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The Notch 3 intracellular domain represses Notch 1-mediated activation through *Hairy/Enhancer of split* (*HES*) promoters

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SUMMARY

The Notch signaling pathway is important for cellular differentiation. The current view is that the Notch receptor is cleaved intracellularly upon ligand activation. The intracellular Notch domain then translocates to the nucleus, binds to Suppressor of Hairless (RBP-Jk in mammals), and acts as a transactivator of *Enhancer of Split* (*HES* in mammals) gene expression. In this report we show that the Notch 3 intracellular domain (IC), in contrast to all other analysed Notch ICs, is a poor activator, and in fact acts as a repressor by blocking the ability of the Notch 1 IC to activate expression through the *HES-1* and *HES-5* promoters. We present a model in which Notch 3 IC interferes with Notch 1 IC-mediated activation at two levels. First, Notch 3 IC competes with Notch 1 IC for access to RBP-Jk and does not activate transcription when positioned close to a promoter. Second, Notch 3 IC appears to compete with Notch 1 IC for a common coactivator present in limiting amounts. In conclusion, this is the first example of a Notch IC that functions as a repressor in *Enhancer of Split/HES* upregulation, and shows that mammalian Notch receptors have acquired distinct functions during evolution.

Key words: Lateral inhibition, Stem cell, Progenitor cell, Transgenic mice, Nervous system

INTRODUCTION

The Notch signaling pathway is an evolutionarily highly conserved mechanism for cell-cell communication, important for many types of cell fate determinations. Notch functions in lateral inhibition, a cell-cell signaling system allowing a differentiating cell to suppress the same differentiation fate in a neighboring cell (the receiving cell). This ensures that the correct number of cells are distributed to distinct cell fates (for reviews, see Lewis, 1996; Simpson, 1997; Beatus and Lendahl, 1998). Notch signaling in the receiving cell is induced by expression of Delta or Serrate ligands in the differentiating cell. Transmission of the signal from the activated Notch receptor in the receiving cell requires the intracellular protein Suppressor or Hairless (Su(H); lag-1 in *C. elegans*; RBP-Jk in mammals) and eventually leads to upregulation of expression of *Enhancer of split* genes (*E(Spl*); *HES* (*Hairy/Enhancer of split*) in mammals) (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). The *E(spl)/HES* genes encode transcriptional repressors of the basic helix-loop-helix type, which in turn repress transcription of another class of genes encoding transcriptional regulators, the *A-Sc* genes (*MASH* genes in mammals) in the receiving cell. As a result of this cascade of gene regulation events, the receiving cells come to express low levels of A-Sc and are forced to take on the secondary fate or remain undifferentiated.

The *Drosophila* Notch receptor is a single transmembrane protein which on the extracellular side contains 36 EGF (epidermal growth factor)-like repeats and an LNR (Lin/Notch/Repeat) region, unique to this class of receptors (Wharton et al., 1985; Kidd et al., 1986). At the intracellular side, Notch is composed of a subtransmembrane domain, a domain containing six ankyrin repeats and, at the C-terminal end, PEST and OPA regions (for a review, see Weinmaster, 1997). By a combination of genetic and molecular analyses in *Drosophila* and *C. elegans*, which contains the two Notch receptors LIN-12 and GLP-1, it has been shown that ligand-induced Notch activation can be mimicked by expression of the intracellular domain of the Notch receptor. The intracellular domain (IC) functions as a constitutively active, ligand-independent form (Lieber et al., 1993; Struhl et al., 1993). Ligand-activation of Notch involves processing of the Notch receptor at the intracellular side, followed by release of the intracellular domain and its transport to the nucleus (Schroeter et al., 1998; Struhl and Adachi, 1998). This process is at least in part controlled by presenilin (De Strooper et al., 1999; Struhl and Greenwald, 1999).

Notch ICs localize to the nucleus (Fortini et al., 1993; Lieber et al., 1993; Struhl et al., 1993; Jarriault et al., 1995; Tamura et al., 1995; Kopan et al., 1996; Schroeter et al., 1998). This is in keeping with the presence of evolutionarily conserved nuclear localization signals on each side of the ankyrin repeats (Lieber et al., 1993). All Notch ICs that have been analyzed bind to the DNA-binding Su(H)/RBP-Jk protein (Fortini and

Artavanis-Tsakonas, 1994; Kato et al., 1996). Binding of Notch IC to Su(H)/RBP-Jk is mediated by a region in the Notch IC referred to as the RAM domain, which is located between the transmembrane domain and the ankyrin repeats (Tamura et al., 1995), although other intracellular regions may also be involved in RBP-Jk binding (Fortini and Artavanis-Tsakonas, 1994; Kato et al., 1997; Wettstein et al., 1997). The binding of *Drosophila* Notch IC, vertebrate Notch 1 IC or Notch 2 IC to Su(H)/RBP-Jk leads to activation of transcription from genes carrying the Su(H) response element, including the *E(spl)/HES* genes (Furukawa et al., 1995; Jarriault et al., 1995; Lecourtois and Schweisguth, 1995; Hsieh et al., 1996; Kopan et al., 1996). This is in accordance with the upregulation of E(spl) observed in lateral inhibition as a response to Notch activation, as described above.

The link between Notch activation and upregulation of *E(spl)/HES* expression is further supported by experiments in *Xenopus* and mouse. During primary neurogenesis in *Xenopus*, expression of *Xenopus* Notch 1 IC leads to reduced neurogenesis (Coffman et al., 1993; Chitnis et al., 1995) and a concomitant upregulation of *Xenopus E(spl)/HES (ESR-1)* (Wettstein et al., 1997). Conversely, expression of dominant negative forms of the ligand Delta or Su(H)/RBP-Jk leads to increased neurogenesis (Wettstein et al., 1997). The functional inactivation of the *Notch 1* or the *Su(H)/RBP-Jk* gene in mice results in downregulation of *HES-5* expression and upregulation of *Math4A*, an *A-Sc* homolog (de la Pompa et al., 1997). These observations point to a strong evolutionary conservation of a molecular mechanism for Notch activation leading to upregulation of *E(spl)/HES* and repression of *A-Sc* expression.

While the two Notch homologs in *C. elegans* can fully substitute for each other in cell-fate decisions (Fitzgerald et al., 1993), the situation could conceivably be more complex in mammals, which harbor four Notch homologs (Notch 1-4). The mammalian Notch 1, 2, 3 and 4 receptors show all the cardinal features of bona fide Notch receptors (for reviews, see Weinmaster, 1997; Beatus and Lendahl, 1998), but the extent to which they perform different functions has not been thoroughly investigated. Support for different functions comes from the observation that mutations in human *Notch 1* and *Notch 3* are associated with distinct diseases. A translocation of the activating domain of Notch 1 leads to T-cell leukemia (Ellisen et al., 1991), while missense mutations in *Notch 3* are the genetic cause of CADASIL, a genetic stroke and dementia syndrome (Joutel et al., 1996, 1997). Furthermore, functional inactivation of the mouse *Notch 1* and *Notch 2* genes generates quite different phenotypes. Notch $1^{-/-}$ embryos die during embryogenesis, presumably from defects in somitogenesis (Swiatek et al., 1994; Conlon et al., 1995; de la Pompa et al., 1997), while Notch $2^{-/-}$ mice die at later embryonic stages (Dr T. Gridley, personal communication).

To test for possible differences in intracellular Notch signaling we have compared the transactivating capacity of Notch 1 IC and Notch 3 IC. Our data demonstrate that Notch 3 IC, in contrast to Notch 1 IC and all other analyzed Notch ICs, is a poor activator of the *HES-1* and *HES-5* promoters, and in fact acts as a repressor of Notch 1 IC-mediated *HES* activation. Notch 3 IC appears to strongly compete with Notch 1 IC for a common coactivator, present in limiting amounts. Furthermore, Notch 3 IC competes with Notch 1 IC for access to RBP-Jk, but Notch 3 IC is incapable of activating transcription from a nearby promoter. In conclusion, the data presented here show that Notch 3 performs functions distinct from that of other Notch receptors.

