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Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway

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SUMMARY

Notch controls cell fate by inhibiting cellular differentiation, presumably through activation of the transcriptional regulator human C promoter Binding Factor (CBF1), which transactivates the *hairy* and *Enhancer of split (HES-1)* gene. However, we describe constitutively active forms of Notch1, which inhibit muscle cell differentiation but do not interact with CBF1 or upregulate endogenous HES-1 expression. In addition, Jagged-Notch interactions that prevent the expression of muscle cell specific genes do not involve the upregulation of endogenous HES-1. In fact, exogenous expression of HES-1 in C2C12 myoblasts does not block myogenesis. Our data demonstrate the existence of a CBF1-independent pathway by which Notch inhibits differentiation. We therefore propose that Notch signaling activates at least two different pathways: one which involves CBF1 as an intermediate and one which does not.

Key words: Notch, CBF1, HES-1, muscle cell differentiation, mouse, myoblast

INTRODUCTION

Studies in vertebrates and invertebrates suggest that the LIN12/Notch receptors inhibit cellular differentiation when activated by members of the DSL (for Delta, Serrate, Lag2) ligand family (reviewed by Greenwald, 1994; Artavanis-Tsakonas et al., 1995; Lewis, 1996). However, while such Notch-mediated inhibition of differentiation has been demonstrated both in vitro and in vivo, the intracellular signaling pathway activated by ligand-Notch interactions is not well understood. Genetic and biochemical studies have implicated the homologous proteins *Drosophila* Suppressor of Hairless [Su(H)], *C. elegans* LAG-1 (Christensen et al., 1996) and mammalian RBP-Jk/CBF1/KBF2 (hereafter referred to as CBF1) in the LIN12/Notch signaling pathway (reviewed by Honjo, 1996; Lewis, 1996). Results from some of these studies suggest that in response to Notch activation the CSL proteins, for CBF1, Su(H) and LAG-1 (Christensen et al., 1996), upregulate expression of the *Enhancer of split* [E(spl)] genes in *Drosophila* or the homologous mammalian *HES-1* gene. Since the *E(spl)* and *HES* genes encode basic helix-loop-helix (bHLH) transcription factors, the CSL proteins may represent an important link between ligand activation of Notch at the cell surface and signal transduction to the nucleus.

Notch signaling has been investigated using either ligand-mediated activation of wild-type Notch or constitutively active, ligand-independent, mutant forms of Notch (reviewed by Greenwald, 1994; Artavanis-Tsakonas et al., 1995; Lewis, 1996). The intracellular domain of Notch produces a dominant, gain-of-function phenotype when expressed in *Drosophila*, indicating that this mutant form of Notch has intrinsic signaling activity similar to that produced by ligand activation of intact Notch. Structurally similar forms of mouse Notch1 have been associated with direct activation of CBF1 and transactivation of the *HES-1* promoter (Jarriault et al., 1995; Tamura et al., 1995; Hsieh et al., 1996). Since these same Notch1 mutant proteins also block MyoD-induced myogenic conversion of fibroblasts, it has been suggested that Notch signaling prevents myogenesis through the upregulation of HES-1 (Jarriault et al., 1995; Kopan et al., 1996), a transcriptional repressor that antagonizes the functional activity of MyoD (Sasai et al., 1992). We have shown that fibroblasts expressing the Notch1 ligand Jagged can suppress the differentiation of Notch1-expressing myoblasts (Lindsell et al., 1995). Specifically, Jagged-mediated activation of Notch1 prevents induction of MyoD and myogenin in contacted myoblasts. Therefore, whether activated in a ligand-independent or ligand-dependent manner, Notch1 activation prevents induction of MyoD, which is required for muscle cell differentiation. Although Notch signaling has been associated with activation of CBF1 and transactivation of *HES-1*, it is not known if these events are

required for the block in myogenesis induced by activated Notch1.

The sequences in Notch1 that are required to activate both exogenous and endogenous CBF1 have been mapped to a stretch of 114 amino acids located between the transmembrane domain and ankyrin repeats (Tamura et al., 1995; Hsieh et al., 1996). Constitutively active forms of Notch that inhibit myogenesis contain all or most of these sequences, consistent with their ability to activate CBF1 and transactivate *HES-1* (Kopan et al., 1994; Nye et al., 1994; Jarriault et al., 1995; Kopan et al., 1996). To determine if CBF1 activity is required for Notch to inhibit myogenesis, we designed truncated cytoplasmic forms of Notch1 that lack most or all of the sequences required for Notch1-CBF1 interactions (reviewed by Honjo, 1996). Here we show that these cytoplasmic forms of Notch1, although unable to activate CBF1 or upregulate endogenous HES-1, nonetheless prevent muscle cell differentiation when stably expressed in C2C12 myoblasts. In fact, expression of only the six Notch1 ankyrin repeats is sufficient to prevent muscle cell differentiation. In addition, we report that interactions between Jagged-expressing fibroblasts and Notch1-expressing myoblasts, while preventing the induction of muscle specific genes, do not lead to the upregulation of endogenous HES-1. Based on these results, we conclude that interactions between Notch1 and CBF1, and the resulting upregulation of endogenous HES-1, are not required for myogenic inhibition induced by Notch signaling. Consistent with this notion, exogenous expression of HES-1 in C2C12 cells does not block myogenesis. Taken together our data imply that Notch signaling involves activation of an undefined CBF1-independent pathway in addition to the CBF1-dependent pathway.

MATERIALS AND METHODS

Constructs, transfections, retroviral infections, and cell culture

The following wild-type and mutant Notch1 cDNA sequences were engineered in the mammalian expression vector pEF1α-BOS (Mizushima and Nagata, 1990) to encode the following amino acids (GenBank accession number X57405): N1, 1-2531; ΔEDN1, 1-182 plus 1470-2531; ZEDN1, 1-24 plus 1712-2531; 0CDN1, 1-1759; CDN1, 1848-2531; and CDCN1T, 1873-2078 in frame with 3 copies of the influenza virus hemagglutinin (HA) epitope YPYDVPDYA. All constructs were confirmed by DNA sequencing. The details of these constructions are available upon request.

