

Invited Review

The role of the epidermal growth factor-like protein *dlk* in cell differentiation

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Summary. This review focuses on the current knowledge about the function of the EGF-like homeotic protein *dlk*. *dlk* is a transmembrane protein that possesses six Epidermal Growth Factor-like sequences at the extracellular domain, a single transmembrane domain and a short intracellular tail. Because of its overall structure and amino acid homology, *dlk* belongs to the EGF-like homeotic protein family. This family includes proteins such as the Notch receptor and its homologues, as well as Notch ligands, such as Delta, Serrate, and their mammalian homologues Dll1, Dll2 and Dll3 and Jagged 1 and Jagged 2. (For a recent review see Fleming, 1998). *dlk* is highly expressed by preadipose cell lines, and neuroendocrine tumors, such as pheochromocytomas and neuroblastomas. *dlk* has been involved in several differentiation processes, such as adipogenesis, hematopoiesis and B cell lymphopoiesis, and neuroendocrine differentiation, including the differentiation of pancreas and the adrenal gland. The extracellular region of *dlk* can be released by action of an unknown protease and this soluble *dlk* variant accumulates in the amniotic fluid and is able to inhibit adipocyte differentiation in vitro. Recent evidence indicates, however, that membrane-associated *dlk* variants play a positive role in the differentiation process. These findings suggest that *dlk* plays an important role in differentiation and tumorigenesis of several cellular types.

Key words: EGF-like, Homeotic genes, Adipogenesis, Neuroendocrine, Differentiation

Introduction

It is sometimes said that some scientific discoveries are made only when the scientific community is ready for them. This might well be the case for *dlk*. At least, as we will see, its discovery reflects the popularization of

certain molecular biology techniques. *dlk* has been discovered or rediscovered not less than six independent times. For that reason, *dlk* has received many names: *pG2* (Helman et al., 1987), *FA-1* (Fay et al., 1988), *Pref-1* (Smas and Sul, 1993), *SCP-1* (Maruyama et al., 1993), *ZOG* (Halder et al., 1998), *dlk* (Laborda et al., 1993). Throughout the article, the name *dlk* will be used mainly for the reason that *Dlk1* has been accepted as the official name for this gene. The many different names used for this gene have created considerable confusion in the literature. In addition, because *dlk* has been discovered or rediscovered in association with diverse biological systems, confusion has also resulted due to each research group, including ours, understandably focussing on the relevance of this gene for the particular biological system under study. This has inadvertently resulted in minimizing the implications of the findings realized by other groups in different biological systems. Consequently, a newcomer to the field would have some difficulty to gather all the literature pertaining to this subject, and arrive to understand the current knowledge about the function of this gene. This review is intended to summarize the current knowledge about the function of *dlk* in the diverse biological systems in which it has been implicated, and to clarify the nomenclature confusion existing in the literature.

The discovery of *dlk*

The first confirmed record of the discovery of *dlk* is more than a decade old. Prior to that date, it is possible that *dlk* could have been detected as an unknown serum or placental protein or tumor antigen. In a paper published in 1987 Helman et al. used differential hybridization techniques to identify genes highly expressed in the adrenal medullar neuroendocrine tumor, pheochromocytoma, but absent from the more immature tumor of the adrenal medulla, neuroblastoma. One of these genes was named *pG2*. As we will see later, this gene resulted to be *dlk*. According to the data presented by this group, *pG2* was highly expressed in adrenal cortex but also in the adrenal medulla, though to be embryologically unrelated to the cortex. These authors proposed that *pG2* was organ-specific, rather than tissue

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specific. They also showed that pG2 expression could be induced in neuroblastoma cells by treatment with glucocorticoids. Since neuroblastoma cells correspond to sympathoadrenal cells arrested at embryonic stages of development, the effect of glucocorticoids on pG2 expression indicated that the expression of this gene might be involved in neuroendocrine differentiation.

A year after the identification of pG2, in 1988, Fay et al. reported the identification of two new fetal antigens presented in the amniotic fluid. They named them FA-1 and FA-2 (fetal antigens 1 and 2). Despite this finding being apparently unrelated to the identification of pG2, FA-1 was later identified as a soluble variant of *dlk*, and therefore of pG2.

The first attempt to characterize the product of pG2 was the sequencing of its cDNA. The pG2 sequence was reported in 1990 by Helman et al. According to their data, the sequence of pG2 was not homologous to any DNA sequence present in the genetic databases at that time. In addition, pG2 encoded a putative protein of 286 amino acids that was no homologous to any other known protein.

We had to wait for three years to discover that the sequencing data published by Helman et al. were incorrect and that the protein encoded by pG2 had, in fact, an important homology with other proteins. The data showing this were published almost simultaneously by three groups, including ours. On March, 1993 appeared the first report by Laborda et al. suggesting that pG2 sequencing data were incorrect. These authors were interested in identifying genes involved in the response to Gastrin Releasing Peptide. This peptide acts as a growth factor in Small Cell Lung Cancer (SCLC), and in Swiss 3T3 mouse fibroblasts, but not in a subclone of Balb/c 3T3 mouse fibroblasts. The authors identified a gene expressed in Swiss but not Balb/c fibroblasts that was expressed also in some SCLC cell lines of human origin. The authors characterized both mouse and human cDNAs of this gene. Sequence data indicated that the mouse gene encoded for a 385 amino acid protein and the human gene encoded for a 383 amino acid protein. This protein would have a molecular mass of around 42 kDa. In vitro translation experiments demonstrated that a protein of similar molecular mass could be produced by in vitro transcribed or hybridization-selected mRNA, using the isolated cDNA as a template either for in vitro transcription or for the selection of an mRNA species expressed in Swiss 3T3 fibroblasts. These data indicated that the open reading frame (ORF) predicted from the sequence data was probably correct. Analysis of the predicted amino acid sequence revealed that the gene encoded a transmembrane protein containing a leading signal peptide at the amino terminus, followed by six Epidermal Growth Factor-like (EGF-like) repeats motifs, a unique transmembrane domain and a short intracellular tail (Fig. 1). Despite the numerous mammalian proteins possessing EGF-like repeats, the EGF-like motifs of the newly identified protein were highly homologous to the EGF-like motifs of the *Drosophila* homeotic protein

Delta, a ligand for the receptor Notch. For that reason, the new gene and protein were named *dlk* (for delta-like).

