DLK1 and the Notch pathway in the liver
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CHAPTER 4

LIVER-SPECIFIC OVEREXPRESSION OF *DLK1* AGGRAVATES HIGH FAT DIET-INDUCED STEATOSIS IN MICE
Delta-like 1 homolog (DLK1) is a noncanonical ligand in the Notch signaling pathway. Soluble DLK1 inhibits adipogenesis, whereas membrane-bound DLK1 enhances adipogenesis, implying that the relative abundance of soluble and membrane-bound DLK1 determines its biological effect. We assessed lipogenesis in \textit{Alfp-Dlk1} transgenic mice, which overexpress DLK1 in liver and have high plasma DLK1 levels. The mice were fed a semi-synthetic low-fat or high-fat diet for 4 weeks. The effect of DLK1 depended on sex and dietary fat content. \textit{Dlk1} overexpression inhibited the expression of \textit{Notch1} and its downstream target \textit{Sox9} in livers of male \textit{Alfp-Dlk1} mice on a low-fat diet, whereas a high-fat diet relieved these inhibitory effects of a low-fat diet and increased the expression of \textit{Hes1} and \textit{Pparγ}. In female \textit{Alfp-Dlk1} mice, a low-fat diet was without effect, but a high-fat diet increased \textit{Notch1}, \textit{Hes1}, and \textit{Sox9} expression, as well as that of key lipogenic transcription factors and enzymes in both liver and fat-pad. The lipogenic effects of circulating (soluble) DLK1 in female fat-pads show that DLK1 exerts a stimulatory effect on lipogenesis that depends on the sex of the mouse and dietary fat content rather than on DLK1 being a membrane-bound or soluble species.

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Keywords
DLK1, Liver, Fat-pad, steatosis, Notch pathway, Pparγ,

List of abbreviations
LFD, low fat diet; HFD, high fat diet
Introduction

Delta-like 1 homolog (DLK1) is a noncanonical ligand in the evolutionarily conserved Delta-Notch signaling pathway, which is involved in stem cell-fate decisions during development (1). It is also known as preadipocyte factor 1 (Pref-1) and Fetal antigen-1 (FA1) (2, 3) and consists of six tandem EGF-like repeats, a juxtamembrane region with a TACE-sensitive cleavage site, a transmembrane domain and a short intracellular tail. The Dlk1 gene is a maternally imprinted, paternally expressed gene on chromosome 14 and 12 in man and mice, respectively (4, 5).

DLK1 interacts with the NOTCH1 receptor in the yeast-two-hybrid system and acts as a negative regulator of Notch signaling in Drosophila, and in the mesenchymal stem cell line C3H10T1/2 (6-8). The best established function for DLK1/Pref-1 is that of an inhibitor of adipogenesis (3, 4, 9, 10): it is highly expressed in murine preadipocytes, whereas its expression is completely abolished in mature adipocytes. In 3T3L1 preadipocyte cells, constitutive expression of soluble DLK1 prevents hormone-induced adipogenic differentiation by inhibiting the expression of the transcriptional regulators peroxisome proliferator-activated receptor γ (Pparγ) and CAAT/enhancer-binding protein α (C/ebpα) through upregulation of Sox9 (3, 4, 11). In agreement, transgenic mice with either adipocyte- or liver-specific overexpression of soluble DLK1 have smaller adipose tissue depots and a lower expression of adipocyte markers in adipose tissues compared to controls (12). This inhibitory effect of soluble DLK1 on adipogenesis in vivo was also found when plasma levels of DLK1 were increased via transfection of a full-length Dlk1-expression plasmid into the liver of adult mice (13). Conversely, mice lacking Dlk1 expression display accelerated adiposity as adults and have enlarged, fatty livers with increased levels of lipogenic regulatory genes fatty-acid synthase (Fas) and Stearoyl-coenzyme A desaturase 1 (Scd1) (14).

However, DLK1 may not only act as an inhibitor of adipogenesis, as membrane-bound DLK1 is required for adipogenesis in the 3T3L1 cell line (15) and overexpression of full-length DLK1 (which generates both soluble and membrane-bound DLK1 species) significantly enhanced the adipogenic response in the mesenchymal stem cell line C3H10T1/2 (8). These findings suggest that DLK1’s role in adipogenesis may depend on the relative abundance of its soluble and membrane-bound species.

