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Preadipocytes proliferate and differentiate under the guidance of Delta-like 1 homolog (DLK1)

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Obesity occurs when an excessive dietary fat intake leads to expansion of adipose tissue, which mainly consists of adipocytes that arise from proliferating and differentiating adipose stem cells, the preadipocytes. Obesity is a consequence of both adipocyte hypertrophy and hyperplasia. Knowledge about preadipocyte differentiation is relatively well established, whereas the mechanism responsible for preadipocyte proliferation is incompletely understood and only in the early stage of comprehension. In this regard, we have recently identified that Delta-like 1 homolog (Dlk1) (also known as Preadipocyte factor 1 [Pref-1]) inhibits preadipocyte proliferation by regulating their entry into G1/S-phase. This novel disclosure, adding to the previous published data on Dlk1 repression of preadipocyte differentiation, has given us the chance to firmly place Dlk1 as a master regulator of preadipocyte homeostasis and adipose tissue expansion. Dlk1 manipulation may, therefore, open new perspectives in obesity treatments.

Keywords: DLK1, preadipocyte proliferation, preadipocyte differentiation, adipose tissue expansion, obesity treatment

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Obesity is one of the world's fastest growing health hazards and among the leading risks for deaths globally. WHO estimates that in 2008, more than 1.4 billion adults were overweight and obesity rates among developed countries have increased substantially during the past 3 decades. The consequences of excess body weight are numerous and include type 2 diabetes, hypertension, coronary artery disease, and many types of cancer.[1-5] With regard to this vast array of health implications, the need to develop new effective strategies for controlling obesity has become more and more crucial.

Obesity can be defined as the excessive accumulation of adipose tissue caused by a chronic imbalance between energy intake and energy expenditure.[6] On the cellular level, adipose tissue enlargement has previously been suggested to occur merely as a result of adipocyte hypertrophy.[7-9] However, by analyzing the integration of ^{14}C derived from nuclear bomb tests in genomic DNA, Spalding et al. elegantly measured adipocyte turnover in humans, and found that adipocyte number is a major determinant of fat mass in adults.[10] The number of mature adipocytes stays constant in adulthood in lean and obese individuals, even after marked weight loss, suggesting that the number of adipocytes is set during the childhood and adolescence.[7] Similar results have been demonstrated in mice.[8] Since the progenitor cells are constantly renewing depending on mechanisms of both proliferation and differentiation, it seems likely that a mechanism such as adipocyte apoptosis negatively balances the number of mature adipocytes. Yet the knowledge about adipose tissue apoptosis is still scarce. Nonetheless, it now seems well documented that obesity is a consequence of both adipocyte hypertrophy and the number of adipocytes.

Adipocytes originate from proliferating and differentiating adipose stem cells, the preadipocytes, located in the stromal

vascular fraction (SVF) of adipose tissue. Whereas much is known for preadipocyte differentiation, the mechanisms regulating preadipocyte proliferation are poorly understood.[11] In this regard, we have recently identified that Delta-like 1 homolog (Dlk1) (also known as Preadipocyte factor 1 [Pref-1]) inhibits preadipocyte proliferation by regulating their entry into the G1/S-phase of the cell cycle.[12] Since Dlk1 has long been considered an important repressor of preadipocyte differentiation,[13] this dual inhibitory function on preadipocytes places Dlk1 as a master regulator of preadipocyte homeostasis and adipose tissue expansion (**Fig. 1**). In this commentary, we therefore highlight our recent findings, and sum up on the dual inhibitory function of Dlk1 in preadipocytes and adipogenesis, knowledge that may not only allow us to understand better the cellular and molecular basis of adipose tissue growth in physiological and pathophysiological states, but also may provide means to develop therapeutic strategies for the treatment and prevention of obesity.

Delta-like 1 homolog (*Dlk1*) is a paternally expressed imprinted gene, encoding a transmembrane glycoprotein with epidermal growth factor (EGF)-like repeats in its extracellular domain.[14] The membrane tethered DLK1 can be cleaved by tumor necrosis α converting enzyme (TACE) at an extracellular juxtamembrane protease recognition site, generating a biologically active soluble form.[15] In the mouse, several isoforms, possessing the protease recognition site or not, are generated through alternative splicing of the *Dlk1* gene.[16] In humans, however, only one cleavable and one non-cleavable isoform is present.[17] The *Dlk1* gene is highly expressed during embryonic development and Dlk1 has been shown to be involved in the differentiation of various tissue types.[18-21] Postnatally, however, Dlk1 expression is downregulated and becomes restricted to cells of neuroendocrine origin and preadipocytes or preadipocyte precursors found within the stromal vascular fraction (SVF) of adipose tissue.[11,22,23] It is well established that Dlk1 acts as an inhibitor of in vitro preadipocyte differentiation. Dlk1 is highly expressed in proliferating preadipocytes, but its expression is abolished upon adipogenic differentiation.[24] Several studies have demonstrated that overexpression of Dlk1 in preadipocytes, as well as treatment with soluble DLK1 inhibits preadipocyte differentiation into adipocytes.[14,25,26] On the other hand, adipocyte differentiation is enhanced when Dlk1 levels are reduced.[27] Likewise, Dlk1 is known to inhibit adipogenesis in vivo. Through targeted disruption of the *Dlk1* gene, Moon et al. have demonstrated increased adiposity in mice lacking Dlk1. They reported that the increased fat mass in *Dlk1*-null mice was the result of both enhanced adipocyte differentiation and fat cell maturation, thus reflecting adipocyte hypertrophy rather than hyperplasia.[28] In agreement, mice overexpressing the large soluble form of DLK1 in adipose tissue display decreased adiposity due to adipocyte hypotrophy.[29]

