Review Article

Hox Targets and Cellular Functions

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Hox genes are a group of genes that specify structures along the anteroposterior axis in bilaterians. Although in many cases they do so by modifying a homologous structure with a different (or no) Hox input, there are also examples of Hox genes constructing new organs with no homology in other regions of the body. Hox genes determine structures through the regulation of targets implementing cellular functions and by coordinating cell behavior. The genetic organization to construct or modify a certain organ involves both a genetic cascade through intermediate transcription factors and a direct regulation of targets carrying out cellular functions. In this review I discuss new data from genome-wide techniques, as well as previous genetic and developmental information, to describe some examples of Hox regulation of different cell functions. I also discuss the organization of genetic cascades leading to the development of new organs, mainly using Drosophila melanogaster as the model to analyze Hox function.

1. Introduction

The amazing variety of animal forms has always attracted the curiosity of scientists and spurred investigations into the underlying cause of such diversity. Comparison of different species, or of serially similar parts within the same animal, led to the concept of homology and to the idea that a basic pattern of development could be variously modified to obtain different structures. Part of the explanation for such diversity, particularly that of serially homologous organs, relies on the activity of Hox genes. These genes have received other names in the past: selector genes or master genes, indicating that they direct a particular developmental pathway, or homeotic genes, because mutations in them frequently cause transformation of one part of the body into another, that is, homeosis. However, these names include genes that do not meet all the characteristics that define Hox genes, as explained below.

Hox genes are a group of genes conserved in evolution that determines the development of different structures along the anteroposterior (A/P) axis of bilaterians [1, 2]. They are usually clustered in gene complexes, although conservation of clustering in evolution is more evident in chordates [3–5]. These genes have been studied in many species, but with more detail in two of them, Drosophila melanogaster and mouse. In Drosophila melanogaster, Hox genes are grouped in two complexes, the bithorax complex (BX-C), comprising the Hox genes Ultrabithorax (Ubx), abdominal-A (abd-A), and Abdominal-B (Abd-B) [3, 6–8] and the Antennapedia complex (ANT-C), including the Hox genes labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr), and Antennapedia (Antp) [9, 10]. In the mouse there are four complexes including the paralogous Hox genes 1 to 13, to a total of 39, although no single complex has the whole repertoire of Hox genes [11–13]. These genes are highly conserved in evolution, and there is a correspondence between particular Hox genes in Drosophila and mouse (and other species) [11, 12, 14]. As in Drosophila, one of the main functions of Hox genes in the mouse is to establish the A/P axis, thus specifying the development of different elements of the axial skeleton, although there is also an important function of Hox genes in limb development [15, 16].

The expression of Hox genes along the A/P axis relates to their position within the cluster. Genes at one end of the cluster are expressed more anteriorly and those at the other end more posteriorly, a phenomenon known as spatial colinearity [3, 17]. In vertebrates, there is also a temporal sequence of activation of Hox genes, so that 5′ genes are
expressed later and more posteriorly in the A/P axis, whereas those located at the other end of the complex are expressed earlier and more anteriorly [18, 19].

Hox genes code for proteins that bind DNA and regulate the expression of different targets. Hox proteins include a highly conserved sequence-specific DNA-binding domain, the homeodomain. The analysis of Hox protein binding activity in vitro identified a short DNA motif bound by the different proteins, with low discrimination between them, although preferences in sequence recognition have been described [20–23]. The relatively unspecific recognition of sequences by Hox proteins posed the problem of how they could bind similarly in vitro but determine different structures in vivo.

Part of the solution of the paradox relies on the fact that Hox proteins do not act alone but are aided by different cofactors and collaborators [23]. The best-characterized cofactors belong to the TALE class of homeoproteins and are encoded by Pbx and Meis genes in vertebrates (extradenticle and homothorax, resp., in Drosophila). The coexpression in the nucleus of Pbx/Exd (and Meis/Hth) and a particular Hox protein increases Hox specificity and affinity for downstream targets [24, 25]. Thus, the collaboration of Exd with different Hox proteins in Drosophila determines the specific recognition of certain sequences and, therefore, provides a means of selecting particular targets for each Hox protein [23, 26, 27].

Hox genes were initially identified in Drosophila by the spectacular transformations (homeosis) observed in Hox mutations [28–30], although homeotic transformations were first described in other species [31]. In many Drosophila Hox mutations, whole segments or structures are transformed into the like of another one within the Drosophila body. For instance, mutations in the Ultrabithorax (Ubx) gene transform the third thoracic segment (T3) into the second one (T2), including the development of four wings in the thorax instead of the wildtype pattern of two wings and two halteres (small dorsal appendages of the T3 needed to fly) [30]; similarly, gain-of-function mutations in Antennapedia transform the antennae into legs [32–34]. In the mouse, the existence of paralogous Hox genes in four different complexes prevents complete transformations when a single Hox gene is inactivated, and only partial transformations along the anteroposterior axis were initially reported [35–37]. However, when all the paralogous Hox genes from the different clusters were simultaneously deleted, complete transformations in the axial skeleton occur [37–39]. Similarly, simultaneous inactivation of Hox genes from different paralog groups frequently leads to phenotypes that are a combination of those of single mutations, although more complex phenotypes are also observed [1, 3, 15].

Such transformations require major changes in the number and properties of cells, such as cell division rates, cell affinities, and cell differentiation. This implies that Hox genes must regulate many genes responsible for cellular functions to elicit the wildtype pattern. Moreover, there must be a coordination in these changes, since the result is the harmonious development of a new structure or organ. Even in the cases in which Hox mutations do not produce clear transformations, regulation of downstream targets is usually quite specific. Because Hox genes are expressed in different tissues, the changes occur in different cell types and require the regulation by Hox proteins of distinct sets of downstream genes. Unraveling how Hox genes regulate cellular functions through specific sets of targets not only is it indispensable for understanding the role of these genes in development but also it has implications in other fields of biology. For example, it was assumed early on that changes in Hox gene expression or activity could account for some morphological changes in evolution [3], and elucidating how different regulation of Hox targets is achieved may explain the generation of new forms.

In this review, I will describe some of the cellular functions performed by Hox genes and the targets they regulate. Although effects of Hox genes have been reported in derivatives of the ectoderm, mesoderm, and endoderm, and in both invertebrates and vertebrates, the main focus will be in the development of epidermal structures and the generation of particular organs or structures. Since the organism where this analysis is more advanced is Drosophila, the fruit fly will be the predominant target of our analysis.

2. Search for the Targets

The classical hypothesis of selector genes of Garcia-Bellido [40] proposed that Hox genes (selector genes at that time, including also genes like engrailed, determining the posterior compartment of segments) acted by regulating a series of targets, called “realizator” genes, which would carry out the critical roles needed to organize different organs. The term “realizator” meant any gene directly connected with basic cellular functions, but it was not precisely defined. Realizer genes would be those required to determine distinct cell affinities, regulate cell proliferation, and establish organ shape [40]. The identification of realizator genes was, therefore, key to understand how Hox genes shape different structures. Different approaches to isolate and characterize Hox targets in Drosophila and vertebrates were initially used to try and identify such genes, including chromatin immunoprecipitation, UV cross-linking and DNA immunoprecipitation, subtractive hybridization, antibody staining in salivary glands, one-hybrid screening in yeast, and analysis of different patterns of expression along the A/P axis (reviewed in [23, 41–49]). Many of the targets were identified just by observing their different segmental expression in the embryo and their response to mutations in Hox genes, and only a few were shown to be directly regulated by Hox genes (see comprehensive lists in [44, 46, 47, 49]). Interestingly, many of the Hox targets proved to be genes coding for transcription factors themselves, suggesting that the architecture of Hox regulation relied on intermediate factors that would subdivide tasks. However, a few of the direct targets first identified, such as centrosomin [50] or connectin [51], conformed to the definition of realizator genes.

More recently, genome-wide techniques such as microarrays [52–73] and chromatin immunoprecipitation (ChIP) [74–80] have been used to determine the genes bound or regulated by Hox proteins in Drosophila, mouse, or cultured cells (Table 1). These methods have shown that hundreds of genes are downstream Hox targets.
Table 1: Microarray expression profile and ChIP methods used to identify Hox targets. The Hox genes are studied in the wildtype, in Hox mutants, or after Hox ectopic expression. Modified from [47].

<table>
<thead>
<tr>
<th>Organism</th>
<th>Hox gene</th>
<th>Tissue</th>
<th>Method</th>
<th>Reference</th>
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<td><em>lab</em></td>
<td>Whole embryo</td>
<td>Microarrays</td>
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<td>Drosophila</td>
<td><em>Antp, Scr, and Ubx</em></td>
<td>Imaginal discs</td>
<td>Microarrays</td>
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<td>Drosophila</td>
<td><em>Scr</em></td>
<td>Leg imaginal discs</td>
<td>Microarrays</td>
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<td>Drosophila</td>
<td><em>Ubx</em></td>
<td>Haltere and wing imaginal discs</td>
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<td><em>Ubx</em></td>
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<td>Drosophila</td>
<td><em>Dfd, Antp, Scr, Ubx, abd-A, and Abd-B</em></td>
<td>Whole embryo</td>
<td>Microarrays</td>
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<td>Haltere and wing imaginal discs</td>
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<td><em>Ubx</em></td>
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<td>Drosophila</td>
<td><em>Ubx</em></td>
<td>Haltere and third leg imaginal discs</td>
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<td>Drosophila</td>
<td><em>Dfd</em></td>
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<td>Mouse</td>
<td><em>Hoxa13</em></td>
<td>Uterus and cervix</td>
<td>Microarrays</td>
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<td>Mouse</td>
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<td>Kidney cell lines</td>
<td>Microarrays</td>
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<td>Mouse</td>
<td><em>Hoxd10</em></td>
<td>Spinal cord</td>
<td>Microarrays</td>
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<tr>
<td>Mouse</td>
<td><em>Hoxc8</em></td>
<td>Embryonic fibroblasts</td>
<td>Microarrays</td>
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<td><em>Hoxa13</em></td>
<td>Embryonic fibroblasts</td>
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<tr>
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<td>Limb and genital tissue</td>
<td>Microarrays</td>
<td>[59]</td>
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<tr>
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<td><em>Hoxa1</em></td>
<td>Embryonic stem cells from blastocysts</td>
<td>Microarrays</td>
<td>[60]</td>
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<tr>
<td>Human</td>
<td><em>Hoxa9, Hoxa10</em></td>
<td>Umbilical cord cells</td>
<td>Microarrays</td>
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<td>Mouse</td>
<td><em>Hoxa11, Hoxd11</em></td>
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<td>Microarrays</td>
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<td>Mouse</td>
<td><em>Hoxa1, Hoxb1</em></td>
<td>Whole embryos</td>
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<tr>
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<td><em>Hoxa2, Hoxb1, Hoxb2, Hoxb3, and Hoxd3</em></td>
<td>Rhombomeres 2–5</td>
<td>Microarrays</td>
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<td><em>Hoxa13</em></td>
<td>NIH 3T3-derived embryonic fibroblasts</td>
<td>ChIP</td>
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<td>Humeral bone chondroblast cell line</td>
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<td><em>Hoxa2</em></td>
<td>Second branchial arch</td>
<td>ChIP</td>
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<tr>
<td>Zebrafish</td>
<td><em>Hoxb1a</em></td>
<td>Whole embryo</td>
<td>Microarrays</td>
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<tr>
<td>Zebrafish</td>
<td><em>Hoxb1b</em></td>
<td>Whole embryo</td>
<td>Microarrays</td>
<td>[70]</td>
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</table>

