DLK1 and the Notch pathway in the liver

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DLK1 PROTEIN EXPRESSION DURING MOUSE DEVELOPMENT PROVIDES NEW INSIGHTS IN ITS FUNCTION

CHAPTER 2
Abstract

Delta-like 1 homolog (DLK1) is a noncanonical ligand in the Delta-Notch signaling 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 post-transcriptional 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 remaining CNS. DLK1 protein expression, therefore, correlates well with the reported Dlk1 mRNA expression pattern, which shows that its expression is mainly regulated at the pre-translational 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.

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Keywords
DLK1, Embryonic development, Notch signaling

List of abbreviations
ED, embryonic day; D, postnatal day
Introduction

Delta-like 1 homolog (DLK1), also known as preadipocyte factor1 (Pref-1), is a transmembrane EGF-like protein consisting of an N-terminal signal sequence, six EGF-like repeats, a short juxta-membrane 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 signaling 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 signaling 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 (55kDa) and soluble (50kDa) 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 in the function of this noncanonical Notch pathway member during development.
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Materials and Methods

Tissue collection
Male and female FVB mice were maintained on a 12 hr light/12hr 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 (D)2 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.

Immunohistochemistry
Embryos were fixed overnight in 4% formaldehyde, embedded in paraffin and sectioned at 7 µm thickness. The sections were deparaffinized, hydrated in graded alcohols, heated for 10 min at 120º C, 1 kPa in 10 mM sodium citrate (pH 6.0) to retrieve antigens, blocked in TENGT (10mM 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, 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.

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 pH8.0, 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 μg 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; diluted 1:500 in TENGT) at 4º C on a platform rocker. After incubation, membranes were washed three times with TBST (SmM Tris/HCl pH 7.5, 0.15M NaCl, 0.1% (v/v) Tween-20), followed by incubation with peroxidase-conjugated donkey-anti-goat secondary antibody (Santa Cruz; diluted 1:5,000 in TBST) for 1.5 hour at RT. Thereafter, membranes were washed 3 times with TBST, followed by visualization with Lumilight substrate (Roche).
Embryonic liver abundantly expresses the 50 and 55 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 -16, also a pronounced 55 kDa band (Figure 1A and B). The 50 and 55 kDa bands represent the cleaved (soluble) and transmembrane 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 (D)2 and D5, DLK1 protein expression is no longer detectable. These observations show that the DLK1-A17 antibody (Santa Cruz) can therefore, be used to demonstrate the presence of DLK1 protein in histological sections.

DLK1 protein distribution in the mouse embryo

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 2A). 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 (Figure 2A-F). ED16 embryo showed the highest overall DLK1 positivity, with the most intense staining in the pituitary gland (Figure 2D). 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-positive organs. By ED18, just prior to birth, DLK1 protein was almost absent from these organs, with only residual positivity in the liver and continued expression in pituitary gland and adrenal medulla (Figure 2G-I).

Figure 2 DLK1 protein expression in the developing mouse embryo
Panels A and B 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 panel A), liver, somites and the tongue, whereas staining is absent in the developing heart. Panels C-F show sagittal sections of the skull base, mouth, thorax and abdominal regions of ED16 embryo, with widely distributed DLK1 positivity. Staining is absent in heart, intestine, stomach and kidneys. Panels G-I show sagittal sections of ED18 embryo in the same anatomical regions as shown for ED16, but now only residual DLK1 positivity is seen in the pituitary gland, liver and adrenal gland. (li) liver, (so) somites, (t) tongue (pl) pituitary, (e) eye, (sa) salivary gland, (st) sternum, (h) heart, (lu) lung, (v) vertebrae, (i) intestine, (pa) pancreas, (k) kidney, (a) adrenal gland. Scale bar in A is applicable for B-I.
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Results

<table>
<thead>
<tr>
<th>Organ</th>
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Table 1 Overview of DLK1 protein expression during mouse embryonic development

NI Not identifiable
+++ Highly positive dark blue staining as shown in Figure 3A
++ Positive blue staining as shown in Figure 3D-E
+ Moderately positive light blue staining as shown in Figure 3G
- No staining

