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DLK1 and the Notch pathway in the liver

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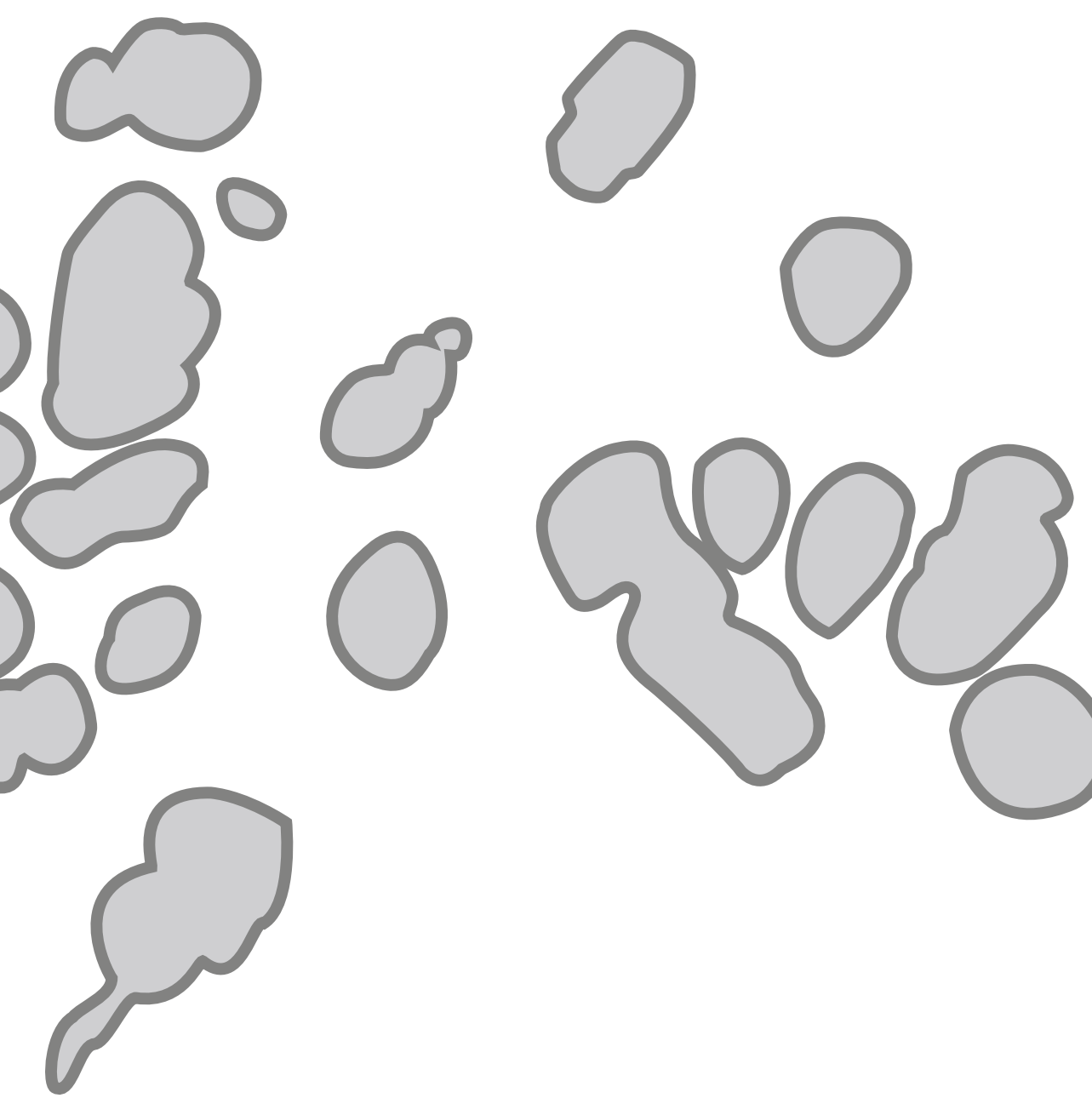
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INTRODUCTION PART I

CHAPTER 1

The Delta-Notch pathway

Notch receptors and ligands

The Delta-Notch pathway is an evolutionarily conserved signaling pathway that controls a broad range of developmental processes including cell-fate determination, terminal differentiation, proliferation and apoptotic events (1-3). Notch receptors and ligands are transmembrane proteins that belong to the Epidermal Growth Factor (EGF)-like family of proteins. After ligand binding, Notch receptors release their intracellular domain (NICD), which is cleaved by γ -secretase and then translocates to the nucleus to initiate signaling. The NICD interacts with the DNA-binding transcriptional repressor C-repeat/DRE Binding Factor 1 (CBF1) also known as Recombination Binding Protein for immunoglobulin kappa J region (RBP-J) and converts it into a transcriptional activator that induces transcription of target genes (Figure 1).