C2C12 mouse myoblasts (ATCC) were cotransfected with the Notch1 constructs described above or with pBOS-HES-1 and the neomycin resistance gene. Stable expressing cell lines were selected with 400 μg/ml G418 (GIBCO) and identified by immunofluorescence, northern and western analyses using protocols described below. The Jagged-expressing Ltk^+ fibroblast cell line (JT) has been described previously (Lindsell et al., 1995). To control for clonal variation, a number of independent cell lines were developed and examined in this study; data from a representative clone are presented for each different Notch1-expressing cell type.

Cells were cultured in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM; GIBCO/BRL) supplemented with 10% fetal bovine serum (FBS) and 5% Cosmic Calf serum (CCS; HyClone). The C2C12 cell fusion and coculture assays have been described in detail previously (Lindsell et al., 1995). Muscle cell differentiation was induced by culturing cells in DMEM containing 10% horse serum (HS).

CDN1 cDNA sequences were subcloned into pSRαMSVtkneo (Muller et al., 1991) in the antisense orientation and infectious virus was produced and used to infect cells at a 0.1 multiplicity of infection, and 48 hours postinfection cells were induced to differentiate for 5 days.

Northern blot analysis

RNA isolation and northern blot analysis was performed as previously described (Weinmaster et al., 1992). After electrophoresis and transfer to nylon membrane (MSI), RNA (10 μg/ml) was stained with methylene blue to verify that equal amounts of RNA were transferred. The myogenin probe corresponds to the 3′ untranslated region of the mRNA from nucleotides 791 to 1486, the Id probe was a 900 bp *Xba*I fragment excised from pBK/RSV-ID1, the MLC2 probe was a 700 bp *Eco*RI fragment released from pV2LC2, and the HES-1 probe was a 1.2 kbp *Xba*I fragment isolated from pBOS-HES1 (a generous gift from R. Kageyama and Y. Sasai).

Generation of Notch-specific antibodies and Western analysis

Notch1-glutathione S-transferase (GST) fusion proteins were engineered to encode Notch1 extracellular amino acids 381-853 (PCR2) or Notch1 intracellular amino acids 2286-2531 (PCR3). The encoded GST-Notch1 proteins were generated and purified using Pharmacia protocols. Polyclonal sera specific for the Notch1 extracellular domain (5261) or Notch1 cytoplasmic domain (93-4) were generated following immunization of rabbits with purified GST-Notch1 fusion proteins using standard procedures.

C2C12 cells and stable Notch1-expressing C2C12 cells (N1, ΔEDN1, 0CDN1, CDN1, and CDCN1T) were grown in 100-mm dishes, washed twice with PBS and lysed in 500 μl hot SDS sample buffer. Specific proteins were identified following SDS/PAGE, transfer to Immobilon-P (Millipore), probing with 5261 (1:5000), 93-4 (1:5000) or 12CA5 (1:1000) and detection using ECL™ (Amersham). Membranes were exposed to BIOMAX film (Kodak) and the resulting images were scanned using ScanMaker III (Microtek) and reproduced for publication using Photoshop (Adobe) software.

CBF1 transactivation assay

Notch1 cDNA sequences encoding N1, ΔEDN1, ZEDN1, 0CDN1, CDN1 or CDCN1T were subcloned into pSRαMSVtkneo and 1 μg of each of these plasmids were analyzed in HeLa cells following cotransfection with 2 μg of either 4×wtCBF1Luc or 4×mtCBF1Luc plasmids. Transient transfection and luciferase assays were performed in triplicate as described previously (Hsieh et al., 1996).

RESULTS

Stable expression of full-length or mutated forms of Notch1 in C2C12 myoblasts

Notch-related genes encode cell surface proteins whose structural motifs have been highly conserved (Fig. 1A; reviewed by Greenwald, 1994; Artavanis-Tsakonas et al., 1995). To identify the structural motifs important for Notch activity we engineered a number of cDNA constructs which express various forms of Notch1 shown in Fig. 1A. Briefly, the N1 construct contains the complete coding sequence of rat Notch1 (Weinmaster et al., 1991), ΔEDN1 lacks more than 80% of the extracellular ligand-binding domain, ZEDN1 encodes the signal peptide and is predicted to initiate 11 amino acids N-terminal to the transmembrane domain, and 0CDN1 lacks almost the entire cytoplasmic domain. CDN1 lacks the signal peptide, the entire extracellular domain, the transmembrane

region, and cytoplasmic sequences containing the first putative nuclear localization signal as well as most of the characterized CBF1-binding sites (Tamura et al., 1994; Hsieh et al., 1996); yielding a soluble cytoplasmic form of Notch1. The CDCN1T construct encodes only the 6 ankyrin repeats followed by a triple tandem repeat of the HA epitope tag. Deletion of the ankyrin repeats in *Drosophila* Notch results in either a loss-of-function or a dominant-negative phenotype, depending upon the genetic background (reviewed by Greenwald, 1994). However, the function of these repeats on their own has yet to be reported. A GLP-1 protein composed primarily of ankyrin repeats is active, but this variant also contains 52 amino acids N-terminal to the ankyrin repeats as well as 33 C-terminal flanking residues, which may have additional functions to those encoded by the ankyrin repeats (Roehl and Kimble, 1993).

The C2C12 mouse myoblast cell line has been widely used as a model system for myogenesis, since the differentiation program of these cells is readily induced, easily monitored, and highly reproducible. Expression of full-length Notch1 in C2C12 cells was detected with both the extracellular domain and cytoplasmic domain antisera by western analysis (Fig. 1B, lanes 2, 6). The calculated relative molecular mass of full-length Notch1 is approx. 300×10^3, and this form migrates in SDS/PAGE well above the 200×10^3 M_r marker (Fig. 1B, lanes 2, 6). The full-length Notch1 (Aster et al., 1994) and Notch2 proteins (Zagouras et al., 1995) undergo processing to yield a smaller molecular mass species of approx. 110-120×10^3. Although the functional significance of this processing is currently unknown, the sequences required for this proteolytic cleavage have been mapped to residues 1655-1661 in mouse Notch1 (Kopan et al., 1996; the position is indicated by ◊ in Fig. 1A). Consistent with these findings a fragment of approx. 120×10^3 M_r is also detected in N1-expressing C2C12 cells (Fig. 1B, lane 2; ∗). The 120×10^3 M_r Notch1 fragment is likely to contain the cytoplasmic domain since it is specifically recognized by antibodies raised against the cytoplasmic sequences of Notch1 (Fig. 1B, lane 2), but not by antibodies directed against the EGF-like repeats of Notch1 (Fig. 1B, lane 6). In addition to the 140×10^3 M_r unprocessed ΔEDN1, the 120×10^3 M_r C-terminal fragment was also detected in cells expressing ΔEDN1 (Fig. 1B, lane 3), consistent with this Notch1 mutant protein encoding the predicted proteolytic sites. Expression of the CDN1 construct in muscle cells produced a protein with a molecular mass of approx. 80×10^3 (Fig. 1B, lane 4).

