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Dual Roles for the Notch Target Gene *Hes-1* in the Differentiation of 3T3-L1 Preadipocytes

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The process of adipogenesis involves a complex program of gene expression that includes down-regulation of the gene encoding Hes-1, a target of the Notch signaling pathway. To determine if Notch signaling affects adipogenesis, we exposed 3T3-L1 preadipocytes to the Notch ligand Jagged1 and found that differentiation was significantly reduced. This effect could be mimicked by constitutive expression of Hes-1. The block was associated with a complete loss of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) induction and could be overcome by retroviral expression of either C/EBPα or PPARγ2. Surprisingly, small interfering RNA (siRNA)-mediated reduction of Hes-1 mRNA in 3T3-L1 cells also inhibited differentiation, suggesting an additional, obligatory role for Hes-1 in adipogenesis. This role may be related to our observation that both Notch signaling and Hes-1 down-regulate transcription of the gene encoding DLK/Pref-1, a protein known to inhibit differentiation of 3T3-L1 cells. The results presented in this study establish a new target downstream of the Notch–Hes-1 pathway and suggest a dual role for Hes-1 in adipocyte development.

The Notch proteins are cell surface receptors activated by the Delta and Jagged/Serrate families of ligands. Interaction with a ligand leads to two proteolytic cleavage events that release the Notch intracellular domain (NICD) from the plasma membrane (3, 23). NICD translocates into the nucleus, where it interacts with the DNA binding protein CSL (CBF-1, Suppressor of Hairless, LAG-1, RBP-J) (1). In the absence of NICD, CSL represses transcription through interactions with a corepressor complex (13, 18, 42). NICD displaces the corepressor complex from CSL and replaces it with a transcriptional activation complex that includes NICD, Mastermind, p300, and possibly PCAF (10, 15, 38, 41). Several genes that are directly activated by the NICD-CSL complex have been identified. The best characterized of these are the *HES* and *HRT* families of genes, all of which encode transcriptional repressors (8, 12, 14, 24).

Hes-1 is a basic helix-loop-helix (bHLH) DNA binding protein related to *Drosophila* Hairy and Enhancer of Split proteins and forms homodimers as well as heterodimers with other HLH proteins (30). Recruitment of the TLE/Groucho family of corepressors through a WPRW motif establishes one mode of transcriptional repression when Hes-1 is targeted to DNA (8, 12). However, functional studies with PC12 neuronal cells indicate that, while DNA binding is critical to Hes-1 activity, the WRPW motif is largely dispensable (4). In this case, Hes-1 may function by simply blocking access of activators that bind to the same promoter or enhancer sites. Finally, Hes-1 may also inhibit transcription by forming inactive heterodimers with other bHLH proteins, such as MyoD and E47 (30).

Notch signaling is generally thought to control cell fate decisions by inhibiting the development of certain lineages and/or promoting the development of others. For neuronal development, when a precursor cell receives a Notch signal, NICD induces Hes-1. Hes-1 directly inhibits the expression of proneural bHLH factors, such as Mash1, and thus inhibits neuronal differentiation (16). By contrast, Notch signaling induces the differentiation of neural precursor cells into many of the glial lineages by an as yet unknown pathway (22, 39). And, while Notch and Hes-1 have been found to phenocopy one another in both gain-of-function and loss-of-function studies of neuronal differentiation, this example is relatively rare. Typically, Hes-1 accounts either for none or for some of Notch's effects (19, 31, 35), underscoring the need to identify additional Notch targets.

Hes-1 was identified among a large group of genes whose expression is down-regulated during adipogenesis in vitro and in vivo (33). Although *Hes-1* expression can be stimulated by growth factors (7), its connection to the Notch pathway prompted us to examine the relationship between Notch signaling and adipogenesis. We found that Notch signaling through Hes-1 can profoundly inhibit the differentiation of 3T3-L1 preadipocytes. Interestingly, artificially reducing *Hes-1* expression also inhibited adipogenesis, and this correlated with the induction of the adipogenic inhibitor DLK/Pref-1. Our data suggest that Hes-1 has two roles in adipogenesis: one promotes adipogenesis, possibly through the down-regulation of inhibitory proteins such as DLK/Pref-1, and the other inhibits adipogenesis at a step prior to the induction of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ).

MATERIALS AND METHODS

Retroviral vectors and transduction. MIGR and MIGR-NICD have been described previously (29). MIGR–Hes-1 was a gift from W. Pear (University of Pennsylvania), and retroviral plasmids pMSCV, pMSCV-PPARγ2, and pMSCV-C/EBPα were from M. Lazar (University of Pennsylvania). The pSIREN-siLUC and pSIREN-siHes-1 retroviral constructs were generated in accordance with the manufacturers' instructions (BD Biosciences and Clontech). The target sequence for the Hes-1 siRNA was 5′-CGACACCGGACAAACCAAA-3′. A detailed

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description of the pSIREN-siHES-1 construct is available upon request. Production of retroviral supernatant fluid and infection of NIH 3T3 and 3T3-L1 cells were performed as described previously (26). Populations of NIH 3T3 and 3T3-L1 cells transduced with MIGR, MIGR–Hes-1, and MIGR-NICD were generated by infection and subsequent sorting for green fluorescent protein-positive cells by fluorescence-activated cell sorting. Cell populations harboring pMSCV, pMSCV-PPARγ2, pMSCV-C/EBPα, pSIREN-siLUC, and pSIREN-siHes-1 were generated by infection followed by selection with 2 μg of puromycin/ml.

Cell culture and differentiation assays. All cells were maintained in Dulbecco's modified Eagle medium (Gibco), supplemented with 10% fetal bovine serum, penicillin-streptomycin, glutamine, and the appropriate selective agent if needed. Details of the coculture assays are available upon request. SUP-T1 cells were treated with 0.01 mM γ-secretase inhibitor X (Calbiochem) or an equivalent amount of dimethyl sulfoxide (DMSO) for 48 h and then harvested for protein or RNA.

