenotype/references
Notch$^{intra\ (Act)}$ overexpression	Chinmo (BTB-POZ Zn finger transcription factor) overexpression	Overgrown tumours in the eye-antennal epithelial tissues [69]
Notch$^{intra\ (Act)}$ overexpression	Fruitless (BTB-POZ Zn finger transcription factor) overexpression	Overgrown tumours in the eye-antennal epithelial tissues [69]
Delta overexpression	Overexpression of transcription factors Psq/Lola (*eyeful* model)	Invasive tumours larval/pupal eye neural-epithelium, which are capable of differentiation to express ELAV [116]
Delta overexpression	Overexpression of Akt or PI3K (Dp110)	Invasive tumours larval/pupal eye neural-epithelium, which are capable of differentiation (ELAV expression) [117]
Delta overexpression Results in repression of *boi* gene expression and reduced Hedgehog signalling	Overexpression of *mir-7* micro-RNA Results in downregulation of *ihog* translation and reduced Hedgehog signalling	Eye-antennal disc overgrowth and invasive cells cable of differentiation [118]
Delta overexpression	Overexpression of *Zfh1* (Zeb1 family transcription factor gene)	Invasive tumours larval/pupal eye neural-epithelium, which are capable differentiation to express ELAV [119]
Delta overexpression Delta with Pipsqueak/Lola overexpression (*eyeful* model)	Knockdown of *atonal* (transcription factor gene) Results in reduced JNK signalling	Invasive tumours larval/pupal eye neural-epithelium, which are capable differentiation to express ELAV [120]
Delta overexpression Delta with Pipsqueak/Lola overexpression (*eyeful* model)	Knockdown of *cut* (transcription factor gene) Results in increased *reaper* expression and elevated PI3K-Akt signalling	Invasive tumours in larval/pupal eye-antennal epithelium, which are capable differentiation to express ELAV [121]
Hippo pathway impairment (Yki overexpression) Results in increased tissue growth through upregulation of cell growth (Myc), proliferation (CycE) and antiapoptotic genes (Diap1), elevation of Upd-Jak-Stat signalling		Increased tissue growth (reviewed by [122])
	BAP (Brahma) complex knockdown (*brm, Snr1, mor, Bap111, osa*) Results in upregulation of Wingless (Wnt) and Dpp signalling	Neoplastic tumour overgrowth in larval wing epithelial tissue [123]
	Taiman (Ecdysone Receptor coactivator) overexpression Results in expression of germline stem cell factors	Hyperplastic tumour overgrowth in larval wing epithelial tissue [124]
Guidance receptors		
Frazzled (Dcc) loss of function and expression of the Caspase inhibitor P35 Results in an invasive phenotype in eye epithelial cells, but cells can differentiate	Inhibition of JNK signalling Requires Rho1	Invasive, but differentiated, tumours in larval/pupal eye-antennal epithelial tissues [125]
Mitotic checkpoint, chromosome instability, DNA damage repair genes		
Nek2 (centrosomal kinase) overexpression	Ret^{MEN2B} overexpression—elevated Ras, PI3K, Src, JNK signalling Csk^{-} Ras^{V12} Results in Rac1, Rho1, Wg signalling and elevated expression of Diap1, MMP1 Results in PI3K signalling	Invasive tumours in larval eye-antennal epithelial tissue [126]
bub3 knockdown Results in aneuploidy	*p35* overexpression to block effector caspase activity	Neoplastic overgrowth of wing epithelial cells [127]
DNA repair or DNA damage checkpoint mutants Depletion of *okra* (*DmRAD54*) or *spnA* (*DmRAD51*) (Homologous recombination of DNA double strand-breaks in G2) *grp* (*chk1*) and *mei-41* (*ATR*) knockdown (DNA damage checkpoint)	Ionizing irradiation and *p35* overexpression Results in JNK activation, which leads to MMP1 and Wg upregulation	Overgrowth and cell delamination/migration in the wing epithelial tissue [128]

survival of tumourous cells and that alternate mechanisms promote cell invasion. In studies where mechanistic insights were obtained, these have indicated the involvement of Rho1 or Rac1, which are known regulators of the actin cytoskeleton in cell migration (reviewed in [177]), and the activation of these small-GTPase may very well be involved in other cases of JNK-independent cell invasion.

In contrast to the above examples that highlight a tumour suppressive role for JNK signalling, in other contexts, the JNK pathway can function as a tumour promoter, by altering cell morphology, driving cell invasion, and blocking differentiation. For example, in *lgl* mutant wing epithelial tissue, JNK activation promotes cell morphology changes that potentiates the loss of apicobasal cell polarity and enables tumour formation [178, 179]. Furthermore, in *scrib, dlg*, or *lgl* mutant Ras^{V12}-expressing clones in the developing eye (see below), inhibition of JNK prevents invasive behaviour of cells into the brain lobes-ventral ganglion and promotes differentiation and pupariation [66, 89–91, 93]. Similarly, in wing epithelial tissues overexpressing the Vha44 component of the V-ATPase, which activates JNK signalling and results in invasive tumours, blocking JNK suppresses the invasive phenotype [169]. Additionally, in eye epithelial tissue activation/overexpression of the Rho1 or Rac1 small GTPases (which regulate actin polymerisation and F-actin/Myosin II contractility) also cooperate with Ras^{V12} to promote invasive overgrowth, dependent upon increased JNK activity [76, 77] (Table 1). In another model, impairment of the Sds22/PP1 phosphatase in Ras^{V12}-expressing cells in the anterior-posterior boundary of the developing wing epithelium, in a JNK-dependent manner, leads to invasive tumours [96] (Table 1). Here, Myosin II activation is also required for invasion, which mechanistically may involve regulation of the JNK pathway by Rho1-Rok-Myosin II signalling, as has been observed in other contexts [61, 76, 77, 149, 180]. Indeed, JNK's oncogenic role in cooperative tumorigenesis is evident in experiments showing that overexpressing JNK pathway genes in combination with Ras^{V12} in the developing eye epithelium induces invasive tumour growth [76, 90, 91, 149]. Moreover, overexpression of the E2 ubiquitin ligase, Ben/dUev1a, which activates JNK signalling, also cooperates with Ras^{V12} to promote invasive tumour growth [94] (Table 1). More recently, loss of function mutations in the PP6 phosphatase have been shown to act upstream of the Tak1 protein kinase, a JNKKK, to induce invasive tumorigenesis in Ras^{V12}-expressing eye-antennal epithelial cells [97]. Furthermore, in the adult *Drosophila* hindgut epithelium, JNK activation through the Egr (TNF) pathway, in response to bacterial infection, also cooperates with Ras^{V12} to promote invasive overgrowth [92, 98] (Table 1).

In summary, the JNK pathway is an important player in cooperative tumorigenesis but dependent on context it can have a tumour-suppressing or tumour-promoting role. Due to this context dependency, which is also observed in mammalian systems [173, 174], the activation of JNK alone in a tumour is not a clear diagnostic or prognostic marker of outcome, and knowledge of other molecular defects is required to predict tumour behaviour.

3.3. Cooperation between Cell Polarity Impairment and Oncogenes. Impairment of cell polarity is a powerful force in tumorigenesis (reviewed in [15, 19, 181]). When cell polarity genes *(scrib, dlg*, and *lgl)* are mutated or knocked down in a clonal context, aberrant mitotic spindle orientation, cell polarity impairment, ectopic cell proliferation, and aberrant differentiation occur, but, despite this, malignant tumours do not form, and the mutant tissue is mostly eliminated by JNK-mediated cell death [23, 90, 91, 136, 141, 182]. However, in arguably the first demonstration of *Drosophila* cooperative tumorigenesis, expression of oncogenic *Ras* (Ras^{V12}) or *Notch* ($Notch^{intra\ (Act)}$) in *scrib* mutant clones prevents their elimination by cell death and instead promotes cell proliferation to produce overgrown undifferentiated and invasive tumours [23, 65, 90, 91] (Table 1, Figure 2(c)). Similar cooperative tumourigenic interactions were also observed for *dlg* and *lgl* mutants and Ras^{V12} [65] and also for *lgl* and $Notch^{Act}$ [72]. In these cooperative interactions, Ras^{V12} and $Notch^{Act}$ promote cell survival and proliferation, whilst *scrib* mutation leads to aPKC activation, which results in impairment of the Hippo negative tissue-growth pathway, leading to the activation of the downstream cotranscriptional activator, Yki, and tissue overgrowth [47, 48, 183]. Additionally, *scrib* mutation promotes JNK activation, which blocks differentiation and progression to the pupal stage and leads to an invasive cell phenotype through upregulation of MMP1 (a metalloprotease, involved in degradation of the extracellular matrix), Paxillin (a regulator of integrin signalling), Robo (a guidance receptor), and various actin-cytoskeletal regulators [45, 66, 90, 184]. More recently, global expression analyses of *scrib* mutant tissue [148], and *scrib* mutant Ras^{V12}-expressing or *scrib* mutant $Notch^{Act}$-expressing epithelial tissues [69, 99, 185–187], has revealed the spectrum of deregulated genes that contribute to cooperative tumorigenesis. In addition to members of the JNK and Hippo pathways, these include Polycomb chromatin-remodelling complex components, the BTB-POZ zinc-finger transcription factor genes, *chinmo* and *fruitless*, the Ets-family transcription factor, *Ets21c*, and the nuclear receptor transcription factor gene, *ftz-F1*. These transcription factors contribute to the switching of the differentiation state of the tissue towards a progenitor cell-like fate, deregulation of signalling pathways, and the promotion of cell proliferation, survival, and invasion. Additionally, genetic screens of *scrib* mutant Ras^{V12}-expressing tumours have revealed the importance of the PI3K signalling pathway [67], and chemical screens have revealed the importance of glutamate utilization enzymes, the TCA cycle, and pyrimidine synthesis [68] for tumour growth. *scrib* mutant Ras^{V12}-expressing tumours, in a JNK-dependent manner, upregulate the diffusible Insulin-like peptide, dILP8 [69]. This, in turn, in the prothoracic gland, leads to the downregulation of the secreted steroid hormone, Ecdysone, which is required for metamorphosis, and therefore pupariation is delayed/prevented, thereby leading to the formation of oversized (giant) larvae [188–190]. In addition, *scrib* mutant Ras^{V12}-expressing tumours secrete the insulin growth factor binding protein, ImpL2, which is an antagonist of Insulin signalling that results in wasting of adipose, muscle, and gonadal tissues in the

larvae [191]. Thus, polarity impairment together with the Ras oncogene leads to a plethora of gene expression changes and perturbed signalling pathways, which together promote the tumourigenic phenotype, as well as affecting other tissues in the larvae. Expression profiling and functional analyses of *lgl* mutant epithelial tissue have revealed that, similar to *scrib* mutants, signalling pathways (Hippo and JNK) and cell fate genes are deregulated [72, 160, 178, 179, 192–194]. However, other signalling pathways, such as Notch, PI3K, and Wingless, are also elevated in *lgl* mutant tissue [72, 160, 194–196], but they have not been reported to be so in *scrib* mutant tissue. Therefore, the cooperative tumorigenesis mechanisms of *scrib* and *lgl* mutants with Ras^{V12} might be slightly different.

Many features of the cooperative tumourigenic interaction between *scrib* mutants and oncogenic Ras are conserved in mammalian epithelial systems, both *in vitro* [197] and *in vivo* in epithelial cells of the mouse prostate, lung, breast, and skin tissue [198–201]. Whilst a complete mechanistic picture is lacking, studies in mammalian cell lines have revealed that Scrib depletion in EGF-stimulated epithelial cells elevates ERK as well as JNK signalling [197], and cell polarity perturbation leads to Hippo pathway impairment [202, 203]. Thus, at least some aspects of the mechanism of cooperation between oncogenic Ras and cell polarity genes mutations have proven to be conserved between *Drosophila* and mammals, and further studies are needed in mammalian systems to reveal whether other downstream events are also conserved. Furthermore, *lgl* mutants cooperate with overexpression of the dMyc transcription factor in the wing epithelium [50], which has also been observed for *scrib* downregulation and Myc in mouse mammary epithelial tissue [204], but whether similar mechanisms are involved is currently not known. Additionally, in the wing epithelial tissue, *lgl* mutant cells that are undergoing cell competition-mediated elimination cooperate with impaired Hippo signalling to generate overgrown neoplastic tumours [49]. However, whether this also occurs in mammalian systems is currently unknown.

Subsequent studies using polarity-impaired epithelial tumour models have revealed novel cooperating genes (see Table 1), which provide insight into mechanisms of tumorigenesis relevant to human cancer. Notable recent examples of these include overexpression of the BTB-POZ transcription factor gene, *abrupt*, which was discovered in a genetic screen to cooperate with *scrib* loss to induce neoplastic tumours in the eye-antennal epithelium [70]. Through target gene identification, *abrupt* overexpression was shown to cooperate with *scrib* mutants in tumorigenesis by downregulation of multiple differentiation genes and deregulation of the Hippo and JNK signalling pathways. Moreover, genes responsive to the steroid hormone, Ecdysone, were downregulated, which contributes to the developmental block at the larval stage, enabling the continuation of invasive tumour growth [70]. Interestingly, the Ecdysone Receptor- (ER-) associated factor, Taiman, which binds to Abrupt in ovarian tissues [205], is required for the growth of *scrib* mutant *abrupt*-overexpressing tumours, and overexpression of *taiman* in *scrib* mutant cells also leads to invasive neoplastic tumours [70]. More recently, Taiman was shown to bind to the Hippo pathway cotranscription factor, Yki, and to control the transcription of a novel set of genes that regulate germline stem cell identity [124], although whether these genes are also deregulated in *taiman* or *abrupt*-overexpressing *scrib* mutant tumours has not been investigated.

Another signalling pathway involved in *scrib* mutant tumorigenesis is the Slit-Robo-Ena pathway. This pathway is involved in the basal extrusion of *scrib* mutant tissue from the epithelium, where they die, and downregulation of this pathway results in overgrown (but noninvasive) tumours within the eye-antennal epithelium [45]. Conversely, hyperactivation of the Slit-Robo-Ena pathway in *scrib* mutant or *wild-type* cells results in a hyperextrusive phenotype, with the apically (lumenally) extruded cells forming overgrown tumours, which might occur by the peripodial membrane epithelium preventing access of the innate cellular immune system cells to the tumour [45, 46]. However, it is also possible that the lumenal microenvironment is conducive to tumour cell growth and survival, which may be dependent on morphogens, such as Dpp, produced from the peripodial epithelium [206].

Scribble module genes, but not other apical-basal cell polarity genes, were identified as *Drosophila* neoplastic tumour-suppressor genes; however the downregulation of Crb and Par modules cell polarity genes together with Ras^{V12} expression in the eye-antennal epithelial also results in neoplastic tumour formation [65]. Furthermore, overexpression of Par1 cell polarity regulator, which inactivates Hippo signalling [74], cooperates with activated Notch signalling in promoting tumourous overgrowth in the eye-antennal epithelium [75], similar to that which occurs with activated *Notch* and *scrib* mutants [23] or *lgl* mutants [72]. It is likely that similar mechanisms are involved in the cooperation of Crb and Par module gene mutants with Ras^{V12}, as well as with *Par1* and $Notch^{Act}$, as occurs with *scrib* mutants with Ras^{V12} or $Notch^{Act}$; however formal demonstration is currently lacking. Interestingly, *canoe* (*cno*, *afadin/AF-6* in mammals), a gene involved in another type of cell polarity, asymmetric cell division [207], has been recently shown to cooperate with *scrib*, *dlg,* or *lgl* depletion in epithelial tumorigenesis [73]. Mechanistically, this synergistic interaction involves the activation of Ras-MAPK signalling, which implicates the *wild-type* function of Cno as well as Scrib, Dlg, and Lgl in the repression of Ras signalling [73], as occurs with the mammalian Cno (Afadin/AF-6) and Scrib [197, 208].

3.4. Cooperative Tumorigenesis Involving Actin-Cytoskeletal Regulators. Deregulation of the actin cytoskeleton leads to cell morphology changes, increased tissue growth through impairment of the Hippo pathway, and reduced cell-cell adhesion and can promote invasive phenotypes [76, 77, 177, 209–212]. However, in a clonal context, tissue growth due to deregulated actin-cytoskeletal gene expression is restrained by JNK-mediated cell death, and therefore cell death blockage or oncogenic activation is required for tumorigenesis. Indeed, the activated small GTPases Rho1 and Rac1, which regulate the actin cytoskeleton, cooperate with Ras^{V12} in tumorigenesis in a JNK-dependent manner [76, 77] (Table 1).

Furthermore, downstream of the Rho1-GTPase, the Rok protein kinase, and activated Myosin II, which regulate F-actin filament contractility, cooperates with Ras^{V12} to promote tumorigenesis [77]. Mechanistically, the contribution of the Rho1-Rok-Myosin II pathway to Ras^{V12}-driven tumorigenesis most likely involves JNK activation [77], and also Hippo pathway impairment, as increased F-actin contractility leads to Yki activation-induced tissue growth (reviewed in [211, 213]). Activation of Rho1, by RhoGEF2 overexpression, also cooperates with overexpression of the Abrupt BTB-POZ transcription factor in inducing neoplastic tumours of the eye-antennal epithelium by blocking expression of differentiation genes [78] (Table 1). It is likely that JNK activation and Hippo impairment are also involved in this cooperative interaction; however this remains to be confirmed.