Western analysis using the Notch1 extracellular domain-specific antibody, detected an N-terminal processed fragment of Notch1 with an apparent molecular mass of approx. 180×10^3 (Fig. 1B, lane 6; ∗). Therefore, using both the extracellular and cytoplasmic domain specific Notch1 antisera we can identify the N-terminal (180×10^3 M_r) and C-terminal (120×10^3 M_r) fragments derived from the processing of full-length Notch1 (300×10^3 M_r). 0CND1 encodes a protein with a predicted molecular mass of approx. 180×10^3 that was readily detected in 0CDN1-expressing cells (Fig. 1B, lane 7). The broad band observed for 0CDN1 probably represents both the full-length and processed forms since the predicted sequences required for this Notch1 processing are encoded by 0CDN1. Cells expressing the CDCN1T construct exhibit a protein of approx. 30×10^3

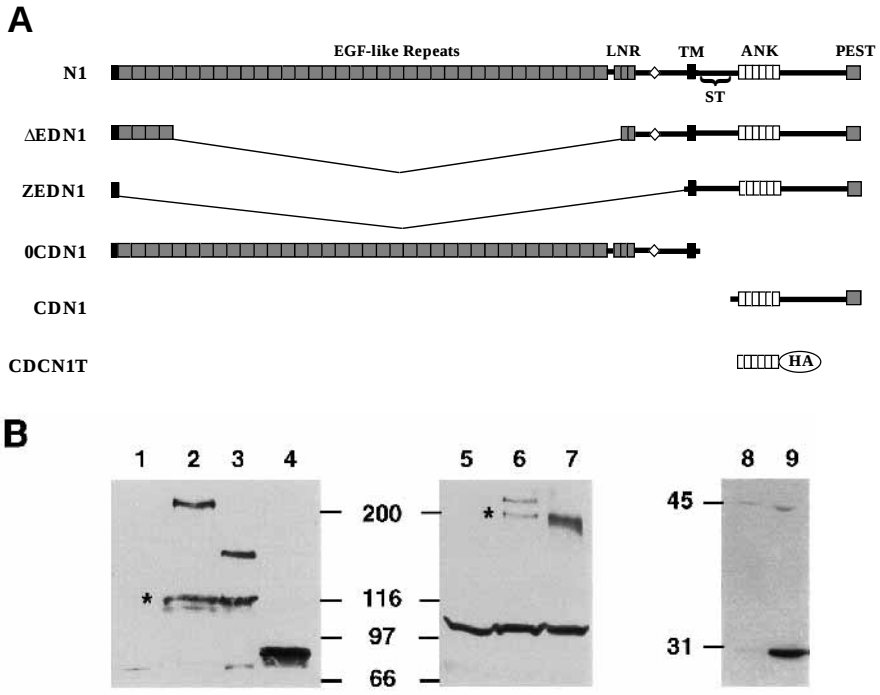

Fig. 1. Schematic representation of full-length and mutant Notch1 proteins and characterization of these proteins expressed in stable C2C12 myoblasts. (A) Approximately two-thirds of the Notch1 protein is extracellular, composed mainly of 36 tandem repeats related to epidermal growth factor (EGF)-like repeats that are required for ligand binding, and 3 cysteine-rich repeats (LNR) that are also found in the related *C. elegans* proteins LIN-12 and GLP-1 (reviewed by Greenwald, 1994). The potential proteolytic cleavage site discussed in the text, is indicated by ◊. The cytoplasmic domain contains 6 repeats, related to a motif found in ankyrin (ANK), which are necessary for function of both the invertebrate and vertebrate LIN12/Notch family members. Physical and functional interactions between CBF1 and Notch1 require sequences (ST) located between the transmembrane domain and the ankyrin repeats (reviewed by Honjo, 1996). All LIN12/Notch proteins terminate with a PEST sequence rich in proline, glutamic acid, serine and threonine. See Materials and Methods for the specific amino acids encoded by these Notch1 constructs. (B) Western analysis of N1, ΔEDN1, 0CDN1, CDN1, and CDCN1T proteins expressed in C2C12 cell lines using Notch1 cytoplasmic (93-4) or extracellular (5261) specific antisera or HA-specific antibodies (12CA5). Whole cell protein lysates from C2C12 cells (lanes 1, 5, 8) or C2C12 cells expressing N1 (lanes 2 and 6), ΔEDN1 (lane 3), CDN1, (lane 4), 0CDN1, (lane 7) or CDCN1T, (lane 9) were resolved by SDS/PAGE in 8% (lanes 1-7) or 10% (lanes 8, 9) gels, electrophoretically transferred to membranes, probed with either 93-4 antiserum (lanes 1-4), 5261 antisera (lanes 5-7) or 12CA5 (lanes 8, 9) and the specific proteins were detected by chemiluminescence. Positions of the molecular mass markers are shown ($M_r\times10^3$) and * indicates proteolytic cleavage fragments.

M_r that was detected using the 12CA5 monoclonal antibody, which recognizes the HA epitope tag (Fig. 1B, lane 9).

Cytoplasmic forms of Notch1 that lack CBF1-interacting sequences block muscle cell differentiation

When C2C12 myoblasts are incubated in medium containing HS they undergo growth arrest and display morphological and molecular changes indicative of muscle cell differentiation. To determine the effects of expression of full-length or mutant forms of Notch1 on C2C12 myoblast differentiation, non-transfected parental cells or cells stably expressing the various forms of Notch1 were first cultured in medium containing 10% FBS and 5% CCS to induce growth and then the culture medium was changed to medium containing 10% HS to induce differentiation. Parental C2C12 cells grew as a flat monolayer when cultured in growth medium (Fig. 2A), but in differentiation medium, they fused to form myotubes within 6 days of culturing (Fig. 2B). The Notch1, ΔEDN1, and 0CDN1 expressing C2C12 cells fused with the same morphology and kinetics as parental C2C12 cells when cultured in differentiation medium (data not shown). In contrast, C2C12 myoblasts expressing either CDN1 or CDCN1T proteins were unable to form multinucleated myotubes in response to the serum change, even after 9-10 days in differentiation medium (Fig. 2D,F).

