Hindawi Publishing Corporation
ISRN Developmental Biology
Volume 2013, Article ID 628962, 10 pages
http://dx.doi.org/10.1155/2013/628962

*Research Article*

# DLK1 Protein Expression during Mouse Development Provides New Insights into Its Function

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Received 8 December 2012; Accepted 26 December 2012

Academic Editors: L. V. Beloussov and M. Lardelli



Delta-like 1 homolog (DLK1) is a noncanonical ligand in the Delta-Notch signalling pathway. Although *Dlk1* mRNA is abundantly present embryonically and declines rapidly just before birth, *Dlk1* knockouts display a relatively mild phenotype. To assess whether this mild phenotype was due to posttranscriptional regulation, we studied the expression of DLK1 protein in mouse embryos and found abundant expression in liver, lung, muscle, vertebrae, pancreas, pituitary, and adrenal gland(s). DLK1 expression was absent in heart, stomach, intestine, kidney, epidermis, and central nervous system. DLK1 protein expression, therefore, correlates well with the reported *Dlk1* mRNA expression pattern, which shows that its expression is mainly regulated at the pretranslational level. The comparison of the reported expression patterns of *Notch* mRNA and those of DLK1 in organs where lineage commitment and branching morphogenesis are important developmental processes suggests that DLK1 is a ligand that prevents premature Notch-dependent differentiation, possibly by competing with canonical ligands.

## 1. Introduction

DLK1, also known as preadipocyte factor 1 (Pref-1), is a transmembrane EGF-like protein consisting of an N-terminal signal sequence, six EGF-like repeats, a short juxtamembrane region containing a TACE-mediated cleavage site, a transmembrane domain, and a short C-terminal cytoplasmic tail [1, 2]. DLK1 is a noncanonical member of the evolutionarily conserved Delta-Notch signalling pathway, which is involved in stem-cell decisions during development [3]. Although DLK1 lacks the Delta-Serrate-Lag2 (DSL) domain for binding with the EGF-like repeats of Notch receptors, which all canonical Notch ligands possess [4, 5], specific interaction of DLK1 with the NOTCH1 receptor was demonstrated with the yeast two-hybrid system and *Notch1* signalling was inhibited by *Dlk1* [6, 7]. Furthermore, in Drosophila, *Dlk1* was shown to regulate the function of the Notch receptor, resulting in an altered cellular distribution of Notch itself and inhibition of expression of Notch target genes [8].

DLK1 function has been studied most in the murine preadipocyte cell line 3T3L1, which expresses both the transmembrane (55 kDa) and soluble (50 kDa) form of the DLK1 protein. Soluble DLK1 acts as an inhibitor of adipogenesis, preventing the differentiation of murine preadipocytes into mature adipocytes [1, 9, 10]. However, recent data show that DLK1 is also able to promote adipogenesis of mesenchymal stem cells [4]. Other proposed roles for DLK1 have been in maturation along the chromaffin lineage in the adrenal gland [11], in hematopoietic supportive abilities [12], in regulation of expansion of muscle progenitor cells [13], and in hepatoblast proliferation [14, 15]. Thus DLK1 seems to inhibit or promote differentiation of immature cells depending on the cellular context.

DLK1 is widely expressed during embryonic development of mammals [9, 14, 16–18], but in the adult, its expression is highly restricted [19–22]. Despite the widespread prenatal expression, DLK1-knockout mice display a relatively mild phenotype with growth retardation, accelerated adiposity, and eyelid and skeletal deformations [22]. Previous

expression studies of DLK1 have focussed on mRNA levels. Because posttranscriptional regulation can be extensive, we studied DLK1 protein expression in mouse embryos with daily intervals from embryonic day (ED) 10 till just after birth, in an attempt to gain more insight into the function of this noncanonical Notch pathway member during development.

## 2. Materials and Methods

*2.1. Tissue Collection.* Male and female FVB mice were maintained on a 12 hr light/12 hr dark cycle with free access to water and food in the animal facility of the AMC, The Netherlands. Noon of the day of the detection of a vaginal plug was considered to be ED 0.5. To further confirm the precise gestational age, the crown-rump length of the embryo was measured and compared with the table of Rugh [23]. Mouse embryos from ED10 till ED18 were collected at daily intervals for immunohistochemistry. Embryonic livers were collected at daily intervals from ED14 till ED19, and at postnatal day D2 and D5 for western-blot analysis and immunohistochemistry. The studies were carried out in accordance with Dutch guidelines for the Care and Use of Laboratory Animals and approved by the AMC supervisory committee.

*2.2. Immunohistochemistry.* Embryos were fixed overnight in 4% formaldehyde, embedded in paraffin, and sectioned at 7 $\mu$m thickness. The sections were deparaffinized, hydrated in graded alcohols, heated for 10 min at 120°C, 1 kPa in 10 mM sodium citrate (pH 6.0) to retrieve antigens, blocked in TENGT (10 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.025% (w/v) gelatin, 0.05% (v/v) Tween-20), and incubated overnight with goat polyclonal DLK1-A17 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1 : 500 diluted in TENGT. After washing 3 times in phosphate-buffered saline (PBS), sections were incubated with alkaline phosphatase-labeled rabbit-anti-goat secondary antibody (Sigma, Zwijndrecht, The Netherlands), diluted 1 : 50 in TENGT, for 1.5 hour. After incubation, sections were washed 3 times in PBS, followed by visualization of alkaline phosphatase with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP 1 : 50; Roche Woerden, The Netherlands). After dehydration in graded alcohols, the sections were mounted with Entellan (Merck, Darmstadt, Germany) and photographed with a Leica DMRA2 microscope equipped with a DC300 camera.