2.1. Differential Expression Studies. The first attempt to identify Hox gene targets in *Drosophila* with microarray technology was done by comparing gene expression in wildtype embryos and in embryos after uniform expression of the Hox gene *lab* [53]. The *lab* gene of the ANT-C is expressed in the head and is required for the development of some head structures [81–83]. The authors probed 1513 genes and found 96 with significant changes in expression levels between the two types of embryos. Among the *lab* downstream genes identified, the most common class belonged to the transcriptional regulation group, followed by the metabolism, proteolytic system/apoptosis, and cell surface receptors (cell adhesion molecules) groups. Interestingly, within the proteolytic systems/apoptosis class, 12 out of 13 genes were upregulated in the embryos overexpressing *lab*, within the cell surface regulators/CAMs/ion changes class, 10 out of 12 were downregulated, and in the cell cycle and transcription/replication/repair class, all the genes differentially expressed in wildtype and *lab*-overexpressing embryos were upregulated. This suggests a similar regulation by *lab* of the elements involved in a particular cellular process.

Since Hox genes differentiate between homologous segments, changes in Hox downstream gene transcription are expected between segments with different Hox expression. Following this logic, two studies [54, 61] compared gene expression between the *Drosophila* leg discs. The three pairs of leg discs, located in the prothoracic (T1), mesothoracic (T2), and metathoracic (T3) segments, express different Hox genes and give rise to the three pairs of legs, with roughly similar shape and size, but differentiating specific patterns. In
the first work [54], the authors found 2 genes differentially expressed in T1 and T3 leg discs, 12 when comparing T2 and T3 discs and 17 in T1 and T2 discs. In the second study [61], the authors focused their analysis on the genes specifically expressed in the T1 leg disc. This disc, the only one requiring the expression of the Hox gene Scr [84, 85], differentiates the T1 leg, which shows some particular structures like the sex comb in males. Barmina and collaborators [61] analyzed 7125 genes expressed in the distal leg discs of 16 h old pupae, and, at a threshold $P < 0.001$, they found 34 genes differentially expressed between T1 and T2 leg discs, 10 of which coded for metabolic enzymes/transporters, 5 for transcription factors, and 5 for proteins of the extracellular ligand/receptor class. Therefore, many of the genes identified in these two studies [54, 61] code for transcription factors or for proteins involved in signaling pathways, but some of the genes are more directly involved in cellular functions.

Other microarray experiments analyzed the most studied homeotic transformation in *Drosophila*, the transformation of halteres into wings caused by mutations in *Ubx*. The halteres are the dorsal appendages of the third thoracic segment (T3) and the wings are homologous dorsal appendages of the second thoracic segment (T2). The structures of the dorsal T3 derive from the haltere disc, which expresses *Ubx*, whereas the dorsal T2 is formed from the wing disc, which lacks *Ubx* expression except for the peripodial membrane [86, 87]. Mutations in *Ubx* transform the haltere disc into the wing disc and, in the adult, T3 into T2, including the transformation of halteres into wings [30, 88]. Three studies have analyzed wing and haltere disc gene expression by using microarrays [64, 66, 73]. They used different approaches to identify genes differentially transcribed either in the whole wing and haltere discs or just in their pouch region (the region of the discs giving rise to the wings and halteres, resp.).

The first two studies [64, 66] identified many genes regulated by *Ubx*, although they could not distinguish direct from indirect targets. By contrast, a more recent microarray analysis made use of the Gal4/Gal80ts system [89] to induce *Ubx* at precise points of development in the wing disc and analyzed the immediate transcriptional response [73]. In this way, the authors could identify genes that were likely to be direct targets and that selectively responded to *Ubx* at different times of development. Their experiments proved that different genes are active at distinct points of larval and pupal development and that most of them respond to *Ubx* at one single stage. They also found, in a more stringent analysis, 308 *Ubx* targets, many of which can be considered as "realizator" genes since they are directly involved in diverse cellular functions: components of the cuticle and extracellular matrix, genes involved in cell specification, cell proliferation, cell survival, cell adhesion, or cell differentiation, structural components of the actin and microtubule filaments, and accessory proteins controlling filament dynamics. Only 10% of the genes identified in this work coincide with those of previous microarray analyses [64, 66] suggesting that many of the latter might not be under *Ubx* direct control. Finally, the authors also found that *Ubx* regulates many genes in a subtle way, with just small differences in levels of expression between the discs expressing or not this Hox protein.

A more extensive analysis of Hox target expression was carried out in the embryo by studying the genes responding to the ubiquitous expression of 6 out of the 8 Hox genes (the genes *pb* and *lab* were not analyzed) [67]. As also found in the study of Pavlopolous and Akam [73] the authors discovered that different targets were activated at different time points, in this case stages 11 or 12 of embryonic development, suggesting a tight temporal control of target gene expression by Hox proteins. The immediate analysis by microarrays after the ectopic expression of Hox genes suggested that many of the genes identified were probably direct targets of Hox proteins (the authors calculate that they are 20%–30% of the total number). Hundreds of genes were found to respond to the ectopic expression of the different Hox genes and about 70% of them were regulated by only one Hox protein. It was also found that the major group of genes identified belonged to the category of realizers and that, within these, the most common group was formed by genes involved in proteolytic processes; other abundant groups, with decreased frequency, were those coding for cytoskeleton proteins, cuticle, chorion and peritrophic membrane, cell cycle and cell proliferation genes, genes involved in apoptosis, and genes coding for cell adhesion proteins [67].

Expression profile analyses have also been carried out in vertebrates or in cultured cells with different Hox activity [52, 55–60, 62, 63, 65, 68–72]. Some studies were done in cell lines mutant for a Hox gene or analyzing the transcriptional response after overexpressing a single Hox product [55, 57, 58, 60, 62]. Other experiments compared the transcriptional profile of a Hox mutant with that of the wildtype in a particular mouse [52, 56, 59, 63, 65, 71, 72] or zebrafish [68, 70] organ. As previously discussed [72], the choice of material is important since no overlap was found in the downstream targets of the same Hox gene (*Hoxal*) in embryonic stem cells or developing embryos [60, 72]. Although in most cases the mutant condition analyzed caused strong transformations, one study compared the transcriptional response in the forelimb and genital eminence of wildtype mice and of mice heterozygous for a deletion in the HoxD locus, which produced just a mild phenotypic effect [59]. In this example, few genes were identified as differentially expressed in the mutant and the wildtype, suggesting a correspondence between this low number and the weak phenotype.

Another study compared gene expression in different wildtype rhombomeres [69], distinct anatomical domains of the vertebrate central nervous system that are specified by Hox genes [90]. The authors found that there were clear differences in transcription profiles in different rhombomeres but also discovered a uniform expression of some Hox targets, with just small differences between rhombomeres. This suggests that the Hox proteins may subtly modulate the transcription of targets and not simply dictate an on/off response. Interestingly, most of the targets were coding for proteins involved in cell communication, cell differentiation, cell death, or cell metabolism, and the authors favored a direct control of realizers by the Hox proteins instead of an indirect one through intermediate transcription factors.

As was also observed in *Drosophila*, the time of the analysis could greatly determine the downstream genes identified.
For instance, in the work of Cobb and Duboule [59] the comparison of the transcriptional profile in forelimb and genitalia led the authors to conclude that many similar targets were regulated by the HoxD genes in the two structures, but at different times of development. In another example, the zebrafish Hoxb1b downstream genes identified at an early time of development, the gastrula stage [68], did not coincide with those recovered in a parallel study done with similar conditions and a paralogous Hox gene (Hoxbla) [70], perhaps because both studies analyzed different developmental stages [70].

2.2. ChIP Experiments. As is the case for the microarray data, ChIP experiments with Drosophila Hox proteins have preferentially studied Ubx, analyzing the binding of the Ubx protein to the haltere disc chromatin [76–78]. One of the studies [76] found 1147 genes bound by Ubx, with a coincidence of 96% with those found in another study [77]. However, only 20% of the genes previously identified as downstream of Ubx in microarray or in situ hybridization analyses in haltere discs were detected in this ChIP experiment. The authors claim that if targets expressed at different times in haltere development were considered, as done in a previous microarray study [73], the figure could be higher. Out of the 1147 genes bound, 154 are associated with cellular functions like cell motility, cell adhesion (shotgun, neuroglian, and cadherin-N), cell communication, or apoptosis (reaper). Out of the 294 genes that were bound and validated, 69 of them (near 25%) belonged to one of these categories. The authors conclude that many genes bound by the Ubx protein are transcription factors or genes belonging to signaling pathways but that there is also a good representation of genes directly regulating basic cellular functions.