Differentiation of 3T3-L1 cells was performed by first growing cells to confluence (day −2) and then shifting them to differentiation medium (DM) after 48 h (day 0). DM contained 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 15 μg of insulin/ml. DM lacking dexamethasone and IBMX was used as a control. Medium was changed to growth medium containing only insulin (GM; 15 μg/ml) after 48 h (day 2). Staining with Oil Red O was typically performed on day 7.

Notch signaling was generated by immobilizing a soluble form of Jagged1 on plates. The expression vector for the Myc-tagged soluble Jagged1 was a gift from W. Pear. Briefly, an expression vector for either a control or a Myc-tagged, secreted form of Jagged1 (amino acids 1 to 393; an extracellular fragment that includes the DSL domain) was transfected into 293T cells, and medium was changed after 24 h. Medium was harvested after 48 h and filtered through a 0.45-μm-pore-size filter. Plates treated with the anti-Myc antibody 9E10 were then treated with control media or media containing secreted Jagged1 for 4 h at 37°C and washed. These plates were then used to grow 3T3-L1 cells.

Western and RT-PCR analyses. Western blotting was performed by standard protocols. The rabbit antibody against cleaved Notch1/NICD (val1744) was from Cell Signaling Technology (Beverly, Mass.). This antibody recognizes the NICD only when it has been appropriately cleaved by γ-secretase at valine 1744. Other antibodies for PPARγ, C/EBPα, SREBP-1/ADD-1, C/EBPβ, Dlk1, and Cdk4 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Total RNA for reverse transcriptase PCR (RT-PCR) was prepared with the RNeasy kit (Qiagen). Semiquantitative RT-PCR assays were performed with various inputs of first-strand cDNA and amplified for 24 cycles in the presence of 5 μCi of [α-^{32}P]dATP. PCR products were separated on a 4% nondenaturing polyacrylamide gel and quantitated with a phosphorimager (Molecular Dynamics). All PCR products were sequenced to verify amplification of the correct cDNA. Primers sequences are available upon request.

Reporters and transfections. To generate the DLK-1400 reporter, a PstI-BsrB1 fragment of the mouse DLK1 promoter, comprising nucleotides −1395 to +138, was cloned into pGL2-Basic (Promega). A KpnI-BsrB1 fragment comprising nucleotides −191 to +138 was subcloned into pGL2-Basic to create the DLK-191 reporter. The genomic clone for the Dlk1 promoter region was a kind gift from J. Schmidt (Northwestern University). Transfections were carried out with Fugene 6 (Roche) in accordance with the manufacturer's instructions. Activities of the reporters (50 ng each) were determined by measuring firefly luciferase activity and normalized to that of *Renilla* luciferase (2.5 ng of pRL-CMV; Promega). All transfections were performed at least in triplicate, and results are shown as the averages ± standard errors of the means.

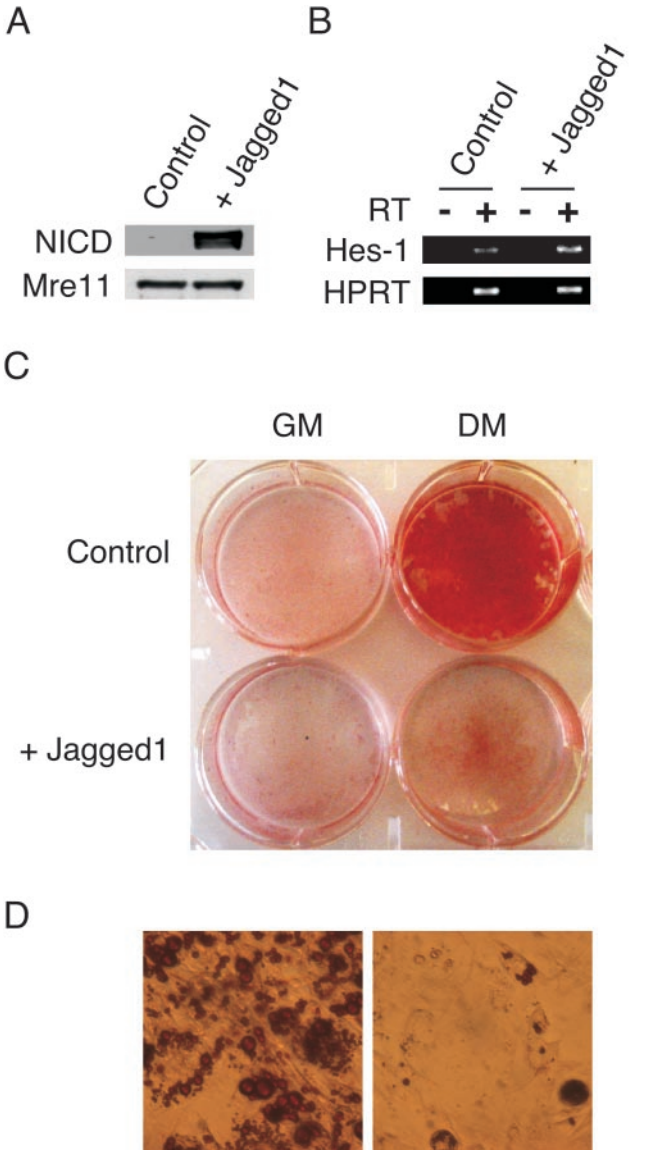

FIG. 1. Constitutive Notch signaling inhibits differentiation of 3T3-L1 cells. (A and B) Analysis of NICD and Mre11 (loading control) protein by Western immunoblotting (A) and Hes-1 and hypoxanthine phosphoribosyltransferase (HPRT; loading control) RNA by RT-PCR (B) in 3T3-L1 cells cultured on control plates or plates containing immobilized Jagged1. RT, reverse transcriptase. (C) Effect of Jagged1 on 3T3-L1 cell differentiation. Cells were transferred to either DM or GM and stained with Oil Red O after 7 days. (D) Micrograph of Oil Red O-stained 3T3-L1 cells from control plates (left) and Jagged1 plates (right).