*2.3. Western-Blot Procedure.* Embryonic livers from ED14 till ED19 and postnatal livers from D2 and D5 were frozen in liquid nitrogen. For protein extraction, liver tissue was homogenized in RIPA buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 500 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100), containing protease inhibitors (Complete, Roche). The protein content was determined with the bicinchoninic acid (BCA) reagent (Pierce, Perbio Science, Etten-Leur, The Netherlands). Fifty $\mu$g of protein per lane was separated on a discontinuous 10% polyacrylamide gel and blotted onto PVDF membrane following the manufacturer's protocol (Biorad, Veenendaal, The Netherlands). After blotting, membranes were blocked with TENGT for 3 hours and incubated overnight with goat polyclonal DLK1-A-17 antibody (Santa Cruz Biotechnology; diluted 1 : 500 in TENGT) at 4°C on a platform rocker. After incubation, membranes were washed three times with TBST (5 mM Tris/HCl pH 7.5, 0.15 M NaCl, 0.1% (v/v) Tween-20), followed by incubation with peroxidase-conjugated donkey-anti-goat secondary antibody (Santa Cruz Biotechnology; diluted 1 : 5,000 in TBST) for 1.5 hour at RT. Thereafter, membranes were washed 3 times with TBST, followed by visualization with Lumi-Light substrate (Roche).

## 3. Results

*3.1. Embryonic Liver Abundantly Expresses the 55 and 50 kDa DLK1 Protein Variants.* Liver lysates from ED14 till ED17 mouse embryos show an intense 50 kDa DLK1 protein band, and particularly at ED15 and ED16 also a 55 kDa band is shown (Figures 1(a) and 1(b)). The 55 and 50 kDa bands represent the transmembrane and cleaved (soluble) variants of the DLK1 protein, respectively [1]. ED18 and ED19 liver lysates show very weak bands, demonstrating a rapid decline in the expression of both variants of DLK1 protein after ED17. After birth, on postnatal day D2 and D5, DLK1 protein expression is no longer detectable. We loaded a relatively high amount of protein to demonstrate the near total disappearance of DLK1 from postnatal liver. To keep the different developmental stages comparable, we loaded equal amounts of protein in all lanes. These observations show that the DLK1-A17 antibody (Santa Cruz Biotechnology) detects a protein which corresponds with the known sizes of the DLK1 protein and can, therefore, be used to demonstrate the presence of DLK1 protein in histological sections.

*3.2. DLK1 Protein Distribution in the Mouse Embryo (Table 1 and Figure 2).* Table 1 provides an overview of the distribution of DLK1 protein during embryonic development. On ED10, DLK1 expression could be detected in liver, hypothalamus, Rathke's pouch (developing anterior pituitary gland), somites, tongue, lung bud, pancreas, and the adrenal gland anlage (Figure 2(A)). From ED11 till ED16, DLK1 protein became even more widely distributed and could additionally be visualized in vertebrae, sternum, muscle, mesenchyme of pancreas, lung, and salivary gland, whereas staining was absent in the skin, heart, stomach, intestine, and kidneys (Figures 2(A)–2(F)). ED16 embryo showed the highest overall DLK1 protein expression, with the most intense staining in the pituitary gland (Figure 2(D)). The eye and masticatory muscles became highly positive for DLK1 at this time point, as well as the epithelial lining of the bronchi and pancreas. After ED16, DLK1 protein content rapidly decreased in all previously DLK1 protein-positive organs. By ED18, just prior to birth, DLK1 protein was almost absent from these organs, with only residual DLK1 protein expression in the liver and continued expression in pituitary gland and adrenal medulla (Figures 2(G)–2(I)).

*3.3. DLK1 Expression Pattern in the Developing Liver.* ED10 liver already showed a very strong expression of DLK1

