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SoxB1 transcription factors and Notch signaling use distinct mechanisms to regulate proneural gene function and neural progenitor differentiation

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The preservation of a pool of neural precursors is a prerequisite for proper establishment and maintenance of a functional central nervous system (CNS). Both Notch signaling and SoxB1 transcription factors have been ascribed key roles during this process, but whether these factors use common or distinct mechanisms to control progenitor maintenance is unsettled. Here, we report that the capacity of Notch to maintain neural cells in an undifferentiated state requires the activity of SoxB1 proteins, whereas the mechanism by which SoxB1 block neurogenesis is independent of Notch signaling. A common feature of Notch signaling and SoxB1 proteins is their ability to inhibit the activity of proneural bHLH proteins. Notch represses the transcription of proneural bHLH genes, while SoxB1 proteins block their neurogenic capacity. Moreover, E-proteins act as functional partners of proneural proteins and the suppression of E-protein expression is an important mechanism by which Notch counteracts neurogenesis. Interestingly, in contrast to the Hes-dependent repression of proneural genes, suppression of E-protein occurs in a Hes-independent fashion. Together, these data reveal that Notch signaling and SoxB1 transcription factors use distinct regulatory mechanisms to control proneural protein function and to preserve neural cells as undifferentiated precursors.

KEY WORDS: CNS development, Neurogenesis, Notch, Proneural bHLH proteins, Sox proteins

INTRODUCTION

In the developing central nervous system (CNS) the generation of a large number of different neuronal and glial cells from a small founding population of self-renewing stem and progenitor cells requires molecular programs acting with high spatial and temporal precision. It is well established that an evolutionary conserved program of proneural bHLH transcription factors is necessary for the progression of neurogenesis and promotes cells to leave the cell cycle, downregulate progenitor characters and upregulate the expression of neuronal markers (Bertrand et al., 2002; Guillemot, 2007). However, to avoid premature depletion of the progenitor pool, neural cells are also subjected to mechanisms that counteract neurogenesis. The Notch signaling pathway and the SoxB1 (Sox1, Sox2 and Sox3) transcription factors have key functions during neurogenesis and maintain neural cells in an undifferentiated state, partly by reducing the activity of proneural bHLH transcription factors (Bylund et al., 2003; Ross et al., 2003). Despite the similar effects exerted by Notch signaling and SoxB1 proteins it is not clear whether these factors control neurogenesis by regulating distinct or common downstream pathways.

The proneural proteins are composed of a family of basic helix-loop-helix (bHLH) transcription factors, which in the vertebrate CNS includes the proteins neurogenin1/2 (Ngn1/2), Mash1 and Math1 (Bertrand et al., 2002). These proteins promote neural cells to initiate a differentiation program that ultimately leads to the formation of mature neurons. The capacity of proneural proteins to promote the progression of neurogenesis has been suggested to be determined by their expression or activity levels (Bertrand et al., 2002). Accordingly, low levels of proneural proteins are compatible with self-renewing progenitors, whereas high levels of proneural proteins are irreversibly committing neural cells to differentiation. Hence, the expression or activity level of proneural proteins determines, at least partly, whether neural cells remain as progenitors or commit to neuronal differentiation.