MATERIALS AND METHODS

DNA constructs and cloning

A nonimmunotagged version of Notch 3 IC was generated by replacing the HA-containing 3′ end of CMVp-Notch 3 IC-HA (Lardelli et al., 1996) with the nonimmunotagged 3′ end of the JJ5 plasmid (Lardelli et al., 1994). The CMV-Notch 3 cdc10 construct encoding the ankyrin/cdc10 region of Notch 3 was amplified by PCR from plasmid CMVp-Notch 3 IC-HA, using a 5′ primer containing an *Eco*RI restriction site, GAATTCGACGGAGTGGATGTCAAC, and a 3′ primer encoding an HA-tag and an *Spe*I restriction site, ACTAGTTCAAAGGCTGGCGTAGTCGGGGACGTCGTAGGGGT AGCACGGATCCTCCAAGTGATCTGTGATCTC. The PCR product was cleaved with *Eco*RI/*Spe*I and ligated into the *Eco*RI/*Nhe*I sites of pCMX-PL-2 (Perlmann and Jansson, 1995).

N-terminally myc-immunotagged Notch 1 IC was subcloned from the pcDNA1.amp-mNotch 1 IC plasmid (*Bam*HI/*Spe*I-blunt ended) to the pGEX-4T3 vector (*Bam*HI/*Sma*I), generating the plasmid GST-Notch 1 IC. To obtain yeast and mammalian two hybrid vectors containing Notch 3 IC-HA and Notch 1 IC cDNAs, we first subcloned the cDNAs from the pGEX vectors to the pBluescript KS vector using *Sal*I/*Not*I (Notch 1) and *Bam*HI/*Not*I (Notch 3), respectively. *Eco*RI linkers were ligated to each pBluescript-Notch IC construct. Notch 3 IC-HA cDNA was subsequently subcloned into *Eco*RI/*Sma*I-digested pGAD424 (yeast GAL4 AD (activation domain) vector, generating Notch 3 IC/GAL4AD); *Eco*RI/*Sal*I-digested pCMXGAL4 (mammalian GAL4 DB (DNA binding domain) vector, generating Notch 3 IC/GAL4DB); and *Kpn*I/*Sal*I-digested pCMXVP16 (mammalian VP16 TA (transactivating domain) vector, generating Notch 3 IC/VP16TA). Notch 1 IC was subcloned from pBluescript-Notch 1 IC into *Eco*RI/*Sal*I-digested pGAD424 (generating Notch 1 IC/GAL4AD), pCMXGAL4 (generating Notch 1 IC/GAL4DB) and *Xba*I/*Sal*I-digested pCMXVP16 (generating Notch 1 IC/VP16TA). The cDNA encoding RBP-Jk was made by PCR with Pfu polymerase and RBP-Jk cDNA as template. The PCR primers contained 5′ extensions containing suitable restriction cleavage sites for later subcloning steps. The RBP-Jk-encoding PCR product was cloned in pGBT9 using *Eco*RI/*Sal*I, generating the plasmid RBP-Jk-GAL4DB. RBP-Jk cDNA was then subcloned from pGBT9-RBP-Jk to *Eco*RI/*Sal*I-digested pGAD424 (generating RBP-Jk/GAL4AD), pCMXGAL4 (generating RBP-Jk/GAL4DB), pGEX-4T1 (generating GST/RBP-Jk) and *Xba*I/*Sal*I-digested pCMXVP16 (generating RBP-Jk/VP16TA). All constructs were sequenced at the 5′ end to ensure that the correct orientation and reading frames were obtained. To generate the HES-5-tk-luc reporter construct, which consists of the *HES-5* promoter linked to the luciferase reporter gene, a 3.7 kb *Xho*I/blunt DNA fragment containing the proximal promoter elements of mouse *HES-5* was cloned into a *Sal*I/*Bam*HI-blunt ended plasmid containing the basal *thymidine kinase* promoter located upstream of the luciferase-encoding gene.

Transfections

JEG cells (a human chorion carcinoma cell line) were transfected using the calcium phosphate precipitation technique. 40,000 cells/well were seeded 6-24 hours prior to transfection. 70 μl DNA was mixed with 70 μl 0.5 M $CaCl_2$ and 140 μl 2× HBS, pH 7.05 (1× HBS=0.28 M NaCl, 0.05 M Hepes, 1.5 M Na_2HPO_4) were added and the mixture was incubated for 5 minutes at room temperature. In each experiment, a total amount of 1 μg DNA was transfected (a vector containing only the CMV promoter was used to adjust the total amount of DNA to

1 μg), including 100 ng of CMV-lacZ as internal control and 200 ng of the *HES-1* reporter plasmid. 36 hours after transfection, the cells were lysed in 150 μl 1× cell lysis buffer (BioOrbit, Turku, Finland). Luciferase activity was assayed using GeneGlow (Biothema, Dalarö, Sweden) and measured in an Anthos Luminoscan Lucy 1. Transfection efficiency was determined by measuring β-galactosidase activity in the cell lysate. HeLa cells were transfected with DOTAP, according to the manufacturer's instructions (Boehringer, Mannheim, Germany). In each experiment, a total amount of 1.5 μg DNA was transfected (a vector containing only the CMV promoter was used to adjust the total amount of DNA to 1.5 μg), including 200 ng of CMV-lacZ as internal control and 400 ng of the reporter plasmid HES-1. Transfections were assayed as described above for JEG cells. Monkey kidney COS-7 cells were transiently transfected using the Lipofectamine PLUS reagent (Life Technologies, Rockville, MA) according to the manufacturer's instructions. Each transfection contained 400 ng plasmid consisting of the the CMV-lacZ plasmid (50 ng), reporter plasmid (HES-1, HES-5 or MH100-tk-luc; 100 ng) and various amounts of different expression plasmids plus mock plasmid. The cells were harvested 24-48 hours post-injection and screened for luciferase and β-galactosidase activity as described above. Expression at the protein level was analyzed by western blot analysis. All experiments were repeated at least three times.

Yeast and mammalian two-hybrid assays

The yeast two-hybrid assay was conducted according to the manufacturer's protocol (Clontech, Palo Alto, CA). Briefly, following transfection of yeast Y190 cells with GAL4DB and GAL4AD fusion protein constructs, yeast cells were assayed for interacting proteins. Yeast colonies were lifted with filter paper from agar plates, subjected to a freeze-thaw cycle and finally submerged in an X-gal-containing phosphate buffer (Clontech, Palo Alto, CA). Protein interactions were monitored by β-galactosidase reporter gene activity. All experiments were repeated at least three times. In mammalian two-hybrid experiments, COS-7 cells were transfected with various constructs encoding GAL4DB and VP16TA fusion proteins in combination with the MH100-tk-luc reporter plasmid, containing the GAL4 DNA binding site coupled to the luciferase gene (Kang et al., 1993) and the CMV-lacZ plasmid. Luciferase activity was monitored, and the activity was normalized for transfection efficiency (β-galactosidase activity), as described above.

GST pull-down assays

E. coli BL21 was transformed with GST-Notch 1 IC, GST-Notch 3 IC or pGEX4T-1 alone. The fusion proteins were induced and extracted according to the manufacturer's instructions (Pharmacia Biotech, Little Chalfont, Bucks, UK). ^{35}S-labelled RBP-Jk Notch 1 IC or Notch 3 IC were synthesized in vitro from the pCMX vector (Perlmann and Jansson, 1995), using the TNT-coupled rabbit reticulocyte lysate system (Promega, Madison, WI). ^{35}S-labelled RBP-Jk was mixed with GST-Notch 1 IC, GST-Notch 3 IC or GST and incubated for 1 hour at 4°C in 200 μl G-buffer (20 mM Tris-HCl, pH 7.9, 150 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$, 0.2% Nonidet P-40 and 10% glycerol). Fusion proteins were washed six times in G-buffer and analyzed by SDS-PAGE. Bound protein was released in 2× sample buffer (2% SDS, 10% glycerol, 80 mM Tris, pH 6.8, 0.72 M β-mercaptoethanol, 0.02% bromphenol blue).