Another study [77] compared the genes bound by the Ubx protein in haltere and third leg discs, the two discs where Ubx is highly expressed. The authors found 3400 genes bound by Ubx in the haltere disc and 779 genes in the third leg disc. Out of the 488 genes previously identified as regulated by Ubx in expression profile experiments in the haltere disc [66], 191 were bound by Ubx in this ChIP experiment. This gives a higher correlation between the two types of experiments than in a previous ChIP analysis [76]. The targets include not only transcription factors (although they make up the majority class) but also genes involved in the Notch, Decapentaplegic (Dpp), and Wingless signaling pathways and genes regulating cellular processes such as cell cycle (dacapo, cyclinE, and E2F). This study also compared targets bound by Ubx in the haltere and leg discs, and also with those bound in the embryo, and found that 2705 genes bound in the haltere disc were not targets in the leg disc and that 84 bound in the leg disc were not targets in the haltere disc. The authors also found that, out of the 4590 binding peaks identified in the haltere disc, only 16% were also detected in the T3 leg disc and 42% in 0–12 h embryos (data provided from the modENCODE project; http://www.modencode.org/). These data indicate a high degree of specificity in binding by the Ubx protein depending on the tissue or organ. This conclusion is supported by the analysis of some genes, like vestigial or cut, with known function in dorsal but not ventral appendages: the authors found binding of Ubx to these two genes in the haltere (dorsal) disc but not in the leg (ventral) disc.

A third study [78] used different methods (Agilent platform, a different Ubx antibody) and found fewer genes [439] bound by the Ubx protein in the haltere disc (more precisely, wing pouch transformed to haltere pouch by a gain-of-function Ubx mutation) than the other two studies. However, the authors found many genes previously identified as being regulated by Ubx, such as connectin, thickveins, spalt major, and scabrous, thus validating their data. By analyzing the function of the genes bound by the Ubx protein the authors concluded that there were less number of genes corresponding to the realizator class (coding for cuticular, chitin-binding, cytoskeletal proteins, etc.) than those coding for transcription factors or forming part of signaling pathways. However, by including genes that are not exactly realizator genes but that determine cellular functions like cell proliferation, cell shape, and apoptosis, the number significantly increases. Comparison with targets obtained in two previous microarray studies [64, 73] showed that only around 5% [64] or 20% [73] of the genes coincide with those identified in this study. For example, out of 542 genes reported in Mohit et al., 2006 [64] as specifically regulated by Ubx in microarray experiments, only 26 are direct targets. This ChIP analysis, as the two previous ones, reveals that many of the genes bound by Ubx code for proteins involved in signaling pathways and this is in agreement with previous data showing the regulation by Ubx of many of these pathways in the haltere disc [64, 66, 91–102].

Finally, ChIP experiments with Drosophila Hox proteins were also carried out looking for genes bound by Dfd in stage 10–12 embryos [79]. The authors combined this experiment with an in silico identification of Dfd-specific Hox response elements. This was done by searching for regulatory sequences with Dfd binding motifs that were conserved in noncoding regions of 12 Drosophila species genomes. In this way, they identified 2012 Dfd enrichment peaks, including those belonging to previously characterized Dfd targets, like reaper. They also showed that Dfd and Ubx bind to different DNA sequences in stage of 10–12 embryos, suggesting a high specificity of binding in vivo.

In vertebrates, McCabe and Innis [74] used a genome-wide screen to identify genes bound by Hoxa13 in mouse embryonic fibroblasts. Among the targets identified they studied particularly Enpp2, a gene highly upregulated in these cells and required for cell motility. Another study [75] used ChIP-on-chip technology to identify genes bound by Hoxd13 in a cell line of mesenchymal derivation that stably expresses the Hoxd13 protein. The authors identified 248 genes, many of which belonged to the category of realizator genes (mainly genes involved in cell cycle, cell proliferation, cell adhesion, cytoskeleton, cell metabolism, and cell signaling). Many of the genes bound and analyzed form part of the regulatory network which determines the formation of the vertebrate limb, including genes such as Bmp2 and Bmp4, Hand2, and Dach1, with some of them being bound only at specific times of development. Finally, another study [80] used ChIP-seq to identify genes bound by the Hoxa2 protein in the mouse second branchial arch, which is affected by inactivation of
Hoxa2, at a precise time in the development (E11.5). The authors found that the Hoxa2 protein binds thousands of genes, with a preference for genes involved in the Wnt pathway.

2.3. General Conclusions of Genomic Approaches to Identify Hox Targets. Microarray and ChIP analyses have significantly contributed to identify Hox downstream genes, providing a more extensive, genome-wide, repertoire of Hox targets than that available from previous studies. Microarrays identify many genes differentially expressed but cannot distinguish between direct and indirect targets, although the former group predominates when the analysis is done just to distinguish between direct and indirect targets, although the rays identify many genes differentially expressed but cannot providing a more extensive, genome-wide, repertoire of Hox targets than that available from previous studies. Microarrays identify many genes differentially expressed but cannot distinguish between direct and indirect targets, although the former group predominates when the analysis is done just after the controlled induction of the Hox gene [67, 73]. On the other hand, ChIP identifies regions bound by Hox proteins, but that does not necessarily mean that the gene expression is regulated at the time of the analysis (or even that it is regulated at all). In fact, some of the targets bound by the Ubx protein at a certain developmental time point are expressed later on [49, 76]. Therefore, it is not surprising that there is no very high coincidence in the Hox targets identified by microarrays or ChIP data for the same Hox protein (Ubx) and in the same tissue (haltere disc) [64, 66, 73, 76–78]. In any case, the data obtained from the microarrays and ChIP data, together with previous studies, allow to draw some conclusions.  

(1) Although many of the direct Hox targets code for transcription factors, a significant fraction of them are realizator genes. ChIP experiments in Drosophila, mouse, or cultured cells have identified many targets that code for proteins carrying out basic cellular functions [75–80], although in one of these studies the proportion is low [78]. Similarly, in those microarray experiments in which a high proportion of the genes identified are likely to be direct targets [67, 73] a significant number of these belong to the category of realizator genes. This suggests that Hox control of morphologies can be achieved, at least in part, without the need for the coordination of realizator genes through secondary transcription factors. However, the analysis of organ specification by Hox genes suggests otherwise (see below). It is possible that realizator genes are controlled both directly by Hox proteins and through intermediate transcription factors.  

(2) Hox proteins govern genes involved in the same pathway in a similar fashion. In two microarrays studies [53, 67] the authors found evidence for coordination in the regulation of Hox targets: genes involved in a similar process, like apoptosis, cell adhesion, or cell cycle, are regulated together in the same direction (activation or repression).  

(3) There is a correspondence between the morphological complexity, with respect to a homologous structure, elicited by a Hox protein, and the number of genes it regulates. The best example is the work of Slattery et al., 2011 [77]. The authors found genes bound by the Ubx protein in the haltere but not the leg disc and vice versa, but the number of genes specifically bound in the leg but not the haltere disc is much smaller than the other way around. This is not unexpected: \( \text{Ubx} \) differentiates T3 legs from T2 legs and halteres from wings (and the corresponding proximal regions), but the morphology of T3 and T2 legs is more similar than that of halteres and wings (this also applies to the domains of the two discs making the non-appendage regions in the adult). The higher morphological complexity of wings with respect to halteres correlates with the data obtained in two microarray studies: in one of them [66] it was found that 174 genes were more abundantly expressed in the wing disc than in the haltere disc but that only 18 genes were predominantly expressed in the haltere disc. In the other study [64], 16 genes were found to be more expressed in the wing disc than in the haltere disc and 7 genes the other way around. In agreement with this reasoning, another study found that just a few genes were differentially expressed in T1 and T2 leg discs (T1 leg is determined by \( \text{Scr} \)) [61], probably because the morphology and size of these two legs are similar. It was also discovered, however, that the same set of Hox proteins can form quite different organs, mouse limbs, and genitalia, through subtle modulation of the amount and temporal expression of a largely common group of targets [59].  

(4) Most of the Hox targets are regulated at particular developmental stages [59, 67, 73]. Some genes are bound by Ubx in the third larval stage but respond to Ubx activity later on [49, 76], suggesting that binding and regulation may not always be coupled. In another example, ChIP analyses done at different developmental times identified different sets of genes [75]. A time difference may also explain why in two studies done with two paralogous Hox genes (Hoxb1a and Hoxb1b) there was no overlap between the identified targets [68, 70]. Time of activation of some Hox genes has been proved, in fact, to be essential for their function [103, 104].  

(5) The number of genes similarly regulated by different Hox proteins has been found to be low [67], although common targets are identified when downstream genes from paralogous Hox genes are compared [65, 68, 72]. Different Hox proteins bind to different targets, as shown when comparing binding by the Dfd and Ubx proteins in the embryo, and the molecular architecture of the sequences that are bound by these Hox proteins is also different [79]. However, the different activity of Hox proteins could be due to some genes being specifically regulated by just one Hox protein or to subtle variations in the regulation of a common set of targets [59, 69]. Moreover, the existence of a certain number of common targets is supported by examples in which different Hox genes can perform the same role in development: \( \text{Ubx} \) and \( \text{abd-A} \) can similarly make gonads [105] or halteres [106], and several Hox genes can make the tritocerebrum [107]. Although in some of these experiments the potential similar role of Hox proteins, rather than their actual function in development, is compared, these analyses, and the similar morphology observed among segments with different Hox expression in Drosophila, suggest that in some cases different Hox genes may regulate common targets.  

(6) The regulation of Hox targets can be highly influenced by the presence of cofactors or collaborators, which help in Hox protein binding and target regulation [23, 102, 108–110]. This may explain why a particular Hox protein regulates some targets at a precise developmental stage and in a restricted number of cells.