DLK1 expression pattern in the developing liver
ED10 liver already showed a very strong expression of DLK1 protein (Figure 3A), with positive staining confined to hepatocytes, whereas red blood cells and endothelium showed no staining (Figure 3D). From ED12 till ED15, all hepatocytes remained DLK1 positive, with a prominent gradient of increasing intensity from the centre of the liver to its periphery underneath the liver capsule (Figure 3B-E). From ED16 onwards, a rapid decline in DLK1 protein expression was observed (Figure 3F-H) and expression had become undetectable by D2 (Figure 3I).

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 4A). While expression was initially also found in the adjacent hypothalamic region (arrowhead in Figure 4A), expression gradually became restricted to the pituitary gland and reached strongest intensity on ED16 (Figure 4B-D). On ED18, the pituitary gland was among the few organs that still expressed DLK1 protein (Figure 4E).
Results

Figure 3 DLK1 protein expression in the developing liver
Panels A-I show sections of ED10, ED12-18 and D2 livers, respectively, with gradually decreasing DLK1 positivity. 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 show absent DLK1 staining at all time points. (e) endothelium, (r) erythrocyte. Scale bar in A is applicable for B-I.

Figure 4 DLK1 protein expression in the developing pituitary gland
Panels A-E show sections of ED10, -13, -15, -16 and -18 pituitary glands, respectively, with on ED10, prominent DLK1 staining in Rathke’s pouch (arrow) and the developing hypothalamic region (arrowhead). From ED13 onwards, DLK1 positivity 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 expresses DLK1 protein. Scale bar in A is applicable for B-E.
Results

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 5A), while the neighbouring mesonephros and gonad did not contain DLK1. By ED13, DLK1-positive cells became restricted to the inner, medullar part of the adrenal gland (Figure 5B). Later in development, on ED16 and -18, DLK1 protein was only found in medullar cells, which predominantly consists of chromaffin cells (Figure 5C,D). In the outer cortex DLK1 protein was not detected.

Figure 5 DLK1 protein expression in the developing adrenal gland
Panels A-D 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 positivity becomes restricted to central part of the adrenal gland (adrenal medulla; arrow in panel B). The developing kidney remains negative at all time points. Scale bar in A is applicable for B-D.

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 Figure 6A and B), whereas the epithelium of the more proximal, bifurcating parts of the bronchial tree were DLK1 negative. Moderate DLK1 positivity 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 6C), while expression was almost completely abolished in the surrounding mesenchyme. On ED18, only residual positivity in the epithelium of the alveoli was detected (inset in Figure 6D).
A comparable, spatio-temporal pattern of DLK1 protein expression was observed during pancreatic development. ED10 pancreas showed strong DLK1 protein expression in all cells (arrow in Figure 7A), with moderate positivity in the surrounding mesenchyme (arrowhead in Figure 7A). Like in embryonic lung, DLK1 positivity had become confined to the distal growing epithelium of the developing pancreas by ED13 (arrows in Figure 7B), with the surrounding mesenchyme still 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 7C) and had disappeared completely from the pancreas by ED18 (Figure 7D).

Figure 6 DLK1 protein expression in the developing lung
Panels A-D 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 panels 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 µm). ED18 lung shows only residual positivity in the epithelium of the alveoli (inset in D; bar: 10 µm). (a) alveolus. Scale bar in A is applicable for B-D.

Figure 7 DLK1 protein expression in the developing pancreas
Panels A-D show sections of ED10, -13, -16 and -18 pancreas, with on ED10 strong DLK1 positivity 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 positivity becomes virtually restricted to the pancreatic mesenchyme (inset in C; bar: 10 µm) and on ED18 the entire pancreas has become negative. (i) islet of Langerhans. Scale bar in A is applicable for B-D.
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 embryonic liver, lung, muscle, vertebrae, pancreas, pituitary - and adrenal gland(s), whereas expression is absent in heart, stomach, intestine, kidney, epidermis and remaining CNS. 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 pre-translational 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 immuno-stainings. 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 signaling during development of these organs.