Transgenic mice and whole-mount in situ hybridization

Transgenic mice carrying the nestinp/Notch 3 IC-HA were generated by the pronuclear injection technique, as previously described (Lardelli et al., 1996). Embryos resulting from the injected eggs were surgically removed from the foster mothers and analysed by whole-mount in situ hybridization for expression of *HES-5*. Transgenicity was established by PCR on extraembryonic tissues as previously described (Lardelli et al., 1996). Whole-mount in situ hybridization was carried out as described by Wilkinson (1993), with the following modifications: proteinase K treatment was performed for 8 minutes, prehybridization for 3 hours (in 50% formamide, 1.3× SSC, 5 mM EDTA, 5 mM Chaps, 0.2% Tween-20, 50 μg/ml tRNA, 100 μg/ml heparin), probe concentration was 1 μg/ml, and incubation temperature 63°C. The probe was digoxygenin-labelled antisense cRNA prepared by T3 transcription from a *Hin*dIII-digested pBluescript plasmid containing an 1.2 kb *Hes-5* fragment (a kind gift from Dr F. Guillemot). The in situ hybridization signal was visualized by overnight treatment with alkaline phosphatase-conjugated anti-digoxygenin antibody (diluted 1:5000) at 4°C followed by 1-3 hours incubation in BM Purple (Boehringer, Mannheim, Germany).

Immunocytochemistry

JEG cells were plated on Chamber Slides (Lab Tek, Nunc, Naperville, Illinois) and transfected with 100 ng of Notch 1 IC, Notch 3 IC RBP-Jk expression plasmids. Notch 1 IC cDNA and RBP-Jk cDNA transfected cells were immunostained with the mouse anti c-myc antibody 9E10 (Pharmingen, San Diego, CA) and Notch 3 IC cDNA-transfected cells were stained with the mouse anti-HA antibody 12CA5 (Berkeley Antibodies Inc, Richmond, CA). A Texas Red-conjugated goat anti-mouse IgG was used as secondary antibody. Immunoreactivity was visualized by fluorescence microscopy, photographed and the pictures were assembled using PhotoShop (Adobe Systems Inc., Mountain View, CA).

RESULTS

Notch 1 IC, but not Notch 3 IC, activates transcription from the *HES-1* promoter

The transcriptional activity of Notch 1 IC and Notch 3 IC was analysed by transfection experiments in cell lines, using expression and reporter constructs summarized in Fig. 1A. We first showed by immunocytochemistry that Notch 1 IC, Notch 3 IC and RBP-Jk were produced and exclusively localized to the nucleus in JEG cells (Fig. 1B-D). This is in keeping with data from other cell lines for Notch 1 IC (Kopan et al., 1996) and Notch 3 IC (Lardelli et al., 1996), and with the reported localization of endogenous RBP-Jk in the mouse (de la Pompa et al., 1997). Protein extracts from the transfected cells contained immunoreactive proteins of the expected sizes (Fig. 1E).

We next compared the transactivating activity of Notch 1 IC and Notch 3 IC by transfecting fixed amounts of each construct and variable amounts of RBP-Jk into JEG cells. We found that the addition of Notch 1 IC caused transactivation at all RBP-Jk concentrations tested, and that the effect was strongest (79-fold) when no exogenous RBP-Jk was added (Fig. 2A). The reduction observed at higher concentrations of RBP-Jk could be explained by the endogenous RBP-Jk being present in JEG cells at levels optimal for Notch 1 IC activation. The addition of more RBP-Jk would then result in an excess of RBP-Jk over Notch 1 IC, leading to the accumulation of free RBP-Jk.

RBP-Jk alone does not possess transactivating activity (Tamura et al., 1995; Hsieh et al., 1996; Kato et al., 1996), but rather functions as a repressor when bound alone to DNA (Hsieh et al., 1996). Excess of free RBP-Jk, not bound to Notch 1 IC, is thus likely to reduce the level of Notch 1-mediated activation.

In contrast to the Notch 1 IC results, transfection of the same amount of Notch 3 IC generated only a very small increase in reporter gene expression (threefold as compared to 79-fold for Notch 1 IC), irrespective of the concentration of RBP-Jk (Fig.

2B). The level of Notch 3 IC was then varied from 50 to 500 ng in the presence of constant levels of RBP-Jk (100 ng), but no further increase in activation was observed (Fig. 2C). We conclude from these experiments that Notch 3 IC is a poor inducer of transcription from the *HES-1* promoter, as compared to Notch 1 IC (Fig. 2D).

Notch 3 IC represses Notch 1 IC-mediated *HES-1* activation

We next tested the hypothesis that Notch 3 IC not only has very low endogenous transactivating activity but directly functions as a repressor of Notch 1 IC-mediated transcriptional activation. Various ratios of Notch 1 IC and Notch 3 IC were contransfected together with a fixed amount of RBP-Jk (100 ng) and the HES-1-luc reporter plasmid into JEG cells. These experiments showed that an increasing amount of Notch 3 IC leads to reduced transactivation of the reporter gene (Fig. 3A). At 50 ng of Notch 3 IC, there was an 88% decrease in reporter gene activity, as compared to transfection of Notch 1 IC alone (100 ng), and at 200 ng, the decrease was 93%. When Notch 3 IC was added in fivefold excess (500 ng) over Notch 1 IC, the Notch 1 IC-mediated transactivation was reduced to the same level as when Notch 3 IC was transfected alone (Fig. 3A). To confirm that the results were not confined only to the JEG cell line, we repeated the experiments in HeLa, COS-7 and P19 cells, and a similar repression was observed (data not shown). All experiments were also performed with DNA constructs encoding Notch 1 IC and Notch 3 IC without immunotags, with very similar results (data not shown). Taken together, these experiments show that Notch 3 IC functions quite differently from Notch 1 IC in regulating transcription from a *HES-1* promoter, and demonstrate that Notch 3 IC represses Notch 1 IC-mediated activation in a concentration-dependent manner.

In a converse experiment, we kept Notch 3 IC at a constant level and tested different levels of Notch 1 IC, to learn whether the Notch 3 IC-mediated repression could be relieved. This led