A cardinal feature of Notch signaling during CNS development is the capacity to counteract neurogenesis and maintain neural cells in an undifferentiated state. At later stages, Notch signaling may also act instructively, to promote gliogenesis and affect functions in mature neurons (Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005). Notch signaling is dependent on cell-cell communication, where the interaction between the Notch receptor and its ligands on adjacent cells induce proteolytic processing of the Notch protein. The final proteolytic cleavage is accomplished by the γ-secretase complex and results in the release and nuclear translocation of the Notch intracellular domain (NICD). In the nucleus, NICD interacts with the DNA-binding protein CSL (CBF1/Suppressor of Hairless/Lag-1), which converts CSL from a transcriptional repressor to an activator by a NICD-induced displacement of a transcriptional co-repressor complex (Bray, 2006). An important transcriptional output of Notch signaling is the upregulation of the bHLH transcription factors Hes1 and Hes5 (Louvi and Artavanis-Tsakonas, 2006). Hes1 and Hes5 function as classical DNA-binding repressors that antagonize the expression of proneural genes (Ohtsuka et al., 1999). However, Hes transcription factors have also been suggested to oppose the progression of neurogenesis by forming non-functional pairs with proneural proteins or E-proteins (Fischer and Gessler, 2007; Sasai et al., 1992). E-proteins are ubiquitously expressed bHLH proteins, which have been proposed to function as obligatory heterodimerizing partner factors of proneural proteins. Thus, the ability of Notch signaling to maintain neural cells in an undifferentiated state appears, at least in part, to be achieved through a reduction in expression and

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activity levels of proneural proteins. However, whether this regulatory mechanism fully explains the capacity of Notch to maintain neural progenitor cells in an undifferentiated state is currently not understood.

The HMG-box transcription factors of the Sox gene family have several regulatory roles during CNS development (Wegner and Stolt, 2005). SoxB1 proteins that are expressed by a majority of neural stem and progenitor cells, both in the developing and adult CNS, counteract neuronal differentiation and maintain neural progenitor specific gene expression (Bylund et al., 2003; Graham et al., 2003). Another HMG-box protein, Sox21, has the opposite activity compared with Sox1-3, and is required for neuronal differentiation (Sandberg et al., 2005). The balance of Sox1-3 and Sox21 activities function as a determinant of whether neural cells should remain as progenitors or commit to differentiation. Interestingly, apart from counteracting the activity of Sox21, SoxB1 transcription factors also suppress neurogenesis by blocking the activity of proneural proteins (Bylund et al., 2003; Guillemot, 2007). Hence, a common feature in the ability of Notch signaling and SoxB1 proteins to regulate the commitment of progenitors to neurogenesis appears to be their capacity to modulate the activity of proneural proteins.

These findings and the functional similarities between Notch signaling and SoxB1 transcription factors evoke the question how do these proteins functionally interact to regulate neurogenesis, if at all? Here, we report that the ability of Notch to maintain neural cells in an undifferentiated state can be explained by its capacity to repress the expression of both proneural bHLH proteins and E-proteins. Notch signaling regulates the expression of these proteins by Hes-dependent and -independent mechanisms, respectively. Based on these findings, we suggest a model in which SoxB1 proteins preserve neural cells in a precursor state by maintaining the expression of progenitor properties, whereas the role of Notch is to control the balance of undifferentiated and differentiated neural cells by regulating the expression levels of proneural bHLH proteins and E-proteins.

MATERIALS AND METHODS

Expression constructs and *in ovo* electroporation

All cDNAs misexpressed in the chick neural tube were expressed from the CMV-IE enhancer/chick β-actin promoter in the pCAGGS vector (Niwa et al., 1991). Myc-tagged mouse N1ICD (*NICD*) (Kopan et al., 1996) and the R218H dominant-negative mouse CSL mutant (*dnCSL*) (Chung et al., 1994), generated by site-directed mutagenesis (QuikChange, Stratagene) of a wild-type CSL template, were both cloned into the *pIRES-EGFP* plasmid (Clontech) prior to subcloning into pCAGGS. *Hes5*$^{\Delta CT}$*-VP16* was generated by replacing the five final codons, encoding the WRPW motif of Hes5, with the VP16 activator domain using the *pSlax-VP16* shuttle vector (Bergsland et al., 2006). *NICD*$^{\Delta CT}$*-EnR* was generated by subcloning the cDNA encoding the N-terminal part of NICD, consisting of the RAM and ankyrin repeat domains [referred to as 1100 in Beatus et al. (Beatus et al., 2001)], to the *pSlax-EnR* shuttle vector (Bergsland et al., 2006). The *Hes5-EGFP* construct was a kind gift from Henrique D. (Fior and Henrique, 2005). The chick *Hes1* construct was a kind gift from Dr J. Ericson (Karolinska Institute). A myc-tagged mouse *E47* construct was subcloned by PCR using IMAGE: 4187386 as a template. *Sox3-myc*, *Ngn2-myc*, *Sox21-myc*, *Sox3-EnR* and *12xCSL-DsRedExpressDR* have been described elsewhere (Bylund et al., 2003; Hansson et al., 2006; Sandberg et al., 2005). DNA constructs were electroporated into the neural tube of HH stage 10 chick embryos. After 5-48 hours, embryos were fixed and processed for immunohistochemistry or in situ hybridization.