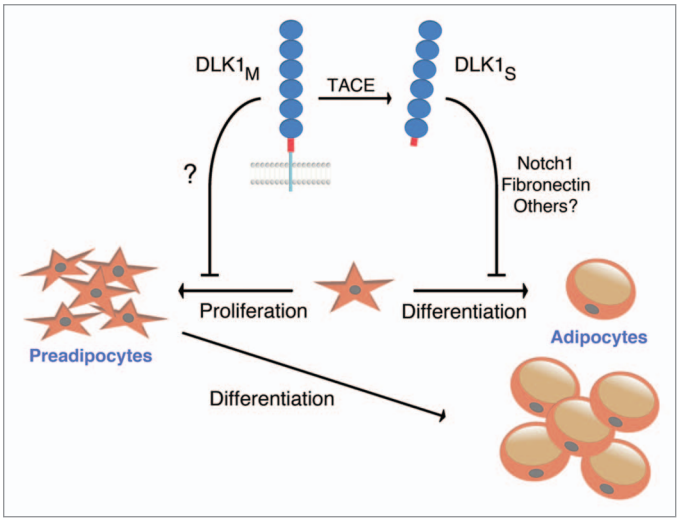

Figure 1. Schematic figure of DLK1's dual function in fat. Whereas the soluble form of DLK1 inhibits preadipocyte differentiation, preadipocyte proliferation is repressed by the membrane tethered DLK1.

Although DLK1 is a member of the EGF-like homeotic family of proteins that include the NOTCH receptors and their ligands, DLK1 differs from the canonical NOTCH ligands by lacking the DSL (Delta/Serrate/LAG-2) domain, involved in receptor binding.[30] Nevertheless, Dlk1 has been suggested to modulate Notch signaling through the DOS (Delta and OSM-11) domain, found in classical and putative Notch ligands.[31] The molecular mechanism by which Dlk1 regulates adipocyte differentiation is controversial and an interaction partner/receptor has yet to be identified with convincing biological and functional evidence. Still, DLK1 has been proposed to inhibit adipocyte differentiation through interaction with either NOTCH1 or FIBRONECTIN (**Fig. 1**).[32,33] However, the mechanism of DLK1 action is debated, and several other interaction partner(s) have been proposed as well.[19,34-38]

Likewise, with some controversies, the inhibitory effect of Dlk1 on adipogenesis is ascribed to occur only by the large soluble form. Mei et al. showed that only the large soluble form is active and sufficient to inhibit adipocyte differentiation, and that neither the small soluble form nor the membrane attached form of DLK1 affects adipogenesis.[25] In disagreement, Garces et al. have suggested that membrane and secreted DLK1 protein variants likely play opposite roles in the control of adipogenesis.[24]

Our recent study[12] aimed to take a step forward regarding the effect of Dlk1 on preadipocyte proliferation in vitro and in vivo, specifically studying whether differential roles apply for the large membrane tethered and soluble DLK1 isoforms in this process.

To accomplish this, we used a novel approach specifically reducing the endogenous level of different DLK1 variants in proliferating preadipocytes that naturally express the protein. We thus designed