RESULTS

Notch signaling inhibits differentiation of 3T3-L1 cells. To investigate the effect of Notch signaling on adipogenesis, we exposed 3T3-L1 cells to the Notch ligand Jagged1. Jagged1 generates a Notch signal if it is expressed on the surface of a neighboring cell or if it is immobilized on a culture dish (36). For convenience and to avoid the complications of a coculture assay, we adopted the latter approach, adhering a secreted form of Myc-tagged Jagged1 to the surface of a culture dish with an anti-Myc antibody. Control plates contained the antibody but lacked the ligand. 3T3-L1 cells grown in the presence of immobilized Jagged1 generated easily detectable amounts of NICD, as measured by Western immunoblotting with an antibody specific for the N-terminal Val^{1744} generated upon cleavage of Notch by γ-secretase (Fig. 1A). They also expressed a higher level of the Notch target *Hes-1* (Fig. 1B). These data confirm that 3T3-L1 cells contain endogenous Notch receptors that can be activated by a ligand.

When 3T3-L1 cells are grown to confluence and then exposed to DM (containing IBMX, insulin, and dexamethasone), they undergo adipogenesis and accumulate lipids, which stain positive with Oil Red O. This process occurred normally when

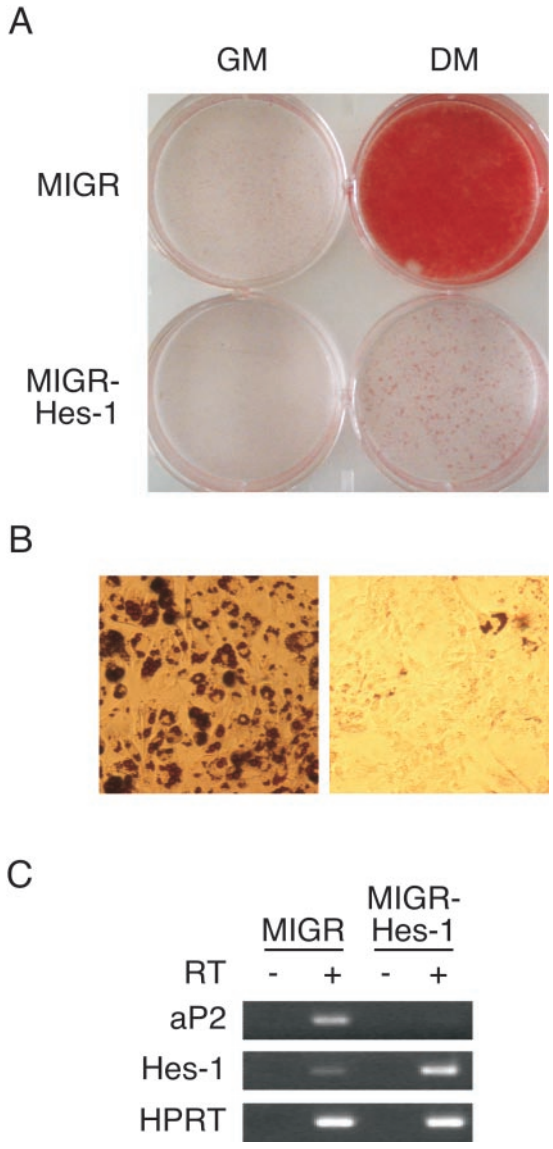

FIG. 2. Constitutive expression of Hes-1 inhibits differentiation of 3T3-L1 cells. (A) 3T3-L1 cell populations transduced with control (MIGR) or Hes-1 (MIGR–Hes-1) retroviruses were induced to differentiate in DM or maintained in GM and stained with Oil Red O after 7 days. (B) Micrograph of MIGR (left) and MIGR–Hes-1 (right) cells cultured in DM for 7 days. (C) RT-PCR analyses of aP2, Hes-1, and hypoxanthine phosphoribosyltransferase (HPRT; loading control) RNAs in the indicated cells grown in DM after 7 days. RT, reverse transcriptase.

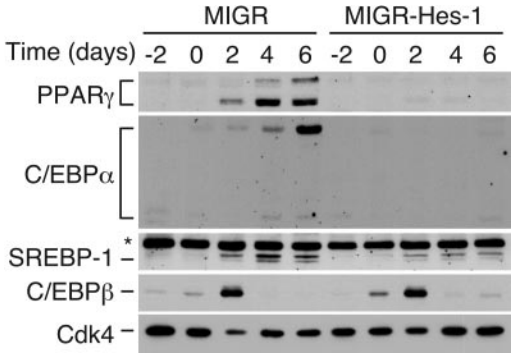

FIG. 3. PPARγ and C/EBPα are not induced in 3T3-L1 cells harboring the MIGR–Hes-1 virus. Control (MIGR) and Hes-1-expressing (MIGR–Hes-1) cells were grown to confluence (day −2) and transferred to DM (day 0), and the expression of PPARγ, C/EBPα, SREBP-1 (ADD-1), C/EBPβ, and Cdk4 (loading control) proteins was determined after the indicated number of days. PPARγ and C/EBPα are each expressed as two isoforms. Asterisk, position of a nonspecific cross-reacting protein.

3T3-L1 cells were grown on plates lacking the immobilized Jagged1 but not on plates containing the ligand (Fig. 1C and D). These data indicate that Notch signaling can repress adipogenesis.

Hes-1 blocks 3T3-L1 differentiation. The ability of Notch to induce *Hes-1*, combined with the observation that *Hes-1* is down-regulated during adipogenesis in vitro and in vivo (33), prompted us to examine the effect of constitutive Hes-1 expression on adipogenesis. After confirming that *Hes-1* is indeed down-regulated during differentiation of 3T3-L1 cells (data not shown), we transduced cells with a Hes-1-expressing retrovirus. Cells harboring the MIGR parental retrovirus differentiated normally, as assayed by Oil Red O staining (Fig. 2A and B). By contrast, differentiation of cells harboring the Hes-1-expressing virus was blocked. While a few cells escaped, we attribute this to the analysis of cell populations (as opposed to clones), with some cells likely expressing very low or no exogenous Hes-1. Importantly, after cells were cultured in DM, the terminal differentiation marker aP2 (fatty acid binding protein) was almost completely absent in Hes-1-expressing 3T3-L1 cells relative to controls (Fig. 2C). We conclude that the Notch target gene *Hes-1* is sufficient to mediate the effects we observe when activating the endogenous Notch receptor in 3T3-L1 cells.