The Src nonreceptor tyrosine protein kinase, a key regulator of the actin cytoskeleton as well as adherens junctions [214], cooperates with several oncogenes to promote tumorigenesis in *Drosophila* (Table 1). Activation of Src through knockdown of its negative regulator, Csk, together with Ras^{V12} also results in invasive overgrown tumours of the eye-antennal epithelium [80–84]. Mechanistically, Src activates JNK and Stat signalling, modulates the actin cytoskeleton, and impairs Hippo signalling to promote invasive overgrowth in cooperation with Ras^{V12} [79–83, 85]. Moreover, on a high sugar diet, Src-activated Ras^{V12}-driven tumours have an altered metabolism and elevate Wg signalling, which leads to upregulation of the Insulin-Receptor gene expression, enabling the tumour cells to become insulin-responsive and aggressively overgrow, whilst other larval tissues are insulin-resistant and hypoplastic [81, 84]. Overexpression of Src64B or Src42A also cooperates with activated Notch signalling to promote tumorigenesis in eye-antennal and wing epithelial tissue, in a mechanism requiring JNK activation in a TNF-independent manner [86]. Given the link between Src and actin-cytoskeletal regulators [85], and the discovery of a mechanism linking Rho1 to JNK activation via the JNKKK, Wallenda [149], a similar mechanism might be involved in the activation of JNK in *Src* $Notch^{Act}$ cooperative tumorigenesis. Additionally, Src64B overexpression cooperates with overexpression of the Abrupt BTB-POZ transcription factor in the eye-antennal epithelium by blocking differentiation genes and promoting a progenitor-like cell fate [78]. Although Src expression changes the repertoire of Notch target gene transcription in the $Notch^{Act}$ tumours [86], whether differentiation blockage is also involved in this tumour type and other Src-driven tumours remains to be determined.

Similarly, when induced in a clonal setting, overexpression of the actin-cytoskeletal regulator, Troponin I, cooperates with $Notch^{Act}$ expression, Ras^{V12} expression, and *lgl* mutant Ras^{V12} expression to promote tumour overgrowth by altering gene transcription [87]. Genes upregulated included those encoding the Insulin Receptor (InR), Rap1 (a Ras-related protein), and Dilp8 (insulin-related peptide), which are likely to affect tumour growth by promoting cell proliferation and in the case of Dilp8 by delaying pupariation through downregulation of Ecdysone production in the prothoracic gland.

3.5. Deregulation of Signalling Pathways in Cooperative Tumorigenesis

EGFR-Ras-MAPK. The mitogenic EGFR-Ras-MAPK signalling pathway is a powerful inducer of tissue growth but also induces differentiation in *Drosophila* (reviewed in [215, 216]). Moreover, this pathway is important in cancer, as mutations in Ras signalling pathway genes that elevate pathway activity are present in ~30% of human cancers (reviewed in [217–219]). Although oncogenic Ras is a potent inducer of tissue growth, high level of pathway flux leads to senescence or differentiation, thereby limiting tumorigenesis (reviewed in [220, 221]). Thus, additional mutations are required for Ras-driven malignant cancer development.

Oncogenic Ras requires EGFR signalling to potently induce tissue overgrowth in both *Drosophila* and human cells [88]. Mechanistically, this occurs through the endocytosis regulator, Arf6, which is important for the trafficking of the Hedgehog morphogen and activation of the Hedgehog signalling pathway. In *Drosophila*, activated Ras signalling cooperates with many pathways to promote tumorigenesis (Table 1). In addition to mutations/overexpression of cell polarity, actin cytoskeletal, and JNK pathway genes that cooperate with oncogenic Ras in tumorigenesis in *Drosophila* (discussed above), many other cooperative interactions have been revealed in various *Drosophila* epithelial tissues that confer either hyperplastic or neoplastic overgrowth (Table 1). Hyperplastic tumourigenic interactions include the cooperation of Ras^{V12} with the overexpression of *chinmo* or *fruitless* BTB-POZ domain transcription factor genes [69], and with impaired Hippo pathway signalling [65, 99], which results in enhanced hyperplastic overgrowth of eye-antennal epithelial tissue. In the cooperation of Hippo pathway impairment with Ras activation, a global transcriptome analysis has provided insight into how the differentiation function of Ras signalling is reprogrammed to promote tumorigenesis, by showing that Yki elevates the expression of the Ras target gene, *pointed*, which is crucial for the synergistic tissue growth [99, 222].

Conversely, in the eye-antennal epithelial tissue, Ras^{V12} cooperates with lysosomal gene loss of function to cause neoplastic overgrowth [100]. Additionally, mutations in the Polycomb complex chromatin-remodelling gene, *polyhomeotic (ph)*, cooperate with Ras^{V12} in a clonal context to induce eye-antennal tissue neoplastic tumours, which depends on Notch pathway activation [102]. However, loss of *ph* and other Polycomb complex genes, when generated in a whole eye-antennal epithelial tissue, results in neoplastic tumours, which in this context is dependent on ectopic Upd-Jak-Stat signalling [223]. These differences might depend on the level of expression and the region of the tissue affected, but, additionally, in the clonal context, the induction of cell competition might affect the cooperative mechanism involved in neoplastic tumour formation.

Interestingly, autophagy gene knockdown cooperates with Ras^{V12} to produce different outcomes depending on context [101]. Knockdown of autophagy genes using *UAS-RNAi* lines via the *eyeless-GAL4* driver, or, clonally, within

the developing eye epithelium, enhances Ras^{V12} hyperplastic overgrowth, whereas using the *eyeless-FLP-out Tubulin-GAL4* system, which results in the strong expression of the transgenes throughout the whole eye-antennal epithelium, autophagy gene knockdown together with Ras^{V12} expression results in neoplastic overgrowth and death at the larval-pupal stage. Mechanistically, the cooperation of Ras^{V12} with autophagy gene knockdown, in both the hyperplastic and neoplastic tissue overgrowth effects, occurs because oncogenic Ras signalling induces autophagy in imaginal disc epithelial tissues, and consequently the blockage of autophagy at any step of the pathway results in ROS accumulation and activation of JNK signalling [101]. This finding may also be relevant to human cancer, since in human pancreatic cancers, where $K\text{-}Ras^{G12V}$ mutations are common, downregulation of several autophagy genes correlates with poor prognosis [101]. Since autophagy inhibitors are being considered for cancer therapy (reviewed in [224]), this study highlights the need for caution with Ras-driven cancers, where inhibiting autophagy might inadvertently exacerbate cancer development.

Overexpression/activation of EGFR also cooperates with several genes in tumorigenesis in *Drosophila* epithelial tissues (Table 1). EGFR cooperates with impaired Hippo pathway signalling, leading to tissue overgrowth [108] and also with the overexpression of the *bantam* micro-RNA (which is a downstream target of Yki [225, 226] and also of EGFR signalling [227]), leading to overgrown invasive tumours [109]. EGFR cooperates with the *bantam* micro-RNA by elevating Jak-Stat signalling due to *bantam* repressing the translation of the Jak-Stat signalling inhibitor, Socs36E. Activated Ras together with knockdown of Socs36E causes similar cooperative effects, showing that, downstream of EGFR, Ras signalling is crucial for this cooperation. Elevated expression of the Snail transcription factor, a driver of the epithelial-to-mesenchymal transition (EMT), also occurred in these tumours, as well as expression of the JNK target, MMP1, suggesting that JNK activation is also involved. This group also discovered that the overexpression of micro-RNAs, *mir-10,* or *mir-375,* cooperates with overexpression of EGFR in promoting invasive overgrown tumours [52]. This cooperation occurs by downregulation of the transcription factor, Pipsqueak (Psq), which leads to increased expression of the extracellular matrix protein, Perlecan (Pcn), resulting in tumour overgrowth by a non-cell-autonomous mechanism involving the surrounding myoblast cells [52, 228] (Figure 2(e), see above). Perlecan promotes Dpp signalling in the myoblasts, supporting their proliferation, and, in turn, the myoblasts provide growth factors that promote epithelial tumorigenesis. More recently, the same group found that overexpression of another micro-RNA and the *miR-200* family member, *miR-8*, cooperates with EGFR overexpression to result in clonal overgrowth, cell polarity loss and invasive phenotypes in the wing epithelial tissue [110]. Curiously, these tumours became polyploid, which was attributed to *miR-8* repressing the translation of the Septin, Peanut, which is required for cytokinesis. However, although Peanut downregulation was required, it was not sufficient for tumorigenesis with EGFR overexpression, suggesting other *miR-8* targets are also involved. These tumours also acquire a supercompetitor phenotype and are able to induce cell death of, and engulf, their neighbours. In mammalian systems, *miR-200* family downregulation induces an EMT in some settings [229]; however its overexpression occurs in ovarian cancers where cells commonly exhibit polyploidy [230–233]. Thus, this unusual cooperative behaviour, identified in *Drosophila*, might have relevance to certain types of human cancer.

Several studies have also focused on directed modelling in *Drosophila* of EGFR-Ras-driven human cancers, such as lung, colorectal, and glioblastoma cancers (Table 1). In a model of Ras-driven lung cancer, Ras^{V12} coexpression with *PTEN* knockdown (which elevates PI3K signalling) in the larval-pupal tracheal epithelial cells results in tracheal cell overgrowth and invasive tumours [103]. Colorectal cancer was modelled by knocking down the *adenomatous polyposis coli (apc)* gene and overexpressing Ras^{V12} in the adult midgut [104, 105], which resulted in hyperplasia. In another study, the adult hindgut was used and Ras^{V12} was expressed together with *p53, apc, pten* knockdown or *dSmad4*, *apc,* and *pten* knockdown (commonly observed mutations in human colorectal cancers), which resulted in invasive tumours [106]. Glioblastoma was modelled in *Drosophila* by expressing constitutively active forms of EGFR and PI3K, which is commonly observed in human glioblastomas [111, 112, 234]. Genetic analysis revealed that dMyc, Cdc25, Cdk4, and the TORC2 regulatory subunits, Sin1 and Rictor, were important in glial cell tumorigenesis in the brain and eye tissue [111]. Moreover, a genetic screen of the kinome led to the identification of RIOK1 and RIOK2 kinases, which promote mTORC-Akt signalling to drive glial tumour growth [113]. A recent study has also revealed cooperative tumorigenesis in glial tumour growth and invasion between Ras^{V12} and overexpression of *pico* (a MRL family gene), *chickadee* (*profilin,* encoding an actin-cytoskeletal regulator) or *Mal* (encoding a cofactor of Serum Response Factor (SRF)) [107], suggesting that SRF signalling might be a novel pathway to investigate in human glioblastomas.

Delta-Notch. The Delta-Notch signalling pathways play multiple roles in tissue growth and development in *Drosophila*, and ectopic activation leads to overgrowth phenotypes (reviewed in [114, 235, 236]). For neoplastic tumour formation, additional gene mutations are required together with Notch-Delta overexpression/activation, as detailed below. Activated Notch was shown to cooperate with overexpression of the transcription factor Mef2, leading to disruption to the actin cytoskeleton and apicobasal cell polarity [115] (Table 1). This cooperative interaction is JNK dependent, requiring upregulation of Egr [115]. In the *eyeful* model, in which *Delta* is overexpressed with the *psq* and *lola* transcription factor genes [116], cooperative tumorigenesis occurs upon downregulation of the *cut* transcription factor gene, which leads to a disruption to adherens junction-mediated cell-cell adhesion and cell-basement membrane β-integrin-mediated adhesion, causing increased invasion [121] (Table 1). In these cooperative interactions of *cut* downregulation with *Delta* overexpression, or with the *eyeful* model, upregulation of the cell death gene, *reaper (rpr),* and elevated PI3K-Akt

signalling are involved [121]. The invasive phenotype of these tumours required MMPs, which is a JNK target, but whether JNK was also involved was not determined. Since caspase activation and JNK signalling have been previously linked to invasive cell behaviour in the wing epithelium [168], it is possible that JNK and caspase activation are also involved in the invasive phenotype of *cut* downregulation in the *eyeful* model. Additionally, PI3K or Akt overexpression has been previously shown to cooperate with Delta overexpression in the eye epithelial tissue to induce an overgrown invasive phenotype, which might be relevant to human cancer, particularly T cell acute lymphoblastic leukaemia, where Notch and Akt pathway activation often occurs [117, 237]. The *Delta*-driven invasive phenotype of the *eyeful* model was suppressible by overexpression of the *miR-200* family micro-RNA, *miR-8* [119]. This tumour-suppressor role for *miR-8* is in contrast to its oncogenic role observed in another study [110] and highlights that, like JNK and caspases, *miR-8* also has a context-dependent role in tumorigenesis. Whilst human *miR-200* family micro-RNAs are considered regulators of the epithelial phenotype and tumour suppressors (reviewed in [238]), these discoveries in *Drosophila* highlight that more research is needed to determine whether the *miR-200* family also have context-dependent effects in human cancer.

Mechanistically, in the *eyeful* model, *miR-8* blocks the invasive phenotype by repressing the translation of the Notch ligand, Serrate, and the zinc-finger transcription factor, Zfh1 (an ortholog of mammalian ZEB1, which is an EMT inducer), and, consistent with this, coexpression of *Delta,* or *Serrate,* with *Zfh1* cooperatively promotes an invasive phenotype [119]. This mechanism might be important in mammalian cancer, since *JAGGED1* (mammalian ortholog of *Delta/Serrate*) is regulated by the *miR-8* orthologs, *miR-200c,* and *miR-141,* in colorectal cancer cell lines [119], and reduced *miR-200* expression is associated with coupregulation of JAGGED1 and ZEB1 proteins in pancreatic and basal-type breast cancer cell lines [239].

The same group also found that another micro-RNA, *mir-7*, when overexpressed, enhances *Delta*-driven tumour overgrowth and promotes invasion in the eye-antennal epithelium, although the cells were still capable of differentiating [118] (Table 1). In this case, the cooperation occurred via blocking Hedgehog pathway signalling, which normally acts to restrict Delta/Serrate-Notch signalling during eye development. *mir-7* reduced translation of the Hedgehog receptor mRNA, *ihog (interference hedgehog)*, whereas Notch signalling blocked transcription of the coreceptor gene, *boi (brother of ihog)*, thereby leading to reduced Hedgehog signalling and enhancing Delta-Notch-driven tumour growth and invasion. Consistent with the mechanism, blocking Hedgehog signalling by knocking down expression of the Hh pathway transcription factor, Ci, also cooperated with *Delta* overexpression to phenocopy the effect of overexpression of *mir-7* and *Delta* [118]. These studies may provide insights into some forms of human cancer, where the *mir-7* ortholog is overexpressed and oncogenic, such as lung and skin cancers [240], or the Ihog orthologs (BOC and CDO) are downregulated or have a tumour-suppressor functions, such as in pancreatic cancer [241] or rhabdomyosarcoma [242].

Hippo. The Hippo tissue-growth control pathway consists of a protein kinase cascade involving Hippo and Warts protein kinases, which when activated, leads to the Warts-mediated phosphorylation and inactivation of the Yki cotranscriptional activator, thereby limiting tissue growth (reviewed in [243]). Hippo is regulated by multiple upstream inputs, including signalling pathways, cell polarity, and mechanical cues (reviewed in [122, 183, 244, 245]). Due to its powerful effect in controlling tissue growth, downregulation of the Hippo pathway is commonly observed in *Drosophila* cooperative tumorigenesis, as well as in human cancers (reviewed in [18, 122, 245, 246]). In addition, to the examples described above, which reveal the cooperation of Hippo pathway impairment in cooperative tumorigenesis with cell polarity impairment and oncogenic Ras, Yki overexpression has also been shown to cooperate with the knockdown of the Brahma (Brm) chromatin-remodelling complex [123] and overexpression of the Taiman transcription regulator [124] (Table 1). Impairment of the Brm-BAP chromatin-remodelling complex (using *brm, snr1,* or *osa* mutants) in epithelial tissues promotes cell cycle entry, alters Ras, Notch, and Dpp signalling, and deregulates Ecdysone responsive genes [247–254]. Brm complex knockdown also deregulates the Hippo pathway in epithelial tissues [255, 256], and therefore it is perhaps surprising that Brm downregulation cooperates with Yki overexpression [123]. Cooperation might occur, due to Brm complex knockdown downregulating the Ras signalling pathway, which decreases cell proliferation and survival [251, 254], and since Yki overexpression provides a strong cell proliferation and survival signal, it would be expected to override decreased cell survival exhibited by Brm complex knockdown alone. However, the recent study showed that Brm-BAP complex depletion, together with Yki overexpression, results in upregulation of Dpp and Wg morphogens leading to neoplastic tumour overgrowth in the larval wing epithelial tissue [123]. Cooperation with Yki and Taiman overexpression occurs by a unique mechanism involving the ectopic expression of germ-line stem cell genes in wing epithelial tissue [124]. This occurs because Yki and Taiman can form a complex leading to upregulation of a new spectrum of Yki targets normally not expressed in imaginal disc epithelial tissue, which alters differentiation.

Mitotic Checkpoints, Chromosome Instability and DNA Damage Repair Genes. Genes important in mitotic checkpoints, DNA repair, and genomic integrity play important tumour-suppressor functions in preventing cancer (reviewed in [157]). Indeed, knockdown of the spindle-assembly checkpoint (SAC) gene, *bub3*, which leads to chromosome instability (CIN) and aneuploidy, results in neoplastic tumorigenesis in the wing epithelial tissue when cell death is blocked [127, 140] (Table 1). The results from one group suggested that the mechanism by which this occurs is a SAC-independent function of Bub3 [127], but the second study revealed a novel mechanism that was induced by aneuploidy and cell delamination [140] (see below). A role for the DNA damage checkpoint and DNA repair after exposure to ionizing radiation (IR) has also been revealed in cooperative tumorigenesis [128] (Table 1). Here, IR together with apoptosis inhibition results

in overgrowth and cell delamination/migration in the wing epithelial tissue, which is enhanced by knockdown of the DNA repair genes, *okra (DmRAD54)* or *spnA (DmRAD51)*, which are involved in homologous recombination of DNA double-strand breaks, as well as by knockdown of the DNA damage checkpoint genes, *grp (chk1)* and *mei-41 (ATR)*.