Since previously characterized transgenic mice with liver-specific overexpression of the full-length DLK1 did not show effects on liver morphology or proliferation (Chapter 7), in this study, we assessed the effects on lipogenesis in the liver and fat-pads of these transgenic (Alfp-Dlk1^tg/^) mice after feeding them either a high-fat diet or a low-fat diet for four weeks. Due to spontaneous cleavage of membrane-bound DLK1, Alfp-Dlk1^tg/^ mice also have elevated circulating levels of DLK1, which enabled assessment of the endocrine effects of soluble DLK1 on the fat-pads. We show that liver-specific overexpression of full length DLK1 stimulates high-fat diet-induced hepatic steatosis, with significant upregulation of lipogenic and Notch-pathway genes. We report that the elevated levels of circulating DLK1 did not exert an inhibitory effect on body and fat-pad weights if the mice were fed a low-fat diet and increased the expression of lipogenic genes in adipose tissue if the mice consumed a high-fat diet, but only if they were females.
Chapter 4 Liver-specific overexpression of Dlk1 aggravates high fat diet-induced steatosis in mice
Materials and Methods

Generation of Alfp-Dlk1\(^{tg/-}\) mice

Full length Dlk1/Pref-1 cDNA was kindly provided by Dr. H.S. Sul (University of California). A pBluescript-based vector was fitted with a multiple-cloning site to accept, from 5′ to 3′, the 2.3 kb murine albumin enhancer and promoter (taken from the Alfp-Cre expression vector (16), a 155 bp human chimeric intron (from the pCIneo vector, Promega, Leiden The Netherlands), the 1,158 bp Dlk1 open-reading frame, a 300 bp fragment containing a bovine growth-hormone polyadenylation fragment (from the pcDNA 3.1 vector), and the 3 core enhancer elements of murine α-Fetoprotein (MERs; 950 bp; (17); Figure 1A). The linearized transgene was injected into the male pronucleus of fertilized oocytes of FVB mice. Transgenic mice were identified by polymerase chain reaction (PCR) analysis on toe genomic DNA with Alfp-Dlk1-specific primers (Supplemental Table 1). From the 5 transgenic lines initially generated, one line with a high and homogeneous DLK1 protein expression in postnatal liver and plasma was selected for the present series of experiments. This transgenic line was infertile in the homozygous condition. Transgenic mice and control littermates were maintained on a 12h light/12h dark cycle with free access to water and food.

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Table S1 Primers used for quantitative PCR analysis

Primers used for genotyping and quantitative PCR analysis. All primers were designed to span an intron.
Materials and Methods

Animals and diets
Ten- to-16 weeks old Alfp-Dlk1<sup>tg/-</sup> mice and their littermate controls (Alfp-Dlk<sup>-/-</sup>) of both sexes (n=8 per group) were fed either a low-fat diet composed of 8 kcal % olive oil, 81 kcal % carbohydrates and 11 kcal% casein (D06092604, Research Diets, New Brunswick, USA) or a high-fat diet composed of 42 kcal % olive oil, 47 kcal % carbohydrates and 11 kcal% casein (D06092602, Research Diets) ad libitum for a period of 4 weeks. To ensure comparable intake in experimental groups, food intake was measured weekly. All mice had ad libitum access to water throughout the experiment. After the 4-weeks diet period, mice were weighed and sacrificed by decapitation after a brief sedation with CO<sub>2</sub>/O<sub>2</sub> (70:30). Systemic blood was immediately collected into heparin-coated microtainer tubes and centrifuged. The resulting plasma was frozen in liquid N<sub>2</sub> and stored at -80°C until analysis. The livers were collected, weighed and parts were either fixed in 4% formaldehyde/PBS or frozen in liquid N<sub>2</sub> and stored at -80°C for histology and RNA isolation, respectively. Epididymal and parametrial fat-pads in male and females, respectively, were removed, weighed, frozen in liquid N<sub>2</sub> and stored at -80°C. The study was carried out in accordance with Dutch guidelines for the Care and Use of Laboratory Animals and approved by the AMC supervisory committee.