Laborda et al. found that the homology between mouse *dlk* and pG2 cDNAs was 81.2% and between human *dlk* and pG2 was 99.5%. Since the protein predicted by *dlk* or pG2 sequences were very different, it was important to see whether pG2 represented a diverse gene related to *dlk* that, however, encoded a very different protein. For that reason, the authors tried to clone pG2 from a mouse adrenal gland cDNA library, but none of the clones selected showed a sequence like pG2. This suggested that the sequence differences reported were not real and that *dlk* and pG2 were the product of one single gene. A careful analysis of the pG2 and *dlk* sequences revealed the regions of discrepancy and demonstrated that pG2 and *dlk* are indeed the same (Lee et al., 1995).

Almost simultaneously to the report by Laborda et al. (1993), Jensen et al. (1993) reported the purification and partial sequencing of FA-1 from second trimester human amniotic fluid. The authors reported that FA-1 is a single chain glycoprotein with a molecular mass of 32-38 kDa, therefore of a different molecular mass than *dlk*. The N-terminal amino acid sequence they reported revealed no homology to other known protein sequences.

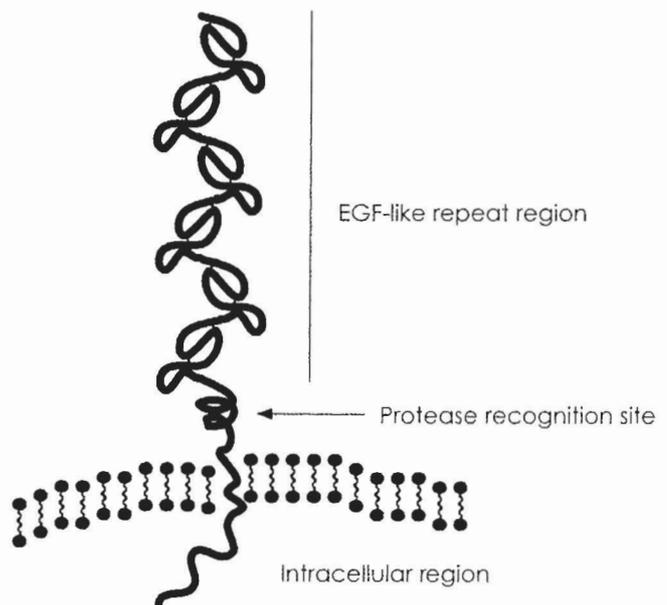


Fig. 1. Cartoon of the structure of the *dlk* protein. The *dlk* protein is a transmembrane protein containing six EGF-like repeats in the extracellular domain, a unique transmembrane domain and a short intracellular tail. This structure is homologous to the structure of EGF-like homeotic proteins that participate in the differentiation process of several invertebrate or vertebrate cells and tissues. The extracellular EGF-like region of *dlk* can be released by action of an unknown protease that cuts the extracellular region near the cell membrane. This soluble form of *dlk* is found in serum and the amniotic fluid and it has been shown to inhibit adipocyte differentiation in vitro.

Role of *dlk* in cell differentiation

However, when the protein sequence was backtranslated into a nucleotide sequence, the DNA sequence could be partially aligned to pG2, although to a region different of the suggested ORF for pG2 (Helman et al., 1990). Despite this discrepancy, and considering the expression patterns of FA-1, highly expressed in the adrenals, the authors suggested that fetal antigen 1 was encoded by pG2. Unfortunately for Jensen and collaborators, the amino acid sequence of the FA-1 region analyzed prevented them to identify homologies with EGF-like proteins. They were, however right in which FA-1 is encoded by pG2, or *dlk*. The differences in molecular mass were explained later with the identification of FA-1 as a secreted soluble variant of *dlk*, therefore lacking transmembrane and cytoplasm regions (Jensen et al., 1994).

Three months after the discovery, or rediscovery, of *dlk* by Laborda and collaborators (Laborda et al., 1993) another manuscript reported the rediscovery of this gene (Smas and Sul, 1993). These authors were interested in identifying new regulators of adipocyte differentiation. To that end, they used differential hybridization screening of a cDNA library prepared from the mouse preadipocyte cell line 3T3-L1 40 hrs after the start of adipocyte differentiation induced by treatment with dexamethasone and methylisobutylxanthine (dex/mix). The library was screened with reverse-transcribed probes, one derived from the same cells, and other derived from 3T3-C2 cells, cells related to 3T3-L1 but unable to undergo differentiation. In that way, they identified a gene, named Pref-1 (pre-adipocyte factor 1),

not expressed in 3T3-L1 cells induced to differentiate but continued to be expressed in 3T3-C2 cells. According to these data, this new gene encoded for a protein of 372 amino acids, instead of the 385 reported for mouse *dlk*. The protein also possessed a signal peptide, six tandem EGF-like repeats in the putative extracellular region, a unique transmembrane domain and a short intracellular tail. Genbank data base search revealed that the cDNA sequence of pref-1 had a high degree of homology to pG2, but, as it should be obvious now, no homologies at the predicted amino acid level.

A comparison between mouse *dlk* and Pref-1 sequences (Lee et al., 1995) revealed several differences between the two. The most important differences clustered at the 3' non-translated region of the mRNA, which suggested the existence of polymorphic variants of the mRNA. The differences at the coding regions were minimal, however, and both proteins displayed virtually identical sequences until the end of the transmembrane region was reached. There, the lack of a guanine in position 1111 of Pref-1 cDNA, present, however, in *dlk*, caused a frame shift that resulted in totally divergent sequences for the intracellular region of both proteins. The characterization of *dlk* from different species, including *Bos taurus*, *Ratus norvegicus*, *Mus musculus* and *Homo sapiens*, has confirmed the lack of a guanine as a sequencing artifact (Fig. 2). However, a recent access to the Genbank database showed that the error present in the Pref-1 mRNA sequence (L12721) is still not corrected.