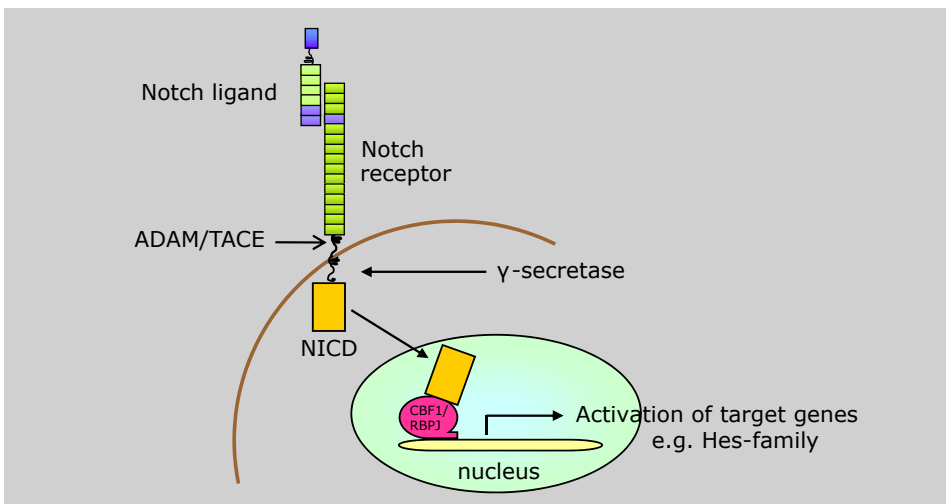


Figure 1 Notch intracellular signaling

Schematic representation of the Notch signaling cascade. Binding of the Notch ligand to the membrane bound Notch receptor leads to a sequence of proteolytic events resulting in cleavage of the Notch extracellular domain by ADAM17/TACE, followed by cleavage of the intracellular domain by γ -secretase (107). The Notch intracellular domain (NICD) then translocates to the nucleus and binds to the DNA binding transcriptional repressor CBF1/RBP-J. This binding converts CBF1 into a transcriptional activator which leads to transcription of target genes like the HES gene family.

The first *Notch* gene was cloned in 1983 and shown to encode a cell-surface receptor in *D. melanogaster* (4). Functional analysis revealed that Notch is important for cell fate decisions during development of *D. melanogaster* (5). Subsequently, two Notch genes (*glp-1* and *lin-12*) were identified in *C.elegans*, whereas four Notch homologs (*Notch1-4*) were identified in vertebrates (6), probably as a result of duplication events. Phylogenetic analysis of vertebrate Notch genes suggested that *Notch1a* and *Notch1b* (in fish) resulted from a duplication near the teleost/mammalian divergence (7). It was further shown that *Notch2* appeared in the first round of vertebrate duplication events and that vertebrate *Notch2* group is closely related to *Notch3* (6). *Notch4* is found only in mammals and is possibly the result of a rapid divergence from *Notch3* (7).

The Delta-Notch pathway

Five canonical mammalian Notch ligands have been described, namely JAGGED1, JAGGED2, Delta-like 1 (DLL1), DLL3 and DLL4. Canonical Notch ligands are characterized by three related structural motifs: an N-terminal Delta-Serrate-LAG-2 (DSL) domain (a cryptic EGF-like repeat), specialized tandem EGF-repeats called the DOS domain and a variable number of EGF-like repeats (Figure 2). Notch ligands can be further classified on the basis of the presence or absence of a cysteine-rich domain into the Jagged/Serrate or Delta-like group (Figure 2). Both the DSL and the DOS domains are involved in receptor binding (8, 9), but DLL3 and DLL4 are DSL-only ligands.

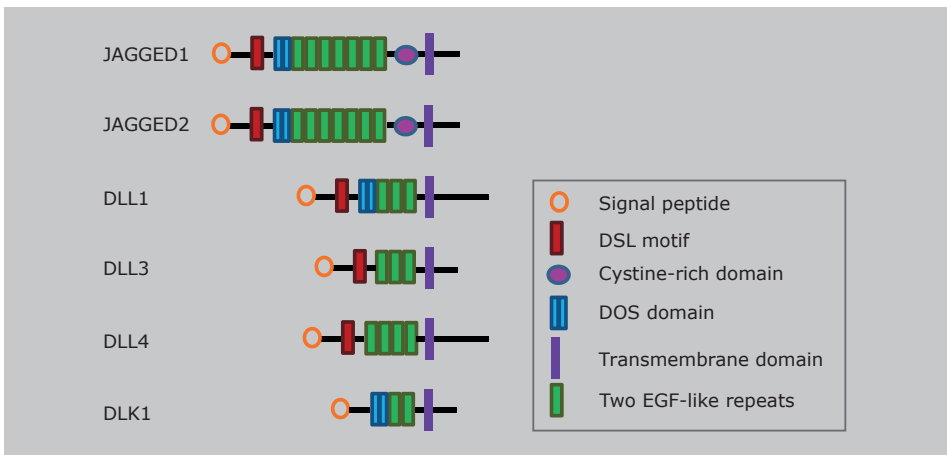


Figure 2 Notch Ligands

Schematic representation of DLK1 protein organization in relation to canonical Notch ligands (Adapted from Kopan *et al* 2009 (9)). Classical Notch ligands contain the DSL (Delta-Serrate-Lag-2), DOS (Delta and OSM-11-like proteins) and EFG (Epidermal growth factor) motifs. DLL3 and DLL4 are considered DSL-only ligands. DLK1 is considered a DOS co-ligand belonging to a subfamily of diffusible ligands. These ligands might act in combination with DSL-only ligands to activate Notch signaling (9).

