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The imprinted gene Delta like non-canonical Notch ligand 1 (Dlk1) is conserved in mammals, and serves a growth modulatory role during tissue development and regeneration through Notch dependent and independent mechanisms

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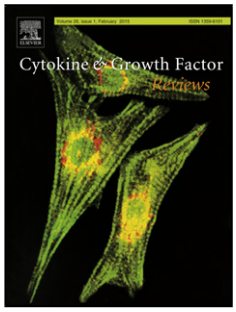

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Title

The imprinted gene Delta like non-canonical Notch ligand 1 (Dlk1) is conserved in mammals, and serves a growth modulatory role during tissue development and regeneration through Notch dependent and independent mechanisms

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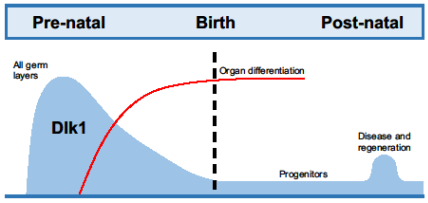
Graphical abstract



Highlights

- Dlk1 expression patterns are similar in man and mouse, making the mouse an appropriate model to study Dlk1
- Dlk1 seems to inhibit both cell proliferation and differentiation but the level of different isoforms may be critical for the overall outcome
- Dlk1 mechanism of action depends on both Notch dependent and independent pathways

Abstract

Delta like non-canonical Notch ligand 1 (Dlk1) is an imprinted gene, mainly known for its involvement in adipogenesis, although it has been associated with many other stem cells/progenitors and is known to be widely expressed during organism development and tissue

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regeneration. In a systematic manner, we have outlined the overall expression pattern of Dlk1 in both man and mouse, and found Dlk1 to be expressed in tissues from all three germ layers. Yet, Dlk1 expression decreases along with increased differentiation as gestation proceeds and in most tissues Dlk1 is absent around birth. Thus, in adults, expression of Dlk1 is restricted to a few tissues and progenitor cells, but is re-expressed during disease and regeneration. Although diffferences exist, we found an overall conservation of Dlk1 expression between mouse and man, and conclude in that sense that the mouse is an appropiate model to study Dlk1. In agreement with the observed Dlk1 expression pattern, we found that the majority of published Dlk1 studies, report Dlk1 to have an inhibitory effect on both cell proliferation and differentiation, but the levels of the different DLK1 isoforms may be critical and have an impact on the overall outcome. This may also be an issue during tissue regeneration where several studies have reported Dlk1's impact during skeletal muscle and liver regeneration without establishing the exact role. Likewise, the underlying mechanism of Dlk1 action is unknown, and seems to depend on both Notch dependent and independent pathways. However, from our data it is intriguing to speculate that the actual role of DLK1 may be to function as a checkpoint to slow down proliferation while forcing cells into the process of differentiation, and thus switch the cell/organ to a state of growth and hypertrophy. This may fit well with its reported impact on growth restiction and body size. Thus, our study which for the first time summarizes reported knowledge on Dlk1 in tissue development and regeneration as well as on the Dlk1 mechanism may provide novel insight to the general role of this remarkable imprinted gene in controlling cell growth, from which new hypotheses can be made in the field of stem cell biology and regenerative medicine.

Keywords

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Mammalian development, Regenerative medicine, Stem cell biology, Differentiation, Proliferation, Delta like non-canonical Notch ligand 1

Introduction

The Delta like non-canonical Notch ligand 1 gene, until recently known as Delta-like 1 homolog (*Dlk1* in rodents and *DLK1* in human) maps to a cluster of imprinted genes, called the *Dlk1-Dio3* gene cluster located on mouse chromosome 12 and human chromosome 14, respectively [1, 2]. The *DLK1/Dlk1* gene, which is expressed from the paternally inherited chromosome [3, 4], encodes a transmembrane glycoprotein, with epidermal growth factor (EGF)-like motifs in its extracellular domain, a juxtamembrane region with a ADAM17/(TNFα converting enzyme (TACE))-mediated cleavage site, a single transmembrane domain and a short cytoplasmic tail [5]. Alternately spliced isoforms of DLK1 are generated, some lacking the protease recognition site in the juxtamembrane region remaining tethered to the membrane, whereas isoforms containing an intact protease recognition site are proteolytically cleaved, generating a large soluble isoform that is released into circulation [6, 7]. In humans only one membrane-tethered and one cleavable isoform is present, whereas in the mouse, two isoforms encompassing the protease recognition site (A and B isoforms) and four isoforms lacking this site (C, C2, D and D2 isoforms) have been identified [8, 9]

Although more than a thousand studies on *DLK1/Dlk1* gene expression have been published so far, the exact function of DLK1 is still uncertain, but it is rigorously considered as a preadipocyte factor [7] with an impact on adipogenesis and metabolism [5, 10-14]. By reviewing the literature, one will however notice a substantial number of papers suggesting that DLK1 also plays an important role in preserving the pool of many types of progenitor cells until it is appropriate for them to differentiate. Stem and progenitor cells are characterized by the

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capacity for proliferation and subsequent differentiation whereby tissues are formed during development, and maintained and repaired upon injury/disease in adulthood [15, 16]. In 2000, Laborda indeed summarized a more general role of DLK1 in cell differentiation [17], and in 2012 Falix et al. extended this by reviewing the impact of DLK1 as a non-canonical Notch ligand involved in tissue development [18]. Yet, in recent years many more studies have demonstrated *DLK1/Dlk1* gene expression and roles at discrete time points during development in large range of tissues, and also unravelled that *DLK1/Dlk1* is transiently re-expressed in damaged adult tissues and here impacts regeneration. This pattern of gene expression is indeed often observed since adult tissue regeneration in many instances is a recapitulation of developmental programs. Thus, to better understand the role of DLK1 in development and regeneration, we here set out to summarize *DLK1/Dlk1* gene expression patterns, functions, and mechanisms from existing studies to enable a more complete atlas of its expression in mammals and to suggest a general functional repertoire of Dlk1 during development and regeneration. In perspective, this may help clarify if Dlk1 remains a candidate to target in regenerative medicine.

Spatial and temporal expression of the *DLK1* gene in humans

Overall, expression of the *DLK1* gene is observed between gestational age (GA) 5 to 25 during human embryonic and fetal development (Table 1).