An unusual example of cooperative tumorigenesis concerns the Nek2 (NimA related kinase 2) centrosome kinase, which is also involved in the SAC, and its loss of function leads to CIN (reviewed in [257]). However, overexpression of Nek2, which is not expected to cause CIN, cooperates with oncogenic pathways to drive neoplastic tumour formation without apparent effects on CIN [126] (Table 1). Here, expression of the activated form of the Ret tyrosine kinase *(Ret^{MEN2B})*, which mimics oncogenic mutations in human thyroid cancer, and Ras^{V12} together with *Nek2* overexpression, leads to invasive overgrowth of the eye-antennal epithelial tissue. Similar cooperativity occurs with mutant *csk*, together with Ras^{V12} and *Nek2* overexpression. The Ret oncogene results in increased signalling through the Ras-MAPK, PI3K, Src, and JNK pathways [258–260]. Nek2 overexpression results in increased Wg signalling and altered expression of Rho1, Rac1, and E-Cadherin, leading to altered cell morphology [126]. Coexpression of Nek2 and oncogenic Ret lead to enhanced local invasion and distant metastases. Mechanistically, Nek and Ret result in elevated expression of MMP1, which is expected to promote extracellular matrix degradation. Additionally, Nek and Ret lead to elevated expression of Diap1 (an antiapoptotic protein, and target of Hippo and Jak-Stat signalling), as well as Wg expression and PI3K signalling, which together are expected to drive tumour growth. A similar cooperative invasive phenotype was observed with elevated Src activity (*csk* mutant) with Ras^{V12} [126]. PI3K signalling was critical for the cooperative invasive phenotype, since inhibiting PI3K suppressed the cooperative behaviour. Although Nek2 is thought to have a tumour-suppressor function due to its role in the SAC and chromosome stability, it is also oncogenic and drugs are being developed to inhibit its function in cancer therapy (reviewed in [257]). Thus, this study in *Drosophila* provides insight into how Nek2 alone, as well as when combined with oncogenic mutations, promotes invasive properties [126], which is relevant to the understanding of Nek2-overexpressing human cancers.

In summary, the above examples of cooperative tumorigenesis in *Drosophila* tissues, and the delineation of the mechanisms involved, provide insights towards the understanding of various human cancers where these pathways are deregulated and present possible novel avenues for therapeutic intervention.

4. The Effect of the Tumour on Normal Tissue Growth and Intertumoural Cooperation

Not only does the mutant cell depend on the surrounding microenvironment for its proliferation and neoplastic transformation, but there are also examples in *Drosophila* where the tumour induces overgrowth of the genetically *wild-type* surrounding epithelial cells (termed non-cell-autonomous overgrowth) (Figure 3). The sophisticated genetics of *Drosophila* have enabled modelling of complex intertumoural cooperation, through generating genetically different populations of epithelial cells. In mammalian systems, tumours also exert non-cell-autonomous effects on cells in their microenvironment that affect tumour development (reviewed in [153–156]). Additionally, cancer heterogeneity, where different populations of tumour cells interact to promote tumorigenesis, is a recognized phenomenon in mammalian cancer (reviewed in [53, 261–264]). We will now discuss *Drosophila* models of cooperative tumorigenesis, where non-cell-autonomous cell proliferation or tumour heterogeneity occurs.

4.1. Non-Cell-Autonomous Cell Proliferation. In clonal settings, the initiation of cell death within mutant tissue results in signalling events that lead to non-cell-autonomous cell proliferation of the surrounding *wild-type* epithelium (reviewed in [28, 33, 265–269]) (Figure 3). In *scrib* mutant clones, non-cell-autonomous proliferative effects on the surrounding *wild-type* epithelial tissue occurs due to JNK-mediated expression of Upd, which in turn induces the Dome-Jak-Stat signalling pathway in surrounding cells [47, 137] (Figure 3(a)). Additionally, there is also evidence that the Hippo pathway is impaired in *wild-type* cells surrounding *scrib* mutant clones [270]. Likewise, clones mutant for the endocytic trafficking genes, *vps25* or *tsg101 (ept)*, also induce proliferation of the surrounding *wild-type* cells, due to impairment of the Hippo pathway and/or upregulation of the Dome-Jak-Stat signalling pathway [47, 137, 270, 271] (Figure 3(a)), which occurs through the aberrant activation of Notch signalling, leading to upregulation of Upd (Dome ligand) in the mutant cells [271, 272]. *vps25* mutant cells also lead to Hippo pathway impairment in the surrounding *wild-type* cells [270, 271], which may partially involve signalling through the Fat atypical cadherin [270]. Ectopic Notch signalling alone also leads to non-cell-autonomous tissue growth, as well as cell-autonomous proliferation [271, 273]. Similarly, activation of the Hh pathway in clones, in a Notch-dependent manner, also results in non-cell-autonomous cell proliferation and expression of the Diap1 cell death inhibitor in the surrounding *wild-type* cells [274, 275]. Since *Diap1* is a transcriptional target of Stat and Yki [276, 277], Jak-Stat or Hippo pathway deregulation may be involved. However, in all these examples, cell proliferation is limited. By contrast, in settings where cell death of the mutant cells is blocked by decreasing or preventing caspase activity, substantial overgrowth of the *wild-type* tissue occurs (Table 2). For example, elevated Hh signalling in clones blocked for cell death induces the Dpp morphogen production in the mutant cells, which activates the Dpp signalling pathway in the surrounding *wild-type* cells to induce non-cell-autonomous tissue growth [135]. In this setting, Yki was also upregulated non-cell-autonomously, and together with the Dpp signalling-activated transcription factor, Mad (Smad), induces expression of the *bantam* micro-RNA to promote non-cell-autonomous tissue growth [135] (Table 2). Another example of non-cell-autonomous tissue overgrowth occurs with "undead cells" [267–269]. Undead cells are generated when cell death is initiated by upregulation of an apoptosis regulator (such as Hid or Rpr), but apoptosis is blocked by

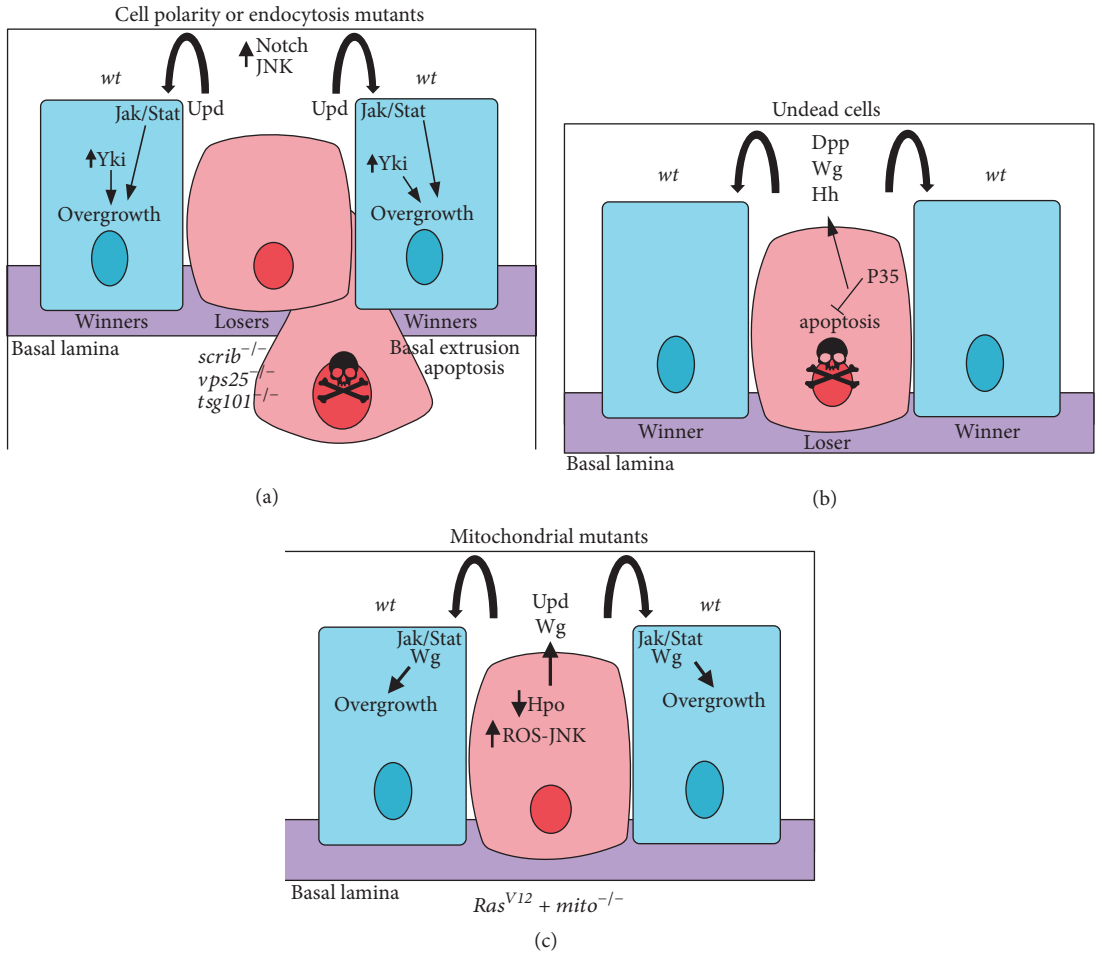

Figure 3: *Non-cell-autonomous overgrowth.* Examples of different types of non-cell-autonomous overgrowth. Mutant cells are in pink, *wild-type* cells are in blue, hemocytes are in grey, and the basement membrane (basal lamina) is in purple. (a) Cell polarity or endocytosis mutant cells are induced by JNK signalling to undergo cell death and induce non-cell-autonomous overgrowth of the surrounding *wild-type* cells. In *vps25* or *tsg101 (ept)* endocytic mutants, which also show apicobasal cell polarity defects, ectopic activation of Notch signalling leads to the expression and secretion of the Dome-Jak-Stat pathway ligand, Upd, which promotes non-cell-autonomous proliferation and overgrowth of surrounding tissue. In *scrib* mutant cells, elevated JNK signalling, and impaired Hippo signalling, leads to transcriptional upregulation of Upd, which activates Dom-Jak-Stat signalling in the surrounding *wild-type* cells, thereby inducing their proliferation. (b) Undead cells, where apoptosis is initiated, but effector caspase activity is blocked, emit morphogens (such as Wg, Dpp, and Hh) that promote proliferation of their *wild-type* epithelial neighbours, thereby leading to non-cell-autonomous overgrowth. (c) Mitochondrial mutants expressing Ras^{V12} lead to non-cell-autonomous overgrowth. The mitochondrial impairment results in the production of ROS, which induces JNK activation, which, in turn, results in Hippo pathway impairment, leading to expression of the Yki targets, Upd and Wg. Upd elevates Jak/Stat signalling and Wg induces Wg pathway signalling in the surrounding *wild-type* cells to promote their overgrowth.

expression of the p35 effector caspase inhibitor [59, 129–134] (Figure 3(b)). The undead cells continually express and secrete the morphogens, Wg, Dpp, or Hh, which act to elevate these signalling pathways in the surrounding *wild-type* cells, thereby inducing their uncontrolled proliferation. The induction of non-cell-autonomous tissue overgrowth by undead cells is dependent on the activation of JNK signalling in the undead cells, which transcriptionally upregulates the expression of the morphogen genes [129, 268] (Figure 3(b), Table 2). Consistent with this, strong activation of JNK together with Raf (protein kinase that functions downstream of Ras signalling) results in non-cell-autonomous overgrowth of surrounding *wild-type* cells [136]. Additionally, overexpression of Ras^{V12} together with the actin-cytoskeletal genes, *RhoGEF2*, *Rac1*, or activated alleles of *Rho1* ($Rho1^{V14}$), can initially induce varying degrees of non-cell-autonomous tissue growth; however tumour growth predominates over time [76, 77]. Similar effects are also observed with overexpression of the *abrupt* transcription factor gene with *RhoGEF2* or *Src64B* [78]. Conversely, overexpression of *abrupt* with *Rac1* leads to a strong non-cell-autonomous tissue overgrowth [78]. The mechanism by which this non-cell-autonomous overgrowth is induced is currently unknown but may involve the cells acquiring an undead-like state.

A different form of non-cell-autonomous cell proliferation occurs without cell death of the mutant cells but instead results in the cells acquiring a senescent secretory phenotype [138, 139] (Figure 3(c); Table 2). This new mechanism was

Table 2: Tumour-wild-type tissue and intertumoural interactions.

Non-cell autonomous overgrowth/tumorigenesis		
1st mutation/mechanism	2nd mutation/mechanism	Phenotype/references
hid/rpr overexpression Initiator caspase (Dronc) activation	p35 expression (blocks effector caspase activity) generates "undead cells" Dependent on ROS production, JNK activation, Dpp, Wg upregulation/secretion	Eye-antennal or wing epithelial tissue non-cell autonomous overgrowth [59, 129–134]
ptc mutant Hh pathway upregulation	*ark* (*apaf1*) mutant Results in upregulation of Dpp secretion from mutant cells and elevated Dpp signalling and Yki activity in the *wild-type* cells	Eye-antennal and wing epithelial tissue non-cell autonomous overgrowth [135]
Raf^{GOF} *overexpression*	Strong activation of JNK (hep^{ACT})	Non-cell autonomous overgrowth and morphology changes of eye-antennal epithelial tissue [136]
Rac1 overexpression	Abrupt (BTB-POZ Zn finger transcription factor)	Eye-antennal epithelial tissue non-cell autonomous overgrowth [78]
Intertumoural cooperation		
Ras^{V12} *overexpression*	*scrib* mutant cells next to Ras^{V12} overexpressing cells Activation of JNK and Upd upregulation and Jak-Stat signalling	Invasive neoplastic tumours of the larval eye neural-epithelium [137]
	Mitochondrial dysfunction in Ras^{V12} overexpressing cells next to Ras^{V12} overexpressing cells Results in ROS production, upregulation of JNK, deregulation of Hippo, secretion of Wg and Upd	Invasive neoplastic tumours of the larval eye neural-epithelium [138, 139]
Chromosome instability Induced by spindle assembly or spindle-assembly checkpoint mutants leading to cell delamination (*rod*, *bub3*, *asp*)	Cell death blockage (*p35* expression) Results in Metabolic stress, ROS induced-JNK activation in epithelial cells promoting cell delamination Results in Secretion of Wg, Upd from delaminated cells, promoting the proliferation of epithelial tumour cells	Invasive tumours in the wing epithelium [61, 140]
Spindle orientation defects Due to mutants/knockdown of genes involved in spindle alignment (*mud*, *scrib, dlg*) leading to cell delamination	Cell death blockage (*p35* expression) with *mud* knockdown Ras^{V12} or *p35* expression with *scrib* or *dlg* Results in Rho1-Wnd induced JNK activation in epithelial cells promoting cell delamination Results in secretion of Wg, Upd from delaminated cells, promoting the proliferation of epithelial tumour cells	Invasive tumours in the eye-antennal or wing epithelium [61, 141, 142]
Dosage compensation mechanism mutants Knockdown of *msl1* or *msl2* in males or *Sxl* in females	p35 overexpression or deletion of *rpr, hid, grim* to block cell death Results in ROS induced-JNK activation Results in MMP1 expression in delaminating cells	Invasive tumours in the wing epithelium [142]

discovered in a genetic screen for mutations that cooperate with Ras^{V12} in the developing eye, which revealed that mutations in mitochondrial oxidative phosphorylation genes together with Ras^{V12} lead to non-cell-autonomous overgrowth of the surrounding *wild-type* tissue. Mechanistically, this involves the generation of reactive oxygen species (ROS) by the mitochondrial-impaired cells, which then lead to JNK pathway activation. In turn, JNK activation results in impairment of Hippo pathway signalling, and, consequently, elevated Yki induces expression of Upd and Wg, which, respectively, induce signalling through the Dome-Jak-Stat and Wg signalling pathways in surrounding *wild-type* cells, leading to tissue overgrowth. Similar mechanisms of non-cell-autonomous tissue growth or tumorigenesis induced by senescent secretory cells are also observed in other settings in *Drosophila* and in human cancers (reviewed in [28, 53]).

4.2. Intertumoural Cooperation. Recent studies in *Drosophila* have revealed mechanisms by which cells of different populations can cooperate to generate neoplastic tumours [61, 137–140, 278, 279] (Figure 4, Table 2). Remarkably, Ras^{V12} cells generated next to *scrib* mutant cells (interclonal), rather than in the same cells (intraclonal, Figure 4(a)), became neoplastically transformed [137] (Figure 4(b)). This occurred by upregulation of JNK signalling and Upd expression in the $scrib^{-}$ cells, which induces Dome-Jak-Stat signalling in the neighbouring Ras^{V12} cells, thereby promoting neoplastic overgrowth. A similar mechanism involving Dome-Jak-Stat signalling, together with Wg signalling, induces neoplastic overgrowth of Ras^{V12} cells when generated next to mitochondrial respiratory chain gene mutant cells that were also overexpressing Ras^{V12} [138] (Figure 4(c)). The Ras^{V12}-expressing mitochondrial gene mutant cells exhibit properties of cellular senescence and acquire a secretory phenotype, through ROS, p53, and JNK upregulation, leading to JNK signalling amplification, similar to that which occurs in response to cellular stress [280], which leads to the transcriptional upregulation of Upd and Wg expression [139].