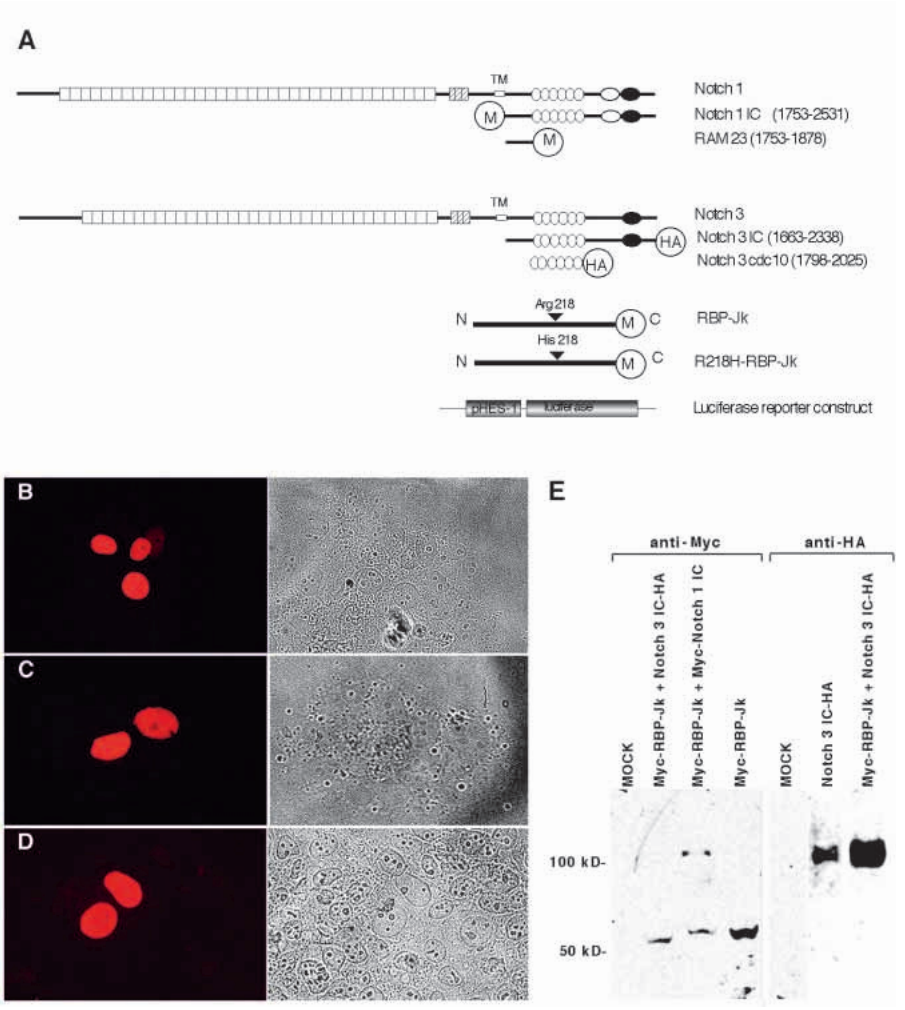


Fig. 1. (A) Schematic depiction of the constructs used in the transfection experiments. The full-length Notch 1 receptor is shown at the top (Notch 1). The Notch 1 ICL-myc construct (a kind gift from Dr R. Kopan) encodes a truncated Notch 1 receptor, which consists of the intracellular Notch 1 domain (Notch 1 amino acid residues in parentheses), and is referred to as Notch 1 IC in the text. It is immunotagged at the N terminus by a c-myc epitope (M) (Nye et al., 1994). The pEF-BOSneo-RAM23-myc construct (a kind gift from Dr T. Honjo) encodes the RAM23 portion of Notch 1, shown below (RAM23) (Kato et al., 1997). A full-length Notch 3 receptor is shown (Notch 3) and below the CMVp-Notch 3 IC-HA construct encoding a truncated Notch 3 receptor (referred to as Notch 3 IC in the text), which is very similar to the truncated Notch 1 receptor shown above. The encoded protein starts at residue 1663 in mouse Notch 3 (Lardelli et al., 1994), i.e. corresponding to a position 8 amino acid residues upstream of the first amino acid residue in Notch 1 IC. The Notch 3 IC contains an HA immunotag at its C terminus (HA) (Lardelli et al., 1996). The CMV-Notch 3 cdc10 construct encodes only the ankyrin/cdc10 region of Notch 3 and a C-terminal HA tag (Notch 3 cdc10). The wild-type RBP-Jk protein (RBP-Jk) and a dominant-negative mutant R218H-RBP-Jk (R218H-RBP-Jk) (kindly provided by Dr T. Honjo) are shown. The wild-type RBP-Jk contains an arginine residue at position 218, while the dominant-negative mutant has an arginine-to-histidine substitution at this position (Chung et al., 1994). Activation of Notch 1-mediated signaling was monitored by a construct containing the *HES-1* promoter linked to the luciferase reporter gene (HES-1-luc, a kind gift from Dr A. Israel), which is known to respond to Notch 1 IC-mediated activation (Jarriault et al., 1995). TM, transmembrane domain. (B-D) Expression and subcellular localization of Notch 1 IC, Notch 3 IC and RBP-Jk. JEG cells transfected with the Notch 1 IC, Notch 3 IC and RBP-Jk constructs show nuclear staining for Notch 1 IC (B), Notch 3 IC (C) and RBP-Jk (D), visualized by the anti-myc (B,D) and anti-HA (C) monoclonal antibodies. To the right are bright field views corresponding to B-D, to show the morphology of the cells. (E) Expression of Notch 1 IC, Notch 3 IC and RBP-Jk were also detected in transfected COS-7 cells by western blot analysis. Notch 1 IC and RBP-Jk were visualized by the anti-myc antibody, and Notch 3 IC with the anti-HA antibody. Notch 1 IC and Notch 3 IC migrate with an apparent molecular mass of approximately 110 kDa, and RBP-Jk of approximately 60 kDa.

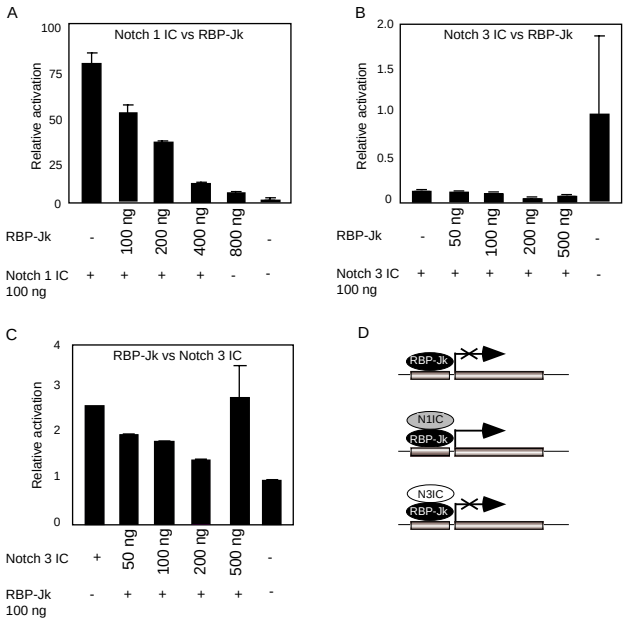


Fig. 2. Transactivating activity of Notch 1 IC and Notch 3 IC. JEG cells were transfected with a luciferase reporter plasmid and different amounts of RBP-Jk, together with Notch 1 IC (A) or Notch 3 IC (B). Transfection of Notch 1 IC results in a 79-fold activation of the reporter gene. The same amounts of transfected Notch 3 IC result in only very weak transactivation or no transactivation at all, irrespective of the amount of RBP-Jk. (C) In the converse experiment, the amount of Notch 3 IC was varied at constant levels of RBP-Jk. Only a threefold activation could be observed, which was independent of the concentration of Notch 3 IC. Values are average + s.d. from three experiments. (D) Schematic representation of the transactivating activities of RBP-Jk alone, Notch 1 IC and Notch 3 IC on the *HES-1* promoter.

to an increase in *HES-1* promoter activation proportional to the amount of Notch 1 IC added (Fig. 3B). This shows that the repressive effect exerted by the Notch 3 IC can be neutralized by elevating the concentration of Notch 1 IC.

The ankyrin/cdc10 domain is the most well-conserved region when all Notch receptors are compared (Weinmaster, 1997), and this region is important for interactions with Su(H) in *Drosophila* (Fortini and Artavanis-Tsakonas, 1994). To learn whether the Notch 3 ankyrin repeat domain alone was sufficient to mediate repression we generated the CMV-Notch 3 cdc10 construct (Notch 3 cdc10), encoding the Notch 3 ankyrin/cdc10 region alone, and cotransfected it with Notch 1 IC (Fig. 3C). Fivefold excess of the Notch 3 ankyrin/cdc10 region reduces reporter gene activation by almost 50%. Thus, the Notch 3 ankyrin/cdc10 region alone can repress Notch 1 signaling, although not as efficiently as the complete Notch 3 IC.

We next compared the repressor activity of Notch 3 IC to that of two previously characterized negative regulators of Notch 1 IC activity: RAM23 and R218H-RBP-Jk (Fig. 1A). When increasing amounts of RAM23 were introduced together with a fixed amount of Notch 1 IC, transactivation was repressed, as expected. At the highest concentration (500 ng), RAM23 blocked expression by approximately 70% (data not shown). The transfection of increasing amounts of R218H-RBP-Jk resulted in a more pronounced repression: 500 ng of R218H-RBP-Jk reduced luciferase activity by 94% (data not shown). These data corroborate previous observations about repressor activities for RAM23 and R218H-RBP-Jk (Kato et al., 1997) and show that Notch 3 IC is a more potent repressor of Notch 1 IC-mediated signaling than RAM23 and equally effective as R218H-RBP-Jk.