Following these discoveries, *dlk* has been still

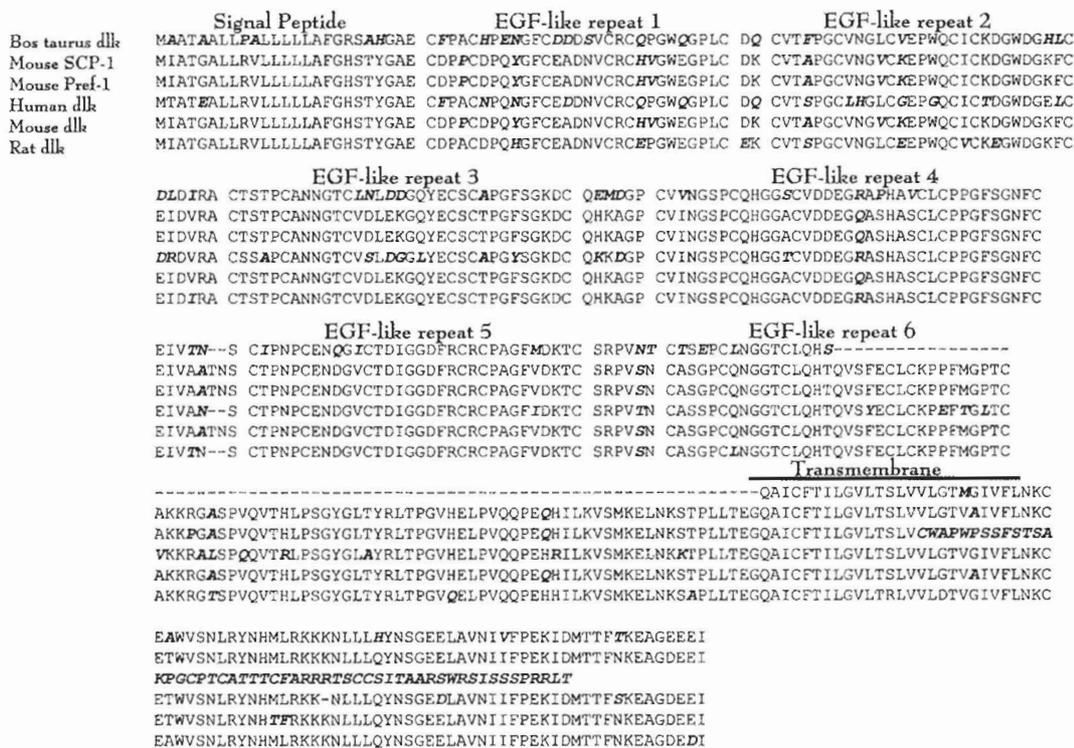


Fig. 2. Alignment of *dlk* protein sequences from different mammalian species. The sequence differences are highlighted. All sequences show a high level of homology among them, the most divergent being *Bos taurus dlk*. The fifth EGF-like repeat appears the most conserved and EGF-like repeats one and two the least conserved among the sequences. Spliced variants involve the sixth EGF-like repeat. The sequence of *Bos taurus dlk* corresponds to one of the spliced mRNA variants also detected in human and mouse. Despite lack of homology with other proteins, the *dlk* intracellular tail is highly conserved, suggesting it might play an important function. The divergent sequence reported for Pref-1 is due to a sequence artifact (see text).

rediscovered three more times. One of them also occurred in 1993 and is only reflected as a Genbank entry (accession number D16847) by Maruyama et al. (1993). These authors called the new gene SCP-1 from Stromal Cell Derived protein-1. They identified this gene from the adipocytic stromal cell line MC3T3G2/PA6 (PA6). According with the Genbank data provided, the gene encodes a transmembrane protein, containing 6 EGF-like repeats in the extracellular region, that is identical to mouse *dlk*.

It is interesting to note that PA6 is a stromal cell line that possess *in vitro* hematopoietic-supporting ability (Kodama et al., 1984). According to their data, Maruyama et al. (1993) also cloned SCP-1 using a differential screening approach. Presumably, they were interested in identifying genes involved in hematopoietic-supportive ability, but a manuscript confirming this assumption has never been published. However, *dlk* was again independently rediscovered as a molecule involved in the hematopoietic-supportive ability of stromal cells. Moore et al. (1997) were interested in dissecting the fetal liver hematopoietic micro-environment into the distinct cellular components that provide the molecular signals that mediate stem cell differentiation and self renewal. To that end they established an extensive panel of stromal cell lines and characterized their hematopoietic-supportive ability *in vitro*. One cell line thus established, AFT024, was able to maintain repopulating stem cells for long periods of time, whereas another cell line, 2018, did not possess this property. By using subtractive hybridization techniques (!) they identified a number of cDNA clones specifically expressed by AFT 204, but absent from 2018. One of these clones was *dlk*.

The final discovery of *dlk*, so far, was reported very recently by Halder et al. (1998). These authors were interested in exploring the differentiation of the rat

adrenal cortex, composed of three zones thought to arise from one single type of pluripotent stem cells. Using subtraction cloning strategies (!), the authors isolated three clones specifically expressed in the glomerulosa zone. One clone, named zone glomerulosa specific clone (ZOG) was identified as the rat counterpart of *dlk*.