Adipogenesis requires the orchestration of a well-defined program of gene expression (28). To ascertain the stage at which cells undergoing Notch signaling or expressing Hes-1 were blocked, we evaluated the expression of several transcription factors known to be regulated during differentiation (Fig. 3). C/EBPβ, which normally is induced early and down-regulated by day 4, was unaltered in the Hes-1-expressing cells. By contrast, PPARγ and C/EBPα, which normally are induced beginning at day 2 and whose expression remains elevated throughout adipogenesis, were virtually absent in the Hes-1-expressing cells. Experiments in which we blocked differentiation with immobilized Jagged1 gave similar results (data not shown). Interestingly, SREBP-1/ADD-1 was induced normally up to day 2 in the Hes-1-expressing cells but never showed the additional increase observed in the control cells. Although the SREBP-1/ADD-1 protein shown in Fig. 3 is the cleaved, transcriptionally active p68 protein fragment, expression of the full-length (~128-kDa) protein showed a similar profile (data not shown).

Expression of PPARγ2 or C/EBPα rescues differentiation of Hes-1-blocked 3T3-L1 cells. To determine if Notch and Hes-1 are epistatic to C/EBPα or PPARγ, we evaluated the abilities of C/EBPα and PPARγ to overcome the differentiation block. We transduced normal 3T3-L1 cells with retroviruses expressing either C/EBPα or PPARγ2 or with a parental control virus (MSCV; Fig. 4A). Transduced populations were then induced

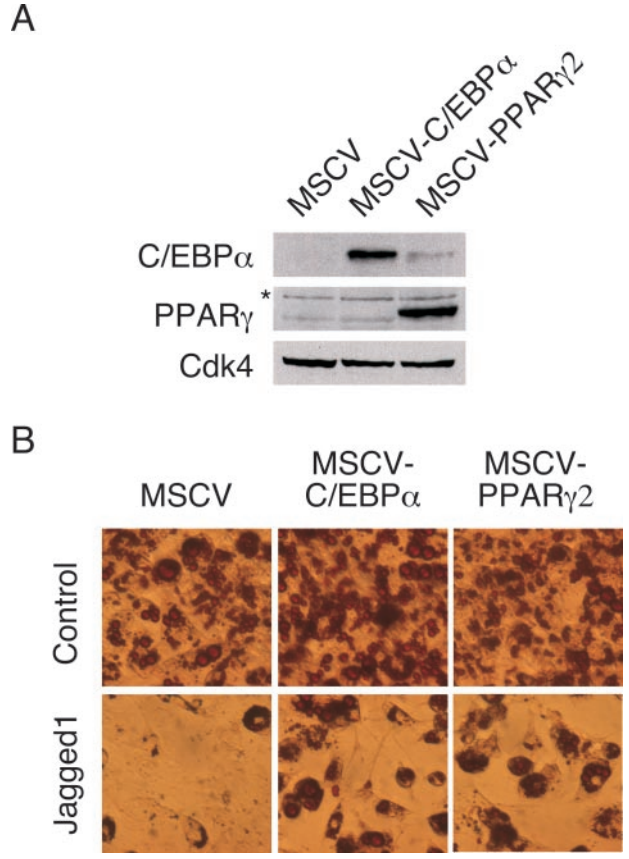

FIG. 4. Constitutive expression of C/EBPα or PPARγ2 allows differentiation of 3T3-L1 cells undergoing Notch signaling. (A) Analyses of C/EBPα, PPARγ, and Cdk4 (loading control) proteins in 3T3-L1 cells transduced with MSCV, MSCV-C/EBPα, or MSCV-PPARγ2. Asterisk, position of a nonspecific cross-reacting protein. (B) Micrographs of Oil Red O-stained 3T3-L1 cells transduced with the indicated retroviruses and induced to differentiate on control plates and those containing immobilized Jagged1.