Histology
Liver histology was assessed by hematoxylin & eosin (H&E) staining. Briefly, fixed livers were embedded in paraffin and sectioned at 7 µm thickness. After staining, sections were dehydrated in graded alcohols and mounted with Entellan (Merck, Darmstadt, Germany). Images were captured by using a Leica DMRA2 microscope equipped with a DC300 camera, at different magnifications.

Triglycerides, total cholesterol and glucose measurements in plasma
Plasma levels of triglycerides, total cholesterol and glucose were measured according to standard procedures of the Laboratory of Clinical Chemistry, AMC.

Quantitative PCR
Total RNA from liver and fat was extracted with Trizol reagent (Invitrogen, Breda, The Netherlands). Two µg purified RNA was reverse transcribed with Superscript III reverse transcriptase (Invitrogen). PCR quantitation was carried out in the LC480 apparatus with SYBR green (Roche, Woerden, The Netherlands). Primers were intron spanning and designed with Primer3 software at the supplied default settings (http://frodo.wi.mit.edu). Primer sequences are supplied in Supplementary Table 1. mRNA levels were normalized to 18S rRNA.
Materials and Methods

Statistical analysis
Data are shown as mean ± SEM of 8 animals per group. Significant interactions between groups were assessed with the 3-way ANOVA test, followed by a 2-way ANOVA in case of significant interactions. When significant interactions were absent, differences between groups were determined by the nonparametric Kruskal-Wallis one-way ANOVA test (18). Significance was set at $P < 0.05$. A trend was defined as $0.05 < P < 0.10$. 
Results

Figure 1 Alfp-Dlk1 expression construct and persisting DLK1 expression in postnatal liver
Panel A shows a schematic presentation of the Alfp-Dlk1 expression construct. The arrows represent the primers used to identify transgenes and for qPCR of Alfp-Dlk1-specific Dlk1 mRNA. Panels B-D show liver sections of Alfp-Dlk1 lines 1.1 (B) and 1.5 (C), and wild-type mice (D) stained for the presence of DLK1. Lines 1.1 and 1.5 show patchy and homogeneous DLK1 expression, respectively (scale bar in B is also applicable for C and D). Panel E shows Western blots of liver homogenates and plasma of the five Alfp-Dlk1 founder lines. The upper panels show the Amidoblack staining of the blots (loading control) and the lower panels DLK1 protein. In the lower left panel, lanes 1-3 and 5-6 show liver homogenates of the five founder lines, with high expression of both the 50 and 55 kDa bands (soluble and membrane-bound DLK1 protein, respectively) in two lines (lanes 5 and 6). Lanes 4 and 7 show liver homogenates of wild-type mice. Lane 8 shows the mainly membrane-bound form of DLK1 protein in 3T3L1 cells used as positive control. M: protein molecular-weight ladder. In the lower right panel, lanes 1-5 show DLK1 protein in plasma of the five founder lines, demonstrating that DLK1 expression in liver correlates with DLK1 levels in plasma; lines 1.1 and 1.5 show low and high plasma levels of DLK1 protein, respectively. Lane 6 and 7 show DLK1 protein in plasma of a wild-type mouse and in culture medium of 3T3L1 cells, respectively.
Results