A strict hierarchical architecture whereby Hox proteins regulate cellular functions through intermediate genes is
probably not a general rule, although in some cases Hox genes seem to follow this regulatory structure ([111]; see below). Nevertheless, even in this case dual regulation, direct and indirect, of some realizator genes, cannot be excluded. The regulation of Hox targets in different tissues and times of development, in collaboration with many different proteins [23, 47, 79, 102, 108–110], suggested that Hox proteins could act more as “micromanagers” than as selector genes at the top of a well-established hierarchy [112]. Whatever the way they act, Hox genes must regulate many downstream targets to control cellular behavior.

3. The Cellular Functions

Since Hox mutations frequently cause major changes in morphology and Hox proteins are also sufficient to develop new organs, it was expected that Hox genes should modify many cellular processes needed for those changes. I describe in what follows some examples of cellular functions regulated by Hox genes.

3.1. Cell Death. Apoptosis, or programmed cell death, is an important mechanism in the development of some organs [113]. Hox genes regulate apoptosis in different tissues and organisms. One of the best-characterized examples involves the regulation of cell death by the Hox gene Dfd in the Drosophila embryo [114]. Dfd is required for the development of the mandibular and maxillary segments, two lobes in the posterior part of the head separated by a groove in the epidermis [115, 116]. Two characteristics of Dfd mutant embryos are that they show an excess of cells in the ventral part of these two segments and that the groove that separates them is eliminated. Expression of the proapoptotic gene reaper (rpr) and subsequent cell death are observed in the cells that form this boundary in wildtype embryos, and embryos homozygous for a deficiency uncovering three proapoptotic genes, rpr, head involution defective (hid), and grim, present a phenotype (absence of the groove) similar to that of Dfd mutants. In Dfd mutants rpr expression (and cell death) is eliminated, and Dfd is sufficient to activate rpr when expressed ectopically in the embryo. It was further demonstrated that Dfd binds to the rpr regulatory region, that this binding is required to activate rpr, and that the expression of rpr is sufficient to make the boundary even in the absence of Dfd [114]. This example demonstrates that cell death is a cellular process required for morphogenesis directly regulated by a Hox gene.

A similar control of cell death and morphogenesis was reported for another Hox gene, Abdominal-B (Abd-B). Abd-B is needed to determine the posterior abdominal segments (A5 to A9) [6, 7]. In mutants for this gene the segment boundaries between the sixth and seventh, or the seventh and eighth, embryonic abdominal segments are partially suppressed, so that a weak fusion between adjacent segments is observed. There is rpr transcription in these boundaries, and this depends on Abd-B. Moreover, Abd-B is able to induce rpr expression ectopically. Therefore, both Dfd and Abd-B are able to activate rpr and cell death, and this activation impinges in morphological changes on the embryo [114]. However, the regulation of cell death by Abd-B is implemented in a dual manner: as I have just described, Abd-B regulates rpr expression and development of the grooves between segments [114]; however, in these same mutants there is ectopic rpr expression at the boundary between the A8 and A9 segments in stage 12 and later embryos [117].

Apart from Dfd and Abd-B, other Hox genes control rpr expression in Drosophila embryos. lab is required for the development of the procephalic lobe [83] and in stage 12 lab mutant embryos rpr expression is increased in this lobe, indicating that lab represses rpr in these cells [117]. Similarly, Scr and Antp, two genes of the ANT-C, repress rpr in stage 13 or 12 embryos, respectively, as detected by the ectopic expression of rpr observed in posterior head or thoracic segments, respectively, in Scr or Antp mutants [117].

Well-characterized examples of the regulation of cell death by Hox proteins occur in the Drosophila embryonic nervous system. In this tissue neuroblasts divide as stem cells, each of them giving rise to another neuroblast and to a smaller cell, the ganglion mother cell, which normally divides only once to give neurons or glia [118, 119]. At postembryonic stages, neuroblasts continue dividing but there are differences in their development along the anteroposterior axis [120]. In the larval ventral cord, a rise in the levels of the Hox protein Abdominal-A occurring in the abdominal segments during the second larval period causes apoptosis through the activation of one, two, or three of the proapoptotic genes, rpr, hid, and grim [121]. Therefore, Abd-A, by controlling apoptosis when the larva enters the third larval stage, limits the number of progeny produced by neural precursors in the larval abdomen [121–123].

The survival of some neurons at the back of the embryonic ventral cord depends on Abd-B [124]. The dMP2 and MP1 pioneer neurons, which are generated along the ventral nerve cord, undergo apoptosis, after their early pioneering function, only in anterior segments. Abd-B prevents the death of the post mitotic neurons dMP2 and MP1 by repressing rpr and grim, and if Abd-B is expressed anteriorly it rescues cell death of more anterior neurons [124]. Another example of Hoxregulated cell death in this tissue is provided by lab. This Hox gene is required in two specific neuroblast lineages of the brain to induce cell death. In lab mutants, there is no cell death and two ectopic neuroblast lineages are formed [125].

The effect of the Abd-B gene in preventing cell death contrasts with its apoptotic-promoting role not only in the ectoderm [114] but also in some other neurons [126]. In the larval VNC, six peptidergic neurons (capability expressing Va neurons) express the Capa neuropeptides, encoded by the capability gene [127]. Generated initially in all the segments, the neurons present in the A5–A8 segments die: here the role of Abd-B is proapoptotic and mediated by the same proapoptotic genes repressed by Abd-B in the dMP2 and MP1 neurons [126]. Similarly, one of the two proteins encoded by the Abd-B gene, AbdBR, triggers programmed cell death of several progeny cells within the NB3-3 lineage of the embryonic ventral cord [128]. Another proapoptotic gene, sickle, is strongly expressed at the back of the ventral nerve cord at late stages of embryogenesis, also suggesting a positive regulation of cell death by Abd-B [117].
The Hox genes *Ubx* and *Antp* provide further examples of Hox genes controlling cell death in the embryonic nervous system. *Ubx* is upregulated in motoneurons of the central nervous system (CNS) at late embryonic stages, and *Ubx* activates *rpr* and induces cell death in these neurons [129]. Contrary to this, *Antp* is required for these cells to survive, and in the presence of the two products *Ubx* function prevails and the cells die [129]. Finally, recent studies have shown that the absence of *Antp* (and *Ubx*) promotes cell death in leg motoneurons [130]. As a summary, these studies not only reveal that Hox genes have a major role in regulating cell death in the *Drosophila* nervous system but also provide evidence of the importance of the context in regulating Hox activity since the same proteins, *Ubx* or *Abd-B*, can be proapoptotic or antiapoptotic in the same tissue.

Apart from *Drosophila*, there have also been reports of Hox genes governing cell death. For example, *Hoxb13* both induces cell death and represses proliferation in the caudal spinal cord of mice [131]. By contrast, inactivation of *Hoxc8* causes an increase of apoptosis in cervical and thoracic (C7-T1) motoneurons in mice [132], and in the absence of *Hoxb1* multiple neurons normally specified within rhombomere 4 are instead programmed for early cell death [133]. Outside the nervous system, one of the most studied examples of programmed cell death is the need of apoptosis to separate digits in the vertebrate limb [134]. Mice lacking the *Hoxa13* gene show reduced levels of cell death and syndactyly [135, 136]. The effect of *Hoxa13* on cell death is mediated, at least in part, not only by the regulation of *Bmp2* and *Bmp7* [136], but also by the control of retinoic acid production, a key step in controlling cell death in the limb [134]. *Aldh1a2*, the gene encoding the enzyme that converts retinaldehyde to retinoic acid and so regulates interdigital programmed cell death by controlling retinoic acid signaling, is directly regulated by *Hoxa13* [137]. *Hoxa13*, therefore, maintains interdigital cell death through the regulation of Bmps and retinoic acid [137].

In *C. elegans*, the Hox gene *lin-39* (orthologous to *Drosophila Scr*) is necessary for the survival of six neurons of the central region of the ventral nerve cord [138] through the repression of the BH3 only cell death gene *egl-1* [139, 140]. By contrast, *mab-5* (orthologous to *Drosophila Antp*) is needed for the programmed cell death of two neurons generated in the P11 and P12 lineages P(11, 12).aaap cells [141, 142].

### 3.2. Cell Migration

The best examples of the control of cell migration by Hox genes are found in the nematode *C. elegans*. In the worm, two neuroblasts, QL and QR, derive from the division of the Q neuroblasts and are located in the posterior part of the embryo. The neuroblast QL and its descendants migrate posteriorly in the wildtype, but in mutants in the Hox gene *mab-5*, they instead migrate anteriorly. A gain-of-function allele of *mab-5* makes QL and QR descendants migrate posteriorly, indicating that *mab-5* is necessary and sufficient for migration [143, 144]. Similarly, the QR neuroblast and its descendants migrate anteriorly in the wildtype but in mutants for *lin-39* this migration does not occur [138, 145].

Transcriptome studies were used to identify *C. elegans* Hox targets involved in migration. RNAseq analysis of *wildtype, mab-5 loss-of-function* and *mab-5 gain-of-function* mutants identified, among the genes putatively regulated by this Hox gene, an enrichment in genes encoding transmembrane and secreted proteins, particularly those involved in cellular matrix formation and rearrangement, but not many genes encoding transcription factors. About one third of the genes identified as being upregulated in *mab-5* gain-of-function alleles and downregulated in *mab-5* loss-of-function mutants had an effect in the migration of Q descendant when inactivated with RNAi [146]. One of the genes required for the anterior migration of neuroblasts is *mig-13*, encoding a transmembrane protein [147]. The effect of *lin-39* in the anterior migration of QR descendants is mediated by its direct control of *mig-13*, which regulates actin accumulation in the leading edge of the cells that migrate; *mab-5* and Wnt signaling, by contrast, repress *mig-13* expression to ensure posterior migration of QL descendants [148].

### 3.3. Cell Affinities

Old experiments in *Drosophila* imaginal discs suggested a role for Hox genes in determining different affinities. Cells from different imaginal discs do not intermingle when put together in culture [149–151] and in some cases this is due to the activity of Hox genes. For example, wing and haltere cells sort out in cultured cells but wing cells mix with haltere cells mutant for *bithorax* or *postbithorax* mutations, which reduce *Ubx* expression in the haltere disc thus transforming it into a wing disc [151].