Extra embryonic tissues

In the extra embryonic tissue published data shows that DLK1 is present as early as GA 5 and remains there until birth [19-22] (Table 1). During this period DLK1 is found within the endodermal cells of the yolk sack [19] and mesenchymal fibroblasts in the placenta [20]. However, we have unpublished data that DLK1 is present already in the fluid of the follicle. Another recent study reports expression of DLK1 in placental endothelium and in the

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syncytiotrophoblast, a placental progenitor [22]. The latter however diasagrees with a previous study showing absence of DLK1 in all the epithelial trophoblasts [19]. Also at the mRNA level *DLK1* is seen in the placenta at the time of birth, where levels appear significantly lower in placental tissue from children that are small for gestational age compared to normal-sized children [21]. In agreement, increased placental expression of *DLK1* is associated with increased birth weight [23]. On the other hand, low maternal levels of circulating DLK1 have been associated with pregnancy complications [22]. Unrestricted somatic stem cells in the umbilical cord blood express *DLK1* mRNA whereas low or no expression resided within the mesenchymal stromal cell population of the same compartment [24]. More clear is it that the soluble form of DLK1 is present at high concentrations in the amniotic fluid during second and third trimester [20] and in the fetal circulation [20, 25] .

Endodermal tissues

Among the endodermally derived structures, DLK1 is mostly expressed in the first and second trimester, except from pancreas and prostate, where its presence has also been documented in adults (Table 1). During pancreatic development DLK1 is intensely expressed at GA 5 [19] and 94% of the glandular epithelial cells are DLK1+ at GA 7 [26], but the number of DLK1 positive cells decreases as pancreatic development progress [19, 26, 27]. In infants, DLK1 still resides in 11% of the glandular cells, but in the adult pancreas DLK1 is restricted to a subpopulation of β-cells within the islets of Langerhans [26, 28]. By contrast, DLK1 appears only weakly expressed in the endocrine cells of the pancreas at GA 7-14. Thus, while DLK1 expression decreases in the glandular cells with development, it increases in the β-cells towards the end of the pregnancy. Thus, whereas glucagon, somatostatin and pancreatic polypeptide immunoreactive cells are DLK1 negative, both during development and in the adult, insulin and DLK1 are co-expressed in adult pancreas [26, 29]. Besides the adult pancreas, adult prostate

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expresses DLK1 in the epithelial cell layers especially in the prostate lumen [30], where DLK1 recently was identified as a marker for multipotent prostate basal stem cells [31]. In the liver, DLK1 has been found in embryonic and fetal hepatocytes from GA 5-17, but the expression decreases at GA 15-17 [19, 26], and is absent in infant and adult liver [26]. In the lung, the opposite picture is seen, where early embryonic and fetal lung tissues of the pseudoglandular phase (GA 7-16) lack DLK1 expression, whereas fetal lung acini at GA 17 is DLK1 positive [19], but adult lung is negative as all other adult endodermal derived structures except for pancreas and prostate as stated above.

Mesodermal tissues

DLK1 gene expression is widely studied in major mesodermal structures such as bone and skeletal muscle. In embryonic bone *DLK1* mRNA appears at GA 6, but expression increases substantially at GA 13 [32], where after it declines towards GA 36, and in the adult bone *DLK1* mRNA levels are only 10% of the amount seen at GA 13 [32](Table 1). In agreement, mesenchymal condensation of the future skeletal system stains positive for DLK1 (GA 5 and 6), but negative when calcification of the matrix is established after GA 11, and only a small number of proliferative chondrocytes in the epiphyseal discs remain DLK1 positive [19]. In the adult bone marrow *DLK1* mRNA expression is detected by PCR, but with a clear inter-donor variation [32]. Previous studies have shown, that DLK1 is largely absent in the human *heart* [19], but a very recent study suggests that DLK1 is expressed in adult cardiomyocytes and fibroblasts [33]. This may however be debatable as we have unpublished data demonstrating that DLK1 is only expressed in cells residing in the adult peri-/epicardium, and not within the myocardium itself. *Skeletal muscle* on the other hand expresses DLK1 in the classical muscle progenitor/stem cell, the satellite cell during development and upon disease/exercise in adults [34-37]. One study recovered 25% more DLK1positive satellite cells in the healthy untrained

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versus trained individuals [35]. In the fetus, DLK1 also resides in the developing multinucleated myotubes (GA 8-23), suggesting a role in myogenesis [34]. Yet, all fully differentiated muscle (skeletal, cardiac and smooth) are DLK1 negative [19]. As with skeletal muscle and bone, also *adipose tissue* is intensely investigated in relation to *DLK1 gene expression*. A decade ago, DLK1 was suggested to be a marker of *in vivo* preadipocytes [38]. Freshly isolated visceral white adipose tissue (v)WAT has a significant lower number of DLK1 positive cells than subcutaneous (s)WAT [39]. A subpopulation of progenitor cells surrounding the small vessels in WAT called adipose-derived stromal cells are also DLK1 positive [39-41] and predicted to give rise to vascular epithelium [39]. Still it remains unclear whether DLK1 in fat primarily exerts its function in blood vessel formation, adipocyte differentiation or both. The developing *kidney* and the cortex of the *adrenal glands* also express DLK1 [19, 42, 43]. Distinctively, DLK1 is expressed in differentiated epithelial cells of the proximal tubules during development (GA 7-25) [19, 42] and likewise *DLK1* mRNA is detected in proximal tubules in the adult [43]. In contrast, no or weak DLK1 expression is seen within progenitor structures of the nephrogenic zone [42], distal tubules and collecting ducts of the kidney [19]. In the adrenal gland, DLK1 is found in all cortical cells during GA 1-10. But after GA 14, DLK1 is limited to the membrane of peripheral cortical cells whereas the adrenal medulla and primitive neuroblasts are DLK1 negative. In the adult adrenal gland, cells in the zona glomerulosa stain positive for DLK1, but the intensity of the staining decreases towards the medulla, which is mainly DLK1 negative [44]. Finally for mesoderm, DLK1 is expressed within the developing and adult *reproductive system*, where interstitial Leydig (testis) cells, theca interna cells (ovary), as well as the hilus of ovary express DLK1 [19, 45]. DLK1 is also present in the corpus luteum and the luteinized cell of premenopausal ovary, but is restricted to cells containing lipid vesicles in the postmenopausal ovaries [45].