In this summary of the many discoveries of *dlk*, one thing the reader has surely not failed to notice is that all discoveries of *dlk* have been accomplished by using differential screening or subtractive hybridization techniques in several cell types. As we will see, this fact alone has implications about how this molecule is expected to function.

dlk belongs to the EGF-like protein superfamily

Based on its structural features, predicted from the mRNA coding sequence, and confirmed by the amino acid sequencing of FA-1 (Jensen et al., 1994), *dlk* belongs to the EGF-like superfamily of proteins. All members of this family are either membrane-associated or released to the extracellular medium (the only known exception being prostaglandin G/H synthase), and all appear to participate in protein-protein interactions through their EGF-like repeats (Carpenter and Zandegui, 1986; Engel, 1989; Muskavitch and Hoffmann, 1990). The EGF-like repeat possesses a structure characterized by six cysteines linked by disulfide bonds in a specific manner. In mammals, there are two generic EGF-like repeat structures, named type A and type B, which differ in the cysteine spacing and conserved amino acid residues (Fig. 3). The structure of the *dlk* EGF-like repeat, despite *dlk* being a mammalian protein, does not correspond to any of the two types, but is more closely related to the structure of the EGF-like repeats of invertebrate homeotic proteins, such as Delta, Serrate, Notch and Lin-12. Recently discovered mammalian homologues of these proteins, unknown by the most part when *dlk* was first discovered, also share a similar EGF-like repeat structure. In addition, the homology between *dlk* and EGF-like homeotic proteins at the amino acid level is greater than with other EGF-like-repeat

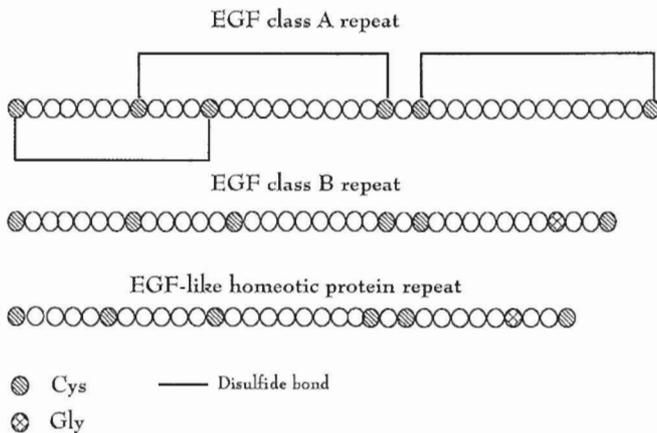


Fig. 3. Schematic structure of several types of EGF-like repeats found in mammalian proteins. The amino acid spacing and overall structure of the *dlk* EGF-like repeats is more closely related to the structure of the EGF-like repeats of vertebrate and invertebrate EGF-like homeotic proteins than to the structures of other EGF-like proteins.

<i>dlk</i>	C	P	C	N	G	G	C	D	Y	C	C	P	G	F	G	C	
Delta	C	P	C	N	G	G	T	C	Y	C	C	G	F	G	G	C	
Serrate	C	P	C	N	G	G	T	C	D	F	C	C	G	G	C	C	
hNotch-1	C	P	C	N	G	G	T	C	D	Y	C	C	G	F	G	C	
DrNotch	C	S	P	C	N	G	G	T	C	D	Y	C	C	G	F	G	C
Xotch	C	P	C	N	G	G	T	C	D	Y	C	C	G	F	G	C	
Lin-12	C	L	C	N	G	C	I	D	Y	C	C	G	Y	G	G	C	

Fig. 4. Alignment of the consensus EGF-like repeat amino acid sequences of *dlk* and several EGF-like homeotic proteins. Despite the fact that *dlk* is not present in invertebrates, it still shows high degree of homology at the amino acid level with proteins present from insects to humans.

containing proteins (Fig. 4) (Laborda et al., 1993). All these considerations clearly place *dlk* as a new EGF-like homeotic protein.

EGF-like homeotic proteins act as receptors or as ligands (Fig. 5) that participate in cell-to-cell interactions involved in cell differentiation decisions leading to a variety of mature tissues, including the nervous and the immune systems (for recent reviews see (Beatus and Lendahl, 1998; Saito and Watanabe, 1998)). It is, therefore, reasonable to speculate, on the basis of structural considerations, that *dlk* may also participate in the differentiation of specific cell types through interaction of its EGF-like repeats with unknown proteins.

It is interesting to notice that *dlk* appears to be present in animals from birds to mammals, but is absent from lower animals (Laborda et al., 1993). Thus, *dlk* appears late in evolution, but is structurally more related to proteins conserved from invertebrates to mammals. This suggests that *dlk* may possess a function specific for higher animals for which the EGF-like homeotic structure is important. *dlk* may be, therefore, an example of how molecular evolution produces new genes possessing conserved primitive structures that, however, acquire new functions necessary for forms of life that are more complex.

Expression of *dlk* in embryonic and adult tissues

The early identification of FA-1 in amniotic fluid drove to the conclusion that *dlk* was probably expressed by some fetal tissues. Indeed, in the mouse embryo, expression of *dlk* can first be detected at 8.5 days of gestational age (Smas and Sul, 1993). Expression in embryonic tissues has been demonstrated in the pituitary, the mesenchymal regions of the developing vertebra, the tongue, the lung and the liver. Expression in the fetal liver was confirmed by Tornehave et al. (1993) and in fetal liver-derived stromal cells by Moore et al. (1997). Expression in the fetal adrenal gland was detected by

Cooper et al. (1990). Finally, *dlk* has been also detected in the glandular cells of the pancreas anlage (Tornehave et al., 1993). In the developing pancreas, *dlk* expression was developmentally regulated. *dlk* was expressed by 94% of the glandular epithelial cells at 7 weeks of gestational age, by 64% of these cells at week 17 and by only 11% four months after birth. Through development, *dlk* was not detected in endocrine glucagon, somatostatin or pancreatic polypeptide cells. Moreover, developing insulin cells that coexpressed glucagon were negative for FA1 (Tornehave et al., 1996). In adult tissues, expression has been detected in the adrenal gland (Laborda et al., 1993; Smas and Sul, 1993), the pituitary (Larsen et al., 1996) the placenta (Jensen et al., 1993, 1994; Laborda et al., 1993) and the pancreas (Tornehave et al., 1993). In this last organ, expression of *dlk* is restricted to the β cells of the islets of Langerhans. This limited patterns of expression in pancreas is probably the reason why expression in this organ was not detected by Laborda et al. (1993). Expression in adult tissues is lacking in differentiated adipose tissue, lung, intestine, stomach, kidney, liver, spleen, brain, muscle, heart and testis (Laborda et al., 1993; Smas and Sul, 1993). The pattern of expression in embryonic and adult tissues suggests a function of *dlk* in the development of hematopoietic and neuroendocrine tissues. Expression of *dlk* in bone marrow, the site of hematopoietic cell production in the adult animal, has not been studied, although bone marrow-derived stromal cells express *dlk* (Bauer et al., 1998).