The morphological differentiation of C2C12 cells shown in Fig. 2 is brought about by the induction of muscle regulatory genes (*MyoD*, *myogenin*, *Myf*5, and *MRF-4*; reviewed by Lassar et al., 1994) that specify muscle cell fate by regulating the expression of muscle structural genes, such as myosin light chain 2 (MLC2). Parental C2C12 cells did not express myogenin at the time of the medium change (day 0); however, after 2 days in medium containing HS, myogenin and MLC2 were induced (Fig. 3). Consistent with the observed fusion with N1, ΔEDN1 and 0CDN1 expressing cell lines, these cells induced both myogenin and MLC2 when cultured in differentiation media (Fig. 3). In contrast, CDN1 resulted in complete inhibition of muscle cell fusion (Fig. 2D) and >90% decrease in expression of myogenin and MLC2 (Fig. 3). Interestingly expression of CDCN1T, which contains only the 6 Notch1 ankyrin repeats, completely inhibited the induction of myogenin and MLC2 (Fig. 3), indicating that the Notch1 ankyrin repeats are sufficient to prevent muscle cell differentiation. Cytoplasmic forms of Notch1 that contain CBF1-interacting sequences have been shown to inhibit the activity of MyoD and Myf-5 when transiently expressed in fibroblasts (Kopan et al., 1994). It is important to note that in contrast to previously reported repressors of myogenesis, both CDN1 and CDCN1T lack sequences required for Notch1 to interact with CBF1 (Tamura et al., 1995; Hsieh et al., 1996).

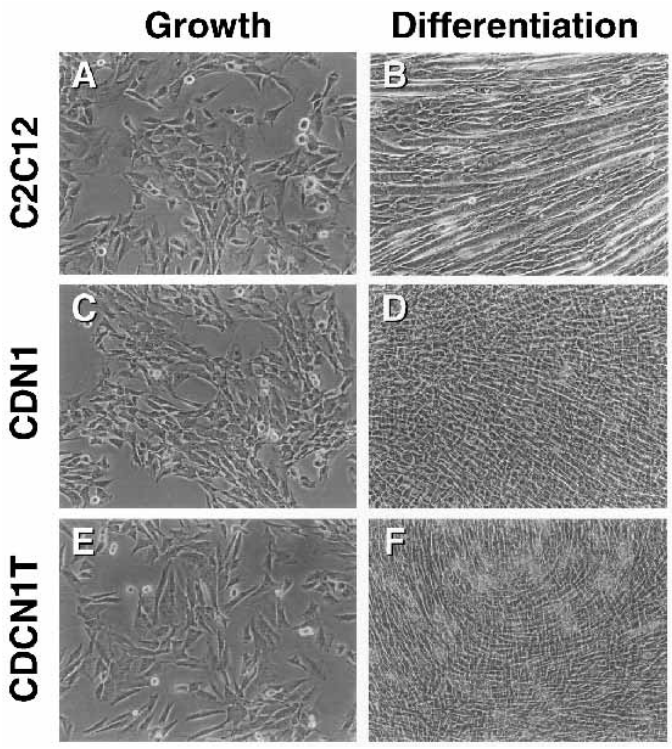

Fig. 2. Activated forms of Notch1 expressed in C2C12 myoblasts inhibit muscle cell fusion. C2C12 cells (A), CDN1 (C), CDCN1T (E) grew as mononuclear myoblasts in medium containing 10% FBS and 5% CCS. These cells were induced to differentiate by replacing the growth medium with medium containing 10% HS. Differentiation medium was replaced daily and after 6 days the cells were examined for the presence of myotubes. Under differentiation conditions C2C12 cells fused to form myotubes (B), while CDN1 (D) and CDCN1T (F) expressing cells were unable to fuse as judged by the lack of myotube formation detected in the monolayers of mononuclear cells.

Expression of CDN1 is required for suppression of muscle cell fusion

It was important to establish whether the block in muscle cell differentiation observed in the isolated CDN1-expressing cell

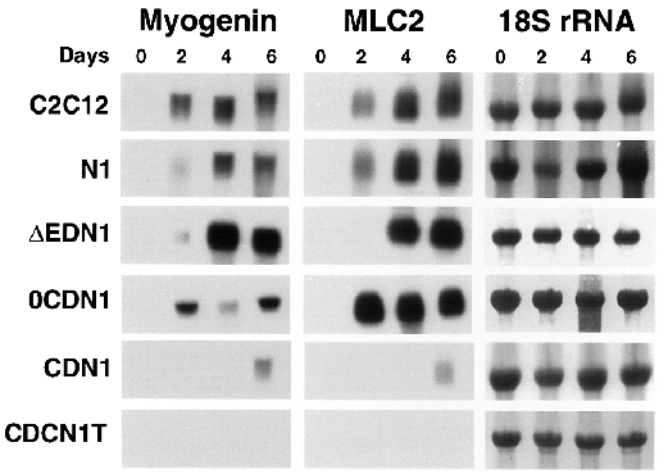

Fig. 3. Activated forms of Notch1 prevent the expression of myogenin and MLC2. Total RNA was isolated from C2C12 cells or N1-, ΔEDN1-, 0CDN1-, CDN1- and CDCN1T-expressing C2C12 cells proliferating in medium containing 10% FBS and 5% CCS (day 0). Three additional sets of these cell lines were incubated in media containing 10% HS to induce differentiation and RNA was harvested every 2 days over a 6 day period (days 2, 4, 6). Isolated total RNAs were analyzed by northern blotting using probes for myogenin and myosin light chain 2 (MLC2). Comparative loading and transfer of RNA was ascertained by methylene blue staining of 18S rRNA.