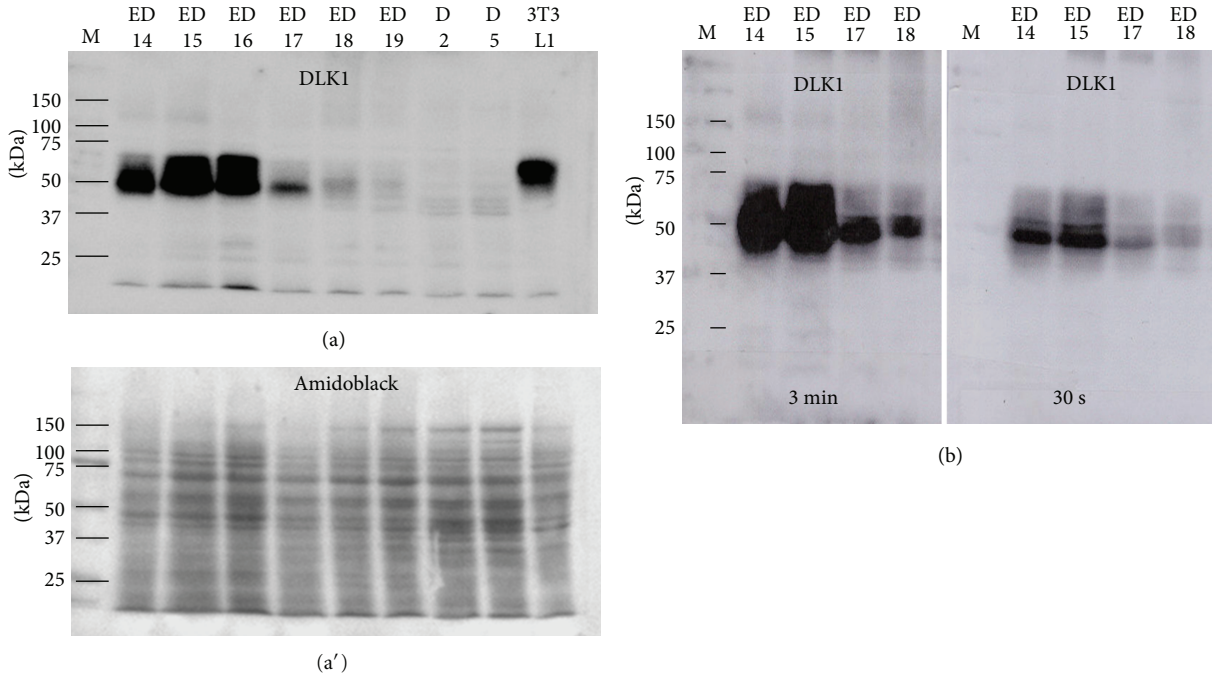


Figure 1: Expression of DLK1 protein in pre- and postnatal liver. (a) It shows a western blot of embryonic liver lysates incubated with DLK1-A17 antibody. DLK1 protein expression in the liver increases up to ED16 and declines thereafter. The most pronounced DLK1 variant is the 50 kDa band (see (b) for a comparison of long and short exposures). Particularly at ED14 and 15, a pronounced 55 kDa band (right panel in (b)) is also present. This 55 kDa band was the only expressed DLK1 protein variant in confluent 3T3L1 cells. The 50 and 55 kDa bands represent the transmembrane and cleaved variants of the DLK1 protein, respectively. (a′) It shows protein loading per lane, visualized with Amidoblack.

Table 1: Overview of DLK1 protein expression during mouse embryonic development.

| Organ | ED10 | ED13 | ED16 | ED18 |
|---|---|---|---|---|
| CNS | ++ | − | − | − |
| Pituitary | ++ | ++ | +++ | + |
| Liver | +++ | +++ | ++ | − |
| Lung | ++ | ++ | ++ | + |
| Pancreas | +++ | ++ | ++ | − |
| Adrenal gland | + | + | ++ | ++ |
| Somites | ++ | | | |
| Muscle | NI | ++ | ++ | − |
| Tongue | +++ | ++ | ++ | − |
| Vertebrae | NI | ++ | ++ | − |
| Mesonephros/kidney | − | − | − | − |
| Heart | − | − | − | − |
| Stomach | − | − | − | − |
| Intestine | − | − | − | − |

NI: not identifiable.
+++: highly positive dark blue staining as shown in Figure 3(A).
++: positive blue staining as shown in Figures 3(D) and 3(E).
+: moderately positive light blue staining as shown in Figure 3(G).
−: no staining.
CNS: central nervous system.

protein (Figure 3(A)), with positive staining confined to hepatocytes, whereas red blood cells and endothelium showed no staining (Figure 3(D)). From ED12 till ED15 (Figures 3(B)–3(E)), all hepatocytes remained DLK1 positive, with a prominent gradient of increasing intensity from the center of the liver to its periphery underneath the liver capsule. From ED16 onwards, a rapid decline in DLK1 protein expression was observed (Figures 3(F)–3(H)) and expression had become undetectable by D2 (Figure 3(I)).

*3.4. DLK1 Expression Pattern in the Developing Pituitary Gland.* Similar to liver, intense expression of DLK1 protein could be detected in Rathke's pouch already on ED10 (arrow in Figure 4(a)). While expression was initially also found in the adjacent hypothalamic region (arrowhead in Figure 4(a)), expression gradually became restricted to the pituitary gland and reached the strongest intensity on ED16 (Figures 4(b)–4(d)). On ED18 (Figure 4(e)), the pituitary gland was among the few organs that still expressed DLK1 protein.

*3.5. DLK1 Expression in the Developing Adrenal Glands Shows a Restricted Pattern.* On ED10, DLK1 protein could be detected in the adrenal gland anlage (arrow in Figure 5(a)), while the neighbouring mesonephros and gonad did not contain DLK1. By ED13, DLK1-expressing cells became restricted to the inner, medullar part of the adrenal gland (Figure 5(b)). Later in development, on ED16 and ED18, DLK1 protein was only found in medullar cells, which predominantly consists of chromaffin cells (Figures 5(c) and 5(d)). In the outer cortex, DLK1 protein was not detected.