Structure and expression of alternately spliced variants of the *dlk* gene

The genomic structure of the mouse *dlk* gene was reported in 1994 by Smas et al. The murine *dlk* gene is composed of five exons separated by four introns of variable length. Exon I is 235 base pairs (bp) in length and comprises the 5' non-translated region and the leading peptide of the *dlk* polypeptide. Exon II, only 64 bp long, is separated from exon I by approximately 1300 bp. Exon II encodes up to the fourth cysteine of the first EGF-like repeat of *dlk*. Exon III is 131 bp long and encodes the remaining of the first EGF-like repeat and the complete second EGF-like repeat. Exon IV is 142 bp long and encodes the third and part of the fourth EGF-like repeats. Exon V is 1061 bp long, encodes the rest of the protein and comprises the 3' non-translated region.

Alternately spliced variants of *dlk* have been detected and, interestingly, all occur within the fifth exon of the gene. That implies that some sequences inside the fifth exon function either as an exon or as an intron. The most common spliced variants detected would produce a *dlk* protein lacking from the third cysteine of the sixth EGF-like repeat to the transmembrane domain, which remains unmodified. Therefore, the putative protein encoded by the mRNA species would remain membrane-associated. Alternately spliced species of *dlk* have been detected in preadipocytes (Smas et al., 1994)

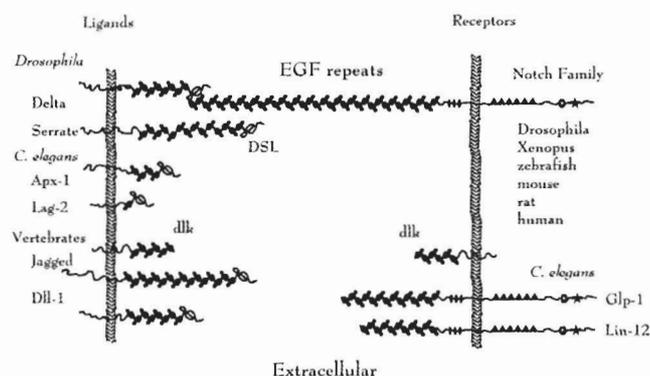


Fig 5. EGF-like homeotic proteins function either as receptor or as ligands. The ligands of this family all possess a DSL domain, so called because it is present in Delta and Serrate, and Lag-2, ligands of Notch and Lin-12, respectively.

and in human neuroendocrine tumors (Laborda et al., 1993). The functional significance of these polymorphic variants of the dlk protein will be discussed later.

Dlk in the differentiation of the neuroendocrine phenotype

Considering the many times dlk has been discovered, it is surprising that the first functional studies on this molecule started even before accurate knowledge about its structure was available. As we will see, these studies suggested that dlk played a role in differentiation. Once its structure was discovered, the presence of EGF-like repeats and the high homology of dlk with EGF-like homeotic proteins reinforced this conclusion. A role of dlk in differentiation has been confirmed, in fact, in several cellular systems.

The first report about the potential function of dlk was published in 1990 by Cooper et al. These authors had previously isolated and characterized pG2 (ignoring yet it was an EGF-like protein) and, therefore, focussed in neuroendocrine differentiation. The authors explored the hypothesis that, as it appear to happen with hematopoietic malignancies, non-hematopoietic neoplasias may contain cells corresponding to those which occur during the differentiation of tissue precursors. To that end, these authors focussed on neuroblastoma tumors as a model. They evaluated the expression of several neuroendocrine differentiation markers, one of which was pG2, in several neuroblastoma cells lines. They also studied the expression of dlk during the development of the chromaffin and neural lineage cells of the human adrenal gland. They observed that dlk was not expressed in adrenal medullar precursors in the first trimester adrenal gland, although it was expressed at high levels by nearly all cells in the fetal adrenal cortex. However, dlk was expressed in the adrenal medulla and outer region of the cortex of a four-week neonatal adrenal gland. This pattern of expression was also observed in the adult adrenal gland, with adrenal cortex dlk-positive cells found in the glomerulosa layer. Further analysis demonstrated that dlk is not detectable in chromaffin medullar precursor cells until twenty weeks post-conception. In contrast, non-chromaffin adrenal neuroblasts show no expression of dlk at the same gestational age. According with the temporal expression of several markers, including dlk, these authors postulated three stages of progressive chromaffin maturation. Then, they showed that neuroblastoma cell lines correspond to adrenal neuroblasts arrested at specific stages during the maturation of adrenal medullar cells. Of the twenty-seven neuroblastoma cell lines studied, ten of them expressed significant levels of dlk, whereas seventeen cell lines were negative. According to the lack of expression of other markers, the negative cells correspond either to undifferentiated cells or to cells differentiating along non-chromaffin lineages. The expression of dlk, therefore, correlated with the maturation along the chromaffin lineage. In addition,

treatment of a neuroblastoma cell line with agents that induce differentiation toward the chromaffin lineage increased the expression of dlk, whereas treatment with differentiating agents inducing a neural phenotype was accompanied of a decrease in dlk expression. This suggests that changes in the expression levels of dlk may mark the plasticity of a cell to mature along both chromaffin and non-chromaffin lineages. The data gathered supported the authors' hypothesis, at least for neuroblastoma tumors. Indeed, metastatic neuroblastoma cells, that share the ability with very primitive cells to migrate and invade other tissues, were negative for the expression of differentiation markers, including dlk.

All the previous data suggested that dlk plays a role in the differentiation of neuroendocrine cells, and perhaps in the increased malignancy of undifferentiated tumors. Additional evidence to this was provided by Laborda et al. (1993), showing that dlk was expressed by SCLC, cell lines, also of neuroendocrine phenotype. In this case, the most undifferentiated cells were also lacking expression of dlk. Further studies (Gaetano et al., 1992) using the differentiation-primitive neuroblastoma cell line SMS-KCNR that lacks expression of dlk, showed that treatment with dibutyryl cyclic AMP (dbcAMP), an agent that induced differentiation along the chromaffin phenotype, induced expression of dlk. This increased expression was inhibited by treatment with retinoic acid (RA), that induces a neuronal phenotype. These data, therefore, are in agreement with those reported previously (Cooper et al., 1990).