In addition to the canonical ligands, noncanonical ligands can bind to Notch receptors. The function of noncanonical ligands is still poorly understood, but soluble noncanonical ligands may act as dominant-negative proteins that block Notch signaling (8, 9). Delta-like 1 homolog (DLK1) is the best studied noncanonical Notch ligand. It resembles DLL ligands, but misses the DSL domain (Figure 2) and was shown to inhibit NOTCH signaling as a DOS co-ligand (10, 11).

The Delta-Notch pathway

One of the most prominent features of canonical Delta-Notch signaling is that the ligand-receptor association occurs only between neighboring cells. This feature becomes accentuated in the process of "lateral inhibition", which occurs when two initially identical progenitor cells adopt different cell fates due to upregulation of the Delta ligand in one cell. This activates the Notch receptor on the neighboring cell, which in turn results in down regulation of *Delta* expression in that same cell, enhancing the divergence between the two cells (12, 13). These cells can then adopt alternative cell fates (Figure 3).

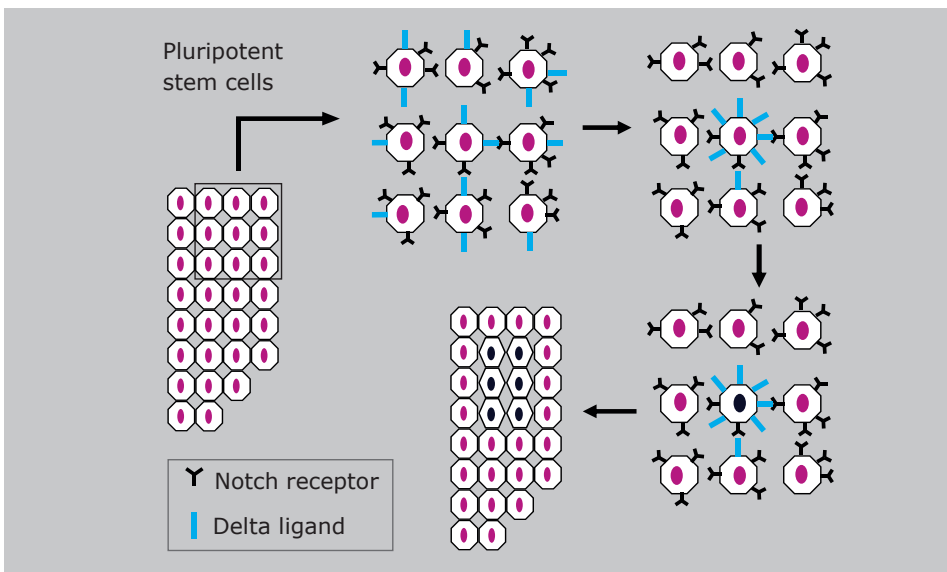


Figure 3 Lateral inhibition

Schematic representation of the process of lateral inhibition by which pluripotent stem cells adopt alternative cell fates. First, an instructive signal leads to upregulation of the Delta ligand in one cell. This leads to activation of Notch on the neighbouring cell, which in turn results in downregulation of Delta in that same cell, by which the first signal is enhanced. The divergence between the two cells can then lead to differentiation into alternative cell fates.

This process is depicted by the central cell which initially expresses two delta ligands and two notch receptors interacting with their right and lower neighbour cells, which express 1 and 2 delta ligands, respectively. After an instructive signal, Delta becomes upregulated in the central cell, depicted by 5 Delta ligands instead of 2, which then leads to downregulation of Delta ligands in its right and lower neighbour cell, which now express 0 and 1 Delta ligand.

The Delta-Notch pathway

Importance of Delta-Notch signaling during development: loss of function studies

Knockout studies for each of the mammalian Notch receptors and ligands have been conducted in mice. Table 1 provides an overview of the resulting phenotypes of these Notch pathway knockouts (14-24).