Transgenic lines with postnatally continued expression of DLK1 in the liver

Transgenic Dlk1 mice (Alfp-Dlk1<sup>tg/-</sup>) were generated by injection of the Albumin-α-fetoprotein/Dlk1 (Alfp/Dlk1) construct (Figure 1A) into the male pronucleus of fertilized oocytes and implantation into pseudopregnant females. Out of the 5 founder lines that expressed the transgene, 3 lines had a low and 2 a high expression of DLK1 protein. The livers of lines with low DLK1 protein levels on Western blot, showed a patchy expression of DLK1 on immunohistochemically stained sections (Figure 1B). This expression pattern is often seen in classic transgenes. Line 1.5, with high DLK1 levels on Western blot, showed a uniform expression pattern, with all hepatocytes staining positive for DLK1 protein (Figure 1C). As expected, wild-type adult livers showed absent DLK1 expression (Figure 1D). DLK1 protein levels in liver correlated with plasma DLK1 levels (Figure 1E). In addition to line 1.5, another infertile line showed very high DLK1 protein expression in liver and plasma. The expression of DLK1 in both lines was more than 10 times higher than that in the other 3 lines (Figure 1E and supplemental Figure 1). Despite the continuous hepatic overexpression of DLK1, we never observed any liver pathology in approximately 40 adult mice of line 1.5 Alfp-Dlk1<sup>tg/-</sup> of up to 1.5 years of age.

Figure S1 High plasma DLK1 levels in transgenic line 1.5
The upper panel shows the Amido black-stained Western blot (protein-loading control) and the lower panel DLK1 protein in liver homogenates of the five Alfp-Dlk1<sup>tg/-</sup> founder lines. Lanes 1-3 show DLK1 protein in liver homogenates of three founder lines displaying patchy DLK1 expression by immunohistochemistry, while lanes 4 and 5 show DLK1 protein in 10x-diluted lysates (see Amido black) of lines 1.5 and line 1.6 with homogeneous DLK1 expression (founder 1.6 was not fertile). Lane 7 shows DLK1 protein in 3T3L1 cells used as positive control. M: protein molecular-weight ladder.
Results

DLK1 overexpression in liver increases liver and fat-pad weight in male mice fed a high-fat diet

Adult male and female control and Alfp-Dlk1<sup>tg/-</sup> mice were analyzed for both local and distant lipogenic effects of DLK1 overexpression. After consuming a low-fat diet for 4 weeks, neither male nor female Alfp-Dlk1<sup>tg/-</sup> mice significantly differed in body, liver, and fat-pad weights compared to littermate controls (Figure 2). Compared to low-fat diet, a high-fat diet increased body, liver, and fat-pad weight in Alfp-Dlk1<sup>tg/-</sup> males (Figure 2A), but no significant differences between Alfp-Dlk1<sup>tg/-</sup> and controls were found in females (Figure 2B).

![Figure 2](image)

Figure 2  Body, liver, and fat-pad weight in Alfp-Dlk1<sup>tg/-</sup> and control mice on low- or high-fat diet

Adult male and female control and Alfp-Dlk1<sup>tg/-</sup> mice (n=8 per group) were fed a low- or high-fat diet for 4 weeks. Panel A shows that male Alfp-Dlk1<sup>tg/-</sup> mice on the low-fat diet (LFD) had similar body, liver, and fat-pad weights as control littermates, although average weights were always higher in transgenic mice. On high-fat diet (HFD), liver weight was significantly increased and fat-pad weight tended to increase in Alfp-Dlk1<sup>tg/-</sup> males (p=0.059), while fat-pad/bodyweight ratio was significantly increased. Panel B shows that body and liver and fat-pad weights in females were comparable in Alfp-Dlk1<sup>tg/-</sup> and control mice, irrespective of the diet. Values are depicted as mean ± SEM. (*: P<0.05, n=8 animals per group).

DLK1 overexpression in liver does not affect plasma glucose, triglycerides and cholesterol

On low-fat diet, plasma glucose, triglyceride and cholesterol levels were comparable in male and female Alfp-Dlk1<sup>tg/-</sup> and control mice. The high-fat diet increased plasma glucose, triglycerides and cholesterol in males compared to females (P = 0.001, 0.01 and 0.01, respectively), but there was no difference in response between Alfp-Dlk1<sup>tg/-</sup> and control mice (Supplemental Figure 2).
Part I