To establish whether Notch 3 IC could repress Notch 1 IC-mediated transcriptional activation on a promoter other than the *HES-1* promoter, we tested an *HES-5* promoter coupled to the luciferase gene in the transfection assay. *HES-5* was chosen because the promoter contains putative RBP-Jk binding sites and the *HES-5* gene responds in vivo to Notch 1 signaling (de la Pompa et al., 1997). The results for the *HES-5* promoter were very similar to the HES-1 promoter results, i.e. Notch 1 IC activates expression, although the activation was lower than for the *HES-1* promoter (Fig. 3D). The activation was repressed

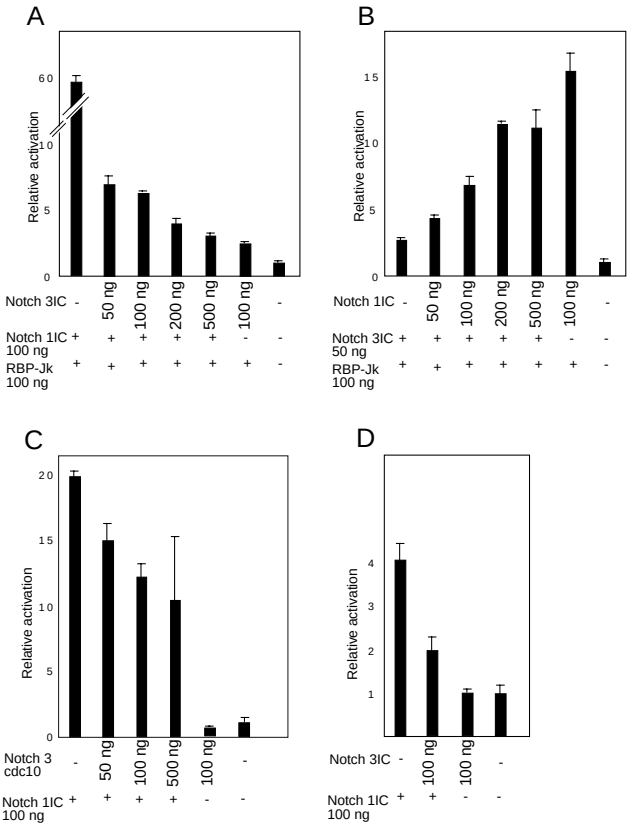


Fig. 3. Repression of Notch 1-mediated activation through the *HES-1* (A-C) and *HES-5* (D) promoters by the addition of Notch 3 IC or Notch 3 cdc10. (A) JEG cells were transfected with a *HES-1* luciferase reporter plasmid alone (rightmost bar) or together with different amounts of Notch 3 IC and/or Notch 1 IC (100 ng). The RBP-Jk expression plasmid was also introduced in all experiments except in the rightmost bar. The luciferase expression induced by Notch 1 IC was repressed to a maximum of 94% in a concentration-dependent manner by the addition of Notch 3 IC. (B) JEG cells were transfected with a luciferase reporter plasmid alone (rightmost bar) or together with a fixed amount of Notch 3 IC and/or varying amounts of Notch 1 IC. (C) When JEG cells were cotransfected with Notch 1 IC and increasing amounts of Notch 3 cdc10, the luciferase expression was decreased by almost 50%. (D) COS-7 cells were transfected with a *HES-5* luciferase reporter plasmid alone (rightmost bar) or together with different amounts of Notch 3 IC and/or Notch 1 IC (100 ng). The induction by Notch 1 IC was repressed 50% by adding 100 ng of Notch 3 IC plasmid. Values are average + s.d. from three experiments.

by cotransfecting Notch 3 IC (Fig. 3D). This demonstrates that Notch 3 IC repression is not exclusively confined to the *HES-1* promoter.

Notch 1 IC and Notch 3 IC interact with RBP-Jk but not with each other

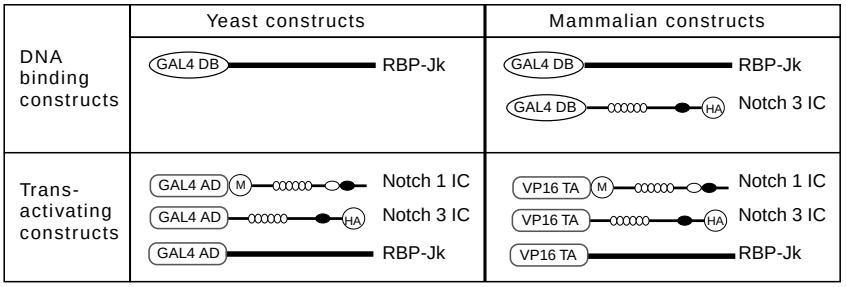
To learn more about the molecular mechanism for Notch 3 IC repression, protein-protein interactions between Notch 1 IC, Notch 3 IC and RBP-Jk were analysed in a yeast two-hybrid assay (Fig. 4A). The Notch 1 IC/GAL4 activation domain (AD) fusion protein showed robust interaction with the RBP-JK/GAL4 DNA binding domain (DB) fusion protein. An interaction, although somewhat weaker, was also observed between Notch 3 IC/GAL4 AD and RBP-Jk/GAL4 DB (Fig. 4B). We next performed similar experiments in a mammalian two-hybrid system in COS-7 cells. Both Notch 1 IC/VP16TA (Notch 1 IC fused to the activation domain of VP16) and Notch 3 IC/VP16 TA interacted with RBP-Jk/GAL4 DB (Fig. 4C). Moreover, Notch 3 IC and RBP-Jk also showed an interaction in the opposite configuration, i.e. when Notch 3 IC/GAL4 DB was tested against RBP-Jk/VP16TA (Fig. 4C). No interaction could be observed between Notch 1 IC/VP16TA and Notch 3 IC/GAL4 DB (Fig. 4C). These data are in agreement with previous reports that Notch 1 IC interacts strongly with RBP-Jk and that the Notch 3 IC RAM23 domain interacts with RBP-Jk (Tamura et al., 1995).

To test the protein-protein interactions between Notch ICs and RBP-Jk by a different approach, we performed pull-down experiments with GST (glutathione-S-transferase) fusion proteins. Bacterially expressed GST-Notch 1 IC and GST-Notch 3 IC fusion proteins (Fig. 5A) were incubated with in vitro transcribed and translated ^{35}S-labelled RBP-Jk, Notch 1 IC or Notch 3 IC. We found that similar amounts of ^{35}S-labelled RBP-Jk were coprecipitated by GST-Notch 1 IC and GST-Notch 3 IC (Fig. 5B). In contrast, neither Notch 1 IC nor Notch 3 IC could be precipitated with GST-Notch 3 IC or GST-Notch 1 IC (Fig. 5B). This further proves that Notch 1 IC and Notch 3 IC can interact with RBP-Jk, but not with each other.

Notch 3 IC represses Notch 1 IC independent of binding to RBP-Jk

The fact that both Notch 1 IC and Notch 3 IC interact with RBP-Jk does not by itself explain why Notch 3 IC represses Notch 1 IC-mediated *HES*-promoter activation. One possibility is that Notch 3 IC may be competing with Notch 1 IC for a common coactivator. To test this, we used a system for transcriptional activation in mammalian cells, which is based on the GAL4 DNA binding domain, and thus is independent of RBP-Jk. A fusion protein between the GAL4 DNA-binding protein and the Notch 1 IC (Notch 1 IC/GAL4 DB) (Fig. 6A) functions as an activator on a UAS-luciferase reporter plasmid, while GAL4 DB alone has no activity (Fig. 6B,C). When the amount of Notch 1 IC/GAL4 DB is kept constant, the addition of increasing amounts of Notch 3 IC lacking the GAL4 DB domain (free Notch 3 IC), leads to repression (Fig. 6B). Interestingly, free Notch 3 IC is a very potent repressor in this system; addition of an equal amount of free Notch 3 IC and Notch 1 IC/GAL4 DB DNA results in 94% repression. We next investigated whether free Notch 1 IC would also repress Notch

A



B

GAL4 DB construct	GAL4 AD construct	β-galactosidase activity
RBP-Jk	Mock	-
RBP-Jk	Notch 1 IC	+
RBP-Jk	Notch 3 IC	+
RBP-Jk	RBP-Jk	-
Mock	Notch 1 IC	-
Mock	Notch 3 IC	-
p53	SV40 T	+