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Ectodermal tissues

Among the ectodermal derived structures, DLK1 is mainly described in the *central nervous system*. Accordingly, DLK1 expression is reported in the anlages of the neuro- and adenohypophysis and in the floor of the 3rd ventricle in pseudostratified and marginal cells in the 6 week old embryo [19]. However, cells from the fetal brain cortex, neural crest, spinal cord and peripheral ganglions were DLK1 negative from week 8 and onwards [19]. In the adult brain stem, DLK1 is found in neurons localized in a section of the mesencephalon [46], whereas in pons, DLK1 resides in neurons in the locus coeruleus and raphe nuclei [46]. Lastly, DLK1 is present in the adult pituitary [47, 48].

***Dlk1* gene expression is conserved in mouse**

Largely, the expression of mouse *Dlk1* is similar, especially in endodermal and mesodermal derived tissues (Table 2), to that reported for humans (Table 1), and thus widely expressed in prenatal development between embryonic day (E) 10.5 and E16.5, but not before E8.0 [49]. This is in agreement with our unpublished data showing that mouse embryonic stem cells in the hanging drop state lack DLK1 expression, whereas ES cells differentiated towards mesoderm start expressing DLK1 at day 7. In adulthood, brain, pituitary, pancreas, adrenals, testes, and lung endothelium have been reported to express *Dlk1*/DLK1 [5, 50-55]. Important diverging results include among others, the presence of DLK1 in a subpopulation of β-cells in humans, a scenario not recapitulated in mice, although it has been observed in rats [56, 57]. In particular, a recent paper demonstrated that , while human DLK1 is specifically expressed in the insulin-positive cells of the islets of Langerhans, in the mouse DLK1 is expressed in glucagon-positive cells [28]. Another issue is the lack of details on *Dlk1* gene expression in the mouse gonads whereas it is well characterized in humans [19, 45]. One study has reported DLK1 in testis (Rockett el al. 2004). In the human kidney, expression of DLK1 is found at

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several locations during the first half of pregnancy [19, 42], but it is only observed in the mouse towards the very end of pregnancy [58, 59] (Table 2).

Regarding ectodermal derived tissues, no *Dlk1* gene expression is seen in the mouse medulla spinalis, although DLK1 is observed in other locations of the mouse brain [19, 46, 49, 59-61] including the pituitary [5, 19, 48, 52, 59, 62]. Despite being shown in mice, expression in the adrenal medulla and the olfactory epithelium has not yet been reported in human. Finally, in the placenta, DLK1 is reported in endothelial cells and trophoblasts in man [22] and mouse [52, 59, 62] although trophoblasts were previously reported negative in humans [19]. Maternal serum levels of DLK1 are increased during pregnancy in both mouse [50] and human [19, 22] and it is the fetus, not the placenta, which is the source of circulating maternal DLK1 [63]. Due to DLK1's high renal clearance, maternal circulating DLK1 most likely originates from the fetal urine [64]. According to a recent study based on mouse and human data, the levels of DLK1 in the maternal circulation can predict fetal growth restriction before birth [63].

Thus, DLK1 is widely expressed in tissues deriving from all three germ layers throughout development in both man and mouse, but is down-regulated either before or right after birth, becoming restricted to either a few tissues or stem/progenitor cells (Table 1 and 2). Hence, in general, the mouse seems a valuable model to study DLK1 expression, and the majority of existing diversities likely arises from a lack of investigation due to ethical restrictions.

The function of *DLK1/Dlk1 gene expression* in tissue development and regeneration

Cell proliferation and differentiation

In general, progenitor cells proliferate to a certain extent and then start differentiating to reach a mature state where their given function is aquired. Since the *DLK1/Dlk11* gene is expressed in many types of immature cells with progenitor characteristica, it is likely that it is involved in these processes. In line, several studies have explored its role in proliferation and differentiation

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by manipulating DLK1 through either knockdown or overexpression (Table 3). From the studies listed in Table 3, it is obvious that DLK1's impact on these processes is complex and likely depends on the tissue/cell type addressed. The vast majority however, report DLK1 to inhibit proliferation [51, 57, 65-70]. In this regard, the membrane bound isoform may be particularly important since multiple studies demonstrate this isoform to excert the inhibiting effect on cell proliferation [65, 67-70]. Only few studies suggest a positive relationship between DLK1 and proliferation [71-73] and it is noteworthy that of these, two where conducted in cancer cell lines [71, 72] suggesting an opposite role for DLK1 in malignancies.

With respect to cell differentiation, the consensus seems to be that DLK1 also inhibits this process (Table 3), although multipotent mesenchymal cells [74] as well as myoblasts in skeletal muscle in certain settings behave differently [66, 69, 70]. It is likely, that some of the conflicting results can be explained by the use of special cell lines or special procedures, for example the use of multipotent cell lines [74] or cells investigated in response to insulin [12, 75] or injury [66, 70]. Also, it is important to emphasize that the differentiation data may be biased by the prior proliferation process. Thus an inbitory effect on proliferation may directly result in less differentiation. Moreover, it has been shown in melanoma and breast cancer cells that low levels of DLK1 increase proliferation whereas high DLK1 levels decrease proliferation [76, 77], and further adding to this complexity the different DLK1 isoforms and the ratio between them may have an impact on the functional outcome. On top of this, DLK1 acts in autocrine, juxtacrine and paracrine manners exerting effects on both DLK1 expression and non-expressing cells, a fact that may hamper conclusions from *in vivo* settings in particular. Results from *Dlk1* transgenic mouse models certainly support a regulatory role for DLK1 in cell proliferation and differentiation but besides minor defects such as skeletal malformations, the most severe phenotype in *Dlk1*-knockout mice is growth retardation [14, 52, 70, 78]. Based on the inhibitory effects on proliferation addressed above, an opposite outcome of *Dlk1*-knockout would have

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been expected. On the other hand, since DLK1 also inhibits differentiation, genetic ablation of *DLK1/Dlk1* is likely to result in an accelerated differentiation process, with terminal differentiation occuring before reaching the full expansion potential of cell mass. This hypothesis would be in line with *Dlk1*-overexpressing mice displaying developmental over-growth [79]. Thus, it appears that low DLK1 expression in general leads to premature differentiation of an organism whereas high expression increases proliferation and/or delays the differentiantion process. Interestingly however, conditional- and tissue specific *Dlk1* deletion does not affect neonatal lethality, nor development of pancreatic β-cells, pituitary somatotroph cells or endothelial cells in the embryo and placenta compared to wild-types [78]. Thus, it seems reasonable to presume that the role of DLK1 is influenced by the cell origin and maturation stage. Collectively the data suggest that DLK1 during development and regeneration regulates/fine-tunes progenitor cell proliferation and cell differentiation to allow proper sizing of organs and the overall organism. Yet, in the differentiated neuroendocrine structures of adults, DLK1 on the other hand participates in regulating overall organism metabolism and glucose homeostasis to carefully adjust these to altered demands along body growth.