Other experiments also argue for an important role of Hox genes in establishing different cell affinities. In *Drosophila*, clones mutant for *Ubx, Abd-B*, or *Dfd* sort out from the rest of the tissue [88, 152–156]. In the eye-antennal disc of *Drosophila*, *Dfd* segregates cells of the maxillary primordium from those in the rest of the disc, establishing a clonal boundary between cells expressing and not expressing *Dfd* [154]. The idea that a different Hox activity can maintain cell lineage restrictions is supported by other experiments. The wing, haltere, and leg discs are subdivided into anterior and posterior compartments from the embryonic stage, and cells from either compartment do not mix with cells from the other one throughout development [157, 158]. Higher expression of myosin II at the boundary between both compartments may help to segregate anterior and posterior cells through local increase in mechanical tension [159, 160]. It has been reported that differences in *Ubx* expression can also increase the levels of myosin at this boundary and maintain segregation of cells from different compartments [155]. The role of Hox genes in regulating cell affinities may be mediated also by cadherins. In the posterior spiracles of the embryo, *Abd-B* regulates the expression of cadherins, which are probably needed to maintain cohesion of the tissue during the morphogenetic movements that results in spiracle development [111, 161].

Targets of vertebrate Hox genes also include cell adhesion molecules. In fact, the first Hox target identified in vertebrates was the mouse neural cell adhesion molecule (N-CAM), which is needed for cell adhesion in the nervous system [162]. In the vertebrate hindbrain there is segregation of populations of cells from adjacent rhombomeres [163], and these structures are characterized by their unique expression...
of Hox genes [90, 164, 165]. Hox genes maintain the normal development of rhombomeres [166] perhaps by providing the tension required to maintain segregation of cells through the control of actomyosin molecules [167]. Therefore, Hox genes help to segregate tissues with different fate and prevent mixing of cells that differentiate into particular structures perhaps through the control of actomyosin and cadherin activity.

3.4. Cell Proliferation. Several examples of the connection between Hox genes and cell proliferation have been reported. For instance, Hoxa10 regulates p21, coding for a cyclin-dependent kinase inhibitor, in differentiating myelomonocytic cells [168]. In Rat-1 cells, Hoxb4 induces cell proliferation through the control of AP-1 activity, which in turn regulates cyclin D1 [169], and in breast cancer cell lines Hoxa5 stimulates the transcription of the tumor-suppressing gene p53 [170] (reviewed in [171]). In Drosophila, Abd-B regulates directly the expression of dacapo, which codes for an inhibitor of CyclinE/Cdk2 complexes, in the Drosophila embryonic epidermis [172]. Within the Drosophila embryonic nervous system, Ubx and abd-A regulate proliferation of neuroblasts, controlling the number of cell divisions they undergo during larval stages [173], and in the Drosophila postembryonic brain lab is required in two neuroblast lineages to end cell division by activating programmed cell death [125]. In C. elegans, lin-39 is necessary for all cell divisions that occur in the vulva after Ras activation [174].

Homoeotic mutations convert all the characteristics of one organ into those of another one; among them is its size. This is most evident in some of the more conspicuous Antennapedia or BX-C mutations. For example, the ectopic expression of the Antennapedia gene in the antennal primordium transforms antennae into legs [32–34]. Antennae are much smaller than legs, and so this transformation involves a change in organ size probably due to changes in cell proliferation. Hox genes, therefore, are likely to regulate the size of different organs by directing changes in cell proliferation [175].

The regulation of growth by Hox genes has been studied in more detail in the control of wing and haltere size by the gene Ubx. Wing discs are about 4-5 times the size of haltere discs at the end of the third larval period, a difference due to Ubx expression [97, 98]. Small Ubx mutant clones induced in the haltere disc, however, grow as the rest of the cells of the disc, indicating that Ubx does not control cell proliferation rate autonomously. However, big Ubx clones can overgrow with respect to surrounding cells, and this is due to the effect of Ubx mutant cells on the expression and activity of the growth factor dpp [97, 98, 101]. The posterior compartment of wing and haltere discs secretes the Hedgehog protein into the anterior cells, activating dpp expression at the anteroposterior compartment boundary. dpp encodes a protein of the BMP2, 4 family that is required for growth in imaginal discs [176, 177]. The Dpp protein, synthesized at the anteroposterior compartment boundary, spreads from this position to both anterior and posterior compartments promoting growth in both of them [177, 178].

The dpp band of expression is wider in the wing disc than in the haltere disc, and in mutations that reduce Ubx expression in the anterior compartment of the haltere disc the dpp band extends anteriorly. Ubx, therefore, reduces dpp expression. In addition, Ubx regulates the spread of Dpp and therefore Dpp activity in the whole disc. This is done through the control of the levels of expression of the main type I receptor of the Dpp ligand, encoded by the gene thick veins, and through the control of dally and dally-like, two genes coding for proteins that contribute to the spread of Dpp [179–184]. Ubx, by increasing thick veins expression in the haltere disc, limits the spread of Dpp, since a substantial amount of ligand molecules are bound to the increased number of receptors, thus reducing Dpp signaling in cells away from the source. Besides, by diminishing dally and dally-like expression, Ubx similarly reduces Dpp diffusion. The end result of these regulations is that Ubx reduces dpp expression and Dpp spread. In Ubx mutations, both expression and spread increase, leading to more cell proliferation and to an increase in haltere disc size comparable to that of the wing disc. The effects on cell proliferation and on cell size (described below) lead to a complete transformation of haltere size into wing size in Ubx mutants [97, 98, 100, 101].

Neuroblasts proliferate in the Drosophila embryo and give rise to a wide range of neurons. After embryogenesis, most neuroblasts of the abdomen die by apoptosis, but most of those in the cephalic or thoracic regions enter into quiescence and reenter mitosis during the larval period. One example of Hox control of neuroblast entry into quiescence has been described with the NB3-3 neuroblasts. Antp is expressed in the NB3-3 thoracic neuroblasts and abd-A in the abdominal ones. In Antp mutants, or after ectopic abd-A expression, some of these thoracic neuroblasts divide at the end of embryonic development instead of entering quiescence. Thus, Antp and abd-A spatially restrict the entry of NB3-3 neuroblasts into quiescence [185].

Outside Drosophila, some experiments also connect Hox gene activity and cell proliferation. For example, the mab-5 and lin-39 Hox genes in C. elegans stimulate cell proliferation in certain cell lineages. Lateral ecderm cells (V cells) require mab-5 for proliferation in the L2 stage [186] and in the formation of the vulva it was found that lin-39 is needed to activate a gene required for cell division [187].

3.5. Cell Size. One good example of how Hox genes regulate cell size is provided by the control by Ubx of cell size differences between wing and haltere. Wing cells are much bigger than haltere cells, and this difference occurs during the pupal stage. During this period, wing cells greatly expand to acquire a much bigger size than those in the haltere. In the pupal haltere disc, Ubx represses this expansion, thus contributing to the great size differences between wings and haleres in the adult [188]. The genes regulated by Ubx to implement this size difference are unknown.

4. Organogenesis

Hox genes may promote new morphologies in at least two major ways [43]: (a) Hox genes can modify a well-defined basic pattern which provides the genetic information defining the general layout of a certain structure, or (b) Hox genes
can promote the development of new structures with a reduced genetic layout. This separation into two classes, however, is not always strict, since the extent of the basic general pattern modified by Hox genes varies with each structure. In the first group are included modifications of serially homologous structures, such as the development of the haltere instead of the wing in Drosophila or the formation of abdominal vertebrae instead of thoracic ones in the mouse. In these cases, Hox genes just modify, although sometimes very significantly, a structure with a well-defined underlying plan. The most famous homeotic mutations, such as the antenna to leg transformation or that resulting in the four-winged fly, are among the best examples of changes in homologous structures due to the different activity of Hox genes. These changes are not simple modifications of the final differentiation state. As I have described for the haltere, Ubx changes the outcome of different signaling pathways and in this way significantly modifies size and pattern. However, even in those cases the basic developmental plan of the haltere and wing disc is very similar, and halteres can just be considered as modified wings, not new structures.

A second class of Hox-mediated organogenesis is provided by the formation of new organs without homology in the rest of the animal. Examples within this group are the formation of salivary glands or posterior spiracles in Drosophila. In this second class, Hox genes do not modify a previously established organ plan but promote the development of a new structure with a reduced background patterning information (defining dorsoventral, anteroposterior, and compartment cues), that is, without a clear homology to any other structure in any other part of the body. Mutations that affect this Hox activity do not give rise, in general, to any recognizable transformation of pattern; thus, mutations in the Drosophila Hox genes Dfd [115, 116] or lab [83] show abnormal or absent structures, but no major homeotic transformations. In some instances, mutations in a single Hox gene can give rise to recognizable transformations or absence of a structure depending on the tissue or organ considered. In any case, Hox genes must coordinate cellular functions to obtain a harmonious development of organs. I will discuss organogenesis in different classes of Hox activity.

4.1. "Classical" Hox Activity

(a) Development of the Drosophila Halteres. Ubx regulates the formation of halteres instead of wings by coordinating several processes during embryonic, larval, and pupal development. At the end of embryogenesis, haltere discs are about half the size of wing discs due to the early action of Ubx in disc size [189, 190]. During larval development, Ubx regulates the activity of different signaling pathways (dpp, wg, EGF, etc.) at different levels within the pathway [64, 66, 91–102]. The regulation of these pathways controls size and pattern, thus promoting haltere development. During pupal stages, Ubx induces substantial changes in cell size and cell differentiation, leading to the final development of the halteres as opposed to wings [73, 188] (Figure 1).

(b) Development of the Drosophila Legs. In contrast to the major differences observed between wings and halteres, the three legs of Drosophila differ just in a few characteristics relating to size and pattern, mainly in bristle arrangement and in the presence of the sex comb in the T1 male leg [191, 192]. The first leg differentiates from the second one by the expression of the Hox gene Scute and the third leg acquires its identity by the activity of Ubx. Accordingly, mutations in Scute transform the T1 leg into the T2 one [84, 85, 193] and Ubx mutations the T3 leg into the T2 one [85, 194–197] (there is also an early function of Ubx to differentiate T2p and T3p from T1p [103, 195]).