Aneuploidy, generated by mutations in spindle-assembly checkpoint (SAC) genes *(asp*, *rod*, and *bub3)* together with blocking apoptosis, also results in cooperative tumorigenesis involving tumour heterogeneity [61, 140, 142, 278] (Figure 4(d)). In this case, CIN induces metabolic stress leading to ROS production, which, via the JNKKK, Ask, activates JNK, leading to cell delamination and basal extrusion of the aneuploid cells. JNK activity also induces Upd and Wg upregulation and secretion from the aneuploid cells, which act on the mutant epithelial cell population to drive their proliferation through the Dome-Jak-Stat and Wg signalling pathways, respectively [61, 140, 142]. The delaminated cells (mesenchymal-like cells) are unable to proliferate but contribute to tumorigenesis by secreting Upd and Wg. Thus, two cellular populations, with the same original genotype, one epithelial and the other mesenchymal-like (which contains aneuploid cells), cooperate to generate the neoplastic tumour. Strikingly, spindle orientation defective mutants, such as *mud* (an ortholog of mammalian *Numa*, which is important for the localization of Dynein/Dynactin motor proteins in spindle orientation), or the cell polarity mutants, *scrib* and *dlg*, also lead to the generation of two cellular populations, which, upon blocking cell death, cooperate to promote neoplastic tumour formation [61, 141]. In this case, spindle misorientation causes the extrusion of cells from the epithelium, where they lose cell-cell adhesion and their epithelial morphology. Thus, these two populations are not genetically different, although, due to the loss of cell polarity and altered signalling pathways in the delaminated cells, they are likely to have different transcriptomes. Mechanistically, cooperation involves induction of JNK signalling, through a Rho1-Wallenda pathway leading to transcriptional upregulation of Upd and Wg [61]. Additionally, knockdown of dosage compensation genes (*msl1* or *msl2* in males, or *Sxl* in females), which result in genome-wide expression changes on the X chromosome similar to aneuploidy, also result in tumour heterogeneity-induced cooperative tumorigenesis when cell death is blocked [142] (Table 2). Here ROS and JNK signalling are induced and the delaminating cells upregulate MMP1. Thus, dosage compensatory gene mutants, when cell death is blocked, show a similar mechanism to CIN, due to SAC gene knockdown, in inducing tumour heterogeneity.

In summary, the analysis of cooperative tumorigenesis in *Drosophila* has revealed several different mechanisms by which tumour heterogeneity is generated and elucidated the mechanism by which two populations of cells can cooperate in promoting neoplastic tumours. Since heterogeneity is a common phenomenon and an important factor in human cancer (reviewed in [53, 261–264]), the findings in *Drosophila* may provide insight into understanding how heterogeneity arises and contributes to human cancer progression.

5. Conclusions and Future Perspectives

We have highlighted in this review how damaged cells are recognized and eliminated in epithelial tissue (cell competition) and the dreadful consequences of the failure of these surveillance mechanisms or of cell death. The persistence of damaged cells, by blocking cell death or by the activation of various oncogenes, drives hyperplastic or neoplastic tumorigenesis by various mechanisms. In cooperative tumorigenesis, which can occur in a myriad of ways, signalling pathways are deregulated to promote tumorigenesis, of which the JNK, Upd (IL6)-Dome-Jak-Stat, and Wg pathways are highly prominent. Interestingly, tissue regeneration also requires these pathways [281], suggesting that normal tissue repair mechanisms are usurped during neoplastic tumorigenesis. As with human cancer, the interaction of the epithelial tumours with their microenvironment plays an important role in neoplastic tumour development in *Drosophila* models. Moreover, non-cell-autonomous cell proliferation induced by the mutant cells affects the surrounding *wild-type* tissues, causing aberrant tissue overgrowth when mutant cell apoptosis is blocked. Additionally, *Drosophila* studies have revealed how tumour heterogeneity arises and has delineated novel mechanisms by which different cell populations are involved in cooperative tumorigenesis. These include the interplay between *scrib* mutant cells juxtaposed to oncogenic Ras-expressing cells and the senescence-induced

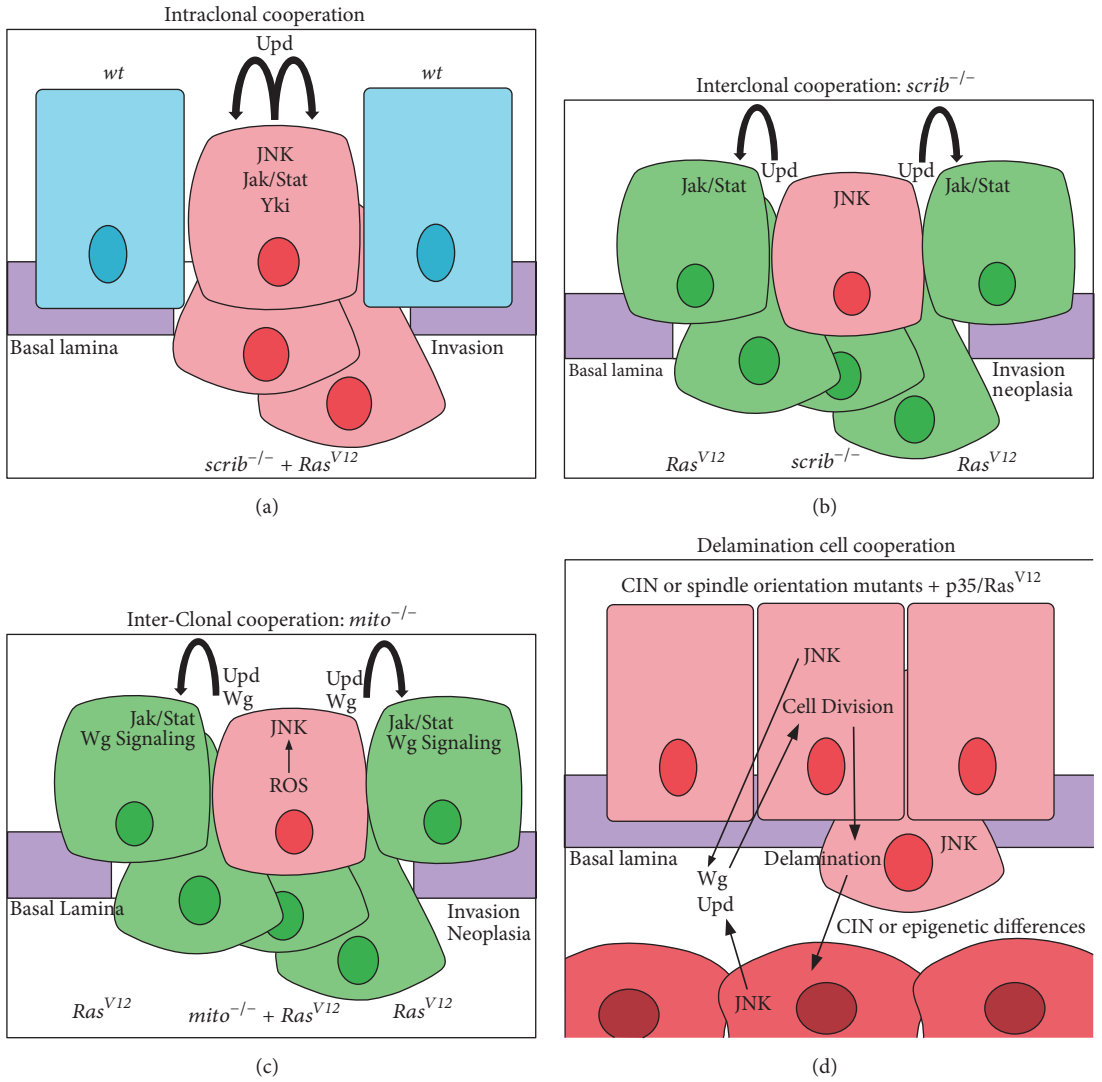

Figure 4: *Different modes of cooperative tumorigenesis*. Examples of different modes of cooperative tumorigenesis. Mutant cells are in pink, Ras^{V12}-expressing cells are in green, *wild-type* cells are in blue, delaminated mutant cells are in dark pink, and the basement membrane (basal lamina) is in purple. (a) Intraclonal cooperation with cell polarity mutants and Ras^{V12}: JNK activation in the tumour cells cooperates with oncogenic Ras signalling to promote tumour overgrowth and invasion. (b) Interclonal cooperation with cell polarity mutants and Ras^{V12}: JNK signalling and Hippo pathway impairment in the *scrib* mutant cells lead to the production of Upd, which induces Dome-Jak-Stat signalling in the surrounding Ras^{V12}-expressing cells, thereby inducing their overgrowth and invasion. (c) Interclonal cooperation with a mitochondrial mutant overexpressing Ras^{V12} and Ras^{V12}-expressing surrounding cells: Upd and Wg are produced by the mitochondrial mutant Ras^{V12}-expressing surrounding cells (see Figure 3(c)), which induce upregulation of Dome-Jak-Stat and Wg signalling, respectively, in the Ras^{V12} cells to induce their neoplastic overgrowth and invasion. (d) Delaminating cells cooperation: in tumours generated by chromosome instability (CIN) mutants *(rod, bub3,* and *asp)* or mutants that effect spindle orientation *(scrib, dlg,* and *mud)*, some cells delaminate, resulting in two populations of cells, which in the case of spindle orientation mutants are not genetically different. The delaminated cell population produces the Wg and Upd ligands to upregulate Wg and Dome-Jak-Stat pathways, respectively, in the nondelaminated cells, thereby inducing their proliferation.

secretory phenotypes generated by mitochondrial respiratory chain mutations together with oncogenic Ras, where each induces neoplastic tumours non-cell-autonomously. Furthermore, cell polarity, spindle orientation, and spindle-assembly checkpoint mutants, which cause delamination of cells, together with blockage of cell death or the activation of oncogenic pathways, lead to tumour heterogeneity and the crosstalk between two cellular populations to promote neoplastic tumorigenesis. Taken together, *Drosophila* studies have revealed novel cooperative gene interactions in tumorigenesis and the mechanisms by which this occurs, which is of relevance to human cancer. In the Omics age of human cancer research, the plethora of information that is being generated is often difficult to fathom, and functional studies are required to reveal the important cancer drivers and how they cooperate. Due to its sophisticated genetics, *Drosophila*

will continue to play an important role in revealing the function of cancer-causing genes *in vivo* and elucidating their mechanisms of action in cooperative tumorigenesis. Moreover, *Drosophila* is now emerging as a highly suitable model organism for the discovery of anticancer compounds against various cancer types, which can be then developed for clinical use, with reduced need for animal models (reviewed in [3, 10, 13, 14, 282, 283]). Thus, in the new age of pharmacogenetics, *Drosophila* will continue to play a fruitful role in elucidating new cooperative gene interactions in cancer and identifying anticancer compounds that then can be harnessed for anticancer therapy.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

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Review Article

Neuronal Proteomic Analysis of the Ubiquitinated Substrates of the Disease-Linked E3 Ligases Parkin and Ube3a

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Both Parkin and UBE3A are E3 ubiquitin ligases whose mutations result in severe brain dysfunction. Several of their substrates have been identified using cell culture models in combination with proteasome inhibitors, but not in more physiological settings. We recently developed the ^{bio}Ub strategy to isolate ubiquitinated proteins in flies and have now identified by mass spectrometry analysis the neuronal proteins differentially ubiquitinated by those ligases. This is an example of how flies can be used to provide biological material in order to reveal steady state substrates of disease causing genes. Collectively our results provide new leads to the possible physiological functions of the activity of those two disease causing E3 ligases. Particularly, in the case of Parkin the novelty of our data originates from the experimental setup, which is not overtly biased by acute mitochondrial depolarisation. In the case of UBE3A, it is the first time that a nonbiased screen for its neuronal substrates has been reported.

1. Introduction

Both Parkin (PARK2) and UBE3A are E3 ubiquitin ligases for which mutations result in severe brain dysfunction, Familial Parkinson's Disease (PD), and Angelman Syndrome (AS). In order to unravel the molecular mechanisms leading to these neurological dysfunctions it is necessary to identify and understand the role of their ubiquitinated substrates. Several substrates of UBE3A and Parkin have been surveyed mostly using cell culture overexpression models in combination with proteasome inhibitors. But more recently, a more physiological setting has been achieved by using an *in vivo* biotinylation strategy to isolate ubiquitinated proteins from *Drosophila* brains. With a label-free mass spectrometry approach, in order to quantify ubiquitinated proteins, we detected substrates of these two E3 ligases in *Drosophila*. This is an example of how flies can be used to reveal physiological substrates of disease-associated proteins. The results, using *Drosophila* as a validated model for neuronal disorders, provide new leads towards the cellular roles of these two disease causing E3 ligases.

2. Intracellular Proteostatic Quality Control Mechanisms: The Ubiquitin-Proteasome System (UPS) and Autophagy

The human genome contains ~20.000 protein-coding genes [1], but the set of proteins (proteome) present in a given cell is specifically determined in a cell type and developmental manner [2, 3]. Currently, the deepest proteomic coverage has identified about 12,000 proteins in mice brain samples [4]. In order to adapt their proteomes according to cellular requirements and warrant appropriate fitness of proteins, cells differentially express and regulate their genome through interconnected pathways of protein synthesis and distinct quality control mechanisms [5]. A plethora of cofactors and chaperones supports newly synthesised proteins to ensure their correct folding into fully functional three-dimensional structures [5]. This is a critical process not only to maintain physiological proteostasis but also to avoid the appearance of toxic protein aggregates [6]. However,

even when proteins are correctly folded and functionally active in their final compartment, various factors can destabilise the proteins and irreversibly impair them. For this purpose, cells possess quality control mechanisms such as the Ubiquitin-Proteasome System (UPS) and autophagy that specifically degrade damaged proteins and organelles [7, 8].

Ubiquitin (Ub) is a small protein (~8.5 kDa) that is specifically attached to target proteins through a sequential enzymatic cascade [7]. Classically, Ub-activating E1 enzymes activate and transfer Ub to Ub-carrier E2 enzymes, which finally covalently modify the target proteins with Ub with the assistance of Ub-ligase E3 enzymes (Figure 1(a)). As is the case with other posttranslational modifications (PTMs), such as phosphorylation, ubiquitination is a reversible process. A fourth family of proteins, called deubiquitinases (DUBs), has the ability to cleave Ub moieties from their substrate proteins, acting as editors and recycling the free Ub pool. Conjugation of a single ubiquitin can be performed to a certain lysine of the target protein (monoubiquitination), or to several lysines simultaneously (multimonoubiquitination). Additionally ubiquitin can also be attached to another preassembled ubiquitin through the N-terminal, or any of its seven internal lysines, building chains (polyubiquitination) of different topology. Depending on which residue of the next ubiquitin is modified, M1, K6, K11, K27, K29, K33, K48, or K63 polyubiquitin chains can be formed. Combinations of alternate lysine residues can result in mixed ubiquitin chains too. Additionally, chains can be branched by other ubiquitin chains. Taken together, all these possible modifications result in a highly diverse set of chain types and ubiquitination types, each of which will have a different readout by the cell, the so-called "ubiquitin code" [9]. Due to this versatility of ubiquitin, the complexity of the UPS is extremely high and is not limited to play a role in protein degradation. Instead, UPS is essential in a plethora of additional key biological processes (Figure 1(a)), including receptor endocytosis and endosomal trafficking [10], cellular progression and chromosome reassembly, transcriptional regulation, signal transduction, and apoptosis [9].

Autophagy refers to the process in which cells engulf their own contents into double-membrane structures (autophagosomes) that ultimately fuse with lysosomes, where cargo is degraded and basic biomolecules are recycled back to the cytosol (Figure 1(b)) [8]. Large cytosolic contents or organelles are typically wrapped into a double membrane (isolation membrane) that expands engulfing cargo into autophagosomes (macroautophagy) [11]. Smaller cytosolic cargo is instead taken up by direct lysosomal invagination (microautophagy) [12], whereas unfolded or aggregated proteins are translocated into the lysosomal lumen by chaperone-mediated autophagy [13]. Interestingly, ubiquitination is also involved in the regulation of autophagy [14–19]. In addition to its other roles, therefore, it is clear that ubiquitination serves as universal tag for substrate degradation, as all intracellular degradation pathways appear to be interconnected and governed by it [20].

3. The UPS Is Essential for Correct Neuronal Homeostasis

Neurons particularly require a tight spatiotemporal regulation of their proteome. The cell body or soma is typically distant from axonal and synaptic connections; and they are constantly receiving, decoding, and transmitting information via synaptic communication. Regulation of protein interaction, sorting, and activity is not only critical for the wellbeing of the neuron itself, but it is also necessary for proper coordinated transfer of the information. Thus, right balance between protein synthesis and degradation is essential for neuronal homeostasis, both for correct neurodevelopment and, at later stages in aged neurons, to protect against stochastic proteotoxicity [21].