Tissue regeneration

In many diseases, endogenous regeneration remains insufficient for full recovery, and genes and progenitors that facilitate tissue repair is therefore desired. Since DLK1 is implicated in proliferation and differentiation of progenitors during development and re-expressed during times of disease and regeneration, the gene itself and the cells in which it is expressed are therefore exciting to pursue for regenerative medicine. Current literature suggests that DLK1 does impact regeneration of adrenal zona glomerulosa, endothelium [80, 81], ear-wound [82], and bone [83, 84]. But the majority of insight to DLK1's role in tissue regeneration derives

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from studies on *DLK1/Dlk1* gene expression and role of DLK1 positive cells in damaged liver and skeletal muscle, which are classical examples of regenerative systems.

The regenerating liver

In the mammalian embryonic and fetal liver, DLK1 is suggested to be a marker of bipotential hepatocytes capable of differentiation to mature hepatocytes or cholangiocytes forming de novo bile ducts. Using DLK1 as a cell surface marker, DLK1 positive bipotential hepatocytes can be isolated from fetal rodent livers [85-87]. In a rat model these cells are shown to repopulate the normal adult liver following a 2/3 partial hepatectomy and differentiate into both mature hepatocytes and cholangiocytes [86]. In contrast, DLK1 positive cells isolated from mouse fetal liver only differentiate into hepatocytes when transplanted into the spleen of recipient adult mice [87]. The different differentiation potentials of rat and mouse DLK1 positive fetal hepatocytes may reflect species differences or alternatively, the different transplantation milieus used in the two studies.

While the normal adult mammalian liver does not express DLK1, re-expression may be observed in certain regenerative scenarios. The adult mammalian liver is known for its exceptional regenerative capacity in response injury and can regenerate its function in different ways depending on the nature and extent of the injury. Following acute injury such as a partial hepatectomy, remaining liver cells will simply replicate to replace missing tissue [88] but in this case, without eliciting a DLK1 response [85, 89, 90]. However, the mammalian liver is also used as a model for the paradigm of regeneration via a facultative stem-cell-mediated recovery. In response to various types of severe or chronic injuries, atypical ductal cells (ADCs) – in the rodent commonly referred to as "oval cells" – accumulate within the liver parenchyma. These ADCs are thought to arise from cholangiocytes or alternatively liver progenitor cells (LPCs) located within the canals of Hering, and thus residing at the interface of the intrahepatic bile

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ducts and hepatocyte-lined canaliculi [91]. In some types of injuries, ADCs display phenotypes of bi-potential fetal hepatocytes and are therefore hypothesized to be able to differentiate into both mature hepatocytes and cholangiocytes [91-95]. In this context, we and others have reported that the expression of DLK1 is re-capitulated in ADCs appearing after severe toxic hepatic injury of rat liver treated with 2-acetylaminofluorene (AAF) (preventing proliferation of existing hepatocytes) and then acutely damaged by 2/3 partial hepatectomy (PHx) [85, 89, 90]. DLK1 positive ADCs appear at day 5 after PHx and reach a significant level at day 9-12, but hereafter declines with a scarce number present at day 18, and none at day 28 [85, 89, 90]. Moreover, DLK1 marks only a subset of ADCs corresponding to 19-, 23-, and 13% at day 10, 14 and 18 after PHx, respectively. These DLK1 positive ADCs localize in clusters at the interface between adjacent DLK1 negative hepatocytes and cholangiocytes [96], and may therefore represent a bi-phenotypic stage in rodent models where hepatocyte proliferation is completely suppressed, and LPCs are a source of new hepatocytes [97, 98]. However, recent lineage tracing studies in the mouse have challenged the paradigm of a facultative stem-cell-mediated liver recovery and reveal an astonishing plasticity of the uninjured liver epithelial cell populations to participate in regeneration [97-103]. Thus, the DLK1 positive ADCs in the AAF/PHx model may alternatively represent an emerging bi-phenotypic stage of mature cholangiocytes transdifferentiating to replenish damaged hepatocytes [97, 98]. Along these lines are our observations in the Retrorsine/PHx protocol where only a few DLK1 positive cells are located in the periportal areas whereas multiple foci of proliferating small DLK1 negative hepatocytes are present (Jensen et al. 2004) possibly representing the hybrid hepatocytes (HybHP) detected in murine regenerating liver by lineage tracing. These hybrid hepatocytes (HybHP) expressing several cholangiocyte-enriched genes are highly efficient in repair of livers deficient in healthy hepatocytes [99]. Finally, our observations on the differential expression of DLK1 in rat and mouse models of liver regeneration with DLK1 positive ADCs appearing in

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rat but not mouse may be due to experimental deficiencies [89]. In our studies, we only used short term liver injury models in the mouse as 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) treatment was provided for a maximum of 6 weeks. Given the observations in the recent lineage tracing study by Deng et al. 2018, we hypothesize that a severe injury induced by long term (24 weeks) treatment with DDC will generate bi-potent DLK1 positive ADCs in the mouseliver also.