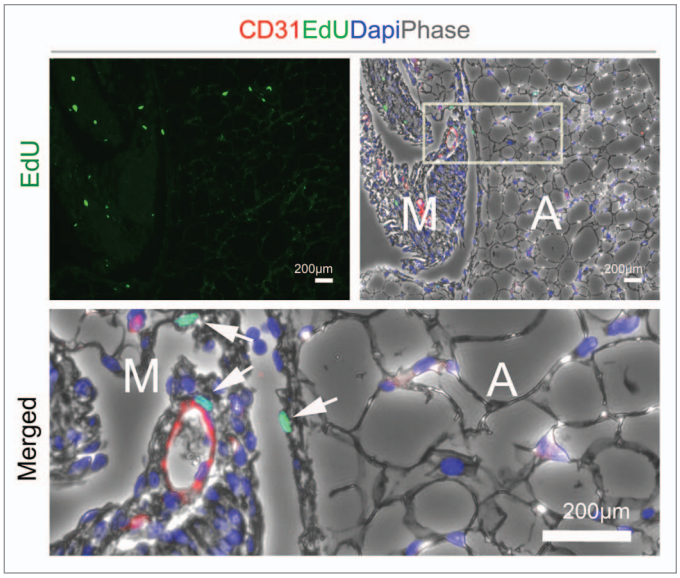

Figure 2. Identification of proliferating cells in developing fat of 6-weeks old C57Bl/6 mice. EdU (5-ethynyl-2'-deoxyuridine) was injected into mice and gonadal fat pads isolated and analyzed one week after. Double fluorescence of EdU, CD31, and Dapi was performed on sectioned fat. Proliferating cells mainly resides within the mesenchyme (M) close to blood vessels rather than in the mature adipose tissue (A).

two different siRNAs, one specifically targeting the protease encoding site, only present in mRNA encoding for the cleavable DLK1 isoform, and another siRNA targeting a mRNA sequence common to all *Dlk1* mRNA variants. By use of this strategy, treated preadipocytes expressed differential levels of the membrane and soluble DLK1 isoforms. Global expression profiling revealed substantial differential regulation of 4 cell cycle-signaling pathways by cleaved and non-cleaved Dlk1 isoforms. These data thus supports the idea, that cleavable and non-cleavable DLK1 isoforms act differently.[24,25] Our data clearly showed that numerous genes in the cell cycle are affected at different time points in the same direction of having more preadipocyte proliferation, when specifically the level of membrane bound DLK1 is reduced. This was confirmed functionally, by a substantial increase in proliferation rate of the preadipocytes in vitro. We did not check adipogenic differentiation of the preadipocytes expressing different membrane and soluble DLK1 isoforms levels. Yet, since initiation of adipogenic differentiation depends on preadipocyte density, it seems likely that adipogenic differentiation will be enhanced in preadipocytes with low levels of membrane DLK1 as these cells will be higher in numbers.

Our study also revealed that in vivo preadipocyte proliferation is enhanced in *Dlk1*$^{-/-}$ mice. We unraveled this by use of EdU (5-ethynyl-2'-deoxyuridine), which is a novel alternative method to detect proliferating cells compared with conventional BrdU (5-bromo-2'-deoxyuridine) assays.[39] The identity of in vivo preadipocytes is still unknown,[11] but they have been demonstrated to be contained within the stromal vascular fraction (SVF). We have unpublished data (**Fig. 2**) showing that proliferating cells within developing adipose tissue mainly reside in proximity with the vasculature and clearly enriched in the adipose tissue mesenchyme (**Fig. 2**). We found that the SVF derived from *Dlk1*$^{-/-}$ mice comprised around 7.0% proliferating cells whereas 3.7% was seen in *Dlk1*$^{+/+}$ SVF cells. In relation to this, Spalding et al. reported that 10% of adipocytes renew annually at adult ages and levels of body mass index. Neither adipocyte death nor generation time is altered in early onset obesity, suggesting a tight regulation of fat cell number in this condition during adulthood.[7] Therefore, pathological disorders may indeed increase adipocyte renewal substantially resulting in obesity. By targeting the unknown "ligand/receptor" for DLK1 in the fat by specific mimicking drugs, we may thus slow down excess adipocyte renewal and inhibit obesity. Such a drug may be specific for either the soluble or the membrane DLK1 or both depending on whether hypertrophy or hyperplasia induced obesity is present. Yet, to obtain a suitable drug to fight hyperplasia induced obesity through DLK1 targeting, the mechanism by which DLK1 represses preadipocyte proliferation needs to be clarified. Our results suggest that membrane DLK1 regulates several components in the G1-S phase of the cell cycle, which is in agreement with membrane DLK1's ability to repress S-phase entry of leukemic cells.[40] Our observation, that soluble DLK1 has no effect on preadipocyte proliferation is in line with a previous study showing that DLK1 mutants encoding only the cleavable DLK1 isoform do not have an impact on hematopoietic cell proliferation.[40] Thus, although the soluble DLK1 may be the only active isoform directly inhibiting the preadipocyte differentiation step of adipogenesis (**Fig. 1**),[28,29] the membrane tethered DLK1 seems to repress adipose tissue expansion likely at an earlier developmental stage by lowering adipocyte numbers (**Fig. 1**).

Yet, several controversies exist on whether both DLK1 forms have an effect on proliferation as well as differentiation of cells in general, and these issues as well as the identification of the DLK1 interaction partner(s) therefore needs to be elucidated in more detail. However, the fact that Dlk1 has a dual role in preadipocytes (**Fig. 1**) firmly places this gene as a major regulator of adipogenesis, and further insights into Dlk1 signaling mechanism, may yield future means to develop therapeutic strategies for the treatment and prevention of obesity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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