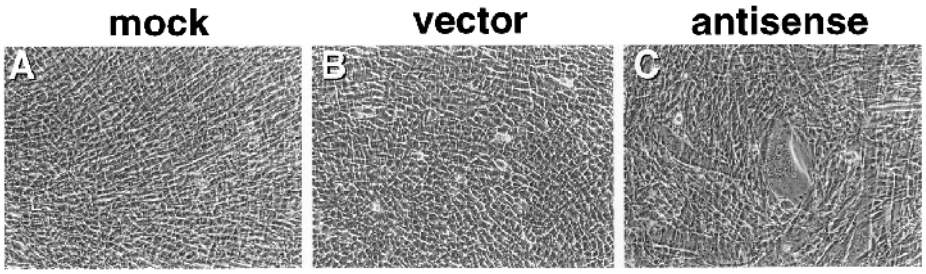

Fig. 4. Infection with CDN1 antisense retrovirus reverses the block in myogenesis induced by the Notch1 cytoplasmic domain. CDN1-expressing C2C12 cells were either mock infected (A) or infected with vector (B) or antisense CDN1 (C) encoding retroviruses. 48 hours postinfection the cells were induced to differentiate by changing the medium to that containing 10% HS and 5 days later the cells were examined for the presence of myotubes. Myotube formation was only detected in cells infected with the antisense virus (C).

lines was due to the expression of the mutant Notch1 protein rather than to the isolation of non-fusing variants from the parental population. For this purpose an antisense CDN1 transcript was expressed from a retroviral vector to determine whether inhibition of CDN1 expression in these lines would allow the cells to differentiate. CDN1-expressing C2C12 myoblasts were mock infected (Fig. 4A) or infected with retroviruses encoding either vector sequences (Fig. 4B) or CDN1 antisense sequences (Fig. 4C), and 48 hours postinfection the medium was changed to that containing HS. Within 5 days, myotubes were obvious on the monolayer of CDN1-expressing cells infected with the CDN1 antisense virus (Fig. 4C) but were not detected in mock-infected cultures (Fig. 4A) or cells infected with virus encoding only vector sequences (Fig. 4B). In addition, the CDN1 cells infected with antisense CDN1 virus and grown in HS expressed high levels of myogenin and MLC2 (data not shown), consistent with the observed cell fusion. These results indicate that the CDN1-expressing cells can differentiate; however, uncompromised expression of CDN1 by C2C12 cells prevents the induction of the muscle cell program.

Activated forms of Notch1 prevent muscle cell differentiation but do not activate endogenous CBF1

Based on previous reports, CDN1 and CDCN1T should not interact with CBF1 (Tamura et al., 1995; Hsieh et al., 1996). However, since these forms of Notch1 repressed myogenesis we determined if CDN1 and CDCN1T could activate endogenous HeLa cell CBF1. To further investigate the relationship between Notch1-induced inhibition of muscle cell differentiation and transactivation by CBF1, the various forms of Notch1 that do not perturb muscle cell differentiation (N1, ΔEDN1 and 0CDN1) were also assayed (Fig. 5).

We have previously reported that the mouse Notch1 cytoplasmic domain, mNotchIC, can interact with endogenous HeLa CBF1 to transactivate a luciferase reporter construct containing multiple CBF1 binding sites (Hsieh et al., 1996). The mouse Notch1 ΔE construct, in which the extracellular domain has been deleted, also interacts with CBF1 to transactivate the *HES-1* promoter (Jarriault et al., 1995) and inhibits MyoD-induced myogenic conversion of 3T3 fibroblasts (Kopan et al., 1994, 1996). Since the rat Notch1 construct, ZEDN1 (Fig. 1A), is similar in structure to ΔE (Jarriault et al., 1995), it serves as a positive control for activation of endogenous CBF1. We assayed the various Notch1 constructs for transactivation of luciferase reporter constructs containing four upstream copies of either wild-type (4×wtCBF1Luc) or mutant (4×mtCBF1Luc) CBF1 binding sites. As previously demonstrated (Hsieh et al., 1996), specific mutation of the CBF1-binding site yields a reporter gene that is not effectively transactivated (Fig. 5). In contrast, the ZEDN1 construct showed an approximate 20-fold transactivation of the wild-type reporter construct (Fig. 5). The high level of transactivation detected with ZEDN1 is consistent with a previous report demonstrating strong transactivation of the HES-1 promoter by mouse Notch1 ΔE (Jarriault et al., 1995). However, the other Notch1 constructs tested, including CDN1 and CDCN1T, transactivated to the same background level produced by the vector with reporter genes containing either wild-type or mutant CBF1 binding elements (Fig. 5). Thus, as predicted CDN1 and CDCN1T do not activate endogenous CBF1 in this assay. The lack of activity detected with CDN1 and CDCN1T corroborates previous data, and

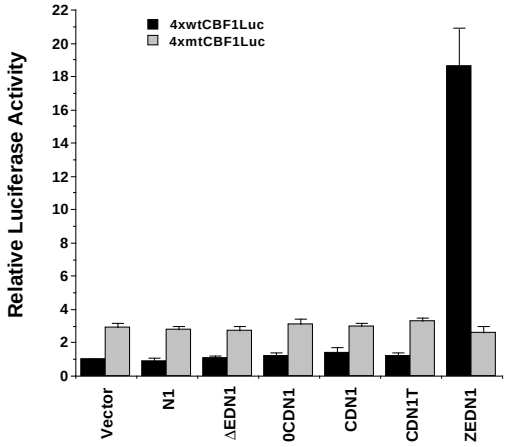

Fig. 5. Constitutively active forms of Notch1 do not transactivate CBF1-luciferase reporter constructs. Transient expression assays with HeLa cells were performed to determine if N1, ΔEDN1, 0CDN1, CDN1, CDCN1T or ZEDN1 expressed in pSRαMSVtkneo could activate endogenous CBF1 to transactivate luciferase reporter constructs carrying either wild-type or mutant CBF1 binding sites. HeLa cells were cotransfected with 1 μg of pSRαMSVtkneo (vector) or the pSRαMSVtkneo Notch1 constructs and 2 μg of either 4×wtCBF1Luc or 4×mtCBF1Luc plasmids. Cell lysates were harvested 48 hours posttransfection and assayed for luciferase activity. The mean and standard deviation from 3 experiments is presented.

confirms that sequences between the transmembrane domain and ankyrin repeats are necessary for the interaction between Notch1 and CBF1 (Tamura et al., 1995; Hsieh et al., 1996). More importantly, these results indicate that CDN1 and CDCN1T function to inhibit muscle cell differentiation of C2C12 myoblasts in a CBF1-independent manner.