The role of DLK1 in liver regeneration is further complicated by the observation that a number of extracellular matrix constituents including Laminins, Collagens and Fibronectin are highly regulated when DLK1 positive ADCs expand in the liver parenchyma after AAF/PHx injury. The DLK1 positive ADCs are draped by extracellular matrix deposition containing Collagen1a1 and laminin [96]. Interestingly, ADCs isolated from mouse liver upregulates *Dlk1* mRNA expression when cultured on Laminin but not on Collagens I, Collagen IV and Fibronectin [104]. As reviewed below, DLK1 has been reported to interact directly with FIBRONECTIN and inhibit cell differentiation [105, 106] wherefore the composition of the extracellular matrix could play an important role in the appearance of bi-potent DLK1 positive ADCs after severe injury. Finally, mesenchymal hepatic stellate cells involved in remodelling of the extracellular matrix during liver regeneration after a PHx [107], increase their *Dlk1* mRNA >4-fold 1 day after the injury, whereafter levels decrease to basal at day 3-7 [73]. *Dlk1*-knockdown studies [73, 108] in rat liver fibrosis pinpoints that the rising DLK1 in hepatic stellate cells activates them from a quiescent state by repressing genes related to quiescence in general [73], which then promotes liver fibrosis. Together these studies underscore that the re-expression of DLK1 during adult liver regeneration depends on both the species and type of injury model. Thus, substantial evidence on *DLK1/Dlk1* gene expression suggests that DLK1 plays an important role during liver regeneration and progenitor cell differentiation although its actual function has not been investigated. Therefore, in light of the recent lineage tracing

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studies in regenerating mouse liver, the specific function of *Dlk1* gene expression in ADCs and liver regeneration needs to be further explored in transgenic mice lacking or overexpressing DLK1

The regenerating skeletal muscle

DLK1 is highly expressed in skeletal muscle during fetal development, whereafter it declines postnatally (Table 1 and 2) [19, 59], but becomes re-expressed in several human myopathies including Duchenne- and Becker muscular dystrophies [34], in mononuclear cells of the muscle following intense exercise [36] and in skeletal muscle following damage [34, 66, 70, 109, 110]. This imply a role for DLK1 in skeletal muscle regeneration that in many ways mirrors embryonic development including satellite cell activation, proliferation and differentiation.

Independent of injury type or species, global *Dlk1* mRNA levels remain unchanged within the first 3 days after induction of regeneration [34, 36, 66, 70, 109, 110]. However, from day 4 levels start increasing significantly reaching peak levels at day 7 post injury [34, 36, 66, 109, 110], which coincides with myoblast differentiation and fusion [34]. Hereafter, *Dlk1* mRNA levels gradually return to normal from day 7-14 [34, 66, 109, 110]. At the cellular level, DLK1 is present in a subpopulation of human satellite cells (SC) and has been suggested to participate in SC proliferation or differentiation although the mechanism has not yet been clarified [37]. However, SCs do not constitute the majority of DLK1 positive cells in regenerating muscle rather DLK1cells represent a heterogeneous population of both myogenic and mesenchymal cells. As such, we and others have shown that in a mouse model for skeletal muscle regeneration, DLK1 cells are present in the perilesional zone from day 5-7 post injury and also in areas harbouring newly formed myotubes. The majority of DLK1 cells though are found outside of the basal lamina and shown to consist mainly of interstitial cells [34, 66, 70]. The day 5-7 peak induction of DLK1 suggests that DLK1 is expressed at the interface between

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myoblast expansion and fusion [34, 109]. However, the specific role of DLK1 in skeletal muscle regeneration is still contradictory [66, 70, 109]. *Dlk1* mRNA knockout in mice injected with cardiotoxin leads to significantly impaired regeneration at day 5-7 where *Dlk1* gene expression normally peaks [70]. In this study, *Dlk1* knockout results in extensive fibrosis, scarification and massive cellular infiltration in the interstitial space, suggesting that *Dlk1* gene expression is important for proper regeneration [70]. Another study however, reveals no differences between $Dlk1^{+/+}$- and $Dlk1^{-/-}$ mice in terms of overall muscle architecture, inflammatory response and scarification day 14 after a stab wound injury [66]. On the contrary, *Dlk1* gene depletion substantially enhances the myogenic program and muscle function, thereby improving regeneration. The latter suggests that DLK1 acts as an inhibitor of adult muscle regeneration [66]. Thus, it appears that like in the liver, DLK1 involvement in skeletal muscle regeneration most likely depends on the type of injury. Both studies however suggest that *Dlk1* knockout increases the number of available myogenic precursor cells [66, 70]. Indeed, Waddell and colleagues proposes that *Dlk1* gene expression influences satellite cell fate in a non-autonomous way since muscle-specific ablation of *Dlk1* leads to inhibition of the early differentiation marker MyoD and facilitated self-renewal of the satellite cell pool instead of myogenic differentiation [70]. Recent studies suggest that the various DLK1 isoforms may have different effects on distinct stages of myogenesis [66, 69, 110] and in addition different levels of *Dlk1* gene expression may have opposite effects on MyoD levels [66]. The membrane bound variants are reported to promote hypertrophy and a higher fusion rate of myotubes [66, 69], which is confirmed by reciprocal values of myostatin, an inhibitor of myogenesis [110]. In contrast, the soluble DLK1 forms may in certain cases promote differentiation of myogenic precursors [66, 70] and in other situations inhibit myotube formation [69, 110]. It is possible that some of these discrepancies can be explained by the use of different soluble forms, ie. monomeric versus dimeric, native versus recombinant or species differences. Overall, *Dlk1*

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gene expression thus seems to influence satellite cell fate in a supposedly non-autonomous way as most DLK1 cells represent a heterogeneous population of both myogenic and mesenchymal cells, which impact myoblast differentiation and fusion.

DLK1 acts through Notch-dependent and –independent mechanisms

Structurally, the DLK1 protein resembles the canonical delta-like ligands, but lacks the DSL (Delta/Serrate/LAG-2) domain, known to conduct interactions between NOTCH receptors and canonical ligands [17, 111]. Yet, DLK1 contains the conserved DOS (Delta and OSM-11) domain thought to be involved in NOTCH/DSL ligand activation [112]. In the yeast two-hybrid screen DLK1 interacts with NOTCH1 (Figure 1) [113], and this interaction either negatively- [51, 74, 113-117], or positively regulates NOTCH signalling [112, 118-121]. Yet, several of these studies rely on indirect evidence based on Hes1 expression that may be regulated by other pathways as well [122]. More direct evidence of an interaction comes from our recent work where DLK1 was shown to directly interact with NOTCH1 in the mammalian two hybrid system and where Dlk1-null mice display modulated NOTCH signalling in several organs during tissue development [123]. In particular, the NOTCH1-DLK1 interaction involves the EGF domains 5 and 6 of DLK1 and EGF domains 10-15 of NOTCH1 [123]. NOTCH ligands are unusual in that they inhibit NOTCH receptors present on the same cell (*cis*-inhibition), while they activate NOTCH receptors present on adjacent cells (*trans*-activation) [124]. Trans-activation by canonical ligands is mediated through EGF repeats 11-12 of the NOTCH receptor [125], thus the same EGF repeats as DLK1 interacts with [113, 123]. It is therefore commonly accepted that at least the inhibitory role of DLK1 on NOTCH signalling is mediated through *cis*-inhibition by competing with canonical ligands. Thus, with a structural resemblance to canonical NOTCH ligands and its proposed involvement in NOTCH signalling, DLK1 is considered a member of a diverse group of molecules, referred to as non-canonical NOTCH