C

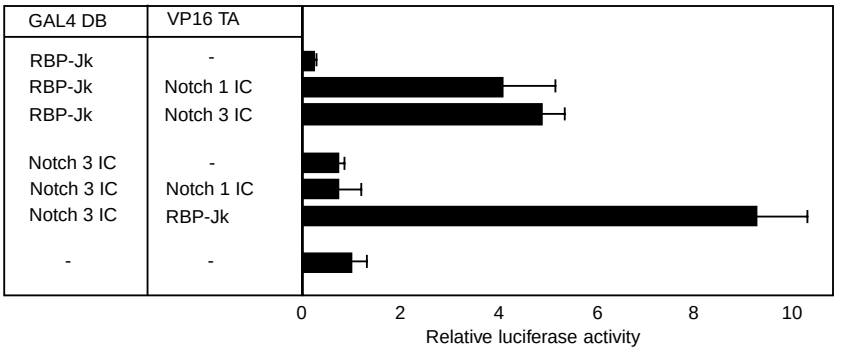


Fig. 4. Protein-protein interactions between Notch 1 IC, Notch 3 IC and RBP-Jk in yeast and mammalian two-hybrid assays. (A) Schematic presentation of the different GAL4DB, GAL4AD and VP16TA fusion protein constructs expressed in yeast and mammalian (COS-7) cells. (B) A summary of the data obtained in the yeast two-hybrid screens. RBP-Jk/GAL4DB interacts with both GAL4AD/Notch 1 IC and GAL4AD/Notch 3 IC but not with RBP-JK/GAL4AD, as measured by β-galactosidase reporter gene activity. As a positive control, interaction was also observed between p53 and SV40 T, which are known to interact. (C) A summary of the data obtained in the mammalian two-hybrid assay. Plasmids encoding GAL4DB and VP16TA fusion proteins were cotransfected with the MH100-tk-luc reporter plasmid into COS-7 cells. The diagram shows luciferase activity in arbitrary units where the activity of mock transfected cells is set to one. All data obtained were normalized for transfection efficiency (β-galactosidase activity, see Materials and methods). Values are the average + s.d. from three experiments.

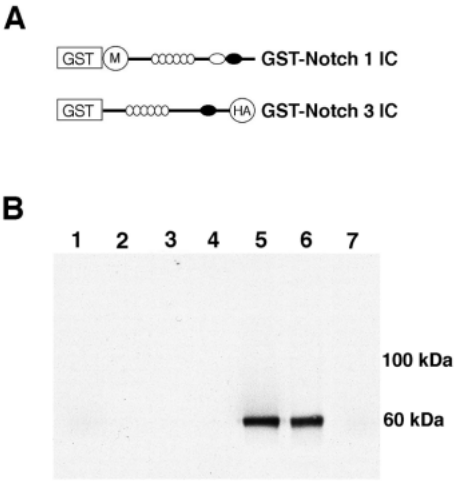


Fig. 5. Analysis of in vitro protein interactions between Notch 1 IC, Notch 3 IC and RBP-Jk. (A) A schematic representation of the GST-Notch 1 IC and GST-Notch 3 IC fusion proteins. (B) Equal amounts of purified Sepharose-GST-Notch 1 IC, Sepharose-GST-Notch 3 IC and Sepharose-GST were mixed with ^{35}S-labeled Notch 1 IC, Notch 3 IC and RBP-Jk. The proteins were precipitated and analyzed by SDS-PAGE in the following order: GST-Notch 1 IC + ^{35}S-Notch 3 IC (lane 1), GST-Notch 3 IC + ^{35}S-Notch 1 IC (lane 2), GST alone + ^{35}S-Notch 1 IC/ ^{35}S-Notch 3 IC (lane 3 and 4), GST-Notch 1 IC + ^{35}S-RBP-Jk (lane 5), GST-Notch 3 IC + ^{35}S-RBP-Jk (lane 6) and GST alone + ^{35}S-RBP-Jk (lane 7). No precipitated ^{35}S-Notch 1 IC or ^{35}S-Notch 3 IC could be detected in lanes 1-4. In contrast, similar amounts of ^{35}S-labeled RBP-Jk, migrating as a band of approximately 60 kDa, was precipitated by GST-Notch 1 IC and GST-Notch 3 IC. No ^{35}S-labeled RBP-Jk could be detected in the GST control.

1 IC/GAL4 DB. This is the case, but the repression appears to be less strong as compared to free Notch 3 IC, i.e. higher concentrations of free Notch 1 IC DNA are required to reach the same level of repression (Fig. 6C). Taken together, these data show that both free Notch 1 IC and Notch 3 IC can successfully repress Notch 1 IC fused to GAL4 DB, but that Notch 3 IC appears to be a considerably more potent repressor. This strongly suggests that Notch 1 IC and Notch 3 IC bind to a common coactivator, which is required for Notch 1, when fused to GAL4 DB, to activate transcription, and that this coactivator is present in limiting amount. We next tested whether Notch 3 IC competes for a coactivator required also for expression mediated by other transactivating factors. To this end, we cotransfected a plasmid encoding the GAL4/VP16 fusion protein, which is a potent transcriptional activator, with increasing amounts of Notch 3 IC and used a GAL4 response element/reporter gene as readout (Fig. 6D). We found that Notch 3 IC only very weakly represses GAL4/VP16 activation and that Notch 3 IC therefore is not likely to function as a general repressor.

An alternative explanation for the Notch 3 IC-mediated repression would be that Notch 3 IC is unable to activate transcription when positioned close to a promoter. To test this idea, we generated a Notch 3 IC/GAL4 DB fusion protein, and compared it with Notch 1 IC/GAL4 DB for activation of the UAS-luciferase reporter gene. Notch 3 IC/GAL4 DB does not activate reporter gene expression, while transfection of Notch 1 IC/GAL4 DB results in a 60-fold increase, as described