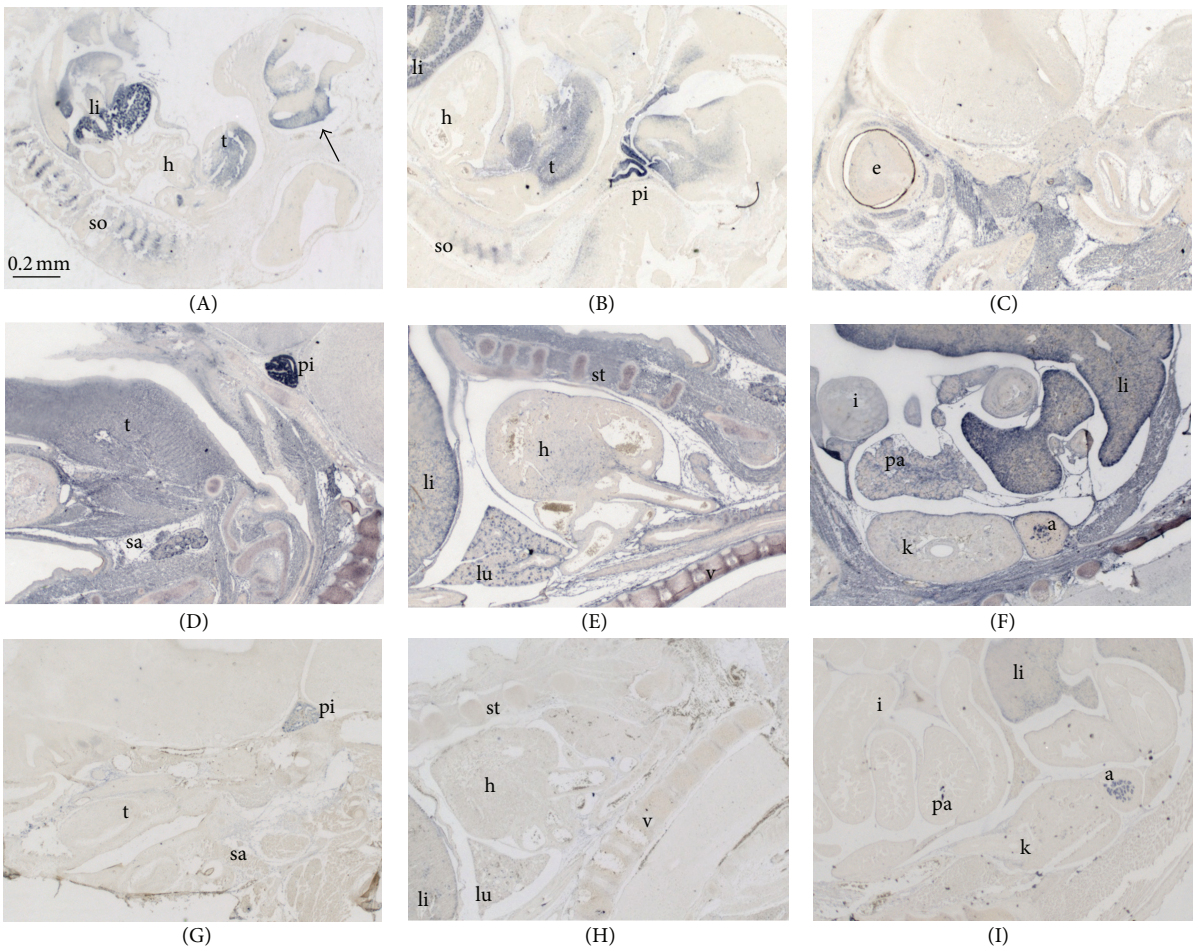


Figure 2: DLK1 protein expression in the developing mouse embryo. (A) and (B) They show sagittal sections of ED10 and ED12 embryos, respectively, that were stained for the presence of DLK1 protein. DLK1 is present in the forebrain region at the location of the developing hypothalamus and pituitary gland (arrow in (A)), liver, somites, and the tongue, whereas staining is absent in the developing heart. ((C)–(F)) They show sagittal sections of the skull base, mouth, thorax, and abdominal regions of ED16 embryo, with widely distributed DLK1 protein expression. Staining is absent in heart, intestine, stomach, and kidneys. ((G)–(I)) They show sagittal sections of ED18 embryo in the same anatomical regions as shown for ED16, but now only residual DLK1 protein expression is seen in the pituitary gland, liver, and adrenal gland. (li) liver, (so) somites, (t) tongue (pi) pituitary, (e) eye, (sa) salivary gland, (st) sternum, (h) heart, (lu) lung, (v) vertebrae, (i) intestine, (pa) pancreas, (k) kidney, and (a) adrenal gland. Scale bar in (A) is applicable for ((B)–(I)).

*3.6. DLK1 Expression in the Developing Lung and Pancreas Shows a Comparable Pattern.* In the developing lung we observed an interesting pattern of DLK1 expression, with ED10 and ED13 lung showing highly positive staining in the distal epithelium of the lung buds (arrows in Figures 6(A) and 6(B)), whereas the epithelium of the more proximal, bifurcating parts of the bronchial tree was DLK1 negative. Moderate DLK1 protein expression was present in the surrounding mesenchyme. On ED16, DLK1 protein expression was still confined to the distally located epithelium of the terminal bronchioli (inset in Figure 6(C)), while expression was almost completely abolished in the surrounding mesenchyme. On ED18, only residual DLK1 protein expression in the epithelium of the alveoli was detected (inset in Figure 6(D)).

A comparable, spatiotemporal pattern of DLK1 expression was observed during pancreatic development. ED10 pancreas showed strong DLK1 protein expression in all cells (arrow in Figure 7(a)), with moderate expression in the surrounding mesenchyme (arrowhead in Figure 7(a)). Like in embryonic lung, DLK1 protein expression had become confined to the distal growing epithelium of the developing pancreas by ED13 (arrows in Figure 7(b)), with the surrounding mesenchyme still staining positive for DLK1 protein. Contrary to the mesenchyme of the embryonic lung, DLK1 staining became almost completely restricted to the pancreatic mesenchyme by ED16 (inset in Figure 7(c)) and had disappeared completely from the pancreas by ED18 (Figure 7(d)).

## 4. Discussion

We studied the expression pattern of DLK1 protein during mouse embryonic development with daily intervals and showed that the DLK1 protein is abundantly present in