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ligands, thought to play an important modulatory role, at least partly responsible for the pleitropic nature of NOTCH signalling. However, the implication of DLK1 in NOTCH signalling is still controversial, and several studies report NOTCH signalling to be unaffected by DLK1 [66, 106, 126, 127] and other interactions partners are also suggested as well (Figure 1). Regarding the latter, evidence in yeast and mammals show that DLK1 interacts with itself [105, 128]. Likewise in yeast, DLK1 has been shown to interact with the cysteine-rich molecules GAS1 and granulin (GRN), involved in the control of cell growth [129], as well as with the IGF binding protein 1 (IGFBP1) [130]. Also, the cysteine-rich FGF receptor (CFR) may bind DLK1 hereby interrupting Fgf18 binding, and thus regulating skeletal development [131], whereas DLK1 interacting with FIBRONECTIN seems to inhibit adipocyte differentiation [106]. Whereas our work in the mammalian two-hybrid system confirms that DLK1 interacts with itself, CFR, and FIBRONECTIN, the IGFBP1-DLK1 interaction seems not recapitulated. Finally, DLK1 is demonstrated to interact with PHB1 and PHB2, in turn regulating mitochondrial functions and possibly self-renewal and clonogenic growth of cancer cells [132].

In particular, the DLK1-DLK1 is mediated through EGF domains 4 and 5, which is distinct from the NOTCH1-DLK1 interaction [105]. Thus, it is likely that DLK1-DLK1 modulates the overall outcome of the NOTCH1-DLK1 interaction and hereby explains some of the context-dependent effect of DLK1 on NOTCH signalling. In line with this, it has been shown that the membrane-tethered DLK1 most potently inhibits NOTCH signalling [114]. Thus, the relative ratio of soluble and membrane-tethered DLK1 may be a level of regulating whether the membrane-tethered DLK1 can exert competitive *cis*-inhibition with canonical ligands or not or the cumulative effect of various DLK1 isoforms may result in no effect on NOTCH signalling observed in some studies. Several lines of evidence already support the notion that DLK1 signalling may be regulated and balanced through the action of the different isoforms. Garcés

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and co-workers demonstrated that membrane-tethered DLK1 was essential for preadipocytic cells to receive and respond to differentiation signals [12], and likewise, in the brain, membrane-tethered DLK1 was shown to regulate stem cell numbers via mechanism that requires soluble DLK1 [126]. Moreover, variations in the levels of membrane-tethered versus soluble DLK1 isoforms are reported to affect cell numbers and cell size [65, 133]. Adding to the complexity, the high and low levels of DLK1 inversely affect cancer cell proliferation through regulation of NOTCH signalling [76, 77]. Lastly, the involvement of the DLK2 protein, highly homologous to DLK1 [134], further complicates the situation, as DLK2 has been shown to interact with itself, with DLK1 and with NOTCH1 and whereas both DLK1 and DLK2 have been demonstrated to independently act as NOTCH antagonists, co-expression of DLK1 and DLK2 increases NOTCH activity [135]. It can thus be speculated that DLK2 can sequestrate the effect of DLK1 on Notch signalling or when DLK1 is absent, DLK2 can compensate for DLK1.

Thus, as with the NOTCH pathway, the underlying mechanism of DLK1 action is highly complex and far from understood.

C**onclusions and perspectives**

Taken together, our near to systematical status shows that the *DLK1/Dlk1* gene is widely expressed in the developing mouse and human organisms and therefore must serve a general function. Its expression is pronounced in the embryonic and early fetal stages, where after it decreases in many tissues along with cellular differentiation and organ maturation (Table 1 and 2). A major limitation of the summarized expression pattern in humans is however the lack of data from the third trimester due to a general lack of abortus and cadaver specimens from the late fetal period as well as during infancy. We however assume that tissues expressing DLK1 in early development as well as in adulthood also express DLK1 in the intervening period.

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Overall, the mouse seems to be a fairly good model to study DLK1, although differences exist. Regarding function, DLK1 generally seems to have an inhibitory effect on both proliferation and differentiation, but the levels of the different DLK1 isoforms may be critical and cause different effects. In regenerating tissues, it is even less clear what the exact role of DLK1 remains. Yet, from our data it is intriguing to speculate that the actual role of DLK1 may be to function as a checkpoint to slow down proliferation while forcing cells into the process of differentiation, and thus switch the cell/organ to a state of growth and hypertrophy. That would fit very well into *DLK1/Dlk1* being in a cluster of imprinted genes that restrict somatic growth [136]. It is noteworthy that in the early period during development most organs are established from cycling progenitors and extremely vulnerable to severe birth defects. Local and paracrine DLK1 levels during this early stage may thus be of vital importance for organ size, and dysregulations, and future studies may specifically address this issue in different organs. Levels of DLK1 is associated with overall children size at birth [63]. Whether DLK1 has another function in (adult) endocrine/neuroendocrine tissues remains to be elucidated, but it is likely that whereas DLK1 during development exhibits a growth modulatory function it may in adulthood serve another more metabolic role. It may also be that DLK1 somehow links tissue growth with metabolism to ensure organism survival. More studies in all areas of DLK1 biology thus seems required, including those on the underlying DLK1 mechanism (Figure 1), in order to take advantage of this exciting imprinted gene in regnerative medicine among others.