Neural explants

Neural tube explants were isolated from the posterior part of HH stage 10 chick embryos, embedded in collagen and cultured in F12 media (Gibco) supplemented with N2 (Gibco). The gamma secretase inhibitor DAPT (Calbiochem) was added at concentrations ranging from 0-6 μM. After culture the explants were subsequently fixed in 4% paraformaldehyde and processed for cryosectioning and antibody staining.

Immunohistochemistry and in situ hybridization

Antibody staining was performed as described previously (Tsuchida et al., 1994). The following antibodies were used; rabbit anti-Sox3 (kindly provided by T. Edlund, Umeå University), rabbit anti-Sox1 (Bylund et al., 2003), rabbit anti-Ngn2 (Sandberg et al., 2005), mouse anti-NeuN (Chemicon), rabbit anti-VP16 (Abcam), mouse anti-PCNA (DAKO), mouse anti-Tuj1 (Covance), mouse FITC-anti-BrdU (Becton Dickson), mouse anti-Myc (Santa Cruz Biotech) and rabbit anti-Myc (Santa Cruz Biotech). In situ hybridization was performed as described (Tsuchida et al., 1994) using chick probes for *Ngn1*, *Ngn2*, *Cash1*, *Hes1*, *Hes5*, *E47*, *Notch1* and *Sox3* (Jasoni et al., 1994; Kamachi et al., 1998; Perez et al., 1999). cDNAs encoding chick *Hes1, Hes5, E47* and *Notch1* were obtained from MRC Geneservice; clone IDs: chEST356J15, chEST382I21, chEST719E2 and chEST891H8.

BrdU incorporation

BrdU (100 μM) was applied to chick embryos in ovo, followed by incubation for 30 minutes at 38°C at which time the embryos were fixed.

Ngn2 and NeuroD promoter activity assay

The Ngn2 promoter construct was generated by PCR amplification of a 1.2 kb upstream sequence of the mouse Ngn2 gene. The sequence, which includes the endogenous proximal promoter together with 91bp of 5′ UTR, was subcloned into the pGL3TK-Basic vector (Promega). Luciferase assays were conducted in transfected 293 HEK or P19 cells using Lipofectamine PLUS (Invitrogen). pGL3Ngn2prom1.2kb was transfected with *NICD*, *Hes5* or *Hes5*$^{\Delta CT}$*-VP16* expression vectors. pGL3NeuroDprom1.0kb (Huang et al., 2000) was transfected with different concentrations of *Ngn2*, *E47* and *Sox3* expression vectors. As an internal transfection control an expression plasmid encoding β-gal was included in all transfections. Twenty hours after transfection, luciferase and β-galactosidase levels were determined as described previously (Castro et al., 1999).