Notch1 repressors of myogenesis that function independently of CBF1 do not upregulate HES-1 RNA

Activated forms of Notch1 that inhibit myogenesis can cooperate with CBF1 to transactivate the *HES-1* promoter in HeLa cells (Jarriault et al., 1995). In addition, HES-1 can functionally antagonize E47 and suppress MyoD-induced myogenic conversion of 10T1/2 cells (Sasai et al., 1992). Taken together, these results have raised the possibility that activated Notch1 inhibits muscle cell differentiation through upregulation of endogenous HES-1. However, we were unable to demonstrate activation of CBF1 with either CDN1 or CDCN1T (Fig. 5). Therefore, we determined whether these activated forms of Notch1 induced the upregulation of HES-1. Interestingly, the levels of HES-1 RNA did not appear to change during differentiation of the C2C12 cells (Fig. 6A, lanes 1-4) indicating that HES-1 expression was not regulated during myogenesis. This result was surprising since HES-1 expression has been reported to be developmentally regulated; being high in embryonic muscle and low in adult muscle (Sasai et al., 1992). In fact, expression of either CDN1 or CDCN1T that inhibited muscle cell differentiation did not increase the level of expression of HES-1 as compared to that detected for parental C2C12 cells (Fig. 6). These results are in contrast to those reported with other constitutively active forms of Notch1, which activate CBF1 and transactivate *HES-1* reporter genes transiently expressed in HeLa cells (Jarriault et al., 1995). Consistent with these reports, we have observed increases in HES-1 expression with cytoplasmic forms of Notch that contain sequences required for CBF1 interactions, demonstrating that activated forms of Notch can indeed upregulate endogenous HES-1 in C2C12 myoblasts (data not shown). However, CDN1 lacks most of the sequences required to activate CBF1, and CDCN1T only encodes the ankyrin repeats which cannot physically or functionally interact with CBF1 (Hsieh et al., 1996; Honjo, 1996). The finding that the activated forms of Notch1 examined here do not upregulate endogenous HES-1 suggests that Notch signaling can act independently of CBF1 and HES-1 to block muscle cell differentiation.

Id is also known to inhibit the activity of MyoD by sequestering E47, which is required for the formation of functionally active MyoD/E47 heterodimers (reviewed by Lassar et al., 1994). Id expression was high in proliferating C2C12 myoblasts (Fig. 6A, lane 1) but declined as they were induced to differentiate over a 6 day period (Fig. 6A, lanes 2-4). A similar pattern of RNA expression was observed for Id in C2C12 cells that expressed either CDN1 (Fig. 6B, lanes 1-4) or CDCN1T (Fig. 6C, lanes 1-4) suggesting that these constitutively active forms of Notch1 do not inhibit muscle cell differentiation by upregulating the expression of Id. Similarly, the levels of E12/E47 RNA measured in CDN1 and CDCN1T-expressing cells were similar to those detected in parental C2C12 cells (data not shown), suggesting that the inhibition of myogenesis observed with these activated forms of Notch1 was not due to a lack of E47, which is necessary for MyoD function (reviewed by Lassar et al., 1994).

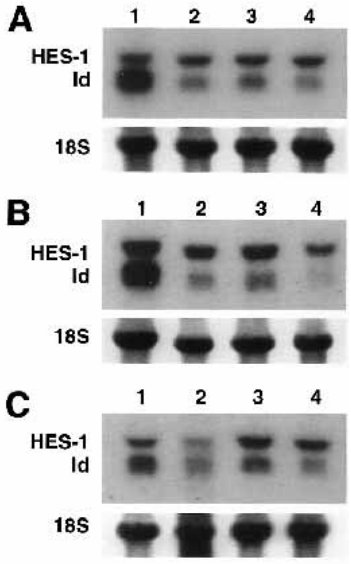

Fig. 6. Activated forms of Notch1 that prevent myogenesis do not upregulate the expression of HES-1 or Id. Total RNA was isolated from proliferating C2C12 cells (A, lane 1), CDN1 (B, lane 1), and CDCN1T-expressing C2C12 cells (C, lane 1). Three additional sets of these cell lines were incubated in medium containing 10% HS for 2 days (A,B,C, lane 2), 4 days (A,B,C, lane 3) and 6 days (A,B,C, lane 4) to induce differentiation. RNA was harvested and analyzed by northern blotting using HES-1 and Id probes. Comparative loading and transfer of RNA was ascertained by methylene blue staining of 18S rRNA.

Ligand-induced activation of Notch1 does not upregulate endogenous HES-1

Jagged activation of full-length Notch1 expressed in C2C12 myoblasts can also block muscle cell differentiation (Lindsell et al., 1995). Therefore, we determined if HES-1 was upregulated in C2C12 cells following ligand-induced activation of Notch1. In this assay, Notch1-expressing C2C12 cells (N1) or parental C2C12 cells were cocultured with either Jagged-expressing fibroblasts (JT) or parental fibroblasts. When these cocultures were incubated in medium containing HS for 5 days, only the combination of JT plus N1 cells was inhibited in differentiation, as determined by the absence of myotubes (Lindsell et al., 1995; data not shown) and MLC2 expression (Fig. 7, lane 6). All other combinations, as well as the C2C12 or N1 cells cultured alone, expressed myogenin and MyoD (data not shown) and MLC2 (Fig. 7, lanes 1-5) in response to the serum change. However, the level of HES-1 RNA detected was similar whether the cells differentiated (Fig. 7, lanes 1-5) or remained as myoblasts (Fig. 7, lane 6). To exclude the possibility that HES-1 RNA was upregulated transiently in response to Notch1 signaling we examined RNA isolated from cocultures of JT and N1 cells or JT and C2C12 cells at 3, 9, 22, 46, and 94 hours following the addition of JT cells. The earliest time point examined was 3 hours to allow time for the JT cells to settle and contact the myoblasts. The levels of HES-1 RNA were unchanged during these time periods (data not shown), suggesting that upregulation of HES-1 expression is not required for the block in myogenesis imposed by ligand activation of Notch1. Thus, neither ligand-independent (Fig. 6) nor ligand-dependent (Fig. 7) activation of Notch1 is associated with an upregulation in endogenous HES-1 expression.