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),( chapter 6), whereas the NOTCH1 receptor, which interacts with DLK1 (6, 8) has shown to stimulate pre- and postnatal bile-duct proliferation in vivo (29). Overexpression of the NICD of Notch in in-vitro differentiating DLK1-positive hepatoblasts resulted in downregulation of hepatocyte-marker genes (albumin, CPS, TAT) and, subsequently, in upregulation of cholangiocyte marker genes (CK7, CK19, HNF1β, integrin β4) (30). Notch signaling may, therefore, confer the capacity to differentiate along the cholangiocyte lineage upon hepatoblasts (30). The rapid downregulation of Dlk1 expression just prior to birth, which coincides with the peak in Notch1, Notch2 and Jagged1 expression, is also compatible with induction of hepatoblast maturation along the cholangiocyte lineage due to activation of NOTCH1/2 receptor signaling. In agreement, we recently showed that Notch2 is indispensable for cholangiocyte differentiation (Chapter 6).
DLK1 and Notch during adrenal gland differentiation

We observed DLK1 protein expression in a variety of endocrine tissues during embryonic development, among which 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 (31, 32), indicative of Notch signaling activity. Furthermore, in neuroblastoma (a pediatric tumor of the peripheral nervous system) -derived cell lines, an inverse relation between DLK1 and the NOTCH3 receptor expression was reported (11, 33). Therefore, it was suggested that overexpression of NOTCH3 in neuroblastoma cell lines corresponds with early precursor stages, whereas overexpression of DLK1 reflects differentiation arrest in a relatively late stage of the chromaffin lineage (11).

DLK1 and Notch during pituitary gland development

During pituitary development in the mouse, between ED11 and ED14 the first pituitary-specific cell types are formed: thyrotroph and corticotroph cells. Thereafter, around ED15 somatotroph, gonadotroph and lactotroph cells are formed with completion of cell specification and differentiation on ED17 (34). 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 (35), similar to DLK1 expression. Notch signaling deficiency resulted in a premature differentiation of the corticotropic lineage and inhibition of the somatotropic and gonadotropic lineages. Furthermore, sustained Notch signaling in somato-/thyro-/lactotropic precursors resulted in a reduction of the prevalence of the respective cell populations (35, 36). These findings show that Notch signaling prevents conversion of the late-arising cell lineages to early-born cell lineages and that attenuation of Notch signaling later in pituitary development is required for proper terminal differentiation of the lineages (35). 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 signaling activity. In agreement, adult pituitary of Dlk1 knockout mice, showed decreased numbers of growth-hormone immuno-reactive cells and reduced follicle stimulating hormone (FSH) and prolactin immuno-reactivity (37).
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 (38). Recently, it was shown that Notch signaling regulates branching morphogenesis in the developing lung (39). Disruption of Notch signaling 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 (39), whereas constitutive Notch signaling prevented the differentiation of alveolar epithelium, with distal cyst formation composed of cells showing upregulated markers of proximal airway epithelium (40). These observations suggest that during mammalian lung development, Notch signaling 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 (41). Analogous to lung development, disruption of Notch signaling during pancreatic development led to pancreatic hypoplasia caused by depletion of pancreatic epithelial precursors (42, 43), while constitutive overexpression of Notch signaling led to impaired branching of the pancreatic epithelium with formation of cyst-like structures, complete absence of exocrine development and repression of endocrine development (44). These findings also demonstrate a need for balanced Notch signaling 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.
Conclusion

We showed that DLK1 protein is expressed in many organs prior to and during terminal differentiation of the parenchymal cells and becomes abolished thereafter. DLK1 seems to be involved in developmental processes, such as branching morphogenesis (lung, pancreas) and terminal differentiation (muscle, liver, pituitary), with as common features among organs stimulation of growth and inhibition of differentiation. Based on its expression pattern during development and its effects upon experimental interventions, DLK1 appears to function as an inhibitory modulator of Notch signaling, either by competing with canonical ligands or by direct interaction with the NOTCH1 receptor, or both.