DLK1 overexpression in liver increases steatosis and steatohepatitis

H&E-stained liver sections of Alfp-Dlk1tg/− mice and controls after 4 weeks on a low-fat diet showed normal liver histology, without signs of steatosis in both sexes (Figure 3A). In agreement with the increased liver weight of Alfp-Dlk1tg/− male mice on high-fat diet, liver sections showed increased lipid accumulation (Figure 3G-I) compared to controls (Figure 3B,C). In controls on the high-fat diet, mainly microvesicular steatosis (19), with small lipid vacuoles in the cytoplasm was observed. Steatosis in livers of Alfp-Dlk1tg/− mice of both sexes on the high-fat diet was mainly of the macrovesicular type (19), with large cytoplasmatic vacuoles that displaced the nucleus to one side of the cell (Figure 3D-I). In some Alfp-Dlk1tg/− livers, steatosis was accompanied by inflammation (Figure 3E,F) and sometimes even necrosis (Figure 3G,H), indicating the development of steatohepatitis (19). In agreement, the mRNA level of the inflammatory marker Mac-1 (integrin β2) tended to be higher in livers of Alfp-Dlk1tg/− than control mice on the high-fat diet, particularly in females (P = 0.08; Supplemental Figure 3). No differences in expression of monocyte chemo-attractant protein-1 (Mcp-1) and myeloperoxidase (Mpo) were found (P= 0.4 and 0.3, respectively). Both wild-type and transgenic females on high-fat diet showed significantly higher mRNA levels of inflammatory markers, compared to males (Supplemental Figure 3).

Results

Figure S2 Plasma concentrations of glucose, triglycerides and cholesterol in Dlk1tg/− and littermate control mice

Plasma values of glucose, triglycerides and cholesterol were significantly higher in male than female mice on a high-fat diet, but there was no difference in response between Alfp-Dlk1tg/− and control mice. Values are depicted as mean ± SEM. (*: P ≤ 0.05; n=8 animals per group).
Chapter 4 Liver-specific overexpression of Dlk1 aggravates high fat diet-induced steatosis in mice

The effects of DLK1 overexpression are influenced by diet and sex

The effects of DLK1 overexpression on genes of the lipogenic and the Notch signaling pathway were modulated to different degrees by the fat content of the diet and the sex of the experimental animal. Table 1 shows the results of a 3-way ANOVA analysis of these effects and their interactions. Diet and sex had strong, independent effects on gene expression in liver, whereas the independent effects of DLK1 overexpression were generally weaker or even absent and replaced with strong interactions between DLK1.
Part I

overexpression and diet. In fat-pad, effects of diet and sex were mostly confined to the lipogenic genes, while the effects of DLK1 overexpression were mostly determined by an interaction between DLK1 overexpression and sex. The statistical analysis, therefore, clearly shows that the fat content of the diet largely determined the effects of DLK1 overexpression in the liver. We, therefore, describe the effects of a low-fat diet and high-fat diet on the effects of DLK1 overexpression separately.

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Table 1 Effects of diet, sex and genotype and their interactions on the expression of lipogenic genes and members of the Notch-signaling pathway

The outcome of a 3-way ANOVA test is shown. Significance was set at P < 0.05 (highlighted in grey). See the main text for a detailed description of these results.
Male Alfp-Dlk1\textsuperscript{tg/-} mice on low-fat diet have decreased Notch1 and Sox9 mRNA levels in liver

To explore the effects of hepatic DLK1 overexpression on adipogenic/lipogenic regulatory genes and the Notch pathway, mRNA abundance of Pparγ, Cebpα, Fas Scd1, Notch1, Hes1 and Sox9 were assessed in livers of Alfp-Dlk1\textsuperscript{tg/-} and control mice on a low-fat diet (Figure 4A-G, LFD bars). Sox9 was recently shown to be a downstream target of both Dlk1 and Notch1 (11, 20). Only in males, both Notch1 and Sox9 mRNA expression was significantly decreased in Alfp-Dlk1\textsuperscript{tg/-} livers compared to controls.

No differences in the mRNA abundance of these genes were found in the gonadal fat-pads of either sex (Figure 5A-G, LFD bars).