As mentioned early, Halder et al. (1998) identified dlk as a gene specifically expressed in the adrenal glomerulosa zone. In basis on immunohistochemical evidence, as well as the studies on dlk expression during adrenal gland regeneration, allowed these authors to conclude that dlk may play a role in adrenal zonal differentiation. Expression of dlk in the developing pancreas (Tornehave et al., 1996) also argues in favor of dlk as having a broad role in the differentiation of neuroendocrine cells and tissues.

Role of dlk in adipocyte differentiation

Adipogenesis still is one of most studied differentiation processes (For a recent review on the subject, see (Loftus and Lane, 1997; Gregoire et al., 1998; Spiegelman, 1998). One of the major interests in studying this process was to identify genes that either became activated or inhibited during the differentiation process. A number of in vitro differentiation systems are being used for the characterization of this process. One of the most popular is the 3T3-L1 cell line. This cell line of fibroblast phenotype is able to undergo differentiation in response to glucocorticoids and insulin/IGF-1 triggering. As mentioned before, Smas et al. (Smas and Sul, 1993) used this cell line and a counterpart cell line, 3T3-C2, unable to differentiate, to isolate and characterize murine preadipocyte factor-1, that resulted to be murine dlk. These authors reported that this gene

was downregulated during adipocyte differentiation. Furthermore, if downregulation of dlk expression was prevented by means of transfection, differentiation was inhibited. This report was the first direct evidence that dlk participates in a differentiation process.

In a recent paper, Smas et al. (1998) showed that dlk mRNA transcription is suppressed during adipocyte differentiation. They described a sequence in the dlk gene promoter region, called SAD element, (for Suppression in Adipocyte Differentiation), responsible for binding a transcription inhibitory factor active in adipocytes but inactive in preadipocytes. This transcription inhibitor has been preliminarily characterized as a protein with a molecular mass of 63 kDa. The molecular mechanism leading to the activation of this factor and the suppression of dlk transcription are unknown.

In a previous manuscript, Smas et al. (1997), reported that membrane-associated dlk is processed to generate a soluble glycosylated protein of around 50 kDa and smaller products of around 25 kDa and 31 kDa. These soluble forms inhibit adipocyte differentiation, as suggested by the fact that addition of recombinant soluble dlk to the differentiation medium results in the inhibition of adipogenesis of 3T3-L1 cells. The authors also report that the soluble dlk products result of the proteolytic cleavage of the membrane associated dlk encoded by the largest spliced dlk mRNA variants, corresponding to the reported complete protein sequence. The authors identified a sequence of 22 amino acids where the cleavage occurs. This sequence is characterized by a particular spacing of leucines, similar to a leucine zipper domain, as well as by the presence every four residues of a basic amino acid. Alternately spliced variants lacking the non-EGF-like repeat extracellular region lack this sequence and probably remain membrane associated. These results suggested that dlk may function as an inhibitor of differentiation in a juxtacrine or paracrine fashion.

If the inhibitory role of soluble dlk on adipocyte differentiation appeared demonstrated, additional data opened new questions. A recent paper (Hansen et al., 1998), reported an increase, rather than a decrease, in dlk expression upon induction of differentiation in primary rat preadipocytes during the first two days of the differentiation process. This increased expression diminished during the course of differentiation, however, and dlk expression virtually disappeared in differentiated adipocytes. Whereas Smas et al. (1993) had reported that the inhibitory role of dlk occurs very early in differentiation, the early increase in dlk expression upon differentiation of rat preadipocytes invited to explain the reason of such an increase and to study how it affected differentiation.

Another question to be answered was whether dlk expression played a role as a switch for the differentiation process to occur. Whereas dlk expression decreases at the end of the differentiation process in 3T3-L1 cells, related cell lines, such as NIH3T3 or

Balb/c 3T3 fibroblasts, express dlk but are unable to downregulate it in response to differentiation agents and are also unable to differentiate (Garcés et al., 1999). This suggested that, in 3T3-L1 and related preadipocyte cell-lines, dlk downregulation at the end of the differentiation may be necessary to complete this process. Garcés et al. (1999) demonstrated that dlk may play a role as a molecule that allows extracellular signals to be interpreted or not as differentiation signals. These authors transfected Balb/c 3T3 cells with antisense dlk constructs and, by that method, they were able to decrease dlk protein expression in these cells. In those conditions, Balb/ 3T3 cells, unable to differentiate to adipocytes in response to insulin/IGF-1 triggering, dramatically increased their differentiation in response to that hormone, as determined by lipid accumulation. The expression levels of dlk appear then to play a role in setting a cellular state permissive or not for differentiation in response to extracellular stimuli.

In the same manuscript (Garcés et al., 1999), Garcés et al. extensively analyzed the time-course of dlk expression along the differentiation process of 3T3-L1 cells. In agreement with the data reported by Hansen et al. in rat preadipocytes (Hansen et al., 1998) these authors observed an increase in dlk mRNA and protein expression at earlier times after differentiation triggering. dlk expression started to decrease only 48 hr after the initiation of the differentiation process to disappear when the differentiation was complete. In addition, the observed increase in dlk mRNA expression corresponded to alternately spliced variants encoding for proteins lacking the putative site of protease action and therefore, encoding for membrane-associated variants of the dlk protein. However, the dlk mRNA variants encoding for potentially released soluble dlk polypeptides, inhibitory for differentiation, were quickly downregulated. These data suggested that membrane-associated forms of dlk might play a positive rather than inhibitory role on differentiation. To prove this point, Garcés et al. sorted 3T3-L1 and Balb/c 3T3 cells negative for membrane dlk expression and studied the differentiation potential. Despite the fact that antisense dlk transfected cells showed an increase in the differentiation response, cells negative for membrane dlk expression showed a decrease. These data are the first to demonstrate that the expression levels of dlk play a role in the way the cell may respond to extracellular signals and to show that expression of membrane-associated dlk play a positive role in adipocyte differentiation.