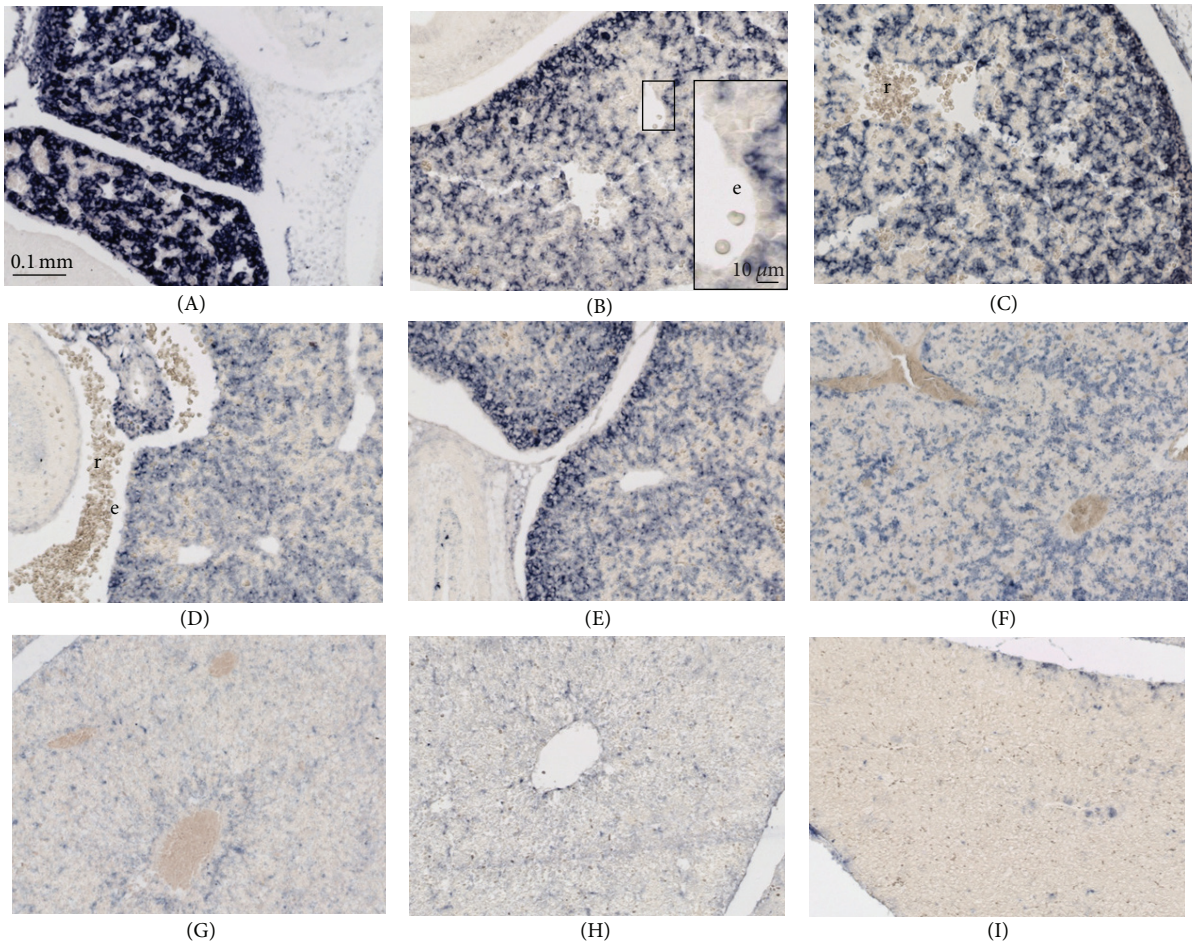


Figure 3: DLK1 protein expression in the developing liver. ((A)–(I)) They show sections of ED10, ED12, -13, -14, -15, -16, -17, 18, and D2 livers, respectively, with gradually decreasing DLK1 protein expression. After ED16, an increasing portion of the hepatocytes becomes rapidly negative for DLK1 staining, with completely absent staining on D2. Red blood cells and endothelium (inset in (B)) do not show DLK1 staining at any time point. (e) endothelium and (r) erythrocyte. Scale bar in (A) is applicable for ((B)–(I)).

embryonic liver, lung, muscle, vertebrae, pancreas, pituitary, and adrenal gland(s), whereas expression is absent in heart, stomach, intestine, kidney, epidermis, and central nervous system. We further showed that, from ED17 onwards, the expression of DLK1 rapidly decreases in all mentioned organs except the pituitary and adrenal gland(s). The expression pattern of DLK1 protein we observed in this study correlates well with the previously reported expression pattern of *Dlk1* mRNA [18], showing that DLK1 expression is mainly regulated at the pretranslational level.

Because of the reported interaction of DLK1 with the Notch1 receptor [6, 8], we applied several NOTCH1 antibodies on the same embryonic sections used for the DLK1 immunostainings. However, a specific NOTCH1 antibody was not available, in agreement with previous findings [24]. Therefore, we compared the expression of DLK1 protein in liver, adrenal and pituitary gland(s), pancreas, and lung with the previously reported (interventions in) Notch expression in the same organs in an attempt to gain more insight into DLK1's role in Notch signalling during development of these organs.

*4.1. DLK1 and Notch during Hepatoblast Differentiation.* Embryonic liver showed early and very high expression of DLK1 protein by both immunohistochemistry and western blot analysis in the ED10 to ED16 liver. The observed DLK1 decline from ED16 onwards coincides with the onset of cholangiocyte formation from hepatocyte precursor cells (hepatoblasts) and the remodeling of the ductal plate into intrahepatic bile ducts. This process starts near the portal veins in the liver hilum at ED16 in mice and progresses towards the periphery of the liver in the next ~10 days [24, 25]. When we relate the observed DLK1 expression pattern to previously reported *Notch* receptor mRNA expression during liver development, DLK1 downregulation after ED16 coincides with upregulation of the *Notch 1* and *-2* receptor mRNA levels [26]. The Notch2 receptor is known to regulate cholangiocyte cell fate and bile-duct morphogenesis [24, 27, 28], (Falix FA, Weeda VB, Labruyere W, et al. Hepatic *Notch2* deficiency in mice causes bile-duct agenesis leading to post-weaning secondary bile-duct formation, 2013, *submitted*), whereas the NOTCH1 receptor, which interacts with DLK1 [6, 8], has shown to stimulate pre- and postnatal bile-duct