Results

Figure 4 Increased mRNA levels of key transcription factors of the Notch-signaling and lipogenesis pathways in livers of Alfp-Dlk1\textsuperscript{tg/-} and control mice

Panels A-G show mRNA levels of lipogenic and Notch pathway genes in livers of male and female mice on a low- and high-fat diet. On a low-fat diet, the mRNA abundance of Notch1 and Sox9 mRNA was significantly decreased in Alfp-Dlk1\textsuperscript{tg/-} males (panels E and G, respectively; LFD bars), whereas on a high-fat diet their levels increased to that of controls (panels E and G, respectively; HFD bars), with also significantly increased mRNA levels of Pparγ and Hes1 (panels A and F, respectively). A low-fat diet did not affect the mRNA levels of the investigated genes in Alfp-Dlk1\textsuperscript{tg/-} females (panels A-F). On a high-fat diet, however, the mRNA levels of Pparγ, Fas, Notch1, Hes1, and Sox9 were significantly increased in female Dlk1\textsuperscript{tg/-} mice compared to their littermate controls (panels A-G, HFD bars). Values are depicted as mean ± SEM. (*: P≤ 0.05; **: P ≤ 0.001; n=8 animals per group).
Results

A high-fat diet increases the expression of lipogenic and Notch pathway genes in Alfp-Dlk1tg/- mice

In agreement with the more severe steatosis observed in Alfp-Dlk1tg/- mice on a high-fat diet, the expression of the lipogenic transcription factor Pparγ was significantly increased in livers of both male and female Alfp-Dlk1tg/- mice compared to control littermates (Figure 4A, HFD bars), while that of Cebpα was not significantly increased (Figure 4B, HFD bars). Furthermore, the expression of the lipogenic enzyme Fas was significantly increased in livers of female Alfp-Dlk1tg/- mice, while that of Scd1 was not (P=0.14; Figure 4C, D, HFD bars). Interestingly, the hepatic mRNA levels of Notch1 and its downstream target Sox9 were no longer downregulated in Alfp-Dlk1tg/- males (as was seen on the low-fat diet) and were significantly upregulated in Alfp-Dlk1tg/- females on high-fat diet (Figure 4E, G, HFD bars). The Notch downstream target Hes1 was significantly upregulated in transgenic livers of both sexes (Figure 4F, HFD bars).

In fat-pads of female Alfp-Dlk1tg/- mice on high-fat diet, the expression of Pparγ, Cebpα, Fas, Scd1, Hes1 and Sox9 were all significantly increased, but these effects were not found in males (Figure 5, HFD bars).

Figure 5 Increased mRNA levels of key transcription factors of the Notch signaling and lipogenic pathways in fat-pads of Alfp-Dlk1tg/- and control mice

Panels A-G show mRNA levels of adipogenic and lipogenic regulatory and Notch-pathway genes in fat-pads of male and female Alfp-Dlk1tg/- and control mice on a low- or high-fat diet. On a low-fat diet, the mRNA abundance of none of the investigated genes was different between Alfp-Dlk1tg/- and littermate control mice. In contrast, the expression of Pparγ, Cebpα, Fas, and Scd1 increased in female Alfp-Dlk1tg/- mice on a high-fat diet (panels A-D, HFD bars). Furthermore, expression of Hes1 and Sox9 increased in female Alfp-Dlk1tg/- mice (panels F,G, HFD bars). No changes in expression of these genes were found in similarly treated Alfp-Dlk1tg/- males. Values are depicted as mean ± SEM. (*: P ≤ 0.05; n=8 animals per group).
Chapter 4 Liver-specific overexpression of Dlk1 aggravates high fat diet-induced steatosis in mice
In the present study, we found that mice with liver-specific overexpression of full-length DLK1 show increased expression of lipogenic genes and increased Notch signaling in livers of both sexes and in adipose tissue of females, when fed a high-fat diet. To assess the adipogenic/lipogenic effects of DLK1 overexpression in liver, we studied a transgenic line with strong, homogeneous DLK1 expression in liver. Despite high circulating levels of DLK1 in Alfp-Dlk1tg/− mice (as a result of TACE-mediated cleavage of membrane-bound DLK1), liver and fat-pad weights were not affected in these mice, if they were fed a low-fat diet. In the liver of Alfp-Dlk1tg/− females on the low-fat diet, the expression of lipogenic or Notch pathway genes was not affected, but the expression of Notch1 and Sox9 was downregulated in livers of male Alfp-Dlk1tg/− mice, without effect on Hes1 expression. Although no other studies reporting an effect of DLK1 on Notch1 expression are known to us, we found earlier (chapter 7) in Alfp-Dlk1tg/−/Mdr2 −/− double transgenic mice that DLK1 overexpression also decreased hepatic Notch1 transcript levels compared to those in Mdr2 −/− livers.