Role of dlk in hematopoiesis

Hematopoiesis is a complex differentiation process by which bone marrow stem cells differentiate into mature blood cells. This process requires both, soluble factors, such as cytokines, and cell to cell interactions (Alexander, 1998; Kincade et al., 1998). As mentioned above, the first insight that dlk may play a role in hematopoiesis was the characterization of Stromal Cell

Derived Protein as dlk. Later, Moore et al. (1997) identified dlk as being expressed in a fetal liver derived stromal cell line with in vitro hematopoietic-supporting abilities, whereas it was not expressed in a non-supporting cell line. Moore et al. also demonstrated that dlk expression was present in several stromal cell lines possessing various degrees of in vitro hematopoietic-supportive ability, but it was absent from cell lines devoid of this property. Therefore, a correlation appears to exist between the stromal cell line's ability to support the growth and prevent the differentiation of hematopoietic stem cells and the expression of dlk by that cell line. However, despite the inhibitory effect of soluble dlk on adipocyte differentiation, addition of soluble dlk to the cultures of progenitor cells in semisolid medium did not affect the cell growth, suggesting that soluble dlk is unable to enhance the growth of progenitor cells in the absence of stromal cells. Addition of soluble dlk, however, had an effect on hematopoietic support when added to dexter-type cultures, in which hematopoietic stem cells are grown in the presence of stromal cells. In those conditions, if the stromal cells supports stem cell growth, "cobblestone area" cell colonies (CSA) develop. When the stromal cell line, BFC012, negative for dlk expression and unable to maintain a significant in vitro hematopoiesis, was used in those conditions, addition of soluble dlk (0.1 μ l/ml) resulted in approximately two-fold increase in the number of CSAs observed. Transfection of BFC012 cells with full-length dlk cDNA increased the hematopoietic support of these cells and resulted in a four to six-fold increase in the number of CSAs observed in dexter-type culture conditions. However, the maintenance of the CSAs was transient, lasting less than two weeks. Individual stable transfected clones of these cells showed various degrees of hematopoietic-support and one of them showed similar levels of support than the supporting, dlk positive, cell line AFT024. However, one clone expressing very high levels of dlk supported even fewer CSA than non-dlk-expressing control cells. These data suggested that dlk was an important molecule participating in the growth and differentiation of hematopoietic stem cells.

Other studies on the role of dlk in hematopoiesis were reported by Bauer et al. (1998). These investigators decided to explore further the old but unexplained observation of the inverse relationship between adipocyte differentiation of bone marrow stromal cells and hematopoietic-supportive abilities of these cells in vitro. Cells that are able to differentiate to adipocytes are generally the best hematopoietic supporters (Dexter et al., 1977; Deryugina and Muller-Sieburg, 1993; Friedrich et al., 1996; Maekawa et al., 1997), but they lose this ability when they differentiate. Adipogenesis in the bone marrow is known to occur by the same mechanisms that regulate adipogenesis of other cells (Gimble et al., 1996a,b). Since dlk was a molecule capable of affecting adipogenesis, Bauer et al. decided to explore whether this molecule could also affect

lymphopoiesis. They used an in vitro system of cultured pre-B cells, which require both interaction with stromal cells and Interleukin-7 to grow and prevent apoptosis or terminal differentiation to B-lymphocytes. Balb/c 3T3 cells were used as stromal cells in this system. These investigators observed that Balb/c 3T3 cells transfected with dlk cDNA expression constructs in sense orientation did not modify the supportive abilities of the stromal cells. Antisense dlk expression constructs had a double effect. First, as observed by Garcés et al. (1999), they decreased membrane dlk expression levels and increased the adipogenic response of the cells to insulin treatment. Second, these cells abrogated the need of IL-7 to maintain the growth of undifferentiated pre-B cells. These results suggested that dlk affects the differentiation or apoptotic signals directed to both stromal cells and lymphocyte precursors.

Potential mechanism for dlk action

The data gathered so far on dlk function are contradictory. Due to its structure and expression pattern, it appears that dlk may play a role in the development and differentiation of a variety of organs or cell types, however it is also highly expressed in some adult tissues. A role for dlk in cells of neuroendocrine phenotype appears likely, but dlk also appears to play a function on cells of other phenotypes, such as preadipocytes and hematopoietic cells. It is difficult to find a common feature in these diverse cellular systems, but it is possible that dlk may influence some common cellular mechanisms that govern their differentiation status or growth. Some important molecules, including members of the EGF-like homeotic family, have been involved in the growth or differentiation of many diverse cells. It is possible that this can also occur in the case of dlk.

So far, no studies on the molecular mechanisms of dlk action have been performed. Considering, however, that dlk affects cell differentiation induced by external molecules that trigger a signaling cascade, it is possible that somehow dlk may affect the signaling outcome or the expression of the receptors or molecules that enable the signaling cascade. Further experimental work is needed to establish this fact.

A question that remains open is whether dlk functions as a ligand or as a receptor. As a member of the EGF-like homeotic family, dlk may function either way. The lack of any significant homology, so far, of the intracellular region of dlk with other proteins prevents us from concluding whether dlk is a ligand or a receptor. The high homology of the dlk extracellular region with Delta and Serrate, ligands of Notch, suggests, however, that dlk may function as a ligand. The fact that soluble dlk inhibits differentiation may also be interpreted in that way, although an alternative explanation compatible with dlk being a receptor is possible. Most ligands of the EGF-like homeotic gene family, however, possess a DSL domain, a cysteine-rich sequence in their extracellular

region, N-terminal to the EGF-like repeat domains (Fig. 5). The DSL domain is involved in the interaction with the corresponding receptor. *dlk* lacks this domain, which places it closer to the receptor subfamily. On the other hand, some experimental data argue also in favor of granting *dlk* a receptor function. Garcés et al. (1999) and Hansen et al. (1998) have reported an increase in *dlk* expression during the first days of adipocyte differentiation. Sorting of cells negative for *dlk* expression results in a cell population impaired in their ability to differentiate. These data suggest that *dlk* may act as a receptor, providing a signal required for differentiation to proceed during the initial days of this process. Soluble *dlk*, blocking the ligand-receptor interaction, would prevent this signal, and, therefore, would inhibit differentiation. Since the cell transfection experiments leading to the conclusion that *dlk* inhibits differentiation have been performed with *dlk* variants that are released to the extracellular medium, these data