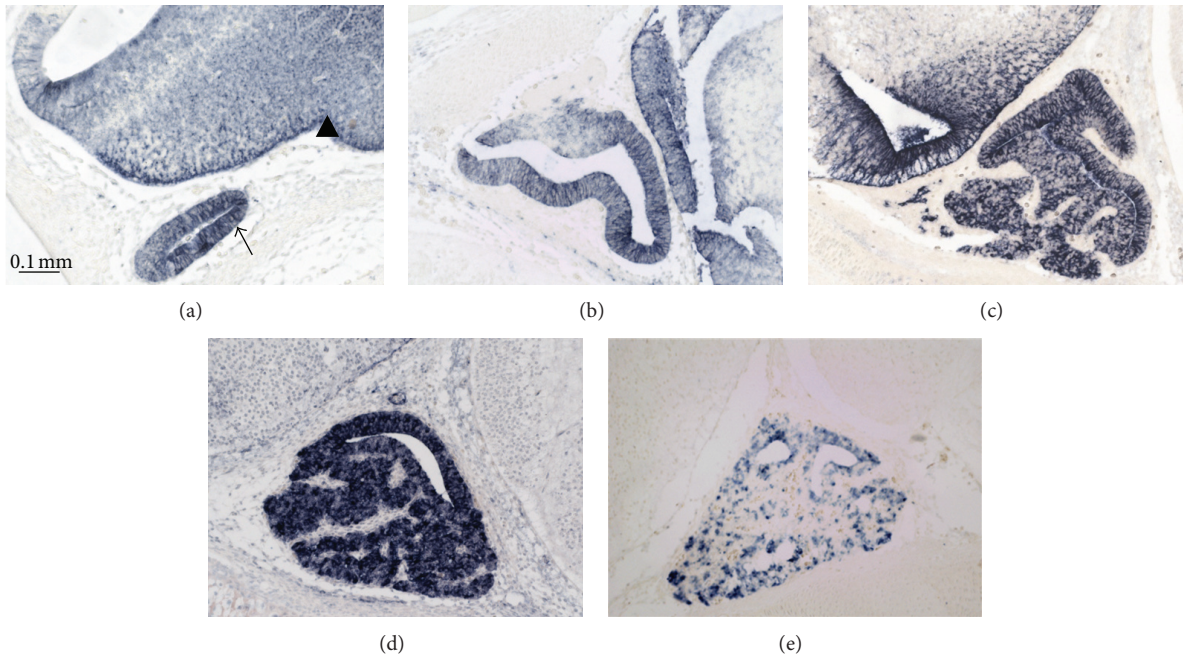


Figure 4: DLK1 protein expression in the developing pituitary gland. ((a)–(e)) They show sections of ED10, -13, -15, -16, and -18 pituitary glands, respectively, with ED10, prominent DLK1 staining in Rathke's pouch (arrow), and the developing hypothalamic region (arrowhead). From ED13 onwards, DLK1 protein expression becomes confined to the developing pituitary gland and reaches the strongest intensity at ED16. On ED18, the pituitary gland is among the few organs which still express DLK1 protein. Scale bar in (a) is applicable for ((b)–(e)).

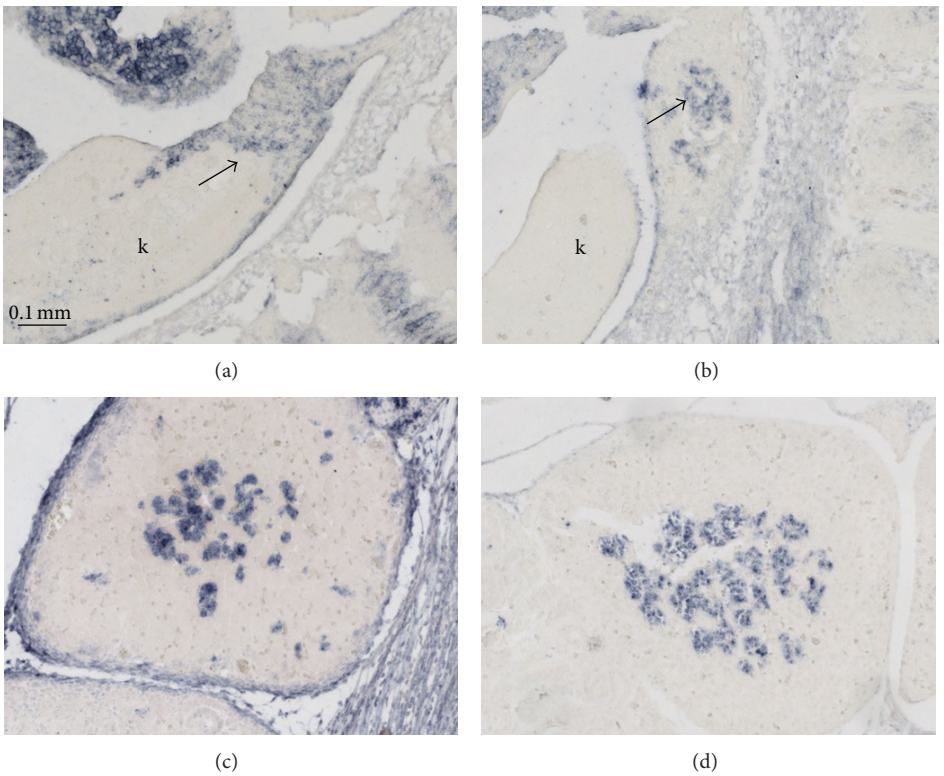


Figure 5: DLK1 protein expression in the developing adrenal gland. ((a)–(d)) They show sections of ED10, -13, -16, and -18 adrenal gland. The ED10 adrenal gland anlage shows diffuse DLK1-positive staining, whereas the neighbouring mesonephros (k) is negative for DLK1. On ED13 and afterwards, DLK1 protein expression becomes restricted to central part of the adrenal gland (adrenal medulla; arrow in (b)). The developing kidney remains negative at all-time points. Scale bar in (a) is applicable for ((b)–(d)).