The first evidence of the involvement of the UPS in the nervous system homeostasis came from the discovery that ubiquitin is present in neurofibrillary tangles of various neurodegenerative diseases [22, 23]. Hereafter, a variety of failures at different levels of the UPS have been linked to several neurodevelopmental and neurodegenerative diseases. For instance, mutations in the UBA1 activating E1 enzyme are associated with X-linked Infantile Spinal Muscular Atrophy [24], whereas UBE2K E2 enzyme has been implicated in the pathogenesis of Huntington's disease [25, 26] and Alzheimer's disease [27]. UBE2H enzyme is associated with autism [28] and loss of Parkin and UBE3A ligase activity is linked to autosomal recessive juvenile parkinsonism and Angelman Syndrome, respectively [29, 30]. Similarly, downregulation of the DUB enzyme UCHL1 has also been linked with Parkinson's and Alzheimer's disease [31, 32]. Additionally, variants of the Ubiquilin1 (UBQLN1) ubiquitin receptor protein are associated with a higher risk of developing Alzheimer's disease [33], whereas disruption of the Rpt2 subunit of the proteasome in mice has been reported to be enough to trigger PD-like neurodegeneration [34]. Ubiquitin-mediated degradation and signalling are of outstanding importance for adequate neuronal function and development. Ubiquitination regulates processes such as neurite growth and guidance [35], synaptic maturation and neurotransmitter release [36, 37], and neurotransmitter receptor internalisation [38] and it is even imperative for neurogenesis to successfully take place [39].

Drosophila has been a valuable tool to shed light on our understanding of the role of ubiquitination in the nervous system. In fact, evidence of a link between UPS and synapse formation has often come first from experiments performed in flies. For example, in the early 90s, the *fat facets (faf)* gene was found to encode a DUB involved in fly eye development [40, 41], while the E2 enzyme coding *bendless* gene was shown to regulate neuronal connectivity [42, 43]. Fly mutants of the E3 ligase gene *highwire* were later reported to have a defective synaptic overgrowth and function in larval neuromuscular junction (NMJ) [44]. Similarly, another E3 ligase, the Anaphase Promoting Complex/Cyclosome, was shown to regulate synaptic size and synaptic transmission at fly NMJ [45]. Over the years, many other *Drosophila* studies have reported evidence of the involvement of the UPS in the nervous system development and function [46–50].

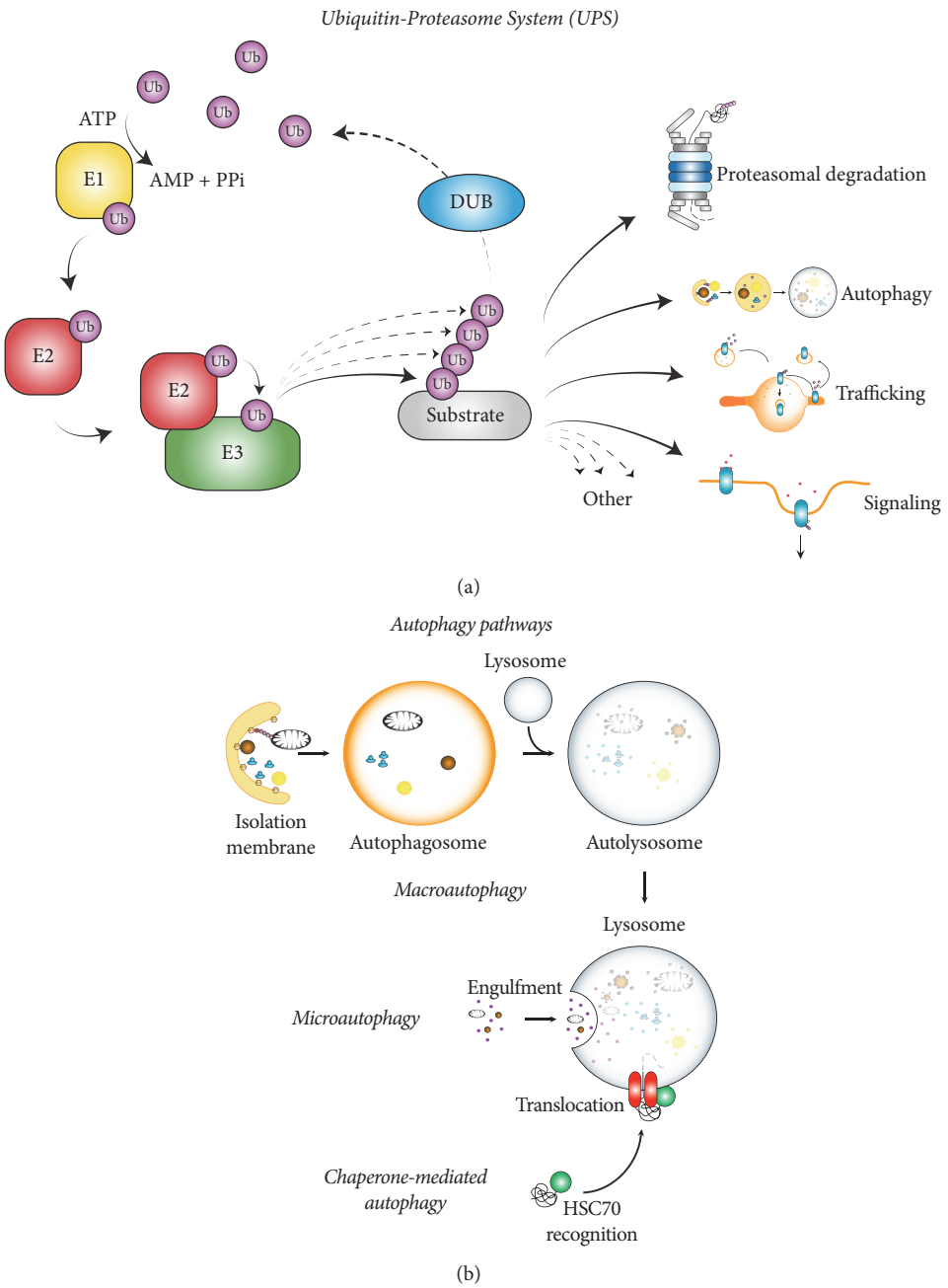

FIGURE 1: *Main intracellular quality control mechanisms: Ubiquitin-Proteasome System (UPS) and Autophagy.* (a) Ubiquitin is attached to target substrates by a sequential enzymatic cascade comprised by E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin-ligase) enzymes. E1 hydrolyses ATP to form an Ub-adenyl intermediate that is subsequently attached to the E1 via a thioester bond. E1-Ub transfers the ubiquitin to the E2, which then interacts with an E3 to transfer the ubiquitin to the substrate. DUBs can cleave ubiquitin moieties to edit ubiquitinated substrates. Protein ubiquitination regulates many biological processes, such as proteasomal degradation, autophagy, endosomal trafficking, and signalling events, and also chromatin assembly, DNA transcription and repair, ribosome biogenesis and translation, cell cycle and division, apoptosis, immunity, and organelle biogenesis. (b) Based on cargo recognition mechanisms, autophagy can be subdivided into macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is the best-studied form of autophagy, in which a double-membrane structure expands around and engulfs large cytosolic contents or organelles, forming an autophagosome. The autophagosome then fuses with a lysosome and the contents are degraded. Microautophagy degrades smaller cytosolic cargo, such proteins and tiny pieces of organelles by lysosomal invagination. CMA is involved in the degradation of unfolded or aggregated proteins that expose a particular degradation motif (KFERQ) that is then recognised by the cytosolic chaperone heat shock cognate protein of 70 kDa (HSC70), which interacts with lysosome-associated membrane protein type 2A leading to the unfolding and translocation of the substrate into the lysosomal lumen where it is degraded. Several macroautophagy subtypes can be distinguished according to cargo: reticulophagy (ER), mitophagy (mitochondria), pexophagy (peroxisome), ribophagy (ribosome), lipophagy (lipid droplets), xenophagy (intracellular pathogens such as bacteria and virus), and aggrephagy (protein aggregates).

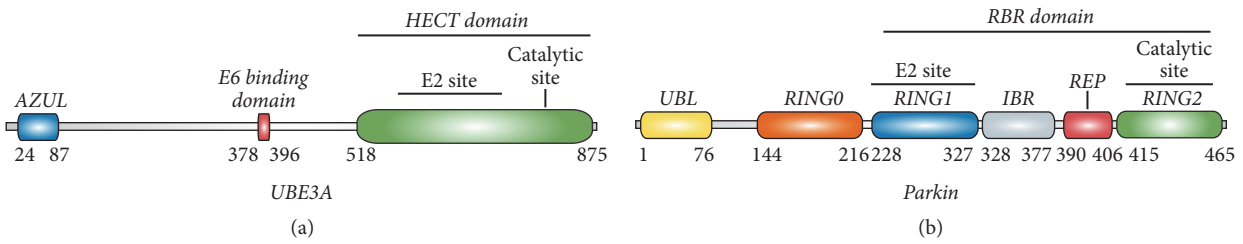

Figure 2: *E3 ligase types, UBE3A and Parkin.* (a) Human UBE3A domain structure. Protein domain structure and amino acid numbering refer to the isoform II. UBE3A contains an AZUL (amino-terminal Zn-finger of UBE3A E3 ligase) domain, thought to play a role in substrate recognition, as well as a HECT domain (Homologue to E6AP Carboxyl Terminus) characteristic of this family of E3 ligases, which was named after its discovery in UBE3A, also known by the name E6AP. The ubiquitin ligating catalytic cysteine is found within this HECT domain. All through the rest of the sequence of UBE3A only a small region known to interact with viral protein E6 has been described. (b) Parkin domain structure. Parkin contains a N-terminal UBL domain followed by a RING-like domain (RING0) and a RBR domain. The RBR domain entails a RING1 domain, which comprises the E2 binding site, a IBR domain, and the catalytic site encompassing RING2 domain. Amino acid numbering is based on human sequences.

4. Studying UBE3A Function and Angelman Syndrome (AS) Employing *Drosophila*

The broad use of *Drosophila* as a model organism since the early years of the 20th century can be explained by its many advantages. First of all they are suitable for genetic studies as their fast reproductive cycle coupled to a great capacity to provide a large amount of eggs guarantees abundant offspring in short periods of time [51, 52]. They are easy and cheap to handle and maintain, which makes large-scale experiments affordable. Moreover, they only contain 4 pairs of chromosomes: the X/Y pair of sexual genes and three pairs of autosomal chromosomes [53], which greatly facilitates the management and interpretation of genetic experiments. In addition, the low genetic complexity of flies implies that there is less redundancy and simplifies biological and mechanistic explanations. Nevertheless, flies contain homologues for ~75% of human genes involved in disease [54], providing a simpler *in vivo* model for the study of their role in the context of many diseases, including neurodevelopmental and neurodegenerative diseases [55, 56].

Angelman Syndrome (AS) is a rare neurodevelopmental disorder with a prevalence of approximately 1/15.000 individuals [57], characterised by a severe intellectual and developmental delay, movement or balance disorders, speech impairment, and a happy demeanour that includes episodes of frequent laughter and easy excitability [58]. Very frequently (>80% of the cases) these symptoms are accompanied by seizures, sleep disturbances, and microcephaly [58, 59]. The underlying molecular cause leading to AS was discovered to be the loss of function of the UBE3A protein in the brain. In particular, mutations leading to truncated forms of UBE3A were found to be enough to develop the syndrome [29, 60]. UBE3A is a HECT-type ubiquitin E3 ligase enzyme (Figure 2(a)) of approximately 100 kDa [61], which according to *in vitro* studies catalyses attachment of K48-linked ubiquitin chains to its substrates, consequently targeting them for proteasomal degradation [62]. Interestingly, duplication of the *UBE3A* gene has been associated with autism [63–65]. Many attempts have been performed in order to identify the neuronal substrates of this enzyme, leading to the proposal of several candidate substrates. Some of the proposed substrates were only validated *in vitro* (Arc, Na+/K+ ATPase, p27, Ring1B, Adrm1, and Rpt5) and therefore cannot be concluded to be neuronal targets of UBE3A [66–70], while others were identified as ubiquitinated by UBE3A using nondenaturing immunoprecipitation approaches (Annexin A1, HHR23A, PSMD2, and Ephexin5), which means that the ubiquitin signal could well belong to any of the coprecipitating proteins [71–74]. Most importantly, *in vivo* validation of any of these candidates has been unsuccessful so far.

Drosophila UBE3A (Ube3a) is ubiquitously expressed during embryogenesis and is broadly detectable in the adult nervous system, particularly in the mushroom bodies, which represent the key region for learning and memory [75]. Different fly models have been generated to study AS and *UBE3A* duplication-based autism cases, reporting that *Ube3a* mutant flies mimic characteristics of human AS [75–78]. *Ube3a* null mutant flies display locomotor impairment, abnormal circadian rhythms, and learning and memory defects, with a particular effect on long-term memory [75]. Furthermore, loss of Ube3a in neurons results in decreased dendritic arborisation of larval peripheral neurons [77] and decreased dopamine levels in adult fly brain [79]. In addition, neuronal overexpression of Ube3a also results in locomotion defects, in an ubiquitin-ligase-dependent manner. Missense mutations found in *UBE3A* alleles of AS patients have been reported to act as loss-of-function mutations also in its *Drosophila* homologue [75]. Fly models overexpressing Ube3a have been shown to display comparable neurotransmission defects to those found in mouse models of duplication 15q autism. Overexpression of wild-type Ube3a, but not its ligase-dead form, compromised the capacity of motor neuron axons to support closely spaced trains of action potentials, while at the same time increasing excitability [78]. Indeed, both overexpression and deficiency for Ube3a alter neurotransmission at the neuromuscular junction in *Drosophila melanogaster* 3rd instar larvae, also inducing in both cases defects in glutamatergic signalling [78]. A study investigating the role of Ube3a in the learning ability of flies using the aversive

phototaxis suppression assay determined that both down- and upregulation of Ube3a are detrimental to learning in larvae and adults [80].

5. Parkin and Parkinson's Disease (PD), Lessons from *Drosophila*

Parkinson's Disease (PD) is the second most common neurodegenerative disease after Alzheimer's. It is considered to affect 1% of people older than 60 years and up to 4% older than 80 years [81, 82]. Parkinsonism englobes numerous neurological syndromes that are mainly characterised by resting tremor, rigidity, and postural disability. PD patients display these motor symptoms, usually accompanied by other nonmotor symptoms, including depression, constipation, hypotension, sleep disorders, and dementia. Pathologically, PD is mainly characterised by loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies, intracytoplasmic proteinaceous inclusions rich in α-synuclein [83, 84]. However, the exact pathophysiological mechanisms leading to the disease are not clear yet and treatments modifying disease progression are not available. PD has been classically considered a sporadic disease linked to aging with an unknown aetiology. However, in about 10% of the cases, mutations in specific genes cause familial forms of PD [85]. Mutations in the RING-Between-RING (RBR) E3 ligase Parkin (Figure 2(b)) are the most frequent cause of all the autosomal recessive forms [86–89]. According to several structural studies, PD-causing mutations in Parkin result in loss of its function by either diminishing the E3 ligase activity or affecting the correct folding of the protein [87, 90–94].

Extensive studies performed employing *Drosophila* have been critical to improve our understanding of PD pathophysiology and Parkin function. In fact, the first hint that Parkin was involved in mitochondrial homeostasis came from the analysis of Parkin null flies (generated through ablation of endogenous *parkin* gene through P-element mutagenesis). *parkin* deficient flies display decreased dopamine content and dopaminergic neurodegeneration; they also reduced longevity, motor deficits, and male sterility [95–98]. Ultrastructural analyses showed that Parkin loss results in abnormally swollen and disorganised mitochondria, leading to apoptotic cell death of muscle tissue and defective spermatogenesis [95, 98]. Transcriptional analysis of *parkin* null flies revealed that mitochondrial electron transport chain genes, as well as genes involved in oxidative stress and innate immune responses, were upregulated [99]. In addition, the c-Jun N-terminal kinase pathway has been suggested to be upregulated in dopaminergic neurons of Parkin deficient flies, resulting in stress-mediated apoptotic neurodegeneration [96]. Ever since, Parkin has been acknowledged as a neuroprotective factor in many *in vitro* and *in vivo* studies [100] and, consequently, Parkin overexpression is associated with improved mitochondrial function, increased lifespan, and reduced proteotoxicity [101]. However, more recent studies in flies and cells are challenging this view, as Parkin overexpression can also have deleterious effects [102–104]. Seminal studies demonstrated that another PD-associated gene, coding for the mitochondrial kinase PINK1 [105], acts in the same pathway upstream of Parkin. *Pink1* null flies display the same defective phenotypes as *parkin* null flies, and Parkin overexpression can rescue *Pink1* loss but not vice versa [106–108]. Subsequent *Drosophila* genetic studies showed that *Pink1* and *parkin* interact with the mitochondrial fission and fusion machinery to regulate mitochondrial dynamics [109–111].

Mammalian cell culture studies first established that PINK1 accumulates on depolarised or damaged mitochondria to recruit and activate latent overexpressed Parkin and dispose of dysfunctional mitochondria via mitophagy [112, 113]. Thereafter, Pink1/Parkin-dependent mitophagy has also been detected in *Drosophila* S2R+ cells [114] and *in vivo Drosophila* models have reinforced mammalian cellular discoveries. Functional studies revealed that Parkin is phosphorylated by Pink1 in *Drosophila* cells, leading to Parkin activation. Parkin phosphorylation status modifies phenotypes typically affected in *Pink1* and *parkin* null mutant flies [115]; and mitochondrially located phospho-Ub (p-Ub) rescued *Pink1* null associated defects, supporting the requirement of both ubiquitin and Parkin phosphorylation for Parkin activation in the Pink1/Parkin pathway [116]. Nevertheless, it remains formally unproven that PINK1 and Parkin promote mitophagy *in vivo* and that defects in the disposal of dysfunctional mitochondria are involved in the progression of the PD.