RESULTS

Elevated levels of Notch and SoxB1 activity block neuronal differentiation

To begin to examine whether SoxB1 proteins and components of the Notch signaling pathway use distinct or similar mechanisms to regulate neurogenesis, we first compared their expression pattern,

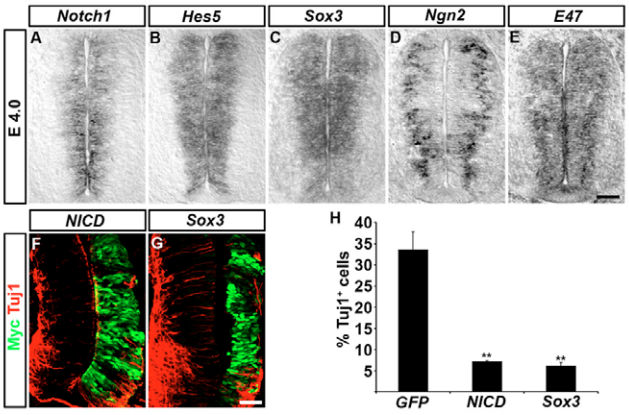

Fig. 1. Both Notch and Sox3 block neurogenesis. (**A-E**) *Notch1* (A), *Hes5* (B), *Sox3* (C), *Ngn2* (D) and *E47* (E) exhibited complementary expression patterns in the embryonic day 4.0 chick spinal cord. (**F-H**) Expression of the intracellular domain of Notch1 (NICD) (F) or Sox3 (G) for 42 hours significantly reduced the generation of Tuj1^{+} neurons (H). Data are represented as percentage of electroporated cells expressing Tuj1 (mean±s.e.m.). $^{**}P<0.01$ relative to *EGFP* control transfected cells, Student's *t*-test. Scale bars: 50 μm.

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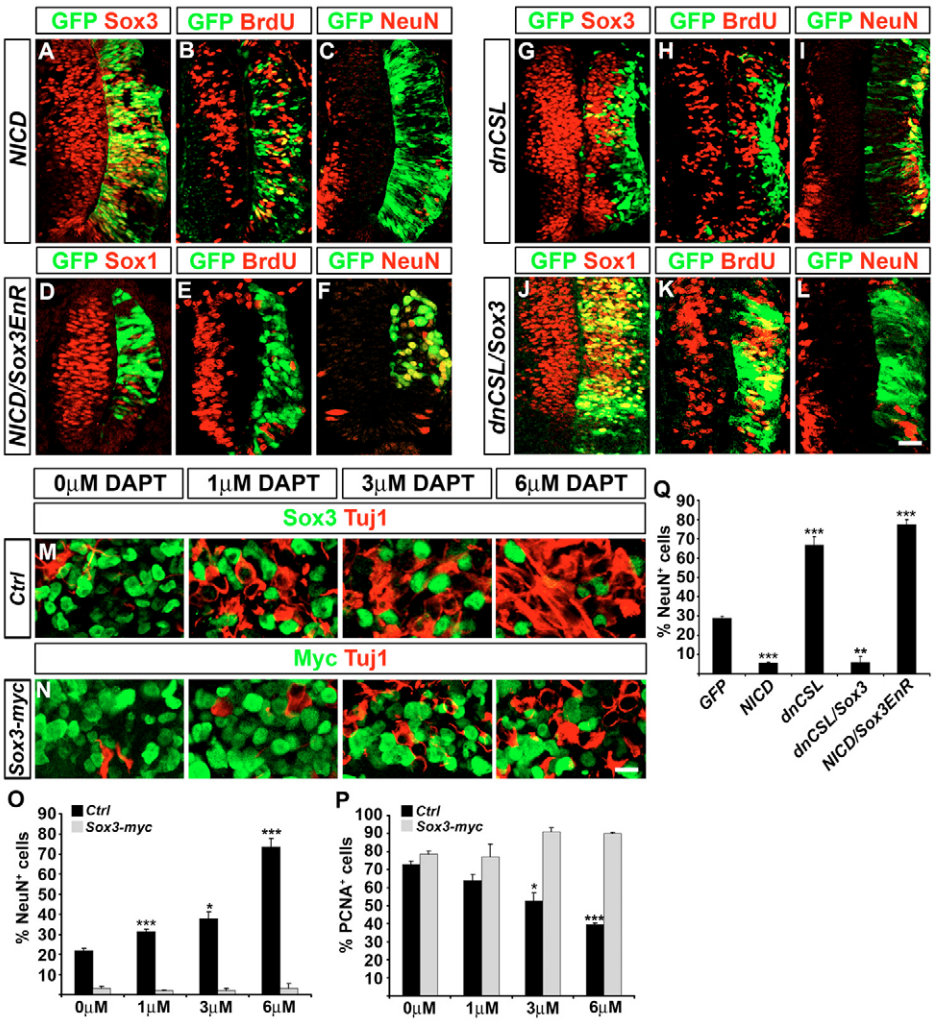