| Disrupted gene | Phenotype |
|-------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Notch1 (Swiatek 1994) | Embryonic lethal at ED 10, widespread cell death, disturbed somitogenesis |
| Notch2 (Hamada 1999) | Embryonic lethal at ED 11.5, widespread cell death, normal somitogenesis |
| Notch3 (Domenga 2004) | Viable and fertile, defects in postnatal maturation of vascular smooth muscle cells |
| Notch4 (Krebs 2000) | Viable and fertile; in combination with Notch1 mutant severe vascular defects |
| Jagged1 (Xue 1999) | Embryonic lethal at ED 10, defects in vascular remodelling of embryo and yolk sac |
| Jagged2 (Jiang 1998) | Perinatally lethal, craniofacial defects, skeletal defects, impaired thymic differentiation |
| DLL1 (Hrabe 1997) | Embryonically lethal at ED12, severe somite patterning defects, hyperplastic CNS |
| DLL3 (Dunwoodie 2002) | Viable with severe axial skeletal defects |
| DLL4 (Gale 2004) | Embryonic lethal from ED10.5, severe vascular remodelling defects in embryo and yolk sac |
| Dlk1 (Moon 2002, Raghunandan 2008, Waddell 2010, Puertas-Avendano 2011) | Increased perinatal lethality, growth retardation, rib deformations, increased adiposity Altered B-cell differentiation in spleen and bone marrow, exaggerated primary T-cell response, reduced skeletal muscle mass, disturbed pituitary cell type development |

Table 1 Phenotypes of mice with targeted disruption of Notch pathway genes

NOTCH1

Homozygous disruption of the *Notch1* gene is fatal around embryonic day (ED)10, indicating that *Notch1* is essential for normal embryonic development. Morphological and histological analysis of homozygous *Notch1*-deficient embryos showed normal pattern formation through the first nine days of gestation. However, histological analysis revealed widespread cell death after this stage, which was attributed to disorganized and delayed somitogenesis (23, 25). To explore the role of NOTCH1 later in development, inducible *Notch1* knockout were made. Mice, in which *Notch* expression was deleted neonatally, were transiently growth retarded, severely deficient in thymocyte development and developed nodular hyperplasia in the liver (26, 27). Inactivation of *Notch1* in mouse skin resulted in epidermal and corneal hyperplasia, followed by the development of skin tumors (28). Additionally, activating *NOTCH1* mutations are associated with human T-cell acute lymphoblastic leukemia (T-ALL) (29). These findings implicate that in the adult stage NOTCH1 is still involved in regulation of cell growth including both tumor suppressor and oncogenic functions.

The Delta-Notch pathway

NOTCH2

Homozygous *Notch2*-deficient embryos show developmental retardation, widespread cell death and embryonic lethality before ED11.5, but have, in contrast to *Notch1* knockouts, normal somitogenesis (17). Mice homozygous for a hypomorphic *Notch2* mutation show defects in development of the kidney, heart and eye vasculature (30). The human Alagille syndrome is associated with mutations in both *NOTCH2* and *JAGGED1*, and is characterized by growth retardation, jaundice due to impairment of intrahepatic bile duct formation and defective development of skeleton, heart, eyes and kidneys (31, 32). Mice doubly heterozygous for a hypomorphic *Notch2* allele and a *Jagged1* null allele exhibit developmental abnormalities that resemble the human Alagille syndrome. Heterozygous *Notch2* mutants show no abnormalities, while heterozygous *Jagged1*-deficient mice exhibit limited eye defects without the other characteristic features of Alagille's syndrome (33). Furthermore, mice with a perinatal, liver-specific complete elimination of *Notch2* (*Notch2^{fl/fl}/Alb-Cre^{tg/-}*) have a paucity of bile ducts and jaundice, demonstrating that Notch2 signaling is responsible for the liver phenotype in Alagille's syndrome (34). Recently, we investigated the effects of early embryonic elimination of *Notch2* in *Notch2^{fl/fl}/Alfp-Cre^{tg/-}* (*Notch2*-cKO) mice and showed that *Notch2* is indispensable for biliary differentiation in mice (Chapter 6). Neonatal *Notch2*-cKO mice were severely jaundiced with livers completely devoid of cytokeratin19 -positive ductal structures. mRNA levels of transcription factors involved in biliary development, including *Hnf6*, *Foxa1*, *Foxa2*, *Hhex*, *Hnf1 β* , *Cebpa* and *Sox9* were either permanently or transiently decreased in postnatal *Notch2*-cKO livers, indicating that during cholangiocyte differentiation, they lie downstream from *Notch2* (chapter 6). The above findings imply that mutations in both *NOTCH2* and *JAGGED1* determine the severity of the phenotype of Alagille's syndrome.

NOTCH3 and NOTCH4

Notch3-null mice are viable and fertile without any apparent phenotypic abnormalities. However, adult *Notch3* knockout mice show obvious arterial defects due to abnormalities in differentiation and maturation of vascular smooth-muscle cells (14, 20). In agreement with a role for *Notch3* in vascular development, mutations in the EGF-repeats of the *NOTCH3* gene in humans cause the cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) syndrome, leading to early stroke and dementia (35). Similar to *Notch3*, *Notch4*-null mice are viable and fertile (20). Involvement of *Notch4* in vascular development is likely because its expression during embryonic development is restricted to vascular endothelial cells (20). Furthermore, *Notch1/Notch4* double knockouts show a more severe phenotype than *Notch1* knockouts, with extensive defects in angiogenic vascular remodeling that affect the embryo, yolk sac and placenta at ED9.5 (20). The aggravation of the phenotype of *Notch1* deficiency by *Notch4* deficiency suggests a partial functional redundancy of *Notch4* for *Notch1*.