These transformations involve just relatively small changes in leg size or in the spatial arrangements of bristles [192–201]. The latter is achieved through the precise control of genes determining bristle development. For instance, in the distal part of the T2 leg there is restricted expression of the genes achaete and scute, responsible for the formation of the bristles, due to the combined activity of the Hairy repressor and the Notch ligand Delta [198, 199]. Scute and Ubx determine the specific pattern of bristles in the T1 and T3 legs, respectively, by regulating Delta, which, in turn, controls achaete expression and bristle development [200]. Ubx also regulates the formation of large macrochetae in T3 legs, at different times of development [201]. These studies highlight the fact that the general control of size and pattern is similar in the three legs (but with some significant differences) and that the major role of Hox genes is to determine changes in bristle pattern at late developmental stages. The absence of major size differences between legs suggests that changes in, for instance, the Dpp signaling pathway, which differentiate haltere and wing disc size, do not operate, or do it much more weakly, in the metathoracic leg discs (ventral T3) than in the haltere disc (dorsal T3), suggesting a different activity of Ubx on Dpp activity ventrally and dorsally within the same segment. As I have explained above, the relatively small differences between legs correlate with the small number of Hox targets that change gene expression among leg discs [54, 61] and with the small number of genes bound by Ubx exclusively in the third leg disc as compared to the haltere disc [77].

(c) Suppression of Structures. An extreme case of modification of one structure by a Hox gene is when such a structure is eliminated. In these examples, mutations in Hox genes prevent the suppression of the structure by promoting the formation of an organ characteristic of a different part of the body.

One example of such Hox activity is the suppression of legs in Drosophila. In the fruit fly, development of the legs requires the expression of the gene Distal-less (Dll) [202, 203]). Dll homologues are also required to specify limbs both in protostomes and deuterostomes [204, 205]. Dll is expressed in the embryonic leg discs, located in the thoracic region [206–208], whereas in the abdomen Dll expression is directly prevented by the Hox genes Ubx, abd-A, and Abd-B, and in mutants for these genes there is ectopic Dll expression in the abdominal region [109, 209–212]. These studies show that Dll is an example of a common Hox target, since it is repressed by three Hox genes, Ubx, abd-A, and Abd-B.
Another example of suppression of a structure mediated by a Hox gene has been described in the abdomen of the fruit fly. In *Drosophila*, females have seven abdominal segments (although the seventh one is smaller than the others) but males lack this segment. The elimination of this metamere depends on the sex determination pathway and on the activity of the Hox gene *Abd-B*: in mutants that convert males into females or in *Abd-B* mutants, a seventh segment develops in the male abdomen [6, 7, 213–215]. The abdominal segments derive from histoblasts, cells that are grouped in discrete clusters called histoblast nests and that do not proliferate during the larval period. These clusters are surrounded by large, polytenic larval cells that during pupal stages are extruded, die, and are replaced by the dividing and expanding histoblast nests [189, 216, 217]. The nests of the male seventh abdominal segment do not differ in any respect from those of more anterior segments or from the female ones until pupation [216]. However, during pupation these histoblasts show a lower proliferation rate than that of the other histoblast nests, are finally extruded, and die [218, 219]. Some genes and pathways are differentially active in the histoblasts of the male A7 and in more anterior segments during pupal stages, and these genes are regulated by *Abd-B*. Thus, *wg*, a Wnt ligand, is expressed in the dorsal male histoblasts except in the A7; similarly, the EGFR pathway is less active in this same segment [218, 219] and the gene *extramacrochetae*, coding for a HLH protein [220, 221], is also required for the suppression of this segment [219]. Since *Abd-B* also modulates the expression of *doublesex*, the gene at the end of the sex determination cascade [219, 222], the cellular functions (cell proliferation, cell extrusion, and cell death) that result in segment elimination ultimately depend on the activity of *Abd-B* in the male A7.

4.2. Formation of New Organs. Hox genes must coordinate different cellular processes to build structures. The complex processes required for this task are being dissected using relatively simple organs where Hox activity could be more easily analyzed. Two examples of this approach are the studies of development of posterior spiracles and of salivary glands, both in *Drosophila*.

4.2.1. Posterior Spiracles. The posterior spiracles (PS) are structures derived from the ectoderm that protrude at the back of the *Drosophila* embryo. They are needed to connect the respiratory system (the longitudinal tracheae) with the outside and are composed of two parts: the spiracular chamber, which makes contact with the trachea, and the stigmatophore, the part that protrudes. Within the spiracular chamber there is a refractile filter called the filzkörper. PS develop in the embryonic eighth abdominal segment (A8).
during 6–13 h of development and their formation requires the coordination of several cellular processes including changes in cell shape, cell rearrangements, cell adhesion, and cell polarity. The combination of simple organization and relatively complex cellular mechanisms makes the PS a tractable model to study the basis of Hox-directed organ formation [111, 223].

At stage 11 of embryonic development some 80–100 epidermal cells at the dorsal part of the A8 undergo apical constriction and invaginate in a sequential order: the anterior cells invaginate first and connect with the trachea, whereas the more posterior cells invaginate later on, developing the more external region of the spiracular chamber. As this chamber is formed, the cells located at the outside, marked by the expression of the gene spalt (sal), surround those making the spiracular chamber, undergo extensive rearrangements, and develop the stigmatophore. During these processes there is no cell death or cell division [111, 223].

Abd-B is the Hox gene expressed in the posterior abdominal segments of the embryo and it is necessary and sufficient for the formation of the PS [6, 7, 215, 224–227]. Although Abd-B is expressed in the whole A8 [228, 229], PS develop only in the anterior part of the segment and in a dorsal position, and the PS induced after ectopic Abd-B expression also develop in similar locations in more anterior metameres. The position where they develop along the dorsoventral axis depends on the activity of the Dpp pathway, and if the activity of this pathway is provided uniformly, genes normally expressed in the PS, and therefore in a dorsal position, are present now in a dorsoventral stripe [111]. Therefore, spatial cues provided by genes that subdivide anteroposterior and dorsoventral axis constrict the activity of Abd-B in forming PS.

As explained above, the activity of Hox genes is frequently modulated by the presence of cofactors, such as those encoded by the genes exd and hth. In the Drosophila embryo, hth and exd expression (and Exd translocation to the nucleus) is repressed in the Abd-B domain [212, 230–232]. This repression is needed for posterior spiracle development, since forcing hth an exd expression at the back of the embryo impedes Abd-B activity and therefore this development [232]. Abd-B, then, regulates all the cellular processes needed for the formation of these structures in the absence of any of the well-known cofactors, although it may use other, still poorly characterized cofactors, like that encoded by the gene lines [233].

(1) Primary Targets. The activity of Abd-B in forming PS is mediated through “intermediate” transcription factors or signaling pathways that carry out different cellular functions. These primary targets are empty spiracles (ems), cut (ct), unpaired, the ligand of the JAK-STAT pathway, and sal [161]. ems, ct, and up are expressed in precursors of the spiracular chamber, in partially overlapping patterns, and sal in the cells giving rise to the stigmatophore, with a pattern complementary to that of ct [161, 223, 234]. The subdivision in two groups of cells developing different structures (spiracular chamber and stigmatophore) and with a complementary expression of two genes, ct and sal, suggests that Abd-B constructs spiracles by the subdivision of tasks. Each of the four primary targets fulfills specific functions in PS development.

Spalt (sal). sal encodes a zinc finger protein [235] and is expressed in the cells forming the stigmatophore. Abd-B activates sal and sal activates the gene grain, which finally determines the rearrangements of the stigmatophore [236]. Mutants in sal form spiricular chambers but no stigmatophore [111].

Empty Spiracles (ems). ems is expressed in PS and is directly, activated by Abd-B [237]. In ems mutants the cells do not invaginate; there is no filzkörper and no connection of the trachea with cells at the surface [238]. The spiracular chamber, therefore, does not form properly in these mutants, but the absence of ems does not affect elongation of the cells of the spiracular chamber [161].

Cut. The gene cut codes for a homeodomain-containing transcription factor [239]. In ct mutants the spiracular chamber is incomplete: the trachea connects to the spiracle but there is no filzkörper [111]. ct controls differentiation of the filzkörper and prevents apoptosis by directly regulating the proapoptotic gene rpr [240]. Like ems mutations, ct mutations do not affect the elongation of the spiracular chamber [161].

Unpaired 1 (upd) and Unpaired 2. These two genes code for two ligands of the JAK-STAT pathway [241, 242] and are expressed in the precursor cells of the spiracles under the control of Abd-B [161, 242]. In mutants that disrupt the JAK-STAT pathway there is no elongation of the cells of the spiracle, and the cells of the spiracular chamber remain at the surface displacing the stigmatophore [161].

Mutations of the four genes completely eliminate spiracles and, importantly, simultaneous expression of the primary targets, ems, ct, upd, and grain (a gene downstream sal), in anterior regions of the embryo, forms ectopic spiracles even in the absence of Abd-B [161]. This indicates that they are the only Abd-B primary targets needed to develop the PS.

(2) Cellular Processes and Secondary Targets. The activity of primary targets regulates other genes (secondary targets) that implement different cellular functions (cell adhesion, cell polarity, etc.) to construct the organ.

Cell Adhesion, Cadherins, and Invagination. Cadherins are calcium-dependent adhesion proteins. Their structure is that of transmembrane proteins with an intracellular domain that can bind beta-catenin (classical cadherins) or not (nonclassical cadherins) [243]. One of the classical cadherins, E-cadherin (E-cadh), is expressed in the whole ectoderm but at higher levels in the presumptive spiracle cells, and four nonclassical cadherins are also expressed in different groups of cells of the spiracle: cad88C and cad96C are present in elongated cells that are deeply invaginated, cad74A is expressed in distal cells of the spiracular chamber, and cells from the stigmatophore express cad86C [161]. The expression
of these cadherins (or their higher levels) depends on Abd-
B, although indirectly: the expression of cad88C and cad96C
depends mainly on ems and also on the JAK/STAT pathway,
that of E-cadhn on ct and and the JAK-STAT pathway, the
expression of cad74A depends on ct, and that of cad86C
depends on sal. The result of the complex regulation of these
genes is a mosaic-type distribution of cadherins in the cells
that develop the PS [161].