Recent findings have identified additional PD-associated genes involved in Parkin-dependent mitophagy. *Fbxo7* genetically interacts with *parkin* in *Drosophila* and is involved in PINK1/Parkin-dependent mitophagy in mammalian cells [117]. In addition, *parkin* has been shown to further genetically interact with *LRRK2* and *Vps35* in flies [118, 119], although the functional implications are yet to be elucidated. Beyond mitophagy, Parkin deficiency has been related to additional dysfunctions [120]. Parkin has been reported to ubiquitinate a broad range of substrates, including several Lewy body components, by interacting with different E2s and catalysing various ubiquitination types, preferentially K6-linked polyubiquitin chains [89, 91, 92, 121–124]. However, most of these studies were performed *in vitro* upon overexpression of the putative substrate and/or Parkin. Although several unbiased quantitative mass spectrometry studies have reported altered protein levels in Parkin deficient *Drosophila* and mice [125–128], *in vivo* Parkin substrates have not been identified so far.

6. Studying Ubiquitin Proteomics with Drosophila

Primary discoveries are usually performed *in vitro* or *in cellulo*, but successive *in vivo* confirmation is required when translation towards human health is sought. *Drosophila* represents an ideal organism to study ubiquitin pathways *in vivo*. Ubiquitin is highly conserved across all eukaryotes [129], *Drosophila* Ub being 100% identical to the human protein. In humans Ub is encoded by four genes: *UBA52*, *RPS27A (UBA80)*, *UBB*, and *UBC* [130, 131], while in *Drosophila* three homologous genes exist: *RpL40 (DUb52)*, *RpS27A (DUb80)*, and *Ubi-p63E* [132].

The *Drosophila* proteome is predicted to contain ~15.000 gene products, of which ~10.000 proteins have been successfully identified employing mass spectrometry (MS) [133, 134]. Studying ubiquitination *in vivo*, however, can be very challenging, particularly in neurons. Due to the low stoichiometry at which ubiquitin-modified proteins are present within cells, it is necessary to enrich the ubiquitinated fraction prior to the MS analysis [135]. For this purpose, several purification methods have been developed so far [66, 136–140]. Nevertheless, most of these enrichment methods require the purification to be performed under native conditions, copurifying contaminants and false positives [141]. Alternatively, ubiquitinated peptides rather than intact ubiquitinated proteins can be enriched prior to the MS analysis. Proteolytic digestion of the sample with trypsin produces a characteristic di-Gly signature in ubiquitinated peptides that is detectable by MS [136]. Specific antibodies that recognised this ubiquitin remnant have been developed in recent years [142] and used for the isolation and subsequent MS-based identification of thousands of putative ubiquitination sites *in vivo* [143, 144]. This approach, however, requires the proteins to be trypsinized preventing any immunoblotting on the purified material. Since other ubiquitin-like proteins, as well as certain experimental conditions, also leave this di-Gly signature in the peptides [145, 146], such orthogonal validations are essential.

To avoid the detection of false positive ubiquitinated proteins, an enrichment process under denaturing conditions is preferred over the usage of physiological buffers. This has been classically performed using poly-histidine tagging [136, 147, 148]. However, a relatively high number of endogenous histidine-rich proteins are found in higher eukaryotes, which are also trapped in the nickel affinity beads, resulting in excessive background. In order to overcome these limitations, we developed the ^{bio}Ub strategy [149], based on a chemical modification performed by biotin holoenzyme synthetase enzymes [150] during the metabolism of fatty acids, amino acids, and carbohydrates [151]. This biotinylation reaction is highly specific and only few proteins are found to be modified with biotin *in vivo* [152]. The minimal length peptide that can be efficiently biotinylated by the *E. coli* biotin holoenzyme synthetase BirA is 14 amino acids long [153]. This can be used as a powerful tool for the generation of fusion proteins that can be easily purified or detected thanks to their biotin tag. The strategy for the *in vivo* isolation of ubiquitin conjugates has so far allowed the purification and enrichment of large amounts of ubiquitin conjugates from flies [104, 149, 154], mice [155], and human cell lines [156].

The ^{bio}Ub system relies on the *in vivo* expression of the ^{bio}Ub construct, which is formed by six ubiquitin-coding sequences in tandem followed by the bacterial bifunctional ligase/repressor BirA enzyme (Figure 3). Endogenous DUBs digest the ^{bio}Ub construct releasing BirA and ubiquitin and mirroring the processing of endogenous ubiquitin gene products [157]. Each ubiquitin contains a 16-amino-acid long biotinylatable motif, which is then recognised and biotinylated by BirA endogenously, resulting in a biotin-tagged ubiquitin moiety (^{bio}Ub) that is efficiently handled by the cascade of ubiquitin-conjugating enzymes and successfully attached to target proteins together with the endogenous ubiquitin. The advantage of having ubiquitinated proteins tagged with biotin is that they can be very efficiently and specifically purified employing avidin-conjugated beads. Biotin-avidin interaction is one of the strongest identified interactions in nature [158, 159], and it allows carrying out the enrichment and washes of ubiquitinated material under very harsh conditions, such as 8 M Urea, 1 M NaCl, and 2% SDS, avoiding coisolation of nonubiquitinated interacting partners [149]. Finally, the isolated material can be subjected to MS or Western blot analysis [104, 149, 154–156].

On our first application of this method, our group detected 121 ubiquitinated proteins in *Drosophila* neurons during embryonic development [149], including several key proteins involved in synaptogenesis and hence suggesting that UPS is important for proper neuronal arrangement. We later compared the ubiquitin landscape between developing and mature neurons in *Drosophila melanogaster* and identified 234 and 369 ubiquitinated proteins, respectively [154], some of which were found in both developmental stages. More interestingly, certain proteins are preferentially ubiquitinated in specific cell types during specific periods of the *Drosophila* life cycle, reinforcing the importance of using the appropriate cell type when studying ubiquitination. For example, Ube3a was found to be active in both developing and adult neurons, while Parkin was found to be enzymatically active in adult neurons only [104, 154]. Recently we have successfully employed this approach to analyze the ubiquitinated proteome of *Drosophila* under different conditions ([104, 154] and Ramirez et al. unpublished data). Altogether and thanks to the usage of more sensitive MS instruments, we have identified a total of ~1700 ubiquitinated proteins in *Drosophila* neurons (Figure 4), which represent ~11% of the total fly proteome (15.000).

7. Label-Free Quantitative Proteomics to Identify E3 Ligase's Ubiquitin Substrates

The ^{bio}Ub strategy can be applied to identify ubiquitin substrates of selected E3 ligases by comparing the levels of ubiquitinated proteins in an E3 ligase-dependent manner (Figure 3). In fact, we have recently been pioneers in deciphering the ubiquitome of flies expressing the biotin-tagged ubiquitin in the context of either gain or loss of function of Parkin [104] and Ube3a (Ramirez et al., 2018 *manuscript under review*) in adult Drosophila neuron. In both cases, to detect the E3-ligase substrates, we followed a label-free quantitative proteomics approach. Ubiquitinated proteins that were enriched using the ^{bio}Ub strategy in each of the experimental conditions were independently analyzed by MS. Resulting MS raw files were subsequently combined for the bioinformatic analysis in which a search engine determined the identity of the proteins in the samples as well as their relative abundance. Consequently, those proteins, which were found to be more abundant in the presence of the wild-type version of the E3 ligase rather than in the presence

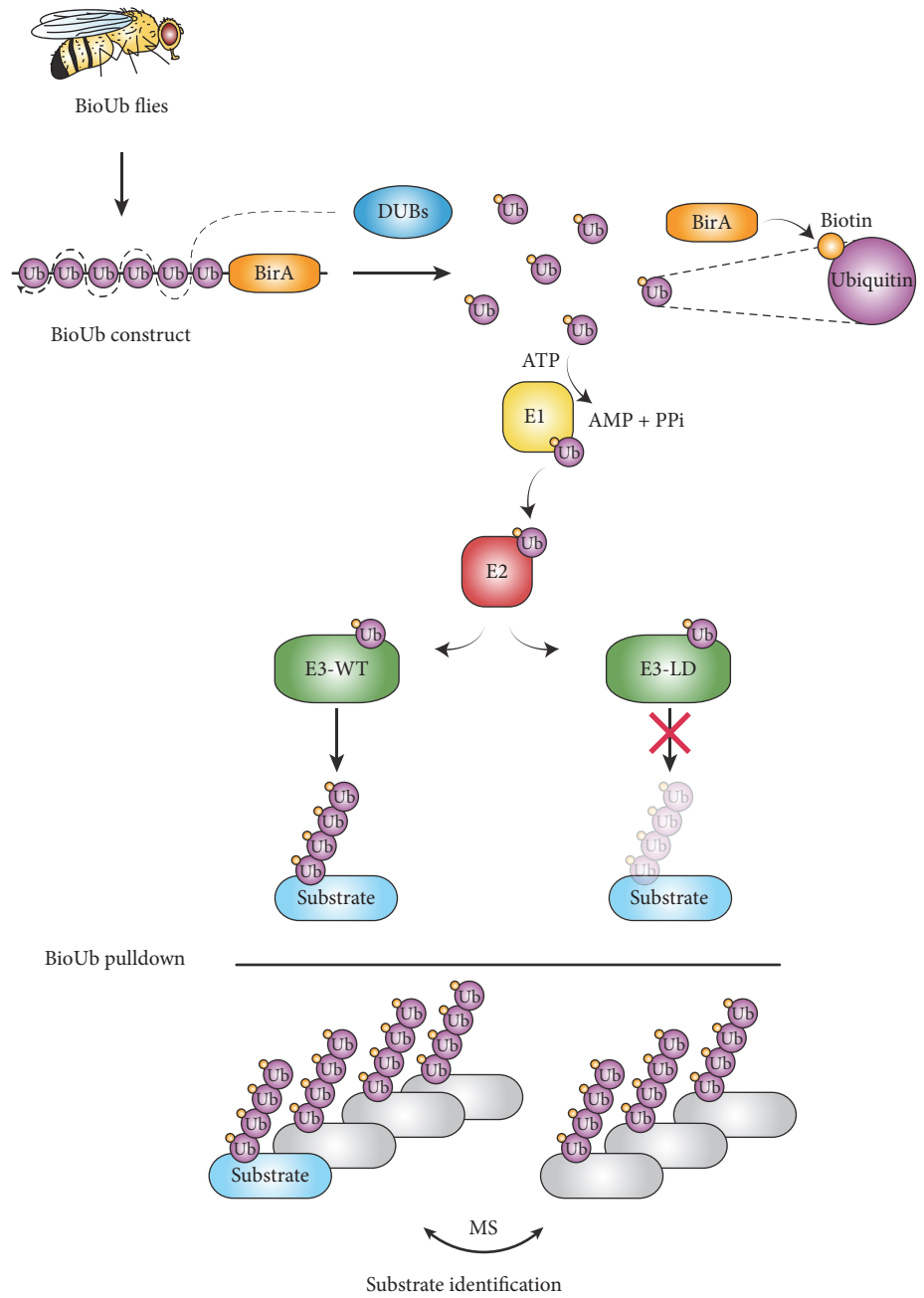

FIGURE 3: *BioUb strategy to identify ubiquitin substrates of E3 ligases in Drosophila neurons.* Scheme of the strategy used to identify proteins ubiquitinated by Parkin and Ube3a in *Drosophila* neurons. Flies were engineered to express endogenously precursor capable of biotinylating ubiquitin in *Drosophila* photoreceptors using the *GMR-GAL4* driver. This biotin modified ubiquitin (^{bio}Ub) is then incorporated within the pool of endogenous ubiquitin, in flies that also overexpress wild-type E3 ligases (E3-WT), Parkin or Ube3a, and in their respective mutant or ligase-dead negative controls (E3-LD). Ubiquitinated material can then be purified using Neutravidin beads and isolated material analyzed by mass spectrometry (MS). Ubiquitinated proteins enriched in Parkin or Ube3a WT overexpressing neurons can then be identified based on both protein LFQ levels and peptide intensities.

of the ligase-dead version of the ligase, were considered putative E3 ligase substrates.

We successfully isolated >1.000 ubiquitinated proteins, identifying, for example, 37 proteins whose ubiquitination is affected by Parkin activity: 35 were more and 2 were less ubiquitinated [104]. These include proteins associated with the endosomal sorting complexes required for transport (ESCRT) machinery (ALiX, Vps4), subunits of the v-ATPase required for endosome and lysosomal acidification, and most importantly the PD-associated retromer component, Vps35. We validated several of these substrates, when *Drosophila* antibodies were available and interestingly showed that most of them were monoubiquitinated by Parkin. Furthermore, in agreement with previous mammalian cellular studies

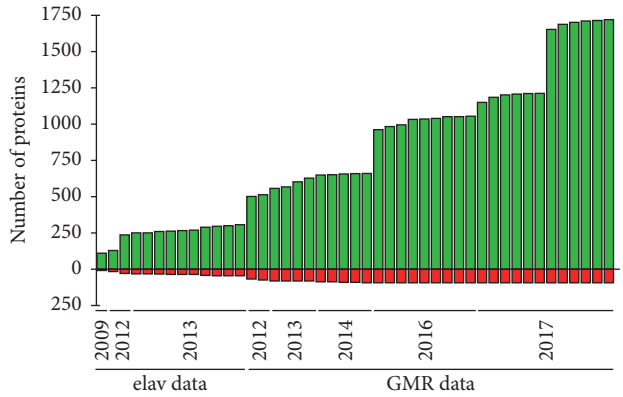

Figure 4: *Proteomic analysis of Drosophila neuronal ubiquitome.* A cumulative number of identified ubiquitinated proteins (green) isolated from *Drosophila* neurons by means of the ^{bio}Ub approach are shown. A cumulative number of proteins that appear in control birA pulldowns, and which are therefore classified as background, are shown in red. The first analyses (until 2013) were obtained from fly embryonic developing neurons (using elav-GAL4 driver). Subsequent analyses were performed with proteins isolated from the *Drosophila* photoreceptor cells (employing GMR-GAL4 driver).

[124], ubiquitin chain-linkage analysis confirmed that Parkin preferentially catalyses K6-Ub chains *in vivo*.

In the case of Ube3a flies, several UPS and autophagy-related proteins were identified to be more ubiquitinated upon Ube3a overexpression, including two proteasomal interacting proteins (Rpn10 and Uch-L5) earlier identified by our lab as Ube3a substrates [160]. Our proteomic data in neuronal tissue corroborate the findings in mammalian cell culture that were earlier reported [70, 73]. That is, UBE3A regulates several proteasomal subunits, which makes it likely that further changes on the Ube3a-altered ubiquitome might be a secondary effect. In any case, several proteins with important roles in neuronal morphogenesis and synaptic transmission have also been detected.

In addition, to detect E3 ligase substrates, our investigation allowed us to gather information about specific ubiquitination sites as well as types of ubiquitination linkages. In most proteomic studies, trypsin is the enzyme of choice to digest proteins and obtain suitable peptides that are further analyzed by MS. When the conjugated ubiquitin is cleaved with trypsin, it leaves a Gly-Gly dipeptide remnant on the conjugated lysine residues that serve as a signature of ubiquitination and allows depicting the specific site of modification. In agreement with *in vitro* studies showing that UBE3A catalyses preferentially the attachment of K48-linked polyubiquitin chains [62, 161], we also observed in *Drosophila* that Ube3a induces K48 and K11 chains on its substrates. Interestingly, not all the validated substrates of Ube3a seem to be targeted for degradation [160] as one would have expected from these ubiquitin chain types.

8. Does Parkin Regulate Something More Than Mitochondrial Homeostasis?

Despite the fact that we identified some outer mitochondrial membrane proteins that have been reported to be ubiquitinated by Parkin during mitophagy, such as VDAC1/2/3, TOM70, and CISD1/2, mitochondrial proteins were not particularly enriched compared to previous studies [162, 163]. The restricted overlap between our dataset and other previous studies indicated that results from artificial cell culture conditions correlate with the biology of the brain within an organism only to a certain degree. Only 8 out of the 35 Parkin substrates identified by us have been identified in previous studies using mitochondrial depolarisation and mitophagy induction. In contrast, we captured the steady state substrates of Parkin *in vivo*, which might be involved in pathways beyond mitophagy. Our proteomic analysis of Parkin substrates revealed that Parkin ubiquitinates a wide range of proteins with no obvious functional connectivity, although endocytic trafficking components, such as Vps35, Vps4, or PDCD6IP/ALiX, were overrepresented. Interestingly, *parkin* has been recently shown to genetically interact with *Vps35* in *Drosophila* [119], and several studies have suggested that Parkin may be involved in endosomal trafficking [164, 165]. Additional studies will in fact reveal whether these substrates are functionally connected in a yet unknown pathway. Moreover, several proteins involved in transport of molecules and proteins; biosynthesis of proteins, carbohydrates, and lipids; ER stress; immunity and apoptosis were also identified in this large-scale ubiquitome study. The heterogeneity in the nature of the putative Parkin substrates detected suggests that the role of Parkin might be much wider than it is actually believed.

It is important to note that our *Drosophila* results, in contrast to previous studies, have not required promotion of Pink1 activity, and therefore we might have identified some Parkin substrates that are Pink1-independent. This opens the question of how Parkin can be activated then. It could be possible that the cleaved cytosolic Pink1 fragment may have a role in the activation of Parkin for other purposes than mitophagy. It can neither be discarded that other kinases have the ability to activate Parkin. Further studies depicting the requirement of Pink1 for the activation and ubiquitination of Parkin will clarify these questions.