The Delta-Notch pathway

Notch canonical ligand knockouts

Homozygous disruption of the Notch ligands results in severe developmental defects. *Jagged1*-null mice exhibit defects in vascular remodelling of the embryo and yolk sac and die at ED10 from extensive hemorrhage (24). Mice homozygous for a *Jagged2* deletion die perinatally because of defects in craniofacial- and limb morphogenesis with cleft palate, fusion of the tongue with the palatal shelves, syndactyly (digit fusions) of the fore and hind limbs, and defective thymus development (19). Homozygous inactivation of *Dll1* causes severe defects in somite patterning and a hyperplastic CNS. *Dll1*-deficient mice become hemorrhagic after ED10 and die around ED12 (18). This implies that *Dll1* expression is a prerequisite for Notch receptors to function during somitogenesis and CNS development. In addition to DLL1, DLL3 is also involved in somitogenesis, but *Dll3*-null mice are viable despite severe axial skeletal defects, which probably result from delayed and irregular somite formation (8, 15). In agreement, mutations in the human *DLL3* gene are associated with spondylocostal dysplasia, that is, with similar vertebrocostal defects as seen in *Dll3*-deficient mice (36). DLL3 differs structurally from the other canonical DSL ligands (Figure 2) and is considered a Notch-signaling antagonist (8). In agreement, *Dll3* expression in the presomitic mesoderm is unable to rescue the *Dll1*-deficient somite phenotype in mouse embryos (8). *Dll4* deficiency causes severe vascular remodeling defects and embryonic lethality even in the heterozygous condition. The phenotype of *Dll4*^{Lz/+} mice is reminiscent of that reported for the homozygous *Notch1/Notch4* double knockout, suggesting that DLL4 is a major physiologic ligand for these receptors and initiates their signaling during vascular development (16). Interestingly, mice lacking *Jagged1* also exhibit a similar phenotype, which suggests an overlapping functional capacity for JAGGED1 and DLL4.

The comparison of the phenotype of Notch receptor- and Notch-ligand knockouts does not reveal extensively overlapping phenotypes, apart from the described DLL4 and NOTCH1/4 vascular phenotypes. In the case of fixed ligand-receptor-pairs, one would expect that the deficiency of either the ligand or the receptor to cause a similar rather than a different phenotype. The existence of non-overlapping phenotypes is, on the other hand, also compatible with particular ligand-receptor pairs, which, upon modification, affect a specific phenotype, but not another. Possibly, therefore, both conditions are met if only a limited number of permutations of ligands per receptor or vice versa are functional. Alternatively, the interactions between receptors and ligands could become unique by having additive effects (the *Notch1/Notch4* double knockout has the same phenotype as the *Dll4* knockout). The relatively mild phenotype of *Notch3* and *Notch4* knockout mice suggests functional redundancy with NOTCH1 and/or NOTCH2, but not vice versa.

DLK1, a noncanonical Notch ligand

Protein structure

Both the human *DLK1* and the murine *Dlk1* genes are maternally imprinted, paternally expressed genes on chromosome 14 and 12 in man and mouse, respectively (37). Delta-like 1 homolog (DLK1), also known as Preadipocyte factor 1 (Pref-1) and Fetal antigen (FA1) (38, 39), is an EGF-like membrane-bound protein. It contains six tandem EGF-like repeats, a juxtamembrane region with a TACE (ADAM17)-mediated cleavage site, a transmembrane domain, and a short intracellular tail (40). TACE-mediated cleavage yields a soluble form of DLK1 with a molecular weight of 50 kDa (40). Alternative DLK1 splicing products have been described in several mammalian species (41-43), which mostly result from in-frame deletions of the juxtamembrane region and the sixth EGF repeat, resulting in membrane bound forms that sometimes lack the TACE-sensitive cleavage site. The biological activity of these splicing variants is yet not fully understood.

The structure and amino-acid sequence of the EGF repeats in DLK1 are closely related to those present in the canonical DLL ligands. However, DLK1 misses the conserved cryptic EGF repeat that is called the DSL domain, which is present at the N-terminus of all canonical Notch ligands (Figure 1). For this reason, DLK1 is considered a DOS (co)ligand (8, 9, 44). Despite of the absence of a DSL domain, interaction between DLK1 and the NOTCH1 receptor was shown in the yeast GAL4 two-hybrid system. In this model system, pairs 10/11 and 12/13 of the NOTCH1 EGF-like repeats interacted with DLK1 EGF repeats 1, 2, 5 and 6. (10). NOTCH1 EGF-like repeats 11 and 12 are those reported to interact with the DOS domain of canonical ligands (45-48). DLK1 behaved as a negative regulator of NOTCH1 signaling in mesenchymal cell lines (10, 11). Furthermore, overexpression of murine *Dlk1* in *Drosophila* altered the cellular distribution of *Notch* and inhibited the expression of *Notch* target genes (49). Recently, a new protein, highly homologous to DLK1, named DLK2, has been discovered that also interacts with the NOTCH1 receptor and inhibits Notch signaling (50, 51). The inhibitory effect of DLK1 and DLK2 on Notch signaling may be mediated by competition of with canonical ligands of the DSL type for the binding site on the Notch receptor (11, 52). DLK1 was also shown to be involved in other signaling pathways, such as the ERK/MAPK pathway via binding to fibronectin (53) and the FGF-signaling pathway by interacting with the FGF-binding molecule Cfr (54). In this review we will focus on its possible roles in the Notch pathway.