Overexpression of HES-1 does not inhibit muscle cell differentiation

HES-1 has been shown to prevent the transcriptional activity

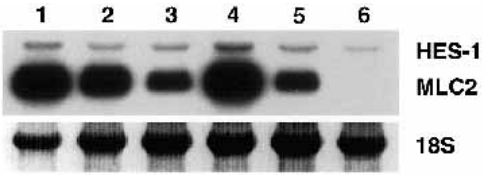

Fig. 7. Jagged-Notch1 interactions inhibit myogenesis but do not upregulate endogenous HES-1. Total RNA was isolated from C2C12 cells cultured alone (lane 1) or cocultured with L cells (lane 2) or Jagged-expressing L cells (lane 3) and from N1-expressing C2C12 cells cultured alone (lane 4) or cocultured with L cells (lane 5) or Jagged-expressing L cells (lane 6) after 5 days incubation in medium containing 10% HS to induce differentiation. RNAs were analyzed by northern blotting using probes for HES-1 and myosin light chain 2 (MLC2). Comparative loading and transfer of RNA was ascertained by methylene blue staining of 18S rRNA.

of MyoD and to inhibit MyoD-induced myogenic conversion of 10T1/2 cells (Sasai et al., 1992). However, the level of HES-1 RNA did not change during the differentiation of C2C12 myoblasts (Fig. 6). As an additional test of the role of HES-1 in C2C12 myoblast differentiation, we assessed whether increased expression of HES-1 in these mouse myoblasts could prevent muscle cell differentiation. Six stable HES-1-expressing cell lines were generated (an example of the level of HES-1 expression obtained with these lines is shown in Fig. 8, lanes 4-6). These lines differentiated with the same morphology and kinetics observed for parental C2C12 cells (data not shown). Proliferating HES-1-expressing C2C12 myoblasts did not express myogenin or MLC2 (Fig. 8, lane 4). However, both the parental cells (Fig. 8, lanes 2-3) and the HES-1-expressing cells (Fig. 8, lanes 5-6) induced the expression of these genes when incubated in differentiation medium for 2 and 4 days. Thus, consistent with the morphological differentiation, HES-1-expressing C2C12 myoblasts induced the expression of muscle specific genes to the same extent and with the same kinetics as parental myoblasts when challenged to differentiate. Therefore, enhanced expression of HES-1 does not prevent differentiation of C2C12 myoblasts. This result provides additional support for Notch1 signaling inhibiting muscle cell differentiation through a CBF1-independent pathway.

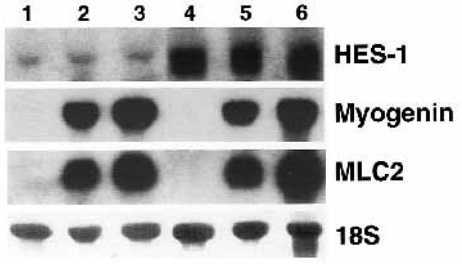

Fig. 8. Overexpression of HES-1 in C2C12 myoblasts does not inhibit muscle-specific gene expression. Total RNA was isolated from proliferating C2C12 cells (lane 1) and C2C12 cells induced to differentiate through incubation in medium containing 10% HS for 2 days (lane 2) and 4 days (lane 3). Total RNA was also isolated from proliferating HES-1-expressing C2C12 myoblasts (lane 4) as well as from these cells after they had been induced to differentiate for 2 days (lane 5) and 4 days (lane 6). RNAs were analyzed by northern blotting using probes for HES-1, myogenin and myosin light chain 2 (MLC2). Comparative loading and transfer of RNA was ascertained by methylene blue staining of 18S rRNA.

DISCUSSION

Activated forms of Notch1 do not require CBF1 or HES-1 function to inhibit differentiation

Constitutively active forms of mouse and human Notch1 physically and functionally interact with CBF1, the mammalian homolog of Su(H) (Jarriault et al., 1995; Tamura et al., 1995; Hsieh et al, 1996; Lu and Lux, 1996). Furthermore, these forms of Notch1 and CBF1 bind *HES-1* promoter sequences to produce transcriptionally active complexes (Jarriault et al., 1995). In addition, these forms of Notch1 also inhibit myogenesis (Kopan et al., 1994; Nye et al., 1994; Jarriault et al., 1995; Kopan et al., 1996). Since HES-1 is a bHLH protein that has been shown to antagonize the function of other bHLH proteins such as MyoD (Sasai et al., 1992), it would follow that HES-1 is responsible for the block in myogenesis induced by Notch1 activation.

However, we have identified activated forms of Notch1, CDN1 and CDCN1T, that block muscle cell differentiation but do not activate CBF1 or upregulate endogenous HES-1. CDCN1T consists of only the 6 Notch1 ankyrin repeats, which are unable to interact physically with CBF1, while the CDN1 protein starts 17 amino acids N-terminal to the ankyrin repeats and thus also lacks sequences required for CBF1-Notch1 interactions (Tamura et al., 1995; Hsieh et al, 1996). Therefore, through our analysis of mutant forms of Notch1, which are constitutive repressors of myogenesis but do not activate CBF1, we have detected the existence of a previously unidentified CBF1-independent pathway for Notch signal transduction. Consistent with this observation, there is evidence for both Su(H)-independent (Lecourtois and Schweisguth, 1995) and LAG1-independent (Christensen et al., 1996) LIN12/Notch signaling in *Drosophila* and *C. elegans*, respectively. Taken together, these results suggest that Notch signaling does not always result in upregulation of endogenous CSL proteins and provide additional support for a CBF1-independent Notch signaling pathway.

HES-1 does not inhibit differentiation of C2C12 myoblasts

We have also found that ligand-mediated activation of Notch1, which functions to inhibit muscle cell differentiation, does not involve upregulation of endogenous HES-1. Consistent with this observation, HES-1 expression did not change as mononuclear C2C12 myoblasts differentiated into multinuclear myotubes. Taken together our results suggest that the block in muscle cell differentiation, induced by activation of the Notch signaling pathway in myoblasts, does not require functional interactions between Notch, CBF1 and the *HES-1* gene. In fact, increased expression of HES-1 in C2C12 myoblasts, through stable expression of exogenous HES-1, did not inhibit myogenic differentiation of these cells. This is in contrast to a previous report in which HES-1 was shown to inhibit MyoD-induced myogenic conversion of 10T1/2 cells, in which both MyoD and HES-1 were ectopically expressed transiently (Sasai et al., 1992). In contrast, our characterization of HES-1 in myogenesis employed permanent HES-1-expressing C2C12

myoblasts which were stimulated to differentiate through the induction of endogenous muscle regulatory genes. It is important to note that C2C12 cells are committed myoblasts, whereas 10T1/2 cells are uncommitted fibroblast-like cells. Perhaps differences in regulation of muscle regulatory genes such as *MyoD*, *Myf5*, *myogenin* and *MRF-4*, obtained in 10T1/2 cells verses C2C12 myoblasts, underlies the differences for HES-1 function in myogenesis in these two cell types.