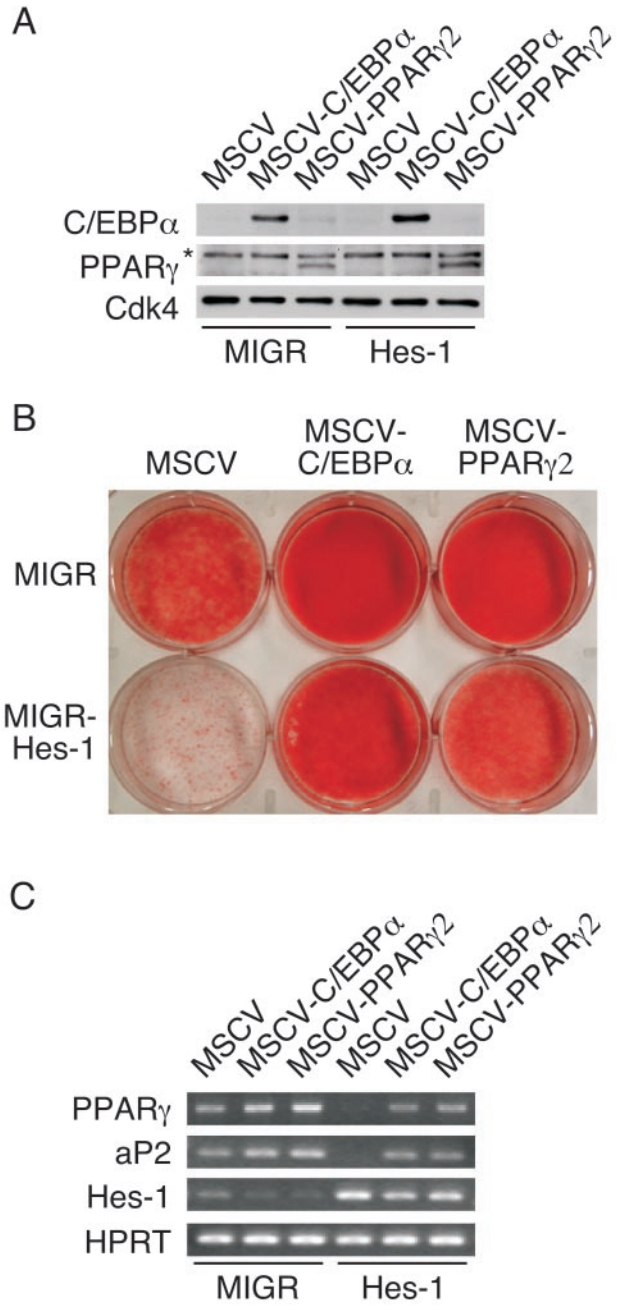

FIG. 5. Constitutive expression of C/EBPα or PPARγ2 allows differentiation of 3T3-L1 cells expressing Hes-1. (A) Analyses of C/EBPα, PPARγ, and Cdk4 (loading control) proteins in 3T3-L1 cells transduced with MIGR or MIGR–Hes-1 and with MSCV, MSCV-C/EBPα, or MSCV-PPARγ2, as indicated. Asterisk, position of a nonspecific cross-reacting protein. (B) MIGR and MIGR–Hes-1 3T3-L1 cells were transduced individually with MSCV, MSCV-C/EBPα, or MSCV-PPARγ2, and puromycin-resistant populations were induced to differentiate and stained with Oil Red O after 7 days. (C) Cells induced to differentiate were evaluated for expression of PPARγ, aP2, Hes-1, and hypoxanthine phosphoribosyltransferase (HPRT; control) RNAs by RT-PCR.

to differentiate in the presence or absence of immobilized Jagged1. 3T3-L1 cells containing the parental retrovirus accumulated significantly less lipid in the presence of Jagged1. By contrast, 3T3-L1 cells containing either the C/EBPα virus or the PPARγ2 virus showed significant lipid accumulation in the presence of Jagged1 (Fig. 4B). We also transduced Hes-1-expressing cells (and control MIGR cells) with the C/EBPα- or PPARγ2-expressing virus (Fig. 5A) and examined the effect on differentiation. Both retroviruses were able to rescue the block imposed by Hes-1, as measured either by Oil Red O staining or by expression of aP2 (Fig. 5B and C). These data indicate that the differentiation blocks imposed by Notch signaling and Hes-1 occur prior to or at the point of C/EBPα and PPARγ induction.

C/EBPα and PPARγ stimulate each other's expression, yet adipogenesis can occur in the absence of C/EBPα but not in the absence of PPARγ (27). Since C/EBPα was able to rescue the blocks imposed by Notch and Hes-1, we reasoned that endogenous PPARγ must have been induced. Indeed, PPARγ was induced in the cells expressing both Hes-1 and C/EBPα (Fig. 5C), indicating that Notch and Hes-1 do not block induction of the PPARγ promoter. Experiments involving transfection of NIH 3T3 cells with reporter plasmids containing the PPARγ1 promoter also failed to show a direct inhibition by Hes-1 in the context of either cotransfected Hes-1 expression vectors or stably integrated *Hes-1* (data not shown).

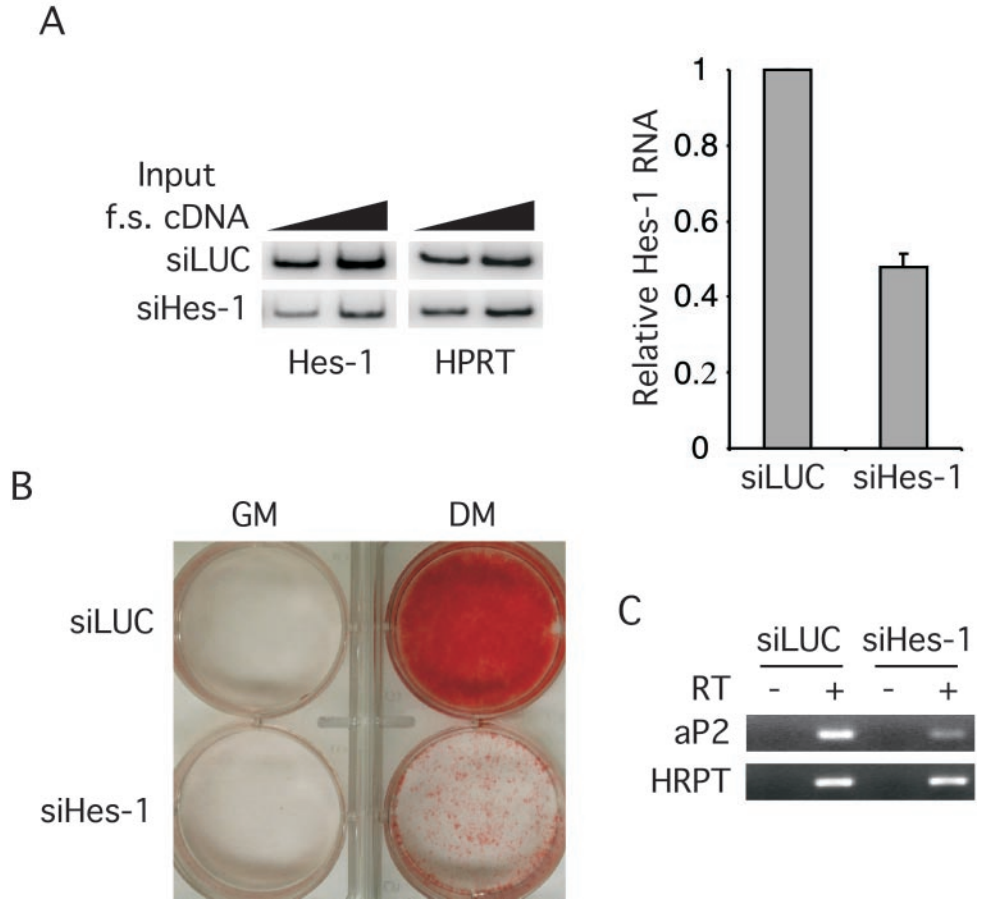

FIG. 6. Reduced Hes-1 expression inhibits differentiation of 3T3-L1 cells. (A) 3T3-L1 cells harboring the pSIREN-siLUC (siLUC) or pSIREN-siHES-1 (siHES-1) retroviruses were analyzed for expression of Hes-1 and HPRT (control) RNA by semiquantitative RT-PCR. The amount of input reverse-transcribed first-strand cDNA (input f.s. cDNA) is represented by black triangles. The bar graph represents the relative levels of Hes-1 mRNA in the pSIREN-siLUC (siLUC) or pSIREN-siHES-1 (siHES-1) 3T3-L1 cells. Hes-1 levels are expressed relative to those in siLUC cells and normalized with hypoxanthine phosphoribosyltransferase (HPRT). (B) 3T3-L1 cells containing control (siLUC) or siHes-1 viruses were transferred to DM or maintained in GM and stained with Oil Red O after 7 days. (C) Cells in panel B were assessed for expression of aP2 and HPRT (control) RNA by RT-PCR. RT, reverse transcriptase.