does not invalidate this assumption. The fact that released *dlk* variants appear to be downregulated very quickly upon induction of differentiation (Garcés et al., 1999) argues also in that direction. A third possibility, however, would be that *dlk* functioned as an inhibitor, or regulator of EGF-like homeotic ligand-receptor interactions. Lacking a DSL domain, *dlk* interaction with a receptor might be unable to signal, but would prevent a signaling interaction between the appropriate ligand and receptor. Stoichiometric relationship between *dlk* and the ligand-receptor couple would be paramount to account for *dlk* function. A problem with this hypothesis, however, is that if true, both membrane and soluble *dlk* variants should equally affect differentiation, which appears not to be the case.

Receptor or ligand, the stoichiometric relationship between soluble and membrane-associated *dlk* appears critical for the outcome of differentiation signals the cells may receive. Changes in the stoichiometric ratio may also explain why Balb/c 3T3 cells with decreased levels of *dlk* expression show an increased differentiation response to insulin/IGF-1. It is possible that soluble *dlk* variants are decreased in a greater extension than membrane-associated ones, or that the change in the expression levels of both is now more favorable to deliver a signal than to block it. Whatever the mechanism, it appears that the expression of *dlk* is important for the cell to decide to respond or not to signals leading to differentiation.

Members of the EGF-like homeotic family, such as Notch and its ligands, participate in differentiation decisions by a mechanism called lateral specification, which functions as a cell-fate-decision fork. In this mechanism (Fig. 6A), two equivalent cells expressing both ligand and receptor molecules interact with each other. Due to random variations in the expression levels of ligand and receptor between the cells, one cell receives more amount of signal than it delivers. This trigger leads to the segregation of the cellular fates. The cell that receives greater signal tends to upregulate the receptor and downregulate the ligand, and becomes a receptor cell that will acquire one of the two possible phenotypes. The other cell does the opposite; upregulates the ligand at the time it downregulates the receptor, and becomes a ligand cell that will acquire the alternate phenotype. This results in a spaced pattern of cells differentiated towards two different mature phenotypes. Another mechanism by which EGF-like homeotic genes control differentiation is called inductive signaling (Fig. 6B). In this case, signals are transmitted between non-equivalent cells. These signals induce neighboring cells to differentiate towards a particular phenotype.

In both types of mechanisms, regulated expression of a membrane-associated ligand-receptor couple is critical. The data accumulated on the effects of *dlk* in differentiation suggest that this molecule may participate in at least one type of differentiation mechanism. Adipocyte differentiation, either *in vivo* or *in vitro*,

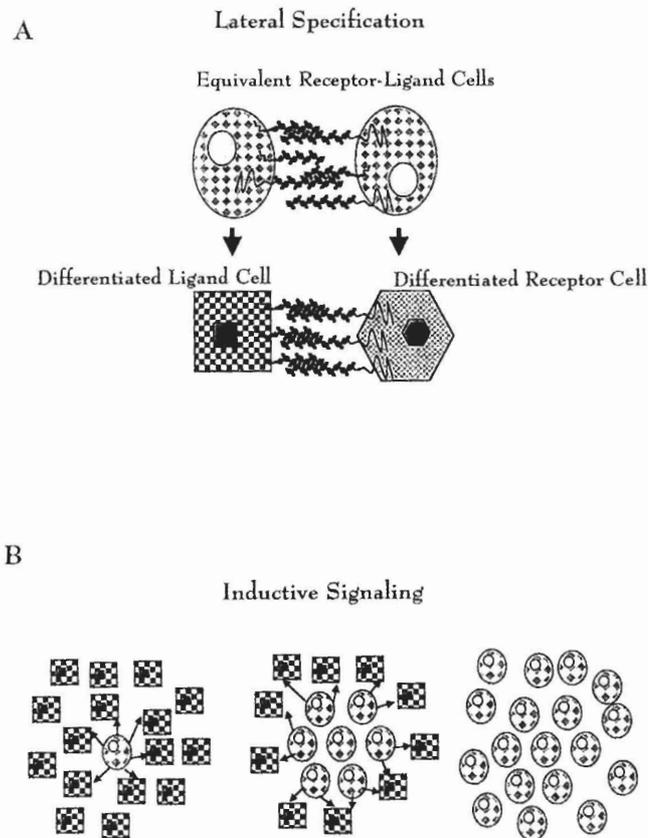


Fig. 6. Mechanisms of cell differentiation by cell-to-cell signaling. **A.** Lateral specification. In this process, equivalent cells expressing both ligand and receptor signal to each other. Random fluctuations in the intensity of the signal sent or received by the cells modify the expression levels of ligand and receptor. One cell becomes a ligand-expressing cell and another becomes a receptor-expressing cell. The cell fates of both cells will differ. A spaced pattern of cells is reached in this way. **B.** Inductive signaling: In this case, cell fates are determined by signals transmitted between non-equivalent cells. No spaced pattern is obtained.

where patches of differentiated cells expand on the culture plates, is more likely to occur by a mechanism of inductive signaling, because no spaced pattern composed of two types of cells is observed in this tissue. Cells that have initiated the differentiation process and are no longer equivalent to the neighbors cells, signal them in turn to initiate their differentiation process. It is possible that *dlk* may participate in this signaling mechanism. Blocking it by means of soluble variants of this molecule, therefore, will prevent differentiation.

Many are the questions still open on the biology and function of this important molecule. Identification of molecular counterparts, including molecules interacting with the EGF-like domain, the protease recognition site domain, or the intracellular domain will provide important clues about the mechanisms in which *dlk* participates. A better understanding of the control of its expression in different tissues and organs would be also important. Finally, generation of a *dlk* knock out mouse, currently underway in our laboratory, may also provide important information about the function of this molecule.

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Role of dlk in cell differentiation

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