Fig. 2. Notch-mediated block of neurogenesis depends on intact SoxB1 function.
(**A-C**,Q) Misexpression of NICD for 42 hours prevented the generation of neurons expressing NeuN (C,Q) but retained expression of the progenitor protein Sox3 (A) and the incorporation of BrdU (B). (**D-F**,Q) Misexpression of HMG^{Sox3}-EnR for 24 hours caused cells to downregulate Sox3 (D), exit the cell cycle (E) and upregulate the expression of NeuN (F,Q), even in the presence of NICD misexpression. (**G-I**,Q) Twenty-four hours after transfection, expression of a dominant-negative version of CSL (dnCSL), which is unable to bind DNA, had induced cells to downregulate Sox3 (G), exit the cell cycle (H) and upregulate the expression of NeuN (I,Q). (**J-L**,Q) Combined expression of Sox3 and dnCSL for 42 hours efficiently blocked the generation of NeuN$^+$ cells (L,Q) and maintained cells in a self-renewing (K) and Sox1 expressing (J) state. Black and white representations of A-L are shown in Fig. S3 (see supplementary material). (**M-P**) The gamma secretase inhibitor DAPT acted in a concentration-dependent manner and caused neural cells to downregulate Sox3 expression (M), exit the cell cycle (P) and upregulate the expression of Tuj1 and NeuN (M,O). Neural explants transfected with *Sox3* did not upregulate neuronal marker expression (N,O) or exit the cell cycle (P), regardless of the DAPT concentration. (**Q**) Statistical representation of NeuN-expressing cells in neural tubes transfected with *EGFP*, *NICD*, *dnCSL*, *dnCSL/Sox3* or *NICD/HMG^{Sox3}-EnR*. Data are represented as mean±s.e.m. $^{**}P<0.01$, $^{***}P<0.001$, Student's *t*-test relative to 0 μM DAPT control in O and P and relative to *EGFP*-transfected control cells in Q. Student's *t*-test. Scale bars: 40 μm in L; 10 μm in N.

both in relation to each other and to proneural bHLH transcription factors and E-proteins. In Hamburg-Hamilton (HH) stage 20 chick spinal cords, *Notch1* and *Hes5* were expressed in progenitor cells within the ventricular zone, in a pattern extensively overlapping with that of the B1-subgroup member *Sox3* (Fig. 1A-C). The genes encoding the proneural bHLH factors Ngn2 and the E-protein E47 were also expressed in a pattern overlapping with that of *Notch*, *Hes5* and *Sox3*, although their expression was most pronounced in progenitor cells located in the lateral margin of the ventricular zone (Fig. 1D,E).

We next compared the effects on neurogenesis of increased levels of Notch or SoxB1 activity. Using chick embryo electroporation, an expression vector containing the intracellular domain of the Notch1 receptor (NICD) (Schroeter et al., 1998), together with an IRES-EGFP cassette, was transfected in the caudal part of the neural tube. Misexpression of NICD significantly decreased the number of cells expressing neuronal markers (Fig. 1F,H). In a similar manner, neural progenitor cells transfected with a myc-tagged version of *Sox3* failed to upregulate the expression of the pan-neuronal marker Tuj1 (Fig. 1G,H). Thus, *Sox3* and components of the Notch signaling pathway have largely overlapping expression patterns in the developing CNS and overexpression of either NICD or Sox3 blocks the generation of differentiated neuronal progeny to an apparently similar extent (Fig. 1H).