DLK1, a noncanonical Notch ligand

DLK1 expression during development

Dlk1 expression is widely distributed during mouse embryonic development, with high expression in placenta, liver, adipose tissue, skeletal muscle, lung, vertebrae, and the pituitary- and adrenal gland(s) (38, 39, 55, 56). In the adult, in contrast, expression becomes restricted to (neuro)endocrine tissues like the pituitary gland, adrenal glands, pancreas, monoaminergic neurons in the central nervous system, testes, prostate and ovaries (38, 55, 57-62). The reported expression pattern, together with its involvement in the Notch pathway, suggests an important role for DLK1 during the maturation of several tissues. However, *Dlk*- null mice display a relatively mild phenotype, with increased perinatal lethality and growth retardation accompanied by accelerated adiposity and developmental defects in the eyelids, ribs and lungs, (21) as well as alterations in B-cell differentiation (22), and pituitary cell type development (63). Furthermore, muscle-specific *Dlk1*-deletion resulted in disturbed muscle development and regeneration(64).

Role of DLK1 and Notch in different cellular systems

Despite of the reported inhibitory action of DLK1 on Notch signaling *in vitro* (10, 11, 49), DLK1 involvement in Notch signaling during development remains poorly understood. DLK1 is expressed in many embryonic tissues, in which active Notch signaling was also reported (65-72). Postnatally, DLK1 expression has disappeared from most of these tissue, but is associated with pediatric malignancies, such as hepatoblastoma, neuroblastoma and nephroblastoma (73-76), and some adult malignancies, such as myelodysplastic syndrome, pituitary tumors, breast, colon and prostate carcinoma (42, 57, 77-79). DLK1 expression in comparison with the Notch pathway will be discussed below in adipogenesis, placental and muscle development, as well as its presence in pediatric carcinogenesis. Its involvement in liver, lung, pancreas, pituitary - and adrenal gland will be discussed in the next chapters.

Adipogenesis

The best established function for DLK1/Pref-1 is that of an inhibitor of adipogenesis, as it prevents the differentiation of preadipocytes into mature adipocytes (39, 40, 80, 81). DLK1 is highly expressed in murine preadipocytes, whereas its expression has become completely abolished in mature adipocytes. The 3T3L1 cell line is a frequently used murine preadipocyte cell line to study the mechanism of adipocyte differentiation after hormonal induction (39, 82). When 3T3L1 cells are induced to differentiate into mature adipocytes, constitutive overexpression of soluble DLK1 prevents adipogenic differentiation by inhibition of the expression of the key transcriptional regulators of adipogenesis, *Cebpa* and *Ppar γ* (39, 80). Similarly, a decrease in body mass due to decreased weight of all adipose tissue pads, including brown fat, is seen in transgenic mice with adipocyte-specific overexpression of *Dlk1*. Conversely, mice lacking *Dlk1* display accelerated adiposity in adulthood and enlarged, fatty livers with increased expression of lipogenic enzymes *Fas* and *Scd1*(21).

It is well possible that soluble and membrane-bound DLK1 differ in function, as membrane-bound DLK1 is required for adipogenesis in the 3T3L1 cell line (83). Furthermore, overexpression of both soluble and membrane-bound DLK1 significantly enhances adipogenic differentiation in the mesenchymal stem cell line C3H10T1/2 (11). Additionally, we recently showed that liver-specific overexpression of *Dlk1* resulted in a sex- and diet-dependent increase of Notch signaling, accompanied by an increase adipogenic (*Cebpa* and *Ppar γ*) and lipogenic genes (*Fas* and *Scd*) in liver and adipose tissue. *Dlk1*-overexpressing female mice on a high-fat diet were most sensitive (Chapter 4).