9. Is UBE3A a Master Regulator of the Proteasome?

The *in vivo* unbiased proteomics approach we have performed has provided for the first time a list of putative Ube3a substrates, whose ubiquitination is enhanced by Ube3a. Additionally, our findings corroborate previous reports performed in cells, indicating that Ube3a interacts with the proteasome and its degradative activity, which results in the accumulation of tens of ubiquitinated proteins of which many are most likely not direct targets of Ube3a. The ubiquitination of proteasomal subunits by UBE3A had been previously reported, but this *Drosophila* study is pioneer in reporting *in vivo* evidence of their ubiquitination in neuronal cells. Complementing previous observations, it appears that the ubiquitination of proteasomal subunits by Ube3a/UBE3A ([70, 73, 160, 166]; Ramirez et al., 2018, *manuscript under review*) places this E3 ligase as a pivotal regulator of the proteasome and proteostasis. This finding opens a new

perspective in which the ubiquitination of other proteins, and thus their levels or activity, can be affected as a downstream effect. The existing working model that UBE3A substrates are targeted for degradation does therefore need to be revised.

10. Concluding Remarks

The ^{bio}Ub approach has been successfully applied for the MS analysis of the ubiquitin landscapes of the embryonic nervous system and *Drosophila* photoreceptor cells, but it has the potential to be implemented to any fly tissue at any stage during the development. The nature of the ^{bio}Ub strategy allows also discerning by Western blot whether such identifications correspond to proteins that are mono- or polyubiquitinated *in vivo*. And most importantly, for the first time it is possible to obtain a list of candidate substrates for any *Drosophila* E3 ligase *in vivo*.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Aitor Martinez and Juanma Ramirez contributed equally to this review.

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Research Article

4-Hydroxy-2-nonenal Alkylated and Peroxynitrite Nitrated Proteins Localize to the Fused Mitochondria in Malpighian Epithelial Cells of Type IV Collagen *Drosophila* Mutants

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Background. Human type IV collagenopathy is associated with mutations within the *COL4A1* and to a less extent the *COL4A2* genes. The proteins encoded by these genes form heterotrimers and are the highest molar ratio components of the ubiquitous basement membrane. The clinical manifestations of the *COL4A1/A2* mutations are systemic affecting many tissues and organs among these kidneys. In order to uncover the cellular and biochemical alterations associated with aberrant type IV collagen, we have explored the phenotype of the Malpighian tubules, the secretory organ and insect kidney model, in *col4a1* collagen gene mutants of the fruit fly *Drosophila melanogaster*. In Malpighian epithelial cells of *col4a1* mutants, robust mitochondrial fusion indicated mutation-induced stress. Immunohistochemistry detected proteins nitrated by peroxynitrite that localized to the enlarged mitochondria and increased level of membrane peroxidation, assessed by the amount of proteins alkylated by 4-hydroxy-2-nonenal that similarly localized to the fused mitochondria. Nuclei within the Malpighian epithelium showed TUNEL-positivity suggesting cell degradation. The results demonstrated that *col4a1* mutations affect the epithelia and, consequently, secretory function of the Malpighian tubules and provide mechanistic insight into *col4a1* mutation-associated functional impairments not yet reported in human patients and in mouse models with mutant *COL4A1*.

1. Introduction

Basement membranes (BMs) are nanoscale sheets of extracellular matrices that play essential roles in multiple organs including muscle homeostasis, structures, and integrity of the dermal and ocular system, neuromuscular junctions, and blood filtration in the kidneys. The most abundant structural components of BMs include laminins, collagen IV, nidogens, perlecan, and agrin [1]. The ubiquitous mammalian BMs consist of heterotrimeric type IV collagens with $(COL4A1)_2COL4A1$ composition. The clinical presentation of patients with *COL4A1* mutation is systemic with numerous affected organs and tissues including the eyes, brain, the vascular system, skeletal muscles, and kidneys [2, 3]. A distinct form of type IV collagenopathy, Hereditary Angiopathy, Nephropathy, Aneurysms, and Muscle Cramps (HANAC) syndrome, is caused by N-terminal mutations within the *COL4A1* gene. The renal manifestation of the same mutations in mouse models includes albuminuria, hematuria, glomerular cysts, and delays in glomerulogenesis and podocyte differentiation [4].

We have identified an allelic series of dominant, temperature-sensitive, antimorph mutations in the cognate *col4a1* gene of the fruit fly, *Drosophila melanogaster*. The $col4a1^{+/-}$ heterozygotes are viable and fertile at permissive temperature of 20°C but die at 29°C. In these mutants, we

have reported severe myopathy [5], tortuous BM, detachment of the gut epithelial and visceral muscle cells from the BM [6], intestinal dysfunction, overexpression of antimicrobial peptides, and excess synthesis of the oxidants hydrogen peroxide and peroxynitrite [7]. Peroxynitrite, by substituting the hydrogen atom by a nitro (-NO_2) group adjacent to the hydroxyl group on the aromatic ring of tyrosine, adversely impacts protein functions and can be detected as a species-independent antigene [8]. Peroxynitrite can also remove a hydrogen atom from polyunsaturated fatty acids resulting in the formation of aldehydes, conjugated dienes, and hydroperoxyradicals that trigger a free radical chain reaction and membrane lipid damage by lipoperoxidation [9].

The main product of membranous polyunsaturated fatty acid peroxidation is the reactive 4-hydroxy-2-nonenal, HNE [10]. The reactivity of HNE with proteins relies on Michael addiction and, by modifying histidine residues, generates alkyl-conjugated polypeptides also detectable as species-independent antigens [11]. As there is no direct laboratory test to estimate lipid peroxidation, measurements of HNE-conjugated protein levels currently serve as surrogates [12]. The bulk of peroxynitrite reacts rapidly with carbon dioxide, present at ~1 mM in cells, forming the unstable product nitrosoperoxycarbonate ($ONOOCO_2^-$), one-third of which decomposes into carbonate ($CO3^{-\bullet}$) and $NO_2^\bullet$ radicals and two-thirds into the neutral NO_3^- and CO_2 [13].

Insect Malpighian tubules serve as secretory organs. These renal tubules lack a vascular blood system and float freely in the hemocoel (blood-filled body cavity). The tubules are surrounded by BM and consist of two epithelial cell types, the metabolically active principal and the intercalated stellate cells [14]. The insect renal system is aglomerular, and urine is formed by active transport rather than by selective reabsorption of ultrafiltrate as in vertebrates. While the insect tubule system represents an intermediate towards the glomerular kidney, it fulfills the same basic functions of transport, excretion, and osmoregulation [15].

We have recently shown that the *col4a1 Drosophila* mutants develop stress fibers in their Malpighian cells and aberrantly express cell-surface integrin receptors [16]. In the present study, we have extended our research to address altered posttranslational protein modifications by peroxynitrite and 4-hydroxy-2-nonenal in the Malpighian tubules. The *col4a1* mutants demonstrated heavy protein tyrosine nitration and protein-histidine alkylation that localized to the enlarged and fused mitochondria as signs of mitochondrial stress. HNE-protein adducts colocalized with the cytoplasmic membrane that was accompanied by cell degeneration in the tubules performing TUNEL-positivity, collectively suggesting that these aberrant processes are integral parts of *col4a1*-associated pathology.

2. Materials and Methods

2.1. Maintenance of Drosophila Strains. Wild-type *Oregon* flies and *col4a1* mutant stock with the *DTS-L3* allele were maintained at 20°C and 29°C on yeast-cornmeal-sucrose-agar food, consisting of nipagin to prevent fungal infection. The mutant stocks were kept heterozygous over the *CyRoi* balancer chromosome. Malpighian tubules were removed under carbon dioxide anesthesia from adults that were grown at both permissive and restrictive temperature for 14 days. Dissected Malpighian tubules were fixed in 4% paraformaldehyde dissolved in phosphate buffered saline (PBS) for 10 min, washed three times in PBS, permeabilized for 5 min in 0,1% Triton X, dissolved in PBS, and washed three times in PBS. Blocking was achieved in 5% BSA dissolved in PBS for 1 hour and washed three times in PBS.

2.2. Immunostaining and Antibodies. Nuclei in the dissected Malpighian tubules were counterstained by 1 μg/ml 4′,6-diamino-2-phenylindole (DAPI) in 20 μl PBS, 12 min in dark. F-actin was stained by 1 unit Texas Red™-X Phalloidin (ThermoFisher) in 20 μl PBS for 20 min. A-Mannopyranosyl and a-glucopyranosyl residues as membrane markers were stained by Concanavalin A, Alexa Fluor™ 594 Conjugate (ThermoFisher) in 20 μl PBS for 1 hour. We used 1 μl mouse monoclonal anti-3-nitrotyrosine [39B6] (Abcam) in 20 μl PBS for 1 hour and stained 4-hydroxynonenal conjugate by 1 μl mouse monoclonal anti-4-hydroxynonenal antibody (Abcam) in 20 μl PBS for 1 hour. Primary mouse antibodies were visualized by 1 μl F(ab′) 2-Goat Anti-Mouse IgG (H + L) Cross Adsorbed Secondary Antibody conjugated with Alexa Fluor 488 (ThermoFisher) in 20 μl PBS for 1 hour and 1 μl Goat Anti-Mouse IgG (H + L) Cross Adsorbed Secondary Antibody, Alexa Fluor 350, in 20 μl PBS for 1 hour. Mitochondria were visualized by the mitochondrially targeted EYFP (mito-GFP) following appropriate crosses [17].

2.3. Confocal Microscopy. Photomicrographs of the Malpighian tubules were generated by confocal laser scanning fluorescence microscopy (Olympus Life Science Europa GmbH, Hamburg, Germany). Microscope configuration was the following: objective lens: UPLSAPO 60x (oil, NA: 1.35); sampling speed: 8 μs/pixel; line averaging: 2x; scanning mode: sequential unidirectional; excitation: 405 nm (DAPI), 543 nm (Texas Red), and 488 nm (Alexa Fluor 488); laser transmissivity: 7% were used for DAPI, 42% for Alexa Fluor 488 and 52% for Texas Red.

2.4. TUNEL-Labelling. Terminal deoxyribonucleotide transferase-mediated dUTP-fluorescein conjugate nick end labelling (TUNEL) was carried out by using the in situ cell death detection kit (Roche) as recommended. Embryos of mutant and control flies were incubated at 20°C or 29°C and L3-stage larvae collected. Nuclei in the Malpighian tubules were counterstained by 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Labellings were visualized by a Hund-Wetzlar fluorescence microscope by using FITC or DAPI filters.

3. Results

3.1. Heavy Protein Nitration in col4a1 Mutants. We have previously demonstrated that the *col4a1* mutant flies synthesize peroxynitrite at higher concentration as part of their antimicrobial immune response under restrictive conditions [7].

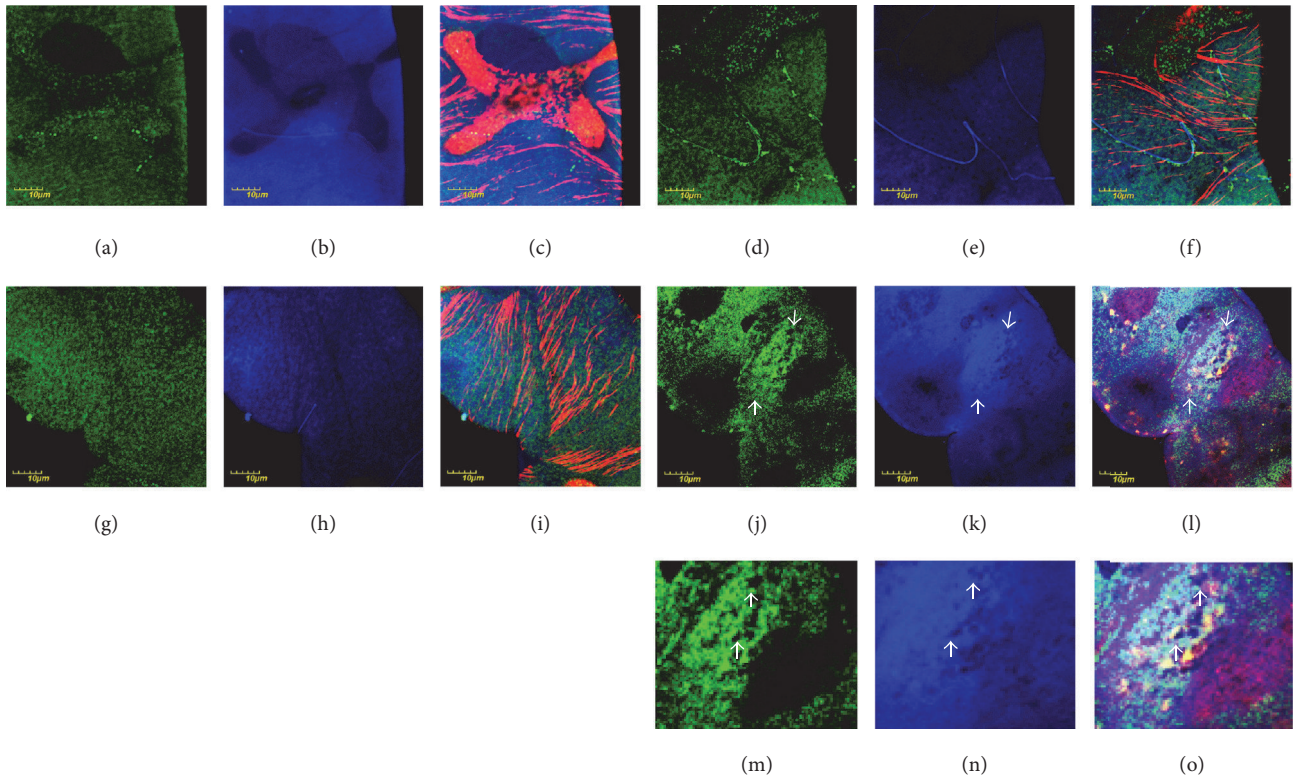

Figure 1: Protein nitration in Malpighian epithelial cells. Color code: mitochondria, green; nitrated proteins, blue; actin, red. (a) Wild-type flies incubated at 20°C, mitochondria, (b) nitrated proteins, and (c) merge. Note a stellate cell in (c). ((d), (e), (f)) Wild-type flies incubated at 29°C and displayed in the same order. ((g)–(i)) Mutant flies, incubated at 20°C, and ((j)–(l)) mutant flies, incubated at 29°C. Photomicrographs are displayed in the same order as in the upper row. Localization of nitrated proteins to mitochondria is shown in (c), (i), (f), and (l). Uneven distribution and fusion of mitochondria are demonstrated in (j). White arrows in (j), (k), and (l) pointing the region displayed in higher magnification in (m), (n), and (o). White arrows in (m), (n), and (o) showing regions with no/few mitochondria and the lack of staining, demonstrating localization to mitochondria with nitrated proteins indirectly.

Peroxynitrite is produced by the diffusion-driven reaction of nitric oxide (NO) in the presence of oxidants such as the mitochondrial-derived radical superoxide anion, $O_2^{\bullet -}$. The sources of NO are at extramitochondrial sites and the dissolved gas diffuses into mitochondria, reacts with $O2^{\bullet -}$, and disrupts protein functions by protein tyrosine nitration [18]. We therefore expected accumulation nitrated proteins in the mitochondria of *col4a1* mutant flies following incubation at 29°C.

We did not observe gross alterations in the Malpighian tubules of the mutants compared to control flies; mitochondria were distributed evenly in the cytoplasm and the fluorescent light intensities used to record nitrated proteins in the mutants were comparable to the control animals (Figures 1(a)–1(c) and Figures 1(g)–1(i)), following incubation at permissive condition. However, under restrictive conditions (29°C), we noted marked differences in the Malpighian tubules of mutant flies. While mitochondria in the epithelial cells of wild-type Malpighian tubules remained evenly distributed with no shape alteration at this temperature (Figure 1(d)), in mutants, mitochondrial fusion and uneven distribution were observed (Figure 1(j)). The level of nitrated proteins was remarkably higher in mutants in comparison with the control (Figure 1(k) versus Figure 1(e)) and these signals localized to the mitochondria (Figures 1(f) and 1(l)).

3.2. High Levels of Alkylated Proteins in the Mutants. The level of lipid peroxidation was determined indirectly by the accumulation of HNE-protein adducts. Results showed comparable amounts of alkylated proteins in the epithelial cells of mutant Malpighian tubules at permissive condition (Figure 2(b) versus Figure 2(h)), and the appearance of mitochondria remained unaffected in both mutants and controls under these conditions (Figure 2(a) versus Figure 2(g)). In mutants under restrictive conditions (29°C), uneven distribution and fusion of mitochondria occurred (Figure 2(j) versus Figure 2(d)), the mutants produced more HNE-protein adducts (Figure 2(k) versus Figure 2(e)), and the alkylated proteins localized to mitochondria (Figures 2(f) and 2(l)).