Hes-1 is required for 3T3-L1 differentiation. Experiments involving ligand-induced Notch signaling and retroviral expression of Hes-1 both represent gain-of-function experiments. As such, they alone do not demonstrate if Notch signaling is a normal regulator of adipogenesis or if Hes-1 expression in preadipocytes is a consequence of Notch signaling. Ideally, we would want also to show that adipogenesis is stimulated by a reduction in Notch signaling and/or Hes-1 activity. Given that Notch signaling requires the action of γ-secretase (2, 5), we treated 3T3-L1 cells with a γ-secretase inhibitor and determined the effect on Hes-1 expression. We found that the relatively low basal level of NICD was extinguished and that Hes-1 RNA was cut in half (data not shown), arguing that Notch signaling accounts for roughly one-half of the Hes-1 RNA expressed in 3T3-L1 preadipocytes. However, we could not assess the effects of lowering Hes-1 levels on differentiation using the γ-secretase inhibitor since its solvent alone (DMSO) adversely affected differentiation. As an alternative, we employed a virus engineered to make a small interfering RNA (siRNA) directed against Hes-1 (siHes-1) and obtained 3T3-L1 cells with about 50% of the normal level of Hes-1 RNA (Fig. 6A). Surprisingly, while the control cells (harboring a retrovirus that generates an siRNA against luciferase [siLuc]) differentiated normally, the siHes-1 cells differentiated poorly (Fig. 6B). This was reflected by the low level of aP2 induction (Fig. 6C). We conclude that, in addition to blocking adipogenesis, Hes-1 is also required for adipocyte differentiation.

The DLK/Pref-1 gene is responsive to Notch/Hes-1 signaling. In parallel studies, an assessment of global changes in gene expression in NIH 3T3 cells expressing NICD identified a significant reduction in the expression of DLK/Pref-1 (D. A. Ross and P. K. Rao, unpublished observations). DLK/Pref-1 is a protein decorated with EGF repeats (hence, Delta-like or DLK); it exists in both membrane-bound and secreted forms and is a potent inhibitor of 3T3-L1 cell differentiation (32, 37). Like Hes-1, DLK/Pref-1 is down-regulated during adipogenesis. Our finding that NICD leads to a reduction of DLK/Pref-1 expression in NIH 3T3 cells suggested the possibility that reduced expression of Hes-1 might result in increased levels of DLK/Pref-1 in 3T3-L1 preadipocytes. Indeed, siHes-1 cells