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Figure 1

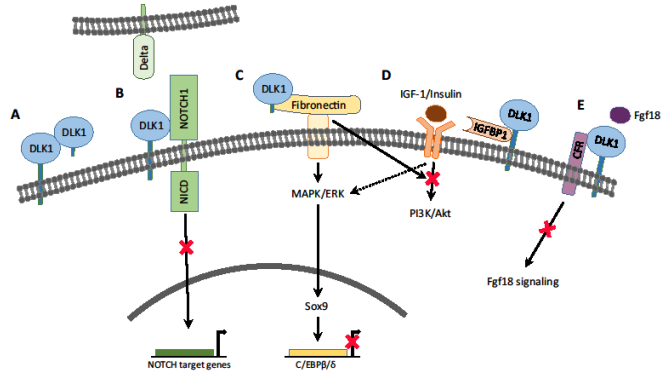

Figure 1. Schematic illustration of the many proposed interaction partners of DLK1 and its complex involvement in signaling pathways. (A) DLK1 has been suggested to interact with itself through specific EGF repeats in its extracellular domain [105, 128]. (B) DLK1 is proposed to interact with the NOTCH1 receptor, hereby competing with canonical Notch ligands (Delta-like/Jagged) [113, 123]. (C) DLK1 has been proposed to inhibit adipocyte differentiation through interaction of its soluble form with FIBRONECTIN [106]. This interaction in turn activates MAPK/ERK signaling, increasing SOX9, hereby blocking expression of adipogenic transcription factors [143]. (D) DLK1 has been reported to modulate MAPK/ERK activation level in preadipocytes in response to IGF-I or insulin [146]. In relation to this DLK1 has been reported to bind to IGFBP1, hereby increasing local concentration of free IGF-1 [130]. Also, DLK1 has been reported to regulate chondrogenic cell differentiation through inhibition of insulin-dependent Akt-kinase activation. Fibronectin may also be in play here, as suppression of FIBRONECTIN expression in DLK1 overexpressing cells rescued this inhibition independently of the Integrin receptor of the Fibronectin protein [147]. (E) Finally, DLK1 has been implicated in FGF (Fibroblast-growth factor) signaling during skeletal development [131].

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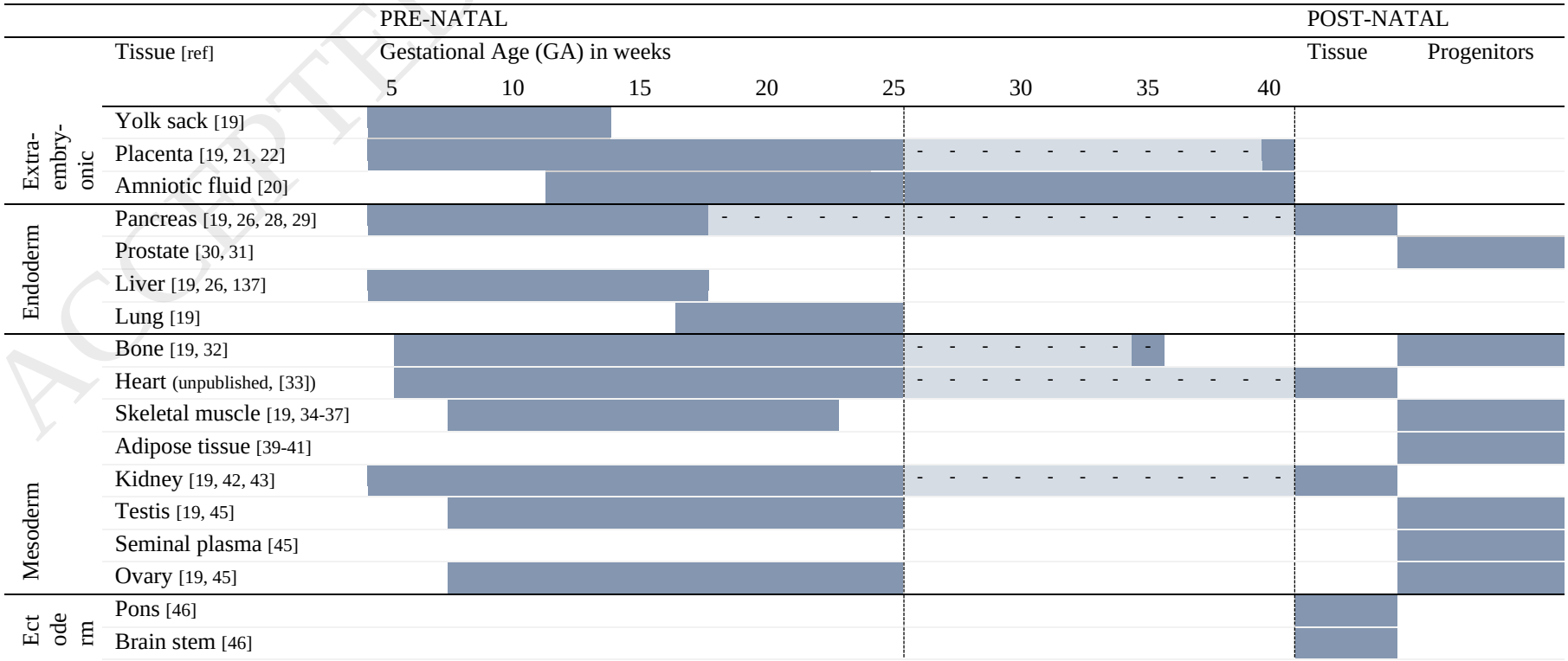
Table 1. Reported findings of spatial and temporal expression of Dlk1 during development and adulthood in human. Dark blue boxes represent findings of Dlk1 by at least one study, while the dashed light-blue boxes represent assumed expression (extrapolation between embryonic/fetal and adult DLK1 expression). Due to limitation of tissue, data is generally lacking from the third trimester of the fetal period (indicated by vertical dotted lines).