Intriguingly, administration of a high-fat diet to Alfp-Dlk1tg/− females caused extensive upregulation of Pparγ, Fas, Scd1, Notch1, Hes1 and Sox9 mRNAs in their livers, and an upregulation of Pparγ, Cebpα, Fas, Scd1, Hes1 and Sox9 mRNAs in their fat-pads. In Alfp-Dlk1tg/− males, the high-fat diet abolished the downregulation of Notch1 and Sox9 mRNA expression in liver that was seen in mice on the low-fat diet and, similar to the transgenic females, significantly increased the expression of Pparγ and Hes1 mRNAs. In the male fat-pads, DLK1 overexpression had no effect on the expression of adipogenic regulatory and Notch pathway genes. These findings suggest that in our Alfp-Dlk1tg/− mice, a high-fat diet induces a stimulatory effect of DLK1 on lipogenesis and Notch signaling and that females are more sensitive to this dietary stimulus, showing lipogenic stimulatory effects in both liver and fat-pad.

The role of the Notch pathway in adipogenesis remains controversial with reportedly both stimulatory and inhibitory roles of Hes1 and Notch1 during adipocyte differentiation (6, 8, 21-23). It was, therefore, proposed that “a proper balance of Notch signaling is critical for adipogenesis to proceed” and that “DLK1 might be a critical factor to control the proper level of Notch signaling for cells to undergo adipogenesis” (6). The present findings which show that the effects of DLK1 overexpression are subject to sex- and diet-dependent modulation may accommodate these earlier findings. The observed effects of sex and diet also bring our findings in line with earlier reports of Dlk1-transgenic mice, which showed that, on a normal chow (that is, low-fat) diet, circulating DLK1 inhibited the development of adipose tissue mass in a dose-dependent way (12, 13). Concentration-dependent DLK1 effects on adipogenesis were found in adult male mice after transfection of the liver with a full-length Dlk1-expressing plasmid (13). Although differences in circulating DLK1 concentration and biological differences between the DLK1/hFc fusion protein and the circulating DLK1 protein in our Alfp-Dlk1tg/− mice may account for some differences between these earlier and our present findings, we propose that the presently available...
information is compatible with our hypothesis that both sex and fat content of the diet determine the effects of DLK1 on lipogenesis and Notch pathway expression in liver and adipose tissue. Based on the finding of upregulated Hes1 levels in the steatotic livers of our Alfp-Dlk1<sup>tg/-</sup> mice on the high-fat diet, we hypothesise that an external lipogenic stimulus, such as a high-fat diet, induces a switch in DLK1 function and turns it into a Notch-signaling activator which can also induce Pparγ expression (23, 24), thereby exacerbating the high-fat diet-induced hepatic steatosis through enhanced activation of lipogenic genes (25, 26). However, the Pparγ upregulation in transgenic males is not accompanied by upregulation of lipogenic enzymes Fas and Scd1, implying that the observed steatosis in transgenic males and females partly develops via alternate mechanisms, probably via other Pparγ downstream targets in males. In agreement with sex-dependent effects on lipid metabolism, previous reports indicate that estrogens might influence the nonalcoholic fatty liver phenotype (27-29).

In summary, we showed that the effects of liver-specific overexpression of full-length Dlk1 depend both on the sex of the mouse and the fat content of the diet, being inhibitory on Notch1 expression in males on a low-fat diet, but stimulatory on Notch and lipogenic gene expression, especially in females on a high-fat diet for 4 weeks. The lipogenic effects of circulating (soluble) DLK1 in the fat-pads of female mice show that the stimulatory effect of DLK1 on adipose tissue is not dependent on the membrane-bound form of DLK1, but, instead, depends on the sex of the mouse and its diet.
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