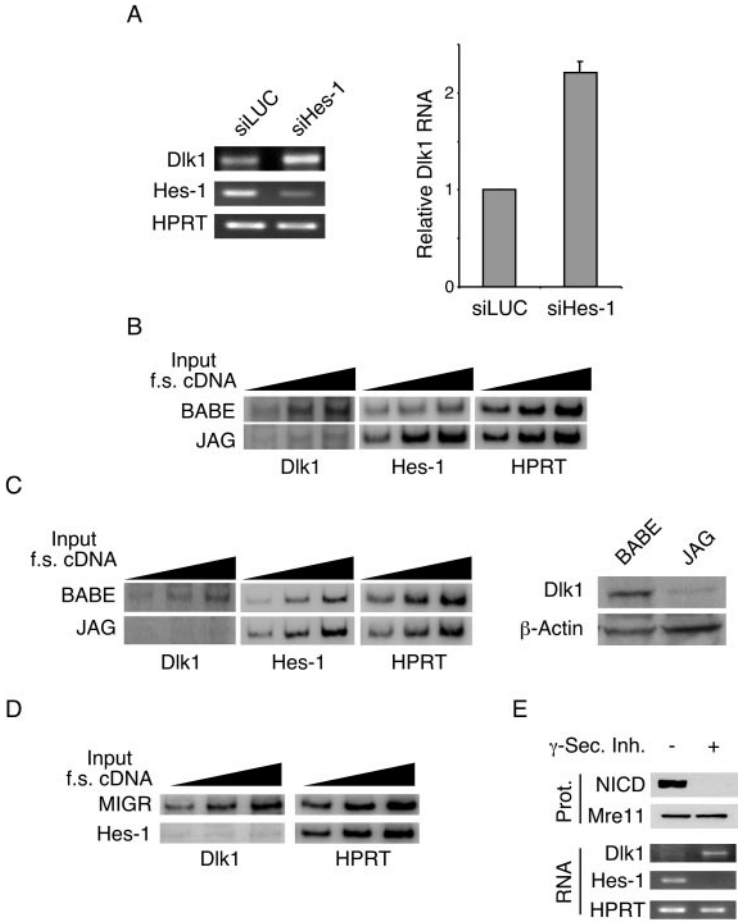

FIG. 7. DLK1 is a Notch/Hes-1 target. (A) 3T3-L1 cells containing low levels of Hes-1 express high levels of Dlk1. 3T3-L1 cells transduced with pSIREN-siLUC (siLUC) or pSIREN-siHES-1 (siHES-1) were assessed for expression of Dlk1, Hes-1, and hypoxanthine phosphoribosyltransferase (HPRT; control) RNA by RT-PCR. The bar graph represents the relative levels of Dlk1 RNA in the siLUC and siHES-1 cells normalized to HPRT, as measured by semiquantitative RT-PCR. (B) Ligand-mediated activation of Notch reduces the level of Dlk1 RNA in 3T3-L1 cells. 3T3-L1 cells were cocultured with control (BABE) or Jagged1 (JAG)-expressing NIH 3T3 cells. Total RNA from the 3T3-L1 cells was assessed for expression of Dlk1, Hes-1, and HPRT (control) RNAs by semiquantitative RT-PCR. Increasing amounts of input reverse-transcribed first-strand cDNA (input f.s. cDNA) are represented by black triangles. (C) Ligand-mediated activation of Notch reduces the level of Dlk1 in NIH 3T3 cells. NIH 3T3 cells were cocultured with control (BABE) or Jagged1 (JAG)-expressing cells and analyzed as described for panel B (left). NIH 3T3 cells were also evaluated for the expression of Dlk1 and β-actin (control) protein by Western immunoblotting (right). (D) NIH 3T3 cells expressing Hes-1 contain reduced amounts of Dlk1. NIH 3T3 cells harboring control (MIGR) or Hes-1-expressing (MIGR–Hes-1) retroviruses were assessed for Dlk1 and HPRT (control) RNA by semiquantitative RT-PCR as for panel B. (E) SUP-T1 cells treated with a γ-secretase inhibitor have increased levels of Dlk1. SUP-T1 T-ALL cells were treated with γ-secretase inhibitor X or DMSO and assessed for expression of NICD and Mre11 (control) protein by Western immunoblotting and for Dlk1, Hes-1, and HPRT (control) RNA by RT-PCR.

contained roughly twice as much DLK/Pref-1 RNA as siLuc cells (Fig. 7A). In addition, while DLK/Pref1-1 is down-regulated during differentiation of siLuc cells, it was not down-regulated in siHes-1 cells (data not shown). To confirm that DLK/Pref-1 is regulated by Notch signaling in 3T3-L1 cells, we carried out an experiment in which 3T3-L1 cells were cocultured with either control NIH 3T3 cells or NIH 3T3 cells engineered to express Jagged1. Exposure of 3T3-L1 cells to membrane-bound Jagged1 led to an increase in Hes-1 and a reduction in DLK/Pref-1 mRNAs (Fig. 7B). DLK/Pref-1 levels were also reduced in 3T3-L1 cells transduced with a Hes-1-expressing retrovirus (data not shown).

The response of DLK/Pref-1 to components of the Notch signaling pathway is not limited to 3T3-L1 cells. Reductions in DLK/Pref-1 protein and/or mRNA levels were also observed in NIH 3T3 cells exposed to Jagged1 (Fig. 7C) or transduced with a virus expressing Hes-1 (Fig. 7D). Treatment of Sup-T1 T-ALL cells, which express NICD constitutively due to a chromosomal translocation that interrupts the Notch1 gene (6, 40), with a γ-secretase inhibitor led to dramatic decreases in NICD and Hes-1 expression, with a concomitant increase in DLK/Pref-1 (Fig. 7E).

We next analyzed the response of reporter plasmids containing the DLK/Pref-1 promoter. NIH 3T3 cells harboring either the parental or NICD-expressing viruses were transiently transfected with the luciferase reporter plasmids pGL2-Pro (containing the simian virus 40 early promoter), CSL-Luc (containing a promoter directly activated by the NICD/CSL complex), DLK-191, or DLK-1400. The last two contain the DLK/Pref-1 promoter extending 191 or 1,400 bp upstream of

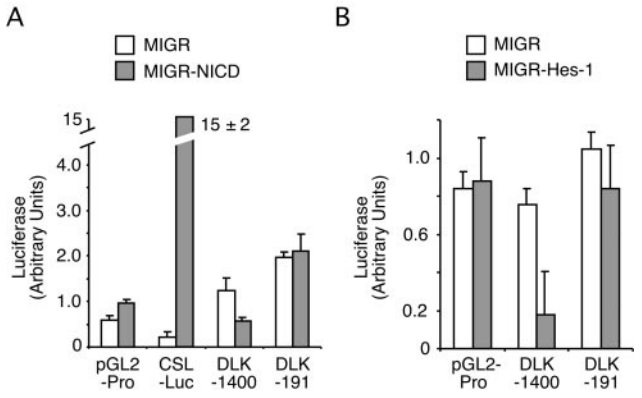

FIG. 8. The Dlk1 promoter is inhibited by NICD and by Hes-1. (A) NIH 3T3 cells harboring either a control (MIGR) or NICD-expressing (MIGR-NICD) retrovirus were transfected with the reporter plasmids pGL2-Pro, CSL-Luc, DLK-1400, and DLK-191. Luciferase values are expressed relative to a control *Renilla* luciferase reporter. (B) Activity of the same reporters was determined in cells containing a Hes-1-expressing virus (MIGR–Hes-1). Mean values and standard errors of the means were determined from at least three individual experiments.

the transcriptional start site, respectively. While pGL2-Pro was minimally affected by NICD, CSL-Luc was activated 75-fold. DLK-1400 was inhibited by roughly 50%, while DLK-191 was unaffected. Similar results were obtained with NIH 3T3 cells harboring a virus expressing Hes-1 (Fig. 8B). We conclude that the DLK/Pref-1 promoter contains a Hes-1 response element(s) that maps between bp −191 and −1400 upstream of the transcriptional start site. Although this region contains several potential Hes-1 binding sites, we have not been able to demonstrate conclusively that Hes-1 binds these sites in vivo. Nevertheless, taken together, our data indicate that the DLK/Pref-1 gene lies downstream of *Hes-1* in the Notch signaling pathway and is repressed either directly or indirectly through its promoter.

DISCUSSION

We have shown that Notch signaling and constitutive Hes-1 expression can each inhibit the differentiation of 3T3-L1 preadipocytes. Because Hes-1 is a direct target of the Notch signaling pathway, we propose that Notch signaling inhibits adipogenesis by stimulating and maintaining Hes-1 expression. The mechanism by which Hes-1 blocks differentiation, however, is not clear. Hes-1 acts distal to C/EBPβ induction and prior to or at the point of PPARγ and C/EBPα induction. Our finding that the Hes-1 block can be rescued by either C/EBPα or PPARγ argues that Hes-1 does not affect the activity of either of these transcription factors per se. Indeed, since PPARγ is required for adipogenesis, the rescue by C/EBPα indicates that its ability to induce PPARγ is not significantly impaired by Hes-1. More than a dozen additional, as yet uncharacterized transcription factors are induced during the early stages of adipogenesis, and any one of these may be a relevant Hes-1 target.