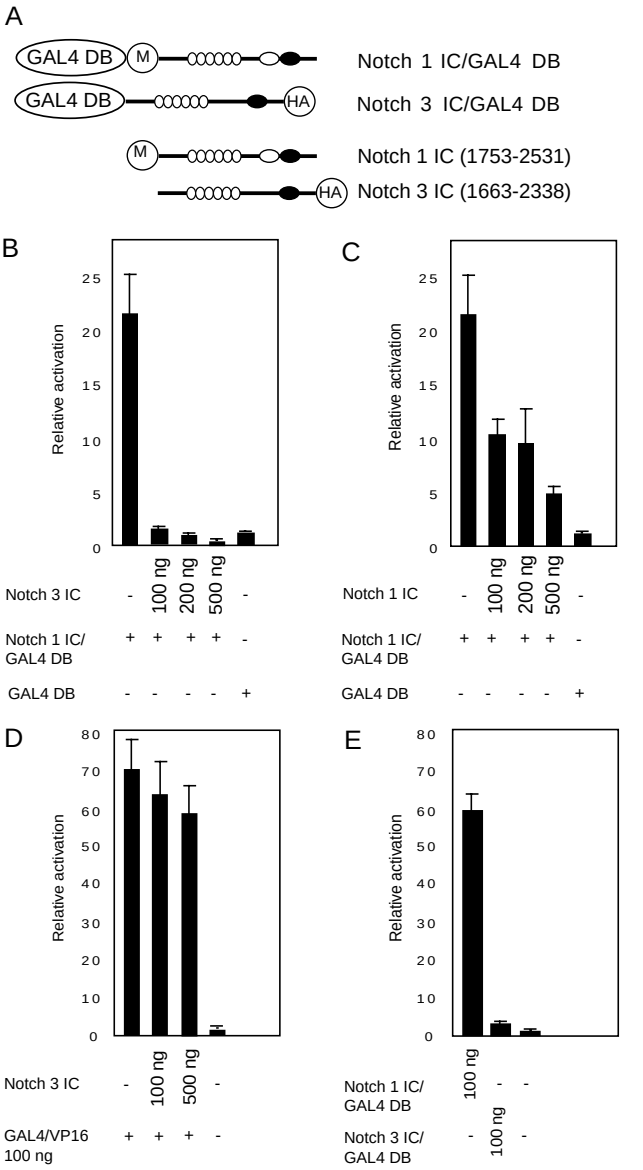


Fig. 6. Notch 3 IC represses Notch 1 IC-mediated activation independently of RBP-Jk. (A) Schematic representation of the different Notch IC DNA constructs used in transfection experiments of JEG (D) and COS-7 (B,C,E) cells. (B) Free Notch 3 IC represses Notch 1 IC-GAL4 DB induced reporter gene activity. COS-7 cells were transfected with either an expression plasmid encoding Notch 1 IC-GAL4 DB (100 ng) alone, or together with increasing amounts of Notch 3 IC DNA. A constant amount of the MH100-tk-luc reporter plasmid was included in each transfection. Reporter gene activity was analysed as described in Fig. 4. (C) The same experiment as in B, except that Notch 1 IC was used instead of Notch 3 IC as competitor. (D) Cotransfection of GAL4/VP16 with variable amounts of Notch 3 IC. Note that increasing amounts of Notch 3 IC lead only to a marginal reduction in transactivation. (E) Notch 1 IC fused to the GAL4 DB (Notch1 IC/GAL4 DB) activates transcription of the reporter plasmid when transfected into COS-7 cells. In contrast, a similar fusion construct of Notch 3 IC and GAL4 DB does not activate transcription. Values are the average + s.d. from three experiments.

above (Fig. 6E). These data suggest that Notch 3 IC is not a potent activator of transcription, even when placed in close proximity to a promoter.

Notch 3 IC represses *HES-5* expression in vivo

Notch 3 and *Notch 1* are coexpressed in many regions during embryonic development in mice (Lindsell et al., 1996; Mitsiadis et al., 1997). Some areas also show an inverse correlation between *Notch 3* and *HES* expression. Thus, high levels of *HES-5* expression are found in the postnatal olfactory bulb and cerebellar Purkinje cells (Akazawa et al., 1992), at time points when Notch 3 expression is downregulated (Lardelli et al., 1994). To directly test whether Notch 3 IC can also act as a repressor in vivo, we analysed *HES* mRNA levels in transgenic mouse embryos expressing Notch 3 IC in the developing CNS. These transgenic mice, which carry the Notch 3 IC under control of the nestin promoter (nestinp/Notch 3 IC), die during embryogenesis and show CNS malformations (Lardelli et al., 1996). We therefore analysed *HES* expression by whole-mount in situ hybridization in transient transgenics, i.e. in embryos derived from injected eggs. We analysed the expression levels of *HES-5* rather than of *HES-1*, since *HES-5* expression is regulated by Notch 3 in vitro (Fig. 3D) and because *HES-5* mRNA levels are reduced in mice targeted for *Notch 1* and *RBP-Jk* (de la Pompa et al., 1997). Of a total of 61 embryos, 10 were transgenic, and of those, one embryo showed a distinct CNS phenotype similar to that previously observed (Lardelli et al., 1996). This E10.5 embryo had a substantially enlarged head (data not shown), and showed a reduction in *HES-5* mRNA levels around the rhombic lip and in the myelencephalic region, but not in more anterior (data not shown) and posterior CNS regions, as compared to E10.5 wild-type controls (Fig. 7). The transgenic embryos that did not show an overt CNS phenotype had normal *HES-5* mRNA levels (data not shown), which suggests that the expression levels of Notch 3 IC may have been too low in these cases to alter *HES-5* expression levels.

DISCUSSION

The data described in this report show that the intracellular domain of Notch 3 has a very low level of transactivating activity compared to Notch 1 IC and that it represses the Notch 1-mediated upregulation of *HES-1* and *HES-5* expression. This is the first example of a Notch intracellular domain acting as a repressor of *Enhancer of Split/HES* expression. Genetic and molecular data from all other analysed Notch homologs in both *Drosophila*, *C. elegans*, *Xenopus*, chick and mammals indicate that Notch intracellular domains are gain-of-function mutants (Coffman et al., 1993; Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993; Chitnis et al., 1995; Dorsky et al., 1995), and both the *Drosophila* Notch IC, as well as *Xenopus* and mouse Notch 1 IC and Notch 2 IC, similarly activate transcription from *E(spl)* or *HES* promoters (Jarriault et al., 1995; Lecourtois and Schweisguth, 1995; Tamura et al., 1995; Hsieh et al., 1996; Kopan et al., 1996; Wettstein et al., 1997). The coexpression of *Notch 3* with *Notch 1* and *2* in many areas during development (Lardelli et al., 1994; Lindsell et al., 1996; Mitsiadis et al., 1997) suggests that *HES* expression in vivo is subject to both positive and negative control from the Notch receptors.

How can Notch 3 IC function as a repressor? Competition between Notch 1 IC and Notch 3 IC for access to RBP-Jk could in theory be the sole explanation for repression. This seems less likely, however, since Notch 1 IC and Notch 3 IC appear to bind RBP-Jk with similar affinity (Fig. 5B), while transfection of equal amounts of Notch 1 IC and Notch 3 IC leads to an 88-fold repression (Fig. 3). To account for this 'powerful' Notch 3 IC repression, we propose a model, in which Notch 3 IC competes with Notch 1 IC at at least two levels, i.e. by competition for access to RBP-Jk and by competition for a common coactivator (Fig. 8). The competition experiments between free Notch 3 IC and Notch 1 IC/GAL4 DB (Fig. 6A) clearly show that Notch 3 IC also functions as a repressor when not bound to DNA. Since Notch 1 IC and Notch 3 IC do not interact, the most parsimonial

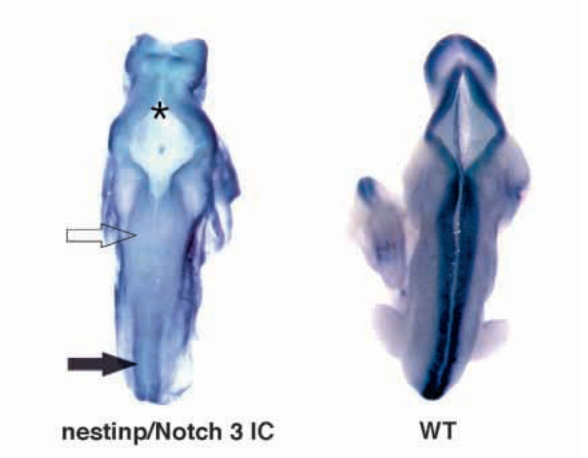


Fig. 7. Reduction of *HES-5* expression in a nestinp/Notch 3 IC transgenic embryo. Dorsal views of an E10.5 transgenic (nestinp/Notch 3 IC) and a wild-type (WT) littermate after whole-mount in situ hybridization for *HES-5* mRNA expression. Note the reduced levels of *HES-5* expression around the rhombic lip (*) and in the myelencephalic region (open arrow). *HES-5* mRNA levels appear not to be significantly altered in the more caudal (closed arrow) and rostral (data not shown) regions of the CNS.