3.3. Protein-HNE Adducts Associate with Cytoplasmic Membrane. We next explored the involvement of the cytoplasmic membrane in *col4a1*-associated pathology. We recorded numerous alkylation sites in form of punctate staining in colocalization with the cytoplasmic side of the membrane

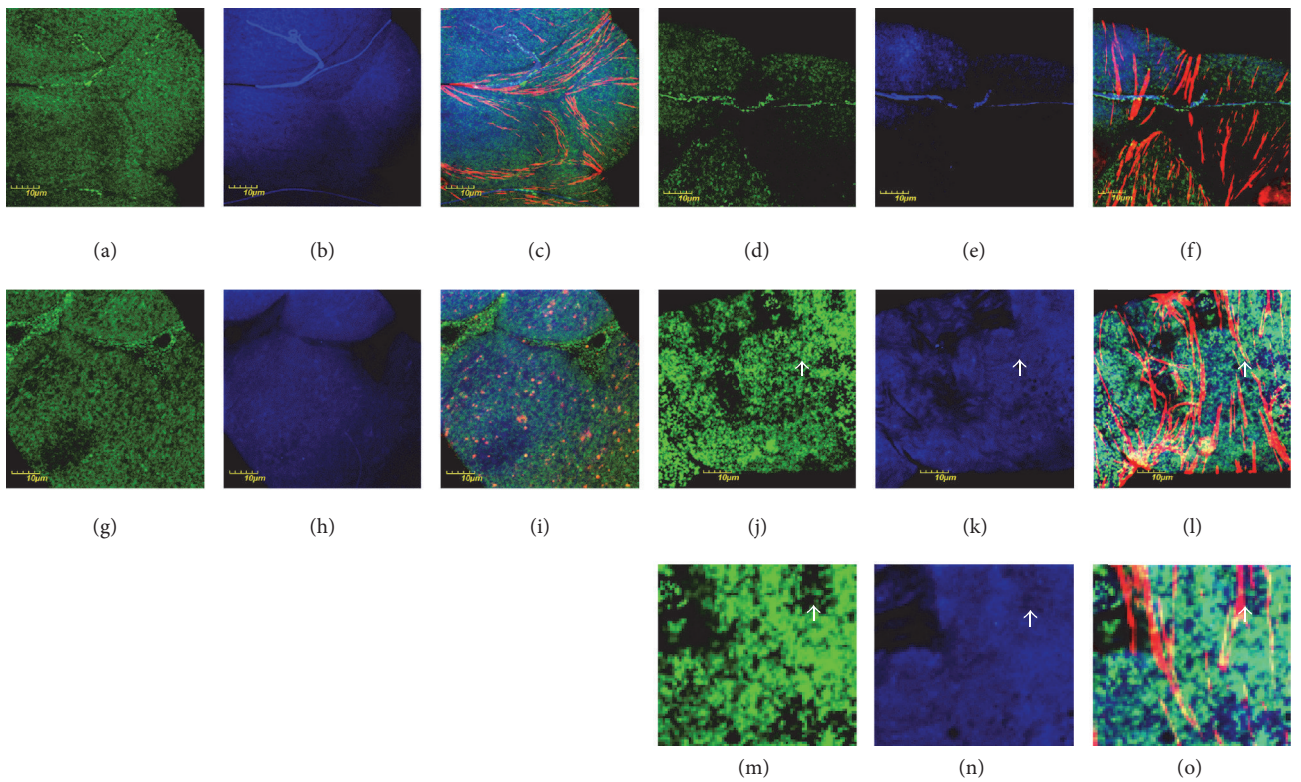

Figure 2: Protein-HNE adducts in Malpighian epithelium. Color code: mitochondria, green; protein-HNE adducts, blue; actin, red. (a) Mitochondria of wild-type flies incubated at 20°C, (b) protein-HNE adducts, and (c) merge. ((d), (e), (f)) Wild-type flies incubated at 29°C, shown in the same order. ((g)–(i)) Mutant flies, incubated at 20°C, and ((j)–(l)) mutant flies, incubated at 29°C. The order of photomicrographs is as in upper row. Note mitochondrial fusion in (j) and actin stress fibers in (l). White arrow in (j), (k), and (l) showing the portion displayed in higher magnification in (m), (n), and (o). White arrow in (m), (n), and (o): point regions with no/few mitochondria and the lack of staining, demonstrating localization of alkylated proteins to mitochondria indirectly.

and apparent perinuclear accumulation in the Malpighian epithelial cells in the mutants at permissive conditions (20°C) (Figures 3(e) and 3(f)). This staining pattern was amplified upon shift to restrictive temperature (29°C) and the HNE-conjugated proteins appeared within the cytoplasmic membrane indicating direct membrane damage by lipid peroxidation (Figures 3(g) and 3(h)). In the control flies the cytoplasmic membrane remained intact and protein-HNE adducts appeared in the vicinity of the membrane at both permissive and restrictive conditions (Figures 3(a)–3(d)).

3.4. Cell Degeneration Detected by TUNEL-Positivity. The epithelial cells of the Malpighian tubules proved to be TUNEL-positive in mutants at 29°C (Figures 4(d)–4(f)), but not at 20°C (Figures 4(a)–4(c)). These observations further supported our earlier observations of cell death affecting multiple tissues in *col4a1* mutants.

4. Discussion

Drosophila models provide useful tools for determining the pathomechanistic details, functional alterations, and some of the genotype-phenotype correlations of human monogenic disorders [19] including mutations associated with disorders of the kidneys as some of the human genes known to be associated with inherited nephrotic syndromes play conserved roles in renal functions from flies to humans [20]. There are nephrotic manifestations of human *COL4A1* mutations of the Hereditary Angiopathy, Nephropathy, Aneurysms, and Muscle Cramps (HANAC) syndrome [21] and recent research revealed glomerular hyperpermeability and adult onset glomerulocystic kidney disease in association with *COL4A1* mutations [4]. Some of the mechanistic elements in context of type IV collagen mutations, such as oxidative stress, have also been demonstrated [22]. However, evidence for chronic inflammation and posttranslational protein modifications are scarce and so far demonstrated only in *Drosophila col4a1* mutants [7, 16].

Mitochondrial fusion occurs under situations of cellular stress. Merging of the contents of partially damaged mitochondria is interpreted as a complementation mechanism rescuing impaired organelles and function [23]. Our prior results demonstrated signs of cellular stress in the form of actin stress fibers in the Malpighian epithelial cells of *col4a1* mutant *Drosophila* [16]. Results of the current study show that mutation-associated stress induced mitochondrial hyperfusion also occurs under restrictive condition with the enlarged organelles unevenly distributed within cells resulting either

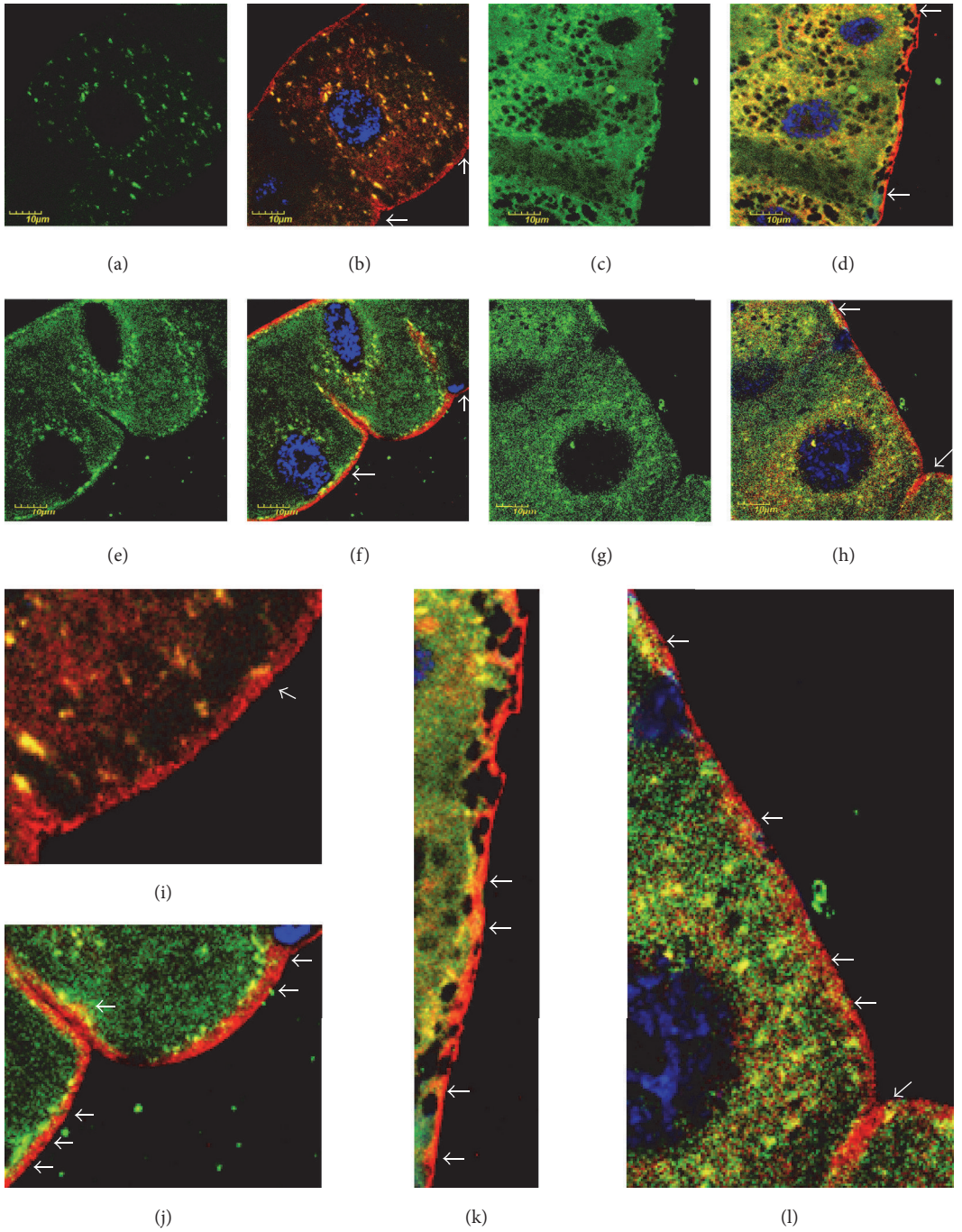

Figure 3: Cytoplasmic membrane-associated HNE-modified proteins in mutants. Color code: protein-HNE adducts, green; cytoplasmic membrane red; nuclei, blue. (a) Protein-HNE adducts in wild-type flies incubated at 20°C. (b) Overlay with membrane staining. (c) Protein-HNE adducts in wild-type flies incubated at 29°C. (d) Merged with membrane staining. ((e), (f), (g), (h)) Representative mutant, incubated at 20 or 29°C presented in the same order as in upper row. White arrows in (b), (d), (f), and (h). Regions presented in higher magnification in (i), (j), (k), and (l), respectively. White arrows in (i), (j), (k), and (l) show association of the cytoplasmic membrane with alkylated proteins. Note the notorious infiltration of HNE-modified proteins into the membrane in (l), which occurs at a less extent in (k). The membrane of wild-type animals is free of alkylated proteins (i), while they associate closely with the membrane in the mutant (j), incubated at permissive temperature.

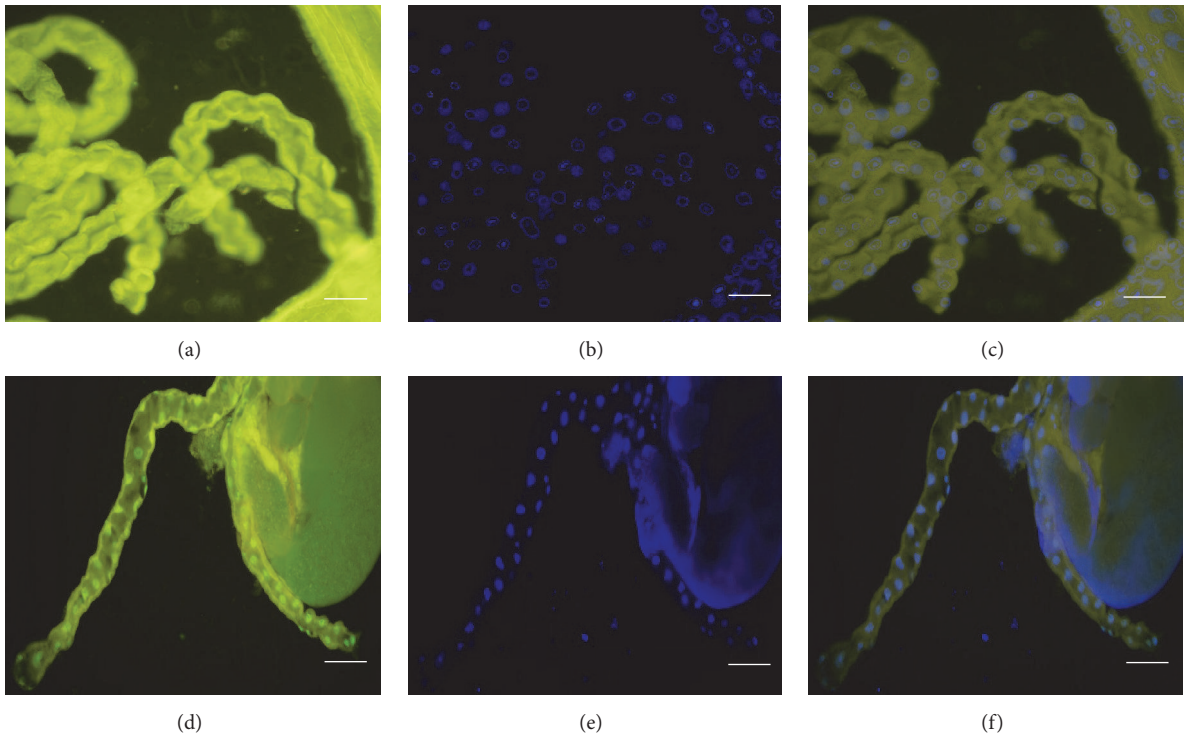

Figure 4: Fluorescence micrographs demonstrating TUNEL-positivity in Malpighian tubules. (a) TUNEL-staining of a Malpighian tubule of *col4a1*$^{+/-}$ L3-larva incubated at 20°C; (b) DAPI-staining; (c) merge, tubules appearing TUNEL-negative. (d) TUNEL-positive Malpighian tubule of a *col4a1*$^{+/-}$ L3-larva incubated at 29°C; (d) DAPI; (e) merge. Scale bars: (a)–(c) 50 μm, (d)–(f) 100 μm.

in areas apparently lacking mitochondria or in organelle-enriched areas. A further consequence of *col4a1* mutation is the accumulation of nitrated and alkylated proteins in the mutants that localize to both normal and fused mitochondria.

This observation indicates a peroxynitrite-mediated nitrosative stress in *col4a1* mutants that produce peroxynitrite at higher concentration [7]. We thus suggest that the elevated peroxynitrite level likely causes excess protein tyrosine nitration; however, this reaction does not deplete peroxynitrite in *col4a1* mutants. Indeed, the still available peroxynitrite can initiate membrane damage by lipid peroxidation producing HNE, which in turn alkylates proteins by the mechanism of Michael addiction [8]. Direct association of alkylated proteins with the epithelial cell membrane of mutant Malpighian tubules supports this scenario. Furthermore, the mutation-induced stress directs the epithelia towards degeneration as demonstrated by the TUNEL-positivity of the nuclei.

The data presented here strongly suggest a central role for peroxynitrite in *col4a1*-associate defects. In wild-type animals and under physiological conditions, the nitrosoperoxycarbonate pathway is the preferential reaction of peroxynitrite, as the main decay products of nitrosoperoxycarbonate, nitrate anion, and carbon dioxide do not exert protein or membrane modification effects (Figure 5) [13]. In mutants, however, peroxynitrite is present above physiological concentrations, and it produces excess protein tyrosine nitration and forms HNE leading to protein alkylation, lipid peroxidation, membrane damage, aberrant mitochondria, epithelial cell death, and Malpighian tubule dysfunction.

5. Conclusions

Drosophila with *col4a1* mutation synthesize peroxynitrite as a part of their stress response above physiological concentrations. The excess peroxynitrite triggers heavy protein tyrosine nitration and protein alkylation adversely affecting protein function; it also initiates membrane lipid peroxidation and mitochondrial fusion. In control animals, these posttranslational protein modifications remain at physiological levels by utilizing the nitrosoperoxycarbonate pathway to neutralize peroxynitrite. We suggest that the posttranslational protein modifications detected in the *col4a1* mutant *Drosophila* model are integral parts of *col4a1*-associated pathology and represent pathomechanistic details that have not yet been addressed in human or mouse *COL4A1* mutants.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

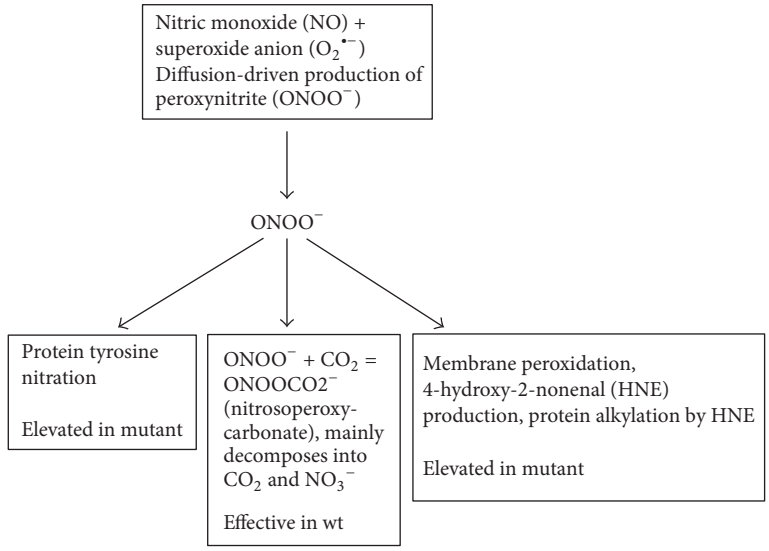

Figure 5: Schematic representation of peroxynitrite effects in wild-type flies shifting towards the neutralizing nitrosoperoxycarbonate pathway and in *col4a1* mutants towards protein nitration and alkylation involving membrane peroxidation.

Acknowledgments

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