Hes-1 is down-regulated during adipogenesis in vitro and in vivo (33). Our data indicate that down-regulation is required for adipogenesis, yet we do not know the particular role of Notch signaling. NICD is expressed at low levels in 3T3 L1 preadipocytes, and roughly 50% of the Hes-1 expression in preadipocytes is dependent on γ-secretase, suggesting that *Hes-1* responds to Notch. The level of Notch signaling must therefore decrease during the course of adipocyte differentiation, and we have found that the level of NICD does in fact fall between days 2 and 6 (data not shown). However, Hes-1 expression can be stimulated by growth factors (7), and its down-regulation during adipogenesis could also be a consequence of reductions in the activity of certain mitogenic signaling pathways. The relative roles played by Notch and by other pathways that impinge on *Hes-1* expression remain to be determined. The ability of Notch to regulate adipogenesis in vivo may define not only the extent of fat cell development but also the tissues where adipocytes form. Bone marrow stromal cells, for example, have the capacity to generate adipocytes but normally do not because of the action of inhibitory cytokines that steer development toward the osteoblast lineage (34). Similarly, the expression of Notch ligands in a particular tissue may insure that resident progenitor cells do not choose an adipocyte fate.

In light of Hes-1's ability to inhibit adipogenesis, we were surprised by our results demonstrating a requirement for Hes-1. The concomitant induction of DLK/Pref-1 provides one explanation. Our data show that the level of Hes-1 in 3T3-L1 cells is sufficient to inhibit gene transcription, specifically transcription of the DLK/Pref-1 gene, and that only a 50% reduction in Hes-1 is needed to increase transcription of Hes-1 targets. We propose that Hes-1 may help keep the expression of adipogenic inhibitors low, thereby promoting the earliest stages of differentiation. In this regard it is interesting that while DLK/Pref-1 is highly expressed in 3T3-L1 preadipocytes and is down-regulated during adipogenesis, it is not expressed at high levels in preadipocytes in vivo (33). This raises the possibility that in vivo Hes-1 is even more effective at keeping the level of DKL/Pref-1 low. There is some dispute as to whether DLK/Pref-1 can inhibit adipogenesis in the presence of insulin (our DM contains insulin), and so the block to differentiation by siHes-1 may be due to increased expression of one or more additional proteins. Again, dozens of uncharacterized genes in addition to the DLK/Pref-1 gene are down-regulated during adipogenesis and all of these represent potential targets of Hes-1 in preadipocytes.

A previous study using antisense Notch-1 constructs and putative inhibitors of ligand-mediated signaling concluded that Notch is required for adipogenesis (11). A plausible explanation for this stems from our observations that 50% of the Hes-1 expression in 3T3-L1 preadipocytes is dependent on Notch and that a 50% reduction in Hes-1 (by using siRNA) is sufficient to block differentiation. Consequently, antisense Notch would be expected to reduce Hes-1 levels and promote the expression of inhibitory proteins, such as DLK/Pref-1. A model consistent with all of these observations is presented in Fig. 9. We propose that Hes-1 is required early in adipogenesis and may function by keeping the expression of adipogenic inhibitors low. However, once differentiation is initiated, Hes-1 levels must fall and allow the induction of an as yet unidentified protein or proteins (protein X) that stimulate adipogenesis. We propose further that such a protein(s) functions upstream of PPARγ and C/EBPα.

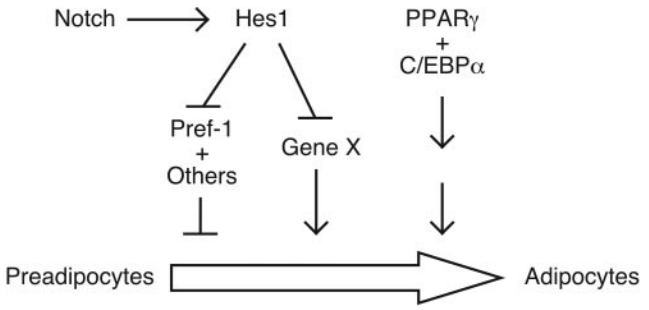

FIG. 9. Model depicting the role of Notch/Hes-1 signaling in differentiating 3T3-L1 cells.

DLK/Pref-1 is a secreted protein whose mode of action is unknown. In addition to its putative role in adipogenesis, DLK/Pref-1 has also been implicated directly in lymphocyte development (17), hematopoiesis (25), and hematopoietic stem cell regeneration (21). It is expressed in most embryonic cell types, and expression declines during development of most, but not all, tissues (9). It is also referred to as fetal antigen 1 due to its presence in amniotic fluid. The gene resides within an imprinted region on mouse chromosome 12 (syntenic with a region of human chromosome 14), and mice lacking DLK/Pref-1 display a variety of phenotypes including morbidity, increased adiposity, and skeletal malformations (20). Some of these phenotypes are consistent with those seen in cases of human maternal uniparental disomy of chromosome 14. Our data identify DLK/Pref-1 as being downstream of Hes-1 and raise the possibilities that DLK/Pref-1 may regulate Notch signaling by feedback and/or mediate some of Notch's known effects on differentiation. The former possibility is suggested by the protein's EGF repeats, which are reminiscent of those found within the extracellular domains of both Notch and its ligands. Despite these structural similarities, however, DLK/Pref-1 affects neither Jagged1 activity nor Notch signaling activity in coculture experiments (D. A. Ross unpublished observations). The possibility that DLK/Pref-1 down-regulation is critical for any of Notch's specific effects on cellular differentiation, including adipogenesis, remains to be determined. Nevertheless, the identification of DLK/Pref-1 as a Notch target should provide insights into the mechanisms though which Notch exerts its diverse effects on cell growth and differentiation.

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