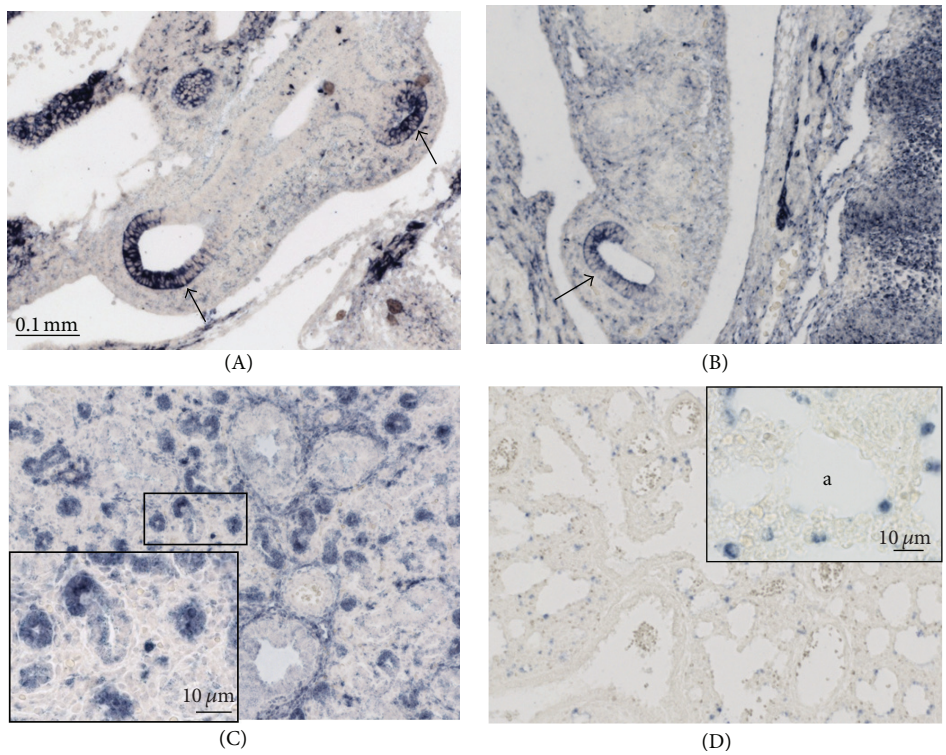


Figure 6: DLK1 protein expression in the developing lung. ((A)–(D)) They show sections of ED10, -13, -16, and -18 lungs. Positive DLK1 staining on ED10- and -13 is located in the distal growing epithelium of the lung buds (arrows in (A) and (B)), with less intense staining in the surrounding mesenchyme. On ED16, positivity is still confined to the distally located epithelium of the terminal bronchioli (inset in (C); bar: 10 $\mu$m). ED18 lung shows only residual positivity in the epithelium of the alveoli (inset in (D); bar: 10 $\mu$m). (a) alveolus. Scale bar in (A) is applicable for ((B)–(D)).

proliferation *in vivo* [29]. Furthermore, recently it was shown that postnatal continued liver-specific overexpression of *Dlk1* did not affect hepatocyte nor cholangiocyte differentiation (Falix FA, Labruyere WT, Lamers WH, et al. Liver-specific overexpression of *Dlk1* aggravates high fat diet-induced steatosis in mice, 2013, *submitted*). The inverse relation between DLK1 expression and *Notch* receptor expression during liver development together with the above findings suggests that DLK1 expression in embryonic liver reflects the hepatoblast phenotype and it can be speculated that DLK1 might regulate the low level of Notch receptor activity before ED16. However, DLK1's precise role in liver development remains to be elucidated.

*4.2. DLK1 and Notch during Adrenal Gland Differentiation.* We observed DLK1 protein expression in a variety of endocrine tissues during embryonic development, such as the pancreas, pituitary, and adrenal gland(s). The pituitary and adrenal gland(s) are the only two organs that remain positive for DLK1 protein expression after birth. In agreement with previous findings [11, 18], expression of DLK1 protein in the adrenal gland was restricted to the adrenal medulla, which is derived from the neural crest. The highly restricted DLK1 expression therefore suggests involvement of DLK1 in differentiation along the chromaffin lineage. Although the expression of Notch receptors in the developing adrenal gland has not been studied, *Notch1* mRNA was detected during development of the peripheral nervous system, another neural-crest derivative [30, 31], indicative of Notch signalling activity. Furthermore, in neuroblastoma-derived cell lines (a pediatric tumor of the peripheral nervous system), an inverse relation between *DLK1* and the *NOTCH3* receptor expression was reported [11, 32], suggesting that neuroblastoma tumors differ in their level of Notch signalling activity.

*4.3. DLK1 and Notch during Pituitary Gland Development.* During pituitary development in the mouse, already at ED12.5 pituitary-specific cell types are formed. First, thyrotroph and corticotroph cells are formed. Thereafter, somatotroph, gonadotroph and lactotroph cells, with completion of cell specification and differentiation on ED17 [33]. This late embryonic timepoint coincides with the significant decrease in DLK1 protein expression in the developing pituitary gland. Expression of canonical Notch pathway members was shown to be differentially regulated during the early stages of pituitary development, with an overall decrease of both *Notch* receptor mRNAs and ligands at late embryonic time points [34], similar to DLK1 expression. Notch signalling deficiency resulted in a premature differentiation of the corticotropic lineage and inhibition of the somatotropic and gonadotropic lineages. Furthermore, sustained Notch signalling in somato-/thyro-/lactotropic precursors resulted in a reduction of the prevalence of the respective cell populations [34, 35]. Therefore, it was suggested that