The effect of inactivating cadherin function was studied by
injecting cadherin dsRNA in embryos. Reducing one or
two non-classic cadherins simultaneously with this method
does not produce any major effect in PS development.
However, reducing both cad88C and cad96C in an embryo
also mutant for E-cadhn duplicates the frequency of PS cells
that do not invaginate as compared to embryos just mutant
for E-cadhn [161].

Regulation of the Cytoskeleton. The Rho family of small
GTPases controls different cellular processes, such as cell
migration or cell rearrangements. They do so by reorganizing
the cytoskeleton through changes in the distribution of
actin and tubulin. They are activated by guanine nucleotide
exchange factors (GEFs), which catalyze the exchange of
GDP for GTP, and inactivated by GTPase activating proteins
(GAPs), which control the ability of the GTPase to hydrolyze
GTP to GDP, thus turning it to the inactive conformation
[244]. There is localized distribution of GEFs and GAPs in
cells of the PS. Thus, RhoGEF64C is located in the apical
domain of PS cells and is activated by Abd-B through ems
and the JAK-STAT pathway [161], RhoGEF2 accumulates
also in the apical side, and the RhoGAP Crossveinless-c
(Cv-c) localizes to the basolateral side of spiracle cells, in a
complementary pattern to that of the two GEFs. RhoGEF2
and cv-c are also regulated by Abd-B [161, 245, 246]. It
has been proposed that Rho1 is GTP-bound apically (where
RhoGEF2 and RhoGEF64C are located) and GDP-bound
on the basolateral side (where Cv-c is present) and, in this
way, apicobasal polarity can be coupled to the control of the
small GTPase Rho function during apical constriction and
invagination of the PS cells [246].

The PS do not invaginate, or the spiracular chamber is
suppressed, when the activity of these GTPases is down-
or upregulated [161, 245, 246]. Similarly, the expression of dom-
inant negative or constitutively active forms of Rho1 prevents
normal spiracle invagination [161, 246], and embryos mutant
for loss- or gain-of function alleles of cv-c show, in a certain
proportion of embryos, defects in invagination of the PS
and abnormal filzkörper [161, 245, 246]. These experiments
demonstrate that the activity of GEFs and GAPs has to be
tightly regulated to allow a normal development of the PS.

Control of Cell Polarity. Morphogenesis of the PS involves
changes in the shape of cells and in the relative amount of
basal or apical membrane surface. Elongating cells of the
spiracular chamber undergo an expansion of the basolateral
membrane, as shown by looking at the expression of apical
proteins like Crumbs (Crb) or Echinoid. Crb is an apical
transmembrane protein required to maintain apical polarity
[247, 248] and accumulates in a subapical region. Crb must be
correctly expressed and localized for the maintenance of the
epithelium because in crb mutants the epithelium collapses
[249]. The expression of crb is upregulated in the cells forming
PS, and this increased expression, directed by a specific
enhancer, is important for cell elongation [161, 250]. The
higher levels of crb may help to maintain apical membrane,
while laterobasal membrane is elongating, although it may
also have a role in the localization of proteins to the apical
membrane [111]. In crb mutants the PS are abnormally
formed; the phenotype resembles that of mutants in the JAK-
STAT pathway, and in fact crb expression is regulated by STAT
[161].

Coordination of the Regulation of Different Processes by Abd-B.
The different processes taking part in PS development require
the activity of secondary genes, as those just described,
and the coordination between different cellular activities.
Several other genes have been shown to be expressed in the
PS primordium and controlled by Abd-B and some of
the primary targets [117]. These include arrowhead, coding
for a LIM-homeodomain transcription factor [251], quail,
coding for a villin-like, actin binding protein [252], pox
neuro, encoding a paired-box transcription factor required
to determine the poly-innervated sense organs in Drosophila
[253,254], senseless, encoding a Zn-finger transcription factor
required in the peripheral nervous system [255], and shifted,
coding for the Drosophila ortholog of the Wnt inhibitory
factor-1, which controls the distribution of Hedgehog in the
wing disc [256, 257]. How the proteins coded by these genes
integrate in the cellular processes described within the PS is
unknown.

A recent publication reports on the coordination of
gene activity required to produce PS [250]. Abd-B increases
the expression of some genes, with otherwise ubiquitous
expression, in cells forming the PS. One of these genes is crb,
whose increased expression is necessary for the elongation
of invaginating cells in the PS. Such increase is deleterious to
cells outside this structure, but in the PS it is compensated
by a concomitant rise in the amount of other proteins like
RhoGEF64C, E-Cadhn, or aPKC. Therefore, Abd-B increases
cv-c expression to help invagination of spiracles but, at the
same time, increases the levels of other proteins (either by
modifying transcription or recycling) to prevent the harmful
effect of the increased activity of Cv-c [250].

4.2.2. Salivary Glands. The salivary glands (SG) of Drosophila
are organs in the fly head secreting proteins that serve to
fix the pupal case to a substrate. SG are formed by two
elongated tubes of epithelial cells with a central lumen that
are connected with a central duct that opens in the ventral
side of the embryo. As PS, SG are relatively simple models of
organogenesis, and their final shape is achieved without cell
proliferation.

At about 4 hours of development, the primordia of the
SG comprise about 100–120 cells in the ventral epidermis
of parasegment 2 (the posterior part of the maxillary segment
and the anterior part of the labial one) [258]. At about
6.5 h of development, two types of cells are distinguished
in this primordium: secretory cells, located more dorsally
and which secrete proteins to the lumen, and duct cells, which connect the secretory tubes with the mouth [259]. The secretory cells form two placodes in the ventral position of the labial segment. During stage II of embryogenesis the cells of the placodes undergo apical constriction, change their cell shape, and internalize sequentially, thus forming the tubes that elongate posteriorly into the thoracic region [259–261]. During this process there is no cell death or cell division [262–264].

The development of the SG depends on the activity of Scr because in Scr mutants the SG do not develop [259, 265]. In addition, the Hox cofactors Exd and Hth are also needed for this specification, as the glands are not formed in exd or hth mutants [266, 267]. The expression of Scr and hth disappears (and the Exd protein is located in the cytoplasm) before invagination of the secretory tubes, at stage II [267], and therefore subsequent development of SG is made without the input provided by Scr. Ectopic Scr expression throughout the embryo leads to new SG in two parasegments anterior to the labial one, parasegments 0 and 1 [259, 265, 268], but not posteriorly, since Scr activity is repressed in posterior segments by the genes tea-shirt and Abd-B [265]. These experiments demonstrate that Scr is necessary and sufficient to make SG but also that its activity is limited to the first stages of SG development.

The opposing activities of Dpp dorsally and the EGFR pathway ventrally confine secretory and duct cells to dorsal and ventral positions, respectively, within the segment. Secretory cells express genes like forkhead (fkh) and huckebein (hkb), whereas duct cells express genes like trachealess (trh) [259, 269]. In the complete absence of Dpp signaling, trh is expressed all over the segment [269] and all ectodermal parasegment 2 cells become SG; conversely, if Dpp activity is present throughout the embryo there are no SG [259, 269–272]. In a reciprocal way, the EGFR pathway blocks fkh expression ventrally and fkh, in turn, prevents the expression of duct cell genes [273, 274]. In the absence of EGFR signaling, duct cells become secretory cells, express genes like fkh, and repress genes like trh [273], normally confined to duct cells by fkh repression [269]. In this way, the opposing activities of the EGFR pathway and fkh distinguish duct cells from gland cells.

1) Primary Targets. The early disappearance of Scr and its cofactors Exd and Hth indicates that the Scr target genes are likely to have a fundamental role in regulating salivary gland development. In fact, many of the Scr primary targets encode transcription factors whose expression in the SG, contrary to that of Scr, persists throughout development. Therefore, Scr dictates the formation of the SG but the cellular functions required to develop SG are implemented through the activity of Scr downstream genes, whose expression is maintained by cross-regulation and autoregulation. There are four primary targets of Scr: fkh, hkb, Cyclic-AMP response element binding protein A (CrebA), and salivary gland-expressed bHLH (sage) [259, 260, 265, 275–277].

forkhead (fkh). fkh encodes a FoxA winged-helix transcription factor orthologous to the mammalian hepatocyte nuclear factor 3β [278]. It is activated by Scr [259] but later on maintains its own expression [279] as well as the expression of other genes like CrebA, senseless, and sage [262, 280, 281]. In fkh mutants, salivary gland cells die by apoptosis [262].

huckebein (hkb). Another gene controlled by Scr is hkb, which codes for an Spi/Egr-like transcription factor [282]. hkb expression is very dynamic and correlates with invagination timing of different cells [260], but its expression in SG is only transitory. In hkb mutants apical constriction and cell shape changes are normal, but the order in which the cells internalize is altered and the shape of the glands is no longer elongated [260].

Cyclic-AMP Response Element Binding Protein A (CrebA). This gene encodes a bZip transcription factor expressed at high levels in the SG throughout the larval period. CrebA is required for the secretion of proteins specific to the SG and for high levels of expression of genes of the early secretory pathway [280, 283]. CrebA is first activated by Scr-Hth-Exd and then maintained by fkh [262, 267, 280], and this regulation is direct [280]. Mutations in this gene reduce secretion, and its ectopic expression can induce the expression of secretory genes in other cell types like tracheae or salivary ducts [283].

Salivary Gland-Expressed bHLH (sage). sage codes for a bHLH protein that is only expressed in the SG [276, 277, 281, 284]. It is initially activated by Scr and fkh but maintained later on partly by autoregulation [281, 284], sage regulates genes coding for proteins that modify other proteins in the secretory pathway or that travel through the secretory pathway (secreted proteins) [281, 284]. In sage mutants the SG cells die at stage 15–16, as revealed by the expression of the proapoptotic genes rpr and hid and by anti-cleaved caspase-3 staining [284].