CBF1 and HES-1 may function in the feedback loop required for lateral inhibition

The DSL ligands and LIN12/Notch receptors mediate a process termed lateral inhibition, which is thought to help specify certain cell fates during *Drosophila* and *C. elegans* development (reviewed by Greenwald and Rubin, 1992; Ghysen et al., 1993). This process involves interactions between a signaling cell and a receiving cell, which ultimately direct these cells to adopt different fates. Initially uncommitted cells are equivalent in their expression of DSL ligands and LIN12/Notch receptors; however, interactions between neighboring cells produce reciprocal changes in ligand and receptor expression. Genetic mosaic analyses have suggested that high DSL ligand expression establishes the signaling cell, while high levels of LIN12/Notch receptor expression define the receiving cell. A feedback mechanism in response to LIN12/Notch signaling has been proposed that would regulate the expression of ligand and receptor, and thus maintain and reinforce the established signaling and receiving potential of these cells (Wilkinson et al., 1994; Christensen et al., 1996; Heitzler et al., 1996).

There is evidence to support the idea that Notch activation of the CSL proteins is responsible for high receptor and low ligand expression in the receiving cell (Wilkinson et al., 1994; Christensen et al., 1996; Heitzler et al., 1996). For example, Notch activation in the receiving cell of *Drosophila*, mediated through interactions with Delta on the signaling cell, activates Su(H) to induce the expression of E(spl) proteins (Jennings et al., 1994). The E(spl) proteins function to repress transcription of the *Achaete-Scute* complex (*AS-C*) genes, which encode bHLH proteins required for Delta expression (Heitzler et al., 1996; reviewed by Lewis, 1996). Thus, loss of AS-C expression in response to Notch signal transduction results in a decrease in Delta expression in the receiving cell. In addition, studies in *Xenopus* have shown that Notch signaling downregulates expression of the DSL ligand X-Delta-1 (Chitnis et al., 1995). The loss in X-Delta-1 expression is probably due to a loss in expression of homologous *Xenopus AS-C* genes such as *XASH-3* and *NeuroD*, since these genes promote the expression of X-Delta-1 (Chitnis and Kintner, 1996). Furthermore, experiments in *C. elegans* not only support the downregulation of ligand expression following receptor activation but also provide molecular evidence for the upregulation of receptor expression in response to signaling (Wilkinson et al., 1994; Christensen et al., 1996). Importantly, this receptor gene regulation is thought to be mediated through the CSL protein, LAG1, activated in the receiving cell (Wilkinson et al., 1994; Christensen et al., 1996). Such positive feedback between receptor activation and expression of the receptor would ensure that the receiving cell continues to express high levels of LIN12/Notch protein.

Extrapolation of these observations to ours and others from mammalian systems raises the possibility that increased HES-1 expression, in response to Notch signaling, regulates the expression of Notch and its ligand in activated cells. In fact, we have found that increased expression of HES-1 in C2C12 cells (either through activated Notch that upregulates endogenous HES-1 or through exogeneous expression of HES-1) increases the expression of Notch in these cells (D. N. and G. W., unpublished data). Therefore, the Notch-activated CBF1 pathway may function to produce high levels of Notch on the surface of cells, thereby sustaining Notch signaling within activated cells.

Notch is thought to prevent differentiation through the propagation of an inhibitory signal. It has been suggested that HES-1 inhibits differentiation by functionally antagonizing bHLH proteins required for cell type specification, for example MyoD in myogenesis and MASH-1 in neurogenesis (Sasai et al., 1992; Ishibashi et al., 1995). However, increases in HES-1 expression may function to increase the expression of Notch on the cell surface and thereby potentiate the cell's capacity to receive signals which prevent its differentiation. Thus, overexpression of either HES-1 or activated Notch would produce the same inhibitory effect on the cell. However, the HES-1 effect would depend upon availability of DSL ligand for activation of upregulated endogenous Notch, while the activated Notch effects would be ligand independent. Our results are consistent with this prediction.

Although we have argued that ligand-mediated Notch signaling results in the upregulation of HES-1, we did not see an upregulation of endogenous HES-1 in cocultures undergoing Jagged-Notch interactions. However, in this system Notch1 expression has been artificially elevated through stable expression of exogenous Notch1. Perhaps high receptor expression in *Drosophila* or *C. elegans* cells accounts for LIN12/Notch signaling in the absence of Su(H) (Lecourtois and Schweisguth, 1995) or LAG1 (Christensen et al., 1996) expression, respectively. However, consistent with the lack of upregulation for HES-1 in the coculture, ubiquitous expression of an activated form of Notch in *Drosophila* embryos does not result in upregulation of endogenous E(spl) proteins in all activated Notch-expressing cells (Jennings et al., 1994). The lack of HES-1 upregulation in the presence of Jagged-induced Notch activation may reflect negative feedback in expression of HES-1 by Notch1. It has been proposed that Notch signaling regulates the rate at which precursor cells differentiate (reviewed by Lewis, 1996). Consistent with a role for Notch in delaying differentiation, Notch expression is downregulated as cells differentiate (Lindsell et al., 1995; Myat et al., 1996). Thus, as a cell becomes determined the expression of Notch must be extinguished to allow differentiation to proceed, a process which might require negative regulation of HES-1. The molecular mechanism responsible for negative regulation of Notch expression during development is unknown; however, HES-1 directly downregulates its own expression (Takabayashi et al., 1994).

In summary, our data imply that Notch signaling prevents differentiation through the activation of more than one pathway in the cell. Substantiated by previous work in other systems, our results provide evidence for a CBF1-independent Notch pathway. This pathway is best illustrated by activated forms of Notch, which inhibit differentiation without the activation of CBF1. However, inhibition of differentiation through ligand-mediated Notch signaling, as seen in normal embryogenesis,

would require activation of CBF1 to upregulate HES-1. This upregulation would in turn induce high levels of Notch, thereby ensuring that an activated cell continues to respond to the contacted ligand-expressing cell. In addition, both ligand-independent and ligand-dependent Notch signaling would activate the CBF1-independent pathway, which would antagonize the differentiation state of the cell. Therefore, our data suggest that Notch signal transduction is more complex than previously proposed (reviewed by Honjo, 1996).

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