Notch-mediated control of differentiation requires Sox3 activity

To further explore whether Notch signaling and SoxB1 transcription factors use similar mechanisms to control neurogenesis, we next determined whether the capacity of Notch to control neurogenesis is dependent on the activity of SoxB1 proteins. Overexpression of NICD alone maintained the expression of progenitor characters (Fig. 2A,B), blocked the formation of neurons (Fig. 2C,Q), but did not increase the endogenous expression levels of SoxB1 proteins (Fig. 2A; and data not shown). By contrast, cells co-transfected with *NICD* and a dominant-negative version of Sox3 (*HMG^{Sox3}-EnR*) downregulated progenitor characters (Fig. 2D,E, data not shown) and instead upregulated the expression of neuronal markers (Fig. 2F,Q, data not shown). Similar results were obtained when *NICD* was co-electroporated with *Sox21* (see Fig. S1 in the supplementary material). Thus, under conditions of blocked SoxB1 activity, NICD is unable to maintain neural cells in an undifferentiated state.

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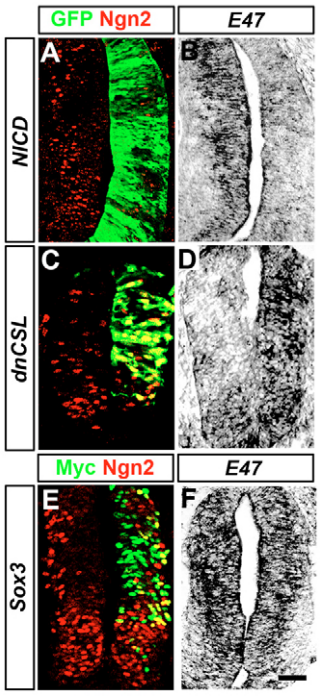

Fig. 3. Notch, but not Sox3, attenuates expression of Ngn2 and *E47*. (**A**,**B**) Expression of NICD for 42 hours attenuated Ngn2 (A) and *E47* expression (B). (**C**,**D**) Transfection of *dnCSL* for 10-20 hours increased Ngn2 (C) and *E47* expression (D). (**E**,**F**) Misexpression of Sox3 for 42 hours did not alter the levels of Ngn2 (E) or *E47* expression. Black and white representations of A-F are shown in Fig. S4 (see supplementary material). Scale bar: 40 μm.

In a converse experiment, we misexpressed a dominant-negative version of CSL (*dnCSL*) either alone or together with Sox3. dnCSL that fail to bind DNA has previously been demonstrated to block Notch signaling by its ability to interact with NICD (Chung et al., 1994; Kato et al., 1997). Indeed, misexpression of dnCSL efficiently caused cells to exit the cell cycle, downregulate progenitor identities and upregulate the expression of pan-neuronal markers (Fig. 2G-I,Q); an effect that could be counteracted by co-electroporated *NICD* (see Fig. S2 in the supplementary material). Notably, neural cells co-transfected with *dnCSL* and *Sox3* remained as self-renewing progenitors (Fig. 2J,K) and failed to upregulate the expression of neuronal markers, even 42 hours after transfection (Fig. 2L,Q). Furthermore, misexpression of NICD fused to the repressor domain of the *D. melanogaster* Engrailed protein (*NICD$^{\Delta CT}$-EnR*) had the opposite activity compared with NICD and caused cells to exit the cell cycle, downregulate the expression of progenitor markers and upregulate the expression of neuronal markers (data not shown). However, cells transfected with *NICD$^{\Delta CT}$-EnR* together with *Sox3* were maintained as self-renewing progenitors despite perturbed Notch signaling (data not shown). Together, these experiments indicate that the ability of Notch to preserve progenitor cells relies on the presence of SoxB1 activity, whereas the