2) Secondary Targets and Cellular Functions. Many of the cellular functions required to make SG are regulated by fkh. In fact, an in situ hybridization analysis revealed that 59% of the genes expressed in SG show different levels of expression in wildtype and fkh mutant embryos [268]. The comparison of microarrays of wildtype and fkh mutant embryos showed that genes regulated by fkh encode proteins involved in many cellular functions [268]. fkh also maintains the expression of Scr primary targets like CrebA or sage [262, 275, 280, 281], thus explaining in part the pleiotropy of fkh mutations. However, contrary to what happens with Scr, ectopic fkh is not able to form extra SG [268]. Some of the cellular functions in which fkh is involved are invagination, tube maintenance, survival, and regulation of secreted cargo, membrane proteins, and enzymes.

Invagination. fkh is necessary for the apical constriction and cell shape changes of invaginating cells in the salivary placode [262, 278, 285]. This apical constriction, like that present in the formation of the ventral furrow [286, 287], requires the apical localization of myosin II and the activity of genes like folded gastrulation, RhoGEF2, and, similar to...
what happens in posterior spiracles [246], the RhoGTPase Rho1 [285]. Another protein that participates in RhoGTPase regulation in the SG is I8-wheeler, a Toll-like receptor protein [288]. I8-wheeler expression in the SG depends on Scr, and in I8-wheeler mutants invagination begins normally but the posterior sequential cell invagination observed in the wildtype is lost: too many cells invaginate at the same time and the whole process takes longer to be completed [289]. I8-wheeler activates the RhoGTPase signaling pathway by inhibiting RhoGAPs like RhoGap5A and RhoGap88C/cv-c.

Therefore, Scr regulates normal cell apical constriction and invagination of SG though fkh and I8-wheeler controlling GTPase activity.

Cell Death. fkh is also required to prevent cell death. In fkh mutants there is ectopic expression of the proapoptotic genes rpr and hid and subsequent cell death [262, 290]. fkh regulates cell death partly through senseless (sens) and sage, whose absence also leads to cell death of secretory cells [277, 284].

Secretion. The SG form two secretory tubes that produce and secrete specific proteins [280]. This secretion requires the expression of fkh, which controls CrebA (first activated by Scr). fkh induces the expression of genes coding for proteins required for the translocation and targeting of specific secretory cargo (like mucins, larval cuticle proteins, and secreted enzymes) in the ER and of genes coding for proteins forming complexes in the endoplasmic reticulum and the Golgi, through CrebA regulation [275, 280]. Microarray studies show that CrebA may regulate some 400 genes, about half of which are related to secretion, targeting of proteins to the membrane, or involved in the secretory pathway and protein transport [283].

Regulation of Lumen Size. Two characterized targets of both sage and fkh are the prolyl-4-hydroxylase genes that encode two endoplasmic reticulum proteins, PH4aSG1 (SG1) and PH4aSG2 (SG2). These proteins hydroxylate proline residues in collagen and other secreted proteins [291]. SG1 and SG2 are needed to maintain the lumen size and when the function of these proteins is reduced, the altered secretory content in SG results in regions of tube dilatation and constriction [281].

Loss of CrebA reduces SG lumen size and content as well as the size and number of apically localized secretory vesicles [283]. CrebA has, therefore, a role in both secretion and tubule maintenance. Rhol also has a dual function; it is needed for salivary gland migration and for regulating the size of the lumen, and it does so through two different mechanisms [285, 292]: on one hand, Rhol controls size of the lumen through regulation of the amount of phosphorylated Moesin at the apical membrane, which is important for the elongation of the apical domain [292]; on the other hand, it regulates cell rearrangements through controlling actin polymerization and distribution [292].

Tube Elongation. hkb, a primary target of Scr, is required for providing additional apical surface membrane (growth of apical membrane) for the elongation of invaginating cells [293]. In hkb mutants, the failure in providing additional apical surface results in reduced membrane length area and a failure of cell elongation. The end result is a change of shape of the organ, with dome-shape instead of elongated glands [260, 293]. hkb mediates tube elongation through two downstream targets that function to increase the apical membrane domain: klarsicht (klar) and crb, klar encodes a putative dynin-associated protein needed for directed movement of organelles [294, 295]; it mediates the polarized delivery of vesicles to the apical membrane through dynin, thus contributing to apical membrane growth and tube elongation. crb encodes a transmembrane protein that functions as an apical determinant [247, 248]; it is required in the apical region of the membranes to polarize cell shape changes during salivary gland invagination [293]. Thus, crb and klar control apical membrane elongation and delivery, respectively.

Tube elongation also requires the activity of ribbon (rib), which encodes a BTB/POZ domain-containing protein [296, 297]. rib mutant cells invaginate but do not complete migration posteriorly [297]. rib (in combination with the gene lola like) is necessary to promote crb expression and to downregulate activation (by phosphorylation) of Moesin, a protein that links crb to the cytoskeleton. The downregulation of the activity of Moesin and the upregulation of crb are necessary for the elongation and maintenance of the tube [298].

Another gene controlling tube elongation is Rhol. Rhol controls cell rearrangement and apical domain elongation by promoting actin polymerization at the basolateral membrane and limiting actin polymerization at the apical membrane. By controlling the distribution of F-actin in apical or basolateral membranes; Rhol regulates lumen size [292]. Rhol regulates Moesin (in parallel to rib) and this control is necessary for the apical domain of the salivary gland cells to elongate and for the proximal gland lumen to narrow [292]. In Rhol mutants there are defects in lumen size and also in cell migration, although the mechanisms whereby Rhol governs these two processes are still unknown [285, 292, 299].

Cell Migration. The salivary cells invaginate, contact the visceral mesoderm, and then turn and migrate posteriorly using the circular visceral mesoderm, and perhaps other tissues, as a substrate [261, 263, 300]. This migration requires Rhol [285] and also the activity of the canonical Wg pathway through Wnt-4, the atypical Wnt receptor Derailed (Drl), and wnt5. In drl or wnt5 mutants the direction of migration changes, curving towards the CNS. drl is expressed at the tip of the SG during migration, and its expression depends on Scr and fkh [301]. Wnt4 is also needed in the second phase of migration, after contacting the visceral mesoderm, and in Wnt-4 mutants the glands curve ventrally [301].

4.2.3. Hox Genes and Organ Formation: Common Themes. The studies on PS and SG development unveil some characteristics, common to both processes, which may illuminate the way Hox genes create different organs.

(1) The organ primordium is subdivided into groups of cells with independent control by Hox genes: in the
PS, stigmatophore and spiracular chamber; in SG, secretory cells and duct cells. This subdivision of the task allows for independent development of the two structures. In spite of this independence, there must also be coordination of the two groups of cells in making the organ.

(2) Hox genes control a limited number of primary targets that, in turn, regulate secondary genes that govern cellular tasks. It is not excluded, however, that in some cases there may be a double input on secondary targets, by primary targets and directly from Hox genes.

(3) Cellular functions are in many cases regulated independently. For example, in the PS, mutations in cadherins disturb cell invagination but cell elongation is not affected [161]. However, some genes can regulate more than one cellular function. For instance, in the SG CrebA is needed for secretion and for regulation of lumen size [280, 283] and Rho1 for migration and for maintaining lumen size [285, 292, 299]. In the PS, ems regulates cell polarity through crb, the cytoskeleton through Gef64C, and adhesion through cad88C [161].

(4) Few steps are needed in the genetic cascade to implement the basic cellular functions. This may have to do with the fact that organs like PS or SG have to develop quickly during embryogenesis, without cell proliferation. It may be that a higher number of steps are needed to make more complex organs during development.

5. Concluding Remarks

The strong transformations observed in some Hox mutations led to the definition of Hox genes as selector genes [40], implying that they are at the top of a gene hierarchy that determines size and pattern in a certain position of the body. Such morphological changes also spurred the interest in the identification of Hox targets, since their altered regulation would be responsible for distinguishing the wildtype structure from the mutated one. The detailed analysis of Hox mutations, however, reveals that in many cases Hox genes change previous genetic information that establishes general body plans. In this way, Hox genes simply modify, in a more evident or subtle way, signaling pathways and developmental routes at different times of development [91]. The fact that Hox genes assist in multiple modifications of gene activity, aiding the regulation of many genes in different tissues, led to the idea that Hox genes act in many cases as "micromanagers" that modify other genes' function to change morphologies rather than uniquely as master genes at the top of a genetic hierarchy [112]. Finally, the potential of Hox genes to construct organs with a minimal basic input of positional information would lead to consider them as "constructor" genes. The different views of Hox genes identify them, nevertheless, as major elements in determining structures in the anteroposterior axis of bilaterians.

The widespread role of Hox genes demands that they control many target genes and also raises the question of whether they regulate many of those directly or by intermediate targets. Detailed microarray studies and ChIP experiments suggested that Hox genes directly regulate many genes. The analysis of the formation of simple organs in Drosophila, however, argues for a direct regulation of a few genes, which in turn would control downstream realizers to carry out diverse cellular functions. The two views can be reconciled if Hox genes can in some instances simultaneously regulate one target both directly and through an intermediate. This double regulation would ensure a more efficient control of downstream genes and cellular functions. It is perhaps relevant also to consider that the analysis or organ formation by Hox genes awaits a detailed study of more complex structures, requiring processes like cell division. It may be that more elaborate regulations are needed in such cases.

The control of Hox downstream genes shows both a specific regulation of a certain target by a particular Hox protein and the existence of targets common to many Hox products. In general, there is a correspondence between the number of genes exclusively regulated by a Hox gene and the unique morphology or development it elicits. Because targets could change throughout development, their regulation by Hox proteins is very dynamic and the existence of particular or common targets will depend on the tissue and stage of development considered. Hox genes regulate targets that participate in different developmental routes, and this allows for an efficient coordination of tasks in order to produce a certain structure. This has implications not only for the development of a certain organism, but also for the evolution of different species. The mechanisms whereby Hox genes, in cooperation with other signals, implement morphological differences in evolution are being actively studied [196, 302–308], and these studies will cast light on how the different regulation of Hox targets has contributed to the morphological diversification of species.

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