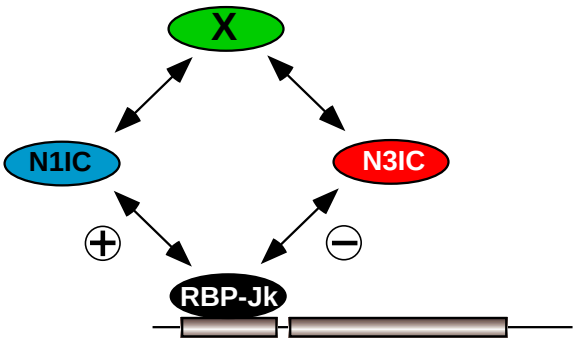


Fig. 8. A model for Notch 3 IC repression of Notch 1-mediated activation of *HES* expression. Repression can occur at two levels of Notch signaling. First, both Notch 1 IC and Notch 3 IC can bind to RBP-Jk and thus compete for access to the binding site on RBP-Jk. Binding of Notch 1 IC leads to activation of *HES* expression (+), while this is not the case when Notch 3 IC is bound to RBP-Jk (–). Second, Notch 1 IC and Notch 3 IC appear to compete for a common coactivator, present in limiting amounts (X). Since Notch 3 IC cannot activate *HES* expression, Notch 3 IC acts as a 'sink' for the coactivator and lowers the amounts of X available for interaction with Notch 1 IC. In order to explain the dramatic repression of Notch 1 IC-mediated activation exerted by Notch 3 IC at very low concentrations, it is plausible that repression is executed by competition at both levels.

explanation is that Notch 3 IC binds to a coactivator that is also required for Notch 1 IC in order to activate transcription, and that this coactivator is present in limiting amounts. It should be noted that considerably smaller amounts of Notch 3 IC DNA were required compared to Notch 1 IC DNA in order to obtain repression. Assuming that the Notch 1 IC and Notch 3 IC DNAs are translated with similar efficiency, this may imply that Notch 3 IC binds the common coactivator with higher affinity than Notch 1 IC. Further support for the existence of a coactivator comes from the finding that the Notch 3 ankyrin repeat construct, which lacks the strong RBP-Jk-binding RAM23 domain, is still able to repress Notch 1 IC-mediated activation (Fig. 3). The common coactivator is most likely not required for all transcriptional complexes, as activation via the GAL4/VP16 fusion protein was not inhibited by Notch 3 IC. In keeping with this, cotransfection of the general coactivators TIF2, SRC1 and p300, did not neutralize Notch 3 IC's repressor activity (data not shown).

Given that both Notch 1 IC and Notch 3 IC bind to RBP-Jk, competition for a common coactivator would, however, not be sufficient to explain repression, unless Notch 3 IC also shows a reduced ability to activate transcription when placed in the proximity of a promoter. We find that a fusion of Notch 3 IC to GAL4 DB is insufficient to activate transcription from the UAS response element, while Notch 1 IC/GAL4 DB is an activator. This supports the notion that Notch 3 IC and Notch 1 IC competes for access to RBP-Jk, and that only Notch 1 IC promotes transcription. The reason why only Notch 1 IC, but not Notch 3 IC promotes transcription still remains to be elucidated, but there are a number of possible explanations. One possibility is that a masking domain in the Notch 3 IC prevents the coactivator from exerting its function. Alternatively, Notch 3 IC, but not Notch 1 IC, might recruit a silencing factor, which quenches transcriptional activation. Finally, it is possible that a second coactivator is required for activation and that only Notch 1 IC, but not Notch 3 IC, binds this factor. In conclusion, our model suggests that Notch 3 IC competes with Notch 1 IC at two levels. First, they compete for access to RBP-Jk, and binding of Notch 3 IC is non-productive in terms of *HES* activation. Second, they compete for binding to a common coactivator. The coactivator is present in limiting amounts, and Notch 3 IC can thus act as a 'sink', and reduce the availability of the coactivator for Notch 1 IC. This two-step model is schematically depicted in Fig. 8.

What is the structural basis for the difference in transactivating capacity between Notch 1 IC and Notch 3 IC? All Notch receptors, including Notch 3, are highly structurally related in the intracellular domains, in particular in the ankyrin repeat region ((Lardelli et al., 1994; see Weinmaster, 1997, for a review). The ankyrin repeat region is important for the transactivating activity in *Drosophila* Notch, LIN-12 and Notch1 (Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993; Jarriault et al., 1995; Tamura et al., 1995; Hsieh et al., 1996; Kato et al., 1996). The high degree of conservation between Notch 1 IC and Notch 3 IC in this domain may at first seem paradoxical. It should however be noted that relatively subtle mutations in the ankyrin repeat region can dramatically alter its transactivation competence (Lieber et al., 1993; Rebay et al., 1993; Schroeter et al., 1998). The RAM23 region is conserved to a somewhat lesser extent, but apparently the conservation is sufficient for both Notch 1 IC and Notch 3 IC to bind to RBP-Jk (Kato et al., 1996). The most obvious differences between Notch 1 IC and Notch 3 IC are found at the C-terminal end, where Notch 3 IC is shorter and lacks the OPA repeats found in other Notch homologs (Lardelli et al., 1994). It remains to be tested, however, whether this region plays a role in transactivation.

Notch 3 IC function in vivo

A partial reduction of *HES-5* expression in the rhombomere region was observed in a nestinp/Notch 3 IC transgenic mouse embryo with a distinct CNS phenotype. This suggests that Notch 3 IC also acts as a repressor of *HES* expression in vivo. Downregulation of HES-5 was evident around the rhombic lip and in the myelencephalic region, but not in more anterior and posterior CNS regions. Interestingly, this is reminiscent of the situation in RBP-Jk and Notch $1^{-/-}$ mice, in which *HES-5* expression is also reduced in this region (de la Pompa et al., 1997). This further supports a role of Notch 3 IC as a repressor of Notch 1-signaling, but also suggests that *HES-5* expression is, at least in part, regulated by other factors in other regions of the CNS.

A role for Notch 3 as a repressor of *HES* expression in vivo receives further support from comparisons of the phenotypes resulting from targeting of *HES-1* and overexpression of Notch 3 IC in transgenic mice. Expression of Notch 3 IC in the developing CNS of transgenic mouse embryos produces an embryonically lethal phenotype (Lardelli et al., 1996). The transgenic embryos have an undulating spinal cord, fail to close the anterior neural pore and exhibit protrusions of neural tissue from the anterior neural pore region (Lardelli et al., 1996). Although initially interpreted differently (Lardelli et al., 1996), the latter phenotype may be a consequence of the open neural pore, in particular considering that the transgenic embryos show a relatively modest increase in proliferative rate in the CNS. Embryos lacking the *HES-1* gene die just after birth, and show a kinked neural tube, open anterior neural pore and an everted neuroepithelium (Ishibashi et al., 1995). Thus, the *HES-1*$^{-/-}$ phenotype shows clear similarities to that observed in embryos overexpressing Notch 3 IC in the early CNS.

Independent support for a function of Notch 3 IC as a repressor comes from recent work on Notch signaling during pancreas development. The Notch signaling pathway plays a critical role in the choice between the progenitor/exocrine fate and differentiation to endocrine cells (Dr H. Edlund, personal communication). Expression of Notch 3 IC from the *Pdx1* promoter in the pancreatic progenitor cells leads to reduced *HES-1* expression and premature differentiation of endocrine cells at the expense of the pool of progenitor cells. The same phenotype is observed in *Delta-1*$^{-/-}$ and *RBP-Jk*$^{-/-}$ mouse embryos and after overexpression of ngn3 (which is negatively regulated by HES) in transgenic mice (Dr H. Edlund, personal communication). These data are in accordance with a role for Notch 3 IC as a repressor of *HES* expression also in the developing pancreas.

The finding that Notch 3 IC acts as a negative modulator of *HES* expression will be important for our understanding of the CADASIL (Cerebral Autosomal Dominant with Arteriopathy and Subcortical Infarcts with Leukoencephalopathy). CADASIL is a familial disease which leads to migraine, subcortical brain infarcts and dementia and is caused by missense mutations in the EGF-repeat region of the human

Notch 3 gene (Joutel et al., 1996, 1997). CADASIL is a dominant disease, but it is not yet known whether the mutations in Notch 3 lead to haploinsufficiency, i.e. that normal function is impaired by loss of one functional allele, or if the CADASIL mutations result in gain-of-function receptors. Since *Notch 1, 2* and *3*, and *HES* genes are expressed in the adult brain (Lardelli et al., 1994; Lindsell et al., 1996), it is conceivable that the function of Notch 3 as a negative modulator of *HES* expression may be affected in the disease. Thus, if CADASIL mutations produce gain-of-function Notch 3 receptors, this would result in decreased *HES* expression. Conversely, in the haploinsufficiency scenario, repression of *HES* expression would be reduced.

In conclusion, our data show that the intracellular domain of Notch 3 behaves differently from all other Notch intracellular domains analysed and acts as a negative modulator of Notch 1-mediated *HES* expression. Mammalian Notch receptors have thus acquired distinct functions during evolution, which may provide a means to create a more fine tuned control of the downstream responses in the Notch signaling pathway.

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