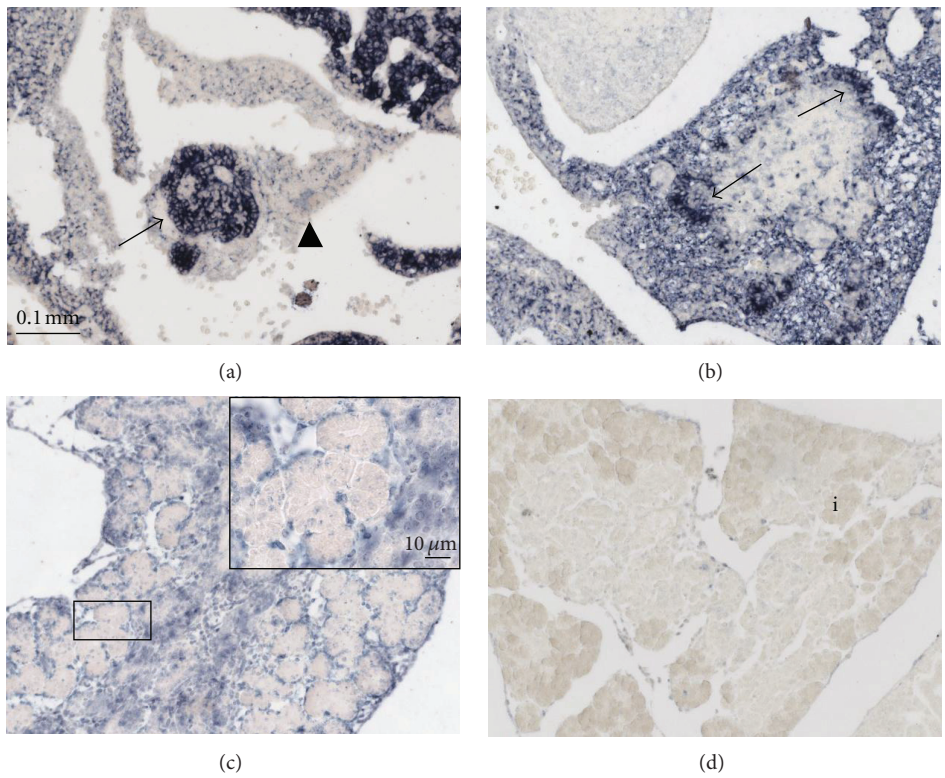


Figure 7: DLK1 protein expression in the developing pancreas. ((a)–(d)) They show sections of ED10, -13, -16, and -18 pancreas, with ED10 strong DLK1 protein expression in the entire pancreas (arrow) and moderate positivity in the surrounding mesenchyme (arrowhead). By ED13, positivity is mainly confined to the distally growing epithelium of the developing pancreas (arrows) and the surrounding mesenchyme. On ED16, DLK1 protein expression becomes virtually restricted to the pancreatic mesenchyme (inset in (c); bar: 10 $\mu$m) and on ED18 the entire pancreas has become negative. (i) islet of Langerhans. Scale bar in (a) is applicable for ((b)–(d)).

Notch signalling controls the formation of diverse precursor subtypes from a progenitor pool and that its subsequent downregulation is required for terminal differentiation [34]. The observed expression pattern of DLK1 in the developing pituitary implies that DLK1 is also involved in regulating the differentiation of pituitary cell types, probably by modulating Notch signalling activity. In agreement, adult pituitary of *Dlk1* knockout mice showed decreased numbers of growth-hormone immunoreactive cells and reduced follicle stimulating hormone (FSH) and prolactin immunoreactivity [36].

*4.4. DLK1 and Notch during Lung and Pancreas Development.* In the developing lung and pancreas, expression of DLK1 protein seemed to demarcate the areas involved in branching morphogenesis, as we observed restricted DLK1 protein expression in the developing lung and pancreas with only positivity in the distal growing epithelia and the surrounding mesenchyme. This finding agrees with earlier assumptions based on its mRNA expression pattern in these organs [18]. Branching morphogenesis is a characteristic process in developing tubular structures that is dependent upon interactions between the distal growing epithelium of the bud and the surrounding mesenchyme [37]. Recently, it was shown that Notch signalling regulates branching morphogenesis in the developing lung [38]. Disruption of Notch signalling during the initial stages of murine lung development resulted in a dramatic expansion of the population of distal progenitors and prevention of the formation of proximal airway structures [38], whereas constitutive Notch signalling prevented the differentiation of alveolar epithelium, with distal cyst formation composed of cells showing upregulated markers of proximal airway epithelium [39]. These observations suggest that during mammalian lung development, Notch signalling regulates the balance between proximal-distal cell fates and thereby regulates branching morphogenesis, with probably involvement of DLK1.

In the developing pancreas, where a comparable DLK1 expression pattern was observed, *Notch* receptor mRNAs are differentially expressed, starting from ED9.5, with a decline after ED15.5. *Notch1* and *-2* mRNA expression was detected in pancreatic epithelium and *Notch3* and *-4* in pancreatic mesenchyme and epithelium [40]. Analogous to lung development, disruption of Notch signalling during pancreatic development led to pancreatic hypoplasia caused by depletion of pancreatic epithelial precursors [41, 42], while constitutive overexpression of Notch signalling led to impaired branching of the pancreatic epithelium with formation of cyst-like structures, complete absence of exocrine development, and repression of endocrine development [43]. These findings also demonstrate a need for balanced Notch signalling during the process of branching morphogenesis and lineage commitment in the developing pancreas. DLK1

protein expression pattern in the developing pancreas again argues for involvement in these processes.

## 5. Conclusion

We showed that DLK1 protein is expressed in a variety of tissues during mouse embryonic development with a rapid decrease during maturation. In some organs, DLK1 protein showed a restricted expression pattern, while in others expression was more uniformly distributed. DLK1 protein is present in embryonic organs where active Notch signalling has previously been reported, regulating developmental processes like lineage commitment, terminal differentiation, and branching morphogenesis. Since these developmental processes also take place in organs that do not show DLK1 protein expression, the reason for its restricted expression pattern remains unclear. The localisation of DLK1 expression often correlated with localisation of reported Notch signalling, for instance in developing lung, pancreas, and pituitary gland. Considering the reported inhibitory action of DLK1 on Notch signalling activity, it can be suggested that DLK1 is a ligand that prevents premature Notch-dependent differentiation, probably by competing with the canonical ligands in a tissue-dependent manner.

## Abbreviations

ED: Embryonic day
D: Postnatal day.

## Acknowledgment

This paper is supported by The Netherlands Organization for Scientific Research (NWO) Mosaic Grant no. 017.001.241 (2004).

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