Role of DLK1 and Notch in different cellular systems

The precise role of the Notch pathway in adipogenesis remains controversial with reportedly both stimulatory and inhibitory roles for *Notch1* and *Hes1* during adipocyte differentiation (10, 11, 66, 84, 85). The effect of DLK1 on the Notch pathway during adipogenesis has been studied *in vitro* in cell lines with (3T3L1 mouse preadipocyte cell line) or without (Balb/c14 mouse fibroblast cell line) endogenous DLK1 expression (10). Increased *Dlk1* expression correlated with a decrease in *Notch1* expression and a concomitant decrease in levels of downstream target *Hes1* in both cell lines, and resulted in inhibition of adipogenesis in 3T3L1 cells (10). Furthermore, in the mesenchymal stem cell line C3H10T1/2, DLK1 overexpression also resulted in Notch signaling inhibition (11). These findings support the hypothesis that DLK1 acts as a negative regulator of Notch signaling. Interestingly, constitutive *Notch1* expression leading to increased *Hes1* mRNA levels resulted in a decrease of *Dlk1* mRNA levels and also prevented adipocyte differentiation in the 3T3L1 cell line (84). Additionally, inhibition of *Notch1* expression prevented the potentiating effects of DLK1 on adipogenesis in C3H10T1/2 cells (11). It was, therefore, proposed that “a proper balance of Notch signaling is critical for adipogenesis to proceed” and that “DLK1 might be a critical factor to control the proper level of Notch signaling for cells to undergo adipogenesis” (10). Collectively, these findings indicate that DLK1 is not only an inhibitor of adipogenesis, but that its role in adipogenesis is dependent on the biological context.

DLK1 and Notch during placental development

The mammalian placenta consists of the maternally derived decidua and fetally derived trophoblast, both with their separate vasculature. (86). In placenta of mice and human, DLK1 is only expressed in the endothelial cells of the fetal vasculature in the umbilical cord and in the mesenchymal fibroblasts of the chorionic villi, respectively, while all other placental derivatives are DLK1-negative (38, 55). Furthermore, soluble human DLK1 (FA1) can be detected in serum during pregnancy, with highest levels from gestational week 20 till 37 and twin pregnancies show significantly higher FA1 levels compared to singleton pregnancies (38). In agreement, maternal serum FA1 levels positively correlated to the number of fetuses in mice (87), suggesting that soluble DLK1 in maternal serum is produced by the fetus.

The canonical Notch pathway members are involved in all stages of preimplantation development (67). In mouse placenta, mutations in *Notch1/Notch4*, *Dll4* and *Rbpj/Cbf1* result in an early block in chorio-allantoic fusion or branching (67). Despite of the lack of disturbed placentation in *Dlk1* knockout mice (21), the uniparental disomy 12 (UDP12) mutant mouse model implies *Dlk1* involvement in placental development. In these embryos both copies of chromosome 12 are derived from either the father (pUDP12) or the mother (mUPD12), which results in loss or overexpression of imprinted genes (88).

Role of DLK1 and Notch in different cellular systems

The mutant embryos show over- and undergrowth of the placenta, respectively, and die during gestation. In pUDP12 where the maternal imprinting is lost, embryos show defects in the fetal vasculature of the placenta and increased *Dlk1* levels (89). These findings suggest that the observed placental defects are at least partly due to overexpression of *Dlk1*, however, whether its role during placental development is exerted via signaling through the Notch pathway, remains to be elucidated.

DLK1 and Notch during muscle development

The callipyge (CLPG) phenotype is an inherited skeletal muscle hypertrophy of sheep. The CLPG mutation occurs in a highly conserved motif between the imprinted *Dlk1* and noncoding *Gtl2* genes (90). This mutation causes abnormally high postnatal *Dlk1* expression in affected muscles, without altering its imprinted status (91). Normally, *Dlk1* expression in muscle is rapidly downregulated after birth in both sheep and mice (90, 91) (*chapter 2*), and becomes re-expressed during muscle injury and chronic myopathies (64, 92). Transgenic mice expressing ovine *Dlk1* under control of the murine myosin light chain 3F promoter have high *Dlk1* expression in type myosin heavy-chain type IIB (MYH4) muscle fibers throughout pre- and postnatal development. Compared to controls, these mice also show increased relative muscle mass and average fiber diameter in both the foreleg and hind-leg muscles (90). Deletion of *Dlk1* in the myogenic lineage resulted, on the other hand, in reduced skeletal muscle mass due to a reduction in the number of myofibers and *Myh4* gene expression and also impaired muscle regeneration. *Dlk1* knockout inhibited the expression of the muscle-determining transcription factor *MyoD*, and facilitated the self-renewal of activated satellite cells. Conversely, *Dlk1* over-expression inhibited the proliferation and enhanced differentiation of cultured myoblasts (64). These findings show that DLK1 participates in the regulation of muscle fiber growth during development and that postnatally persisting *Dlk1* expression in skeletal muscle contributes directly to the muscular hypertrophy observed in CLPG sheep. Notch signaling inhibits myogenic differentiation by suppression of *MyoD* expression, which is critical for the proper expansion of muscle progenitors during development (70, 72, 93, 94). Mice carrying either a hypomorphic allele of the Notch ligand *Dll1* or a myocyte-specific deletion of the Notch downstream transcription factor *Cbf1* both display severe muscle hypotrophy due to uncontrolled premature differentiation of the muscle progenitor cell pool, with increased expression of myogenic regulatory factors *MyoD* and *Myogenin* and a reduced number of muscle progenitor cells (70, 94). Comparison of the DLK1 and Notch muscle phenotypes shows that DLK1 and Notch have opposite effects on myogenesis, which is compatible with the putatively inhibitory effect of DLK1 on Notch signaling.