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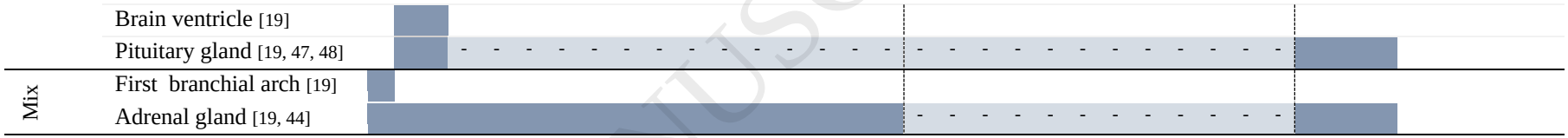

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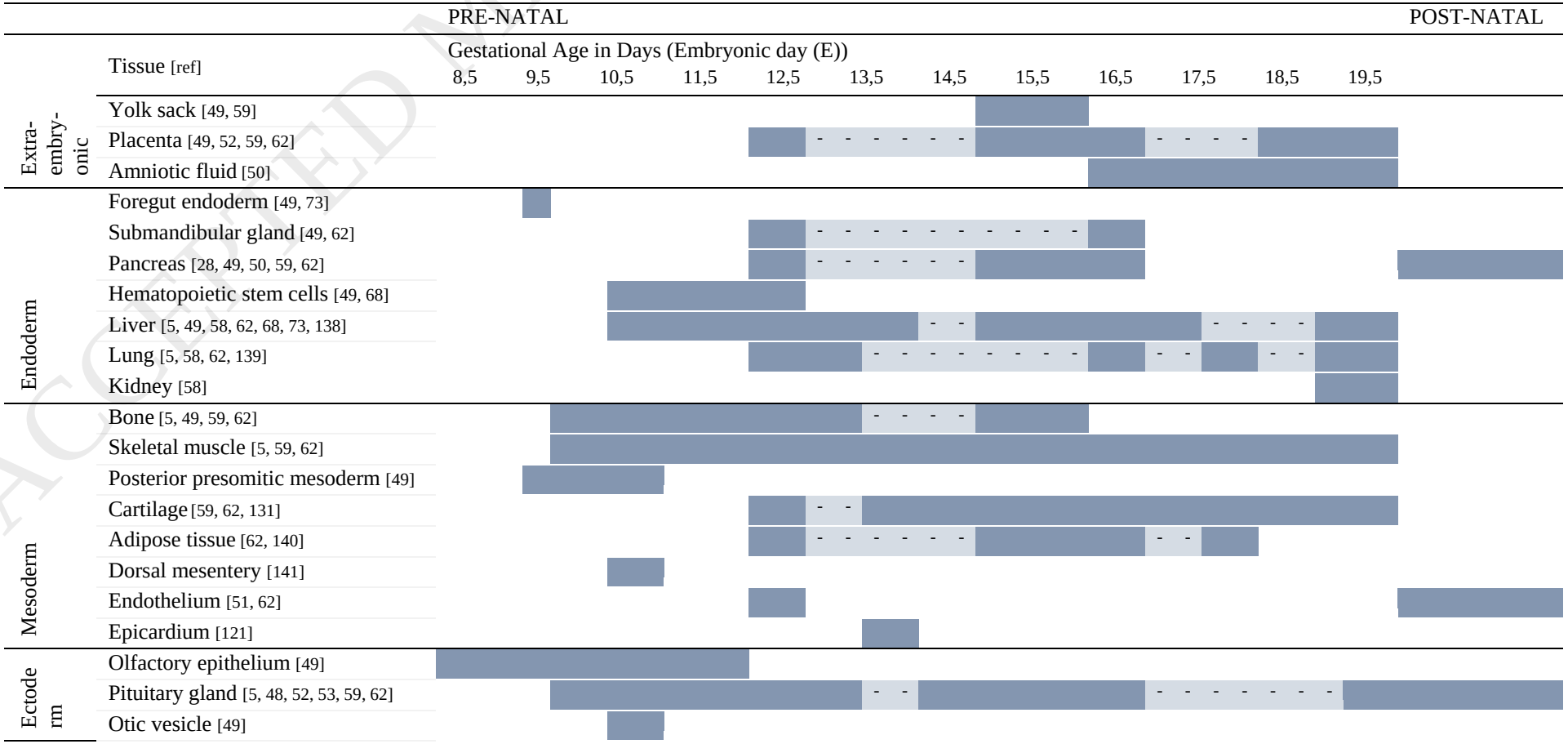
Table 2. Reported findings of spatial and temporal expression of Dlk1 during development and adulthood in mouse. Dark blue boxes represent findings of Dlk1 by at least one study, while the dashed light-blue boxes represent assumed expression.



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	Brain [53, 54, 59-61]	
Mix	First branchial arch [49]	
	Second brancial arch [49]	
	Adrenal glands [5, 59, 62]	

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Table 3. The function of Dlk1 related to differentiation or proliferation in models manipulating Dlk1 with either knockout or overexpression. The table demonstrates overall effect of Dlk1 on proliferation or differentiation. Knockout (decrease of Dlk1) leads to enhanced differentiation and proliferation, suggesting an inhibitory effect. Overexpression (increase of Dlk1) leads to decrease of differentiation and proliferation also suggesting an inhibitory effect. A few studies found the opposite or no effect. *Some studies have investigated the role of full-length (FL) Dlk1, whereas others have specifically studied the role of either the membrane-tethered (M) or the soluble (S) isoform. ↓ inhibitory effect, ↑ enhancing effect. NA, not addressed.

	PROGENITOR/CELLS	Isoform	Proliferation	Differentiation
Dlk1 Knockdown	Myogenic mononuclear cells [66]	FL	↓	↓
	Satelite cells [70]	FL	↓	No change
	Endothelial cell [51]	FL	↓	NA
	Stromal cells [68]	FL/M	↓	NA
	Preadipocyte [65]	FL/M	↓	NA
	Neuroblastoma cells [71]	FL	↑	↓
	Hepatocellular carcinoma cells [142]	FL	↑	↓
	Hepatic stellate cells [73]	FL	↑	NA
	Predipocytes BALB/c [12, 75]	FL	NA	↓
	Mouse embryo fibroblast [143]	FL	NA	↓
	Neuronal precursor cells [61]	FL	NA	↓
	Adipose derived stem cells [40]	FL	NA	↓
	Preadipocytes BALB/c [12]	M	NA	↑
	Multipotent C3H10T1/2 [74]	FL/S	NA	↑
Dlk1	Endothelial cells [51]	FL	↓	↓
	Hematopoietic stem cells [67]	FL/M	↓	↓
	Myoblasts [69, 70]	M	↓	↑
	Pancreatic β-cells [57]	FL	↓	NA
	Neuroblastoma [71]	FL	↑	↓

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	Leukemia cell line K562 [72]	FL	↑	NA
	Myoblast [69, 110]	FL/S	No change	↓
	Mesenchymal stem cells [32, 144]	FL	No change	↓
	Myoblast [66]	FL	NA	↑
	Multipotent C3H10T1/2 [74]	FL/S	NA	↑
	Osteoblasts [83]	FL	NA	↓
	Preadipocyte [12]	S	NA	↓
	Preadipocytes [145]	FL	NA	No change