DLK1 and Notch in pediatric malignancies

Pediatric tumors like neuroblastoma, hepatoblastoma and nephroblastoma (Wilms tumor) are believed to arise from cellular populations that have not completed the process of differentiation. Signal-transduction pathways involved in embryonic development, like the Wnt/beta-catenin pathway, are frequently upregulated in these tumors (95, 96). Recently, both DLK1 and the Notch pathway have also been associated with pediatric malignancies (73, 75, 76, 97-99).

Neuroblastoma

Neuroblastoma, an embryonic tumor originating from immature sympathetic neuroblast, displays a remarkable spectrum of clinical and biological behavior, ranging from spontaneous regression of metastases to rapid and fatal progression despite intensive therapy (74). High expression of *DLK1* and the *NOTCH3* receptor was reported in subsets of neuroblastoma tumors and cell lines (98). *DLK1* expression correlated perfectly with dopamine β -hydroxylase (*DBH*) expression, an enzyme which is normally highly expressed in the chromaffin cells of the adrenal medulla and converts dopamine to noradrenaline (98). During early embryonic development, *DLK1* expression is detected throughout the adrenal gland, while later during development expression becomes restricted to the chromaffin cells, one of the few cell types that maintains postnatal *DLK1* expression (55, 58). Interestingly, the reported *DLK1* expression in neuroblastoma cell lines was inversely correlated to *NOTCH3* expression (98). 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 (98).

Hepatoblastoma

Hepatoblastoma, a malignant pediatric liver tumor, is believed to derive from hepatoblasts, because of the stem-cell like appearance of the hepatoblastoma cells (100-102). Hepatoblastomas are characterized by a diversity of epithelial and often mesenchymal patterns of differentiation, with some epithelial variants that morphologically resemble embryonic or fetal hepatocytes (103, 104). Recently, increased expression of DLK1 was found to be a consistent feature among hepatoblastomas (75, 76, 95, 97). *DLK1* was significantly elevated in all histological subtypes when compared to normal liver, sometimes even higher than in fetal liver (75). We recently showed that serum DLK1 levels were significantly elevated in hepatoblastoma patients compared to age-matched controls, even in the youngest patients, in whom serum α -fetoprotein levels are often in the same range as the still elevated control levels (*chapter 3*). These findings make DLK1 a candidate serum marker to diagnose hepatoblastoma in the young infant age group. *NOTCH2* receptor expression was increased in 92% of hepatoblastomas compared to normal liver tissue. *HES1*, the best studied Notch downstream target, was also elevated in hepatoblastomas, especially in the pure fetal subtype (75, 105). These findings indicate that active Notch signaling occurs in hepatoblastoma tumors and might regulate tumor growth. The abrupt disappearance of DLK1 expression in late liver development, together with its re-appearance in hepatoblastoma, imply a role for

DLK1 and Notch in pediatric malignancies

DLK1 in hepatoblastoma pathogenesis. However, we showed recently that transgenic mice with hepatocyte-specific overexpression of DLK1 do not develop liver tumors up to 1.5 years of age (*chapter 4*). These findings imply that increased Notch signaling, probably via the NOTCH2 receptor, is more likely to be involved in the pathogenesis of hepatoblastoma.

Wilms tumor

Nephroblastoma is a pediatric tumor of the kidney, also known as Wilms tumor. Loss of imprinting (LOI) of the reciprocally imprinted *H19/IGF2* domain is a common feature of Wilms tumor, where *H19* is a non-coding gene and *IGF2* an important regulator of fetal growth (73, 106). The *DLK1* gene is similarly arranged by formation of an imprinted domain with a noncoding gene called *GTL2* (73). *DLK1* expression is absent in developing kidney, but interestingly, high *DLK1* expression was detected in 11 out of 30 Wilms tumors with prominent myogenic differentiation and blastemal components. The imprinting status of the *DLK1/GTL2* domain was shown to be retained (73). Since DLK1 is associated with muscular growth and development (90), *DLK1* expression in Wilms tumor may only reflect the presence of myogenic differentiation in a significant proportion of the tumor cells (see section 3.2).

Aim and outline of the thesis

The aim of the research described in this thesis was to characterize the Notch pathway in mouse liver, specifically the roles of DLK1 and Notch2. Furthermore, we wanted to investigate the value of DLK1 as a serum marker for hepatoblastoma.

Chapter 2 describes the DLK1 expression pattern during embryonic development and its relation to the Notch pathway. The usefulness of DLK1 as a novel serum marker for the pediatric liver tumor hepatoblastoma is discussed in chapter 3. The effects of liver-specific overexpression of DLK1 are described in chapter 4. Chapter 5 provides an introduction into biliary development and involved genes/transcription factors including the Notch pathway. The indispensable role of *Notch2* in biliary differentiation is described in Chapter 6. The inhibitory effects of *Dlk1* overexpression on bile duct proliferation are described in chapter 7. Chapter 8 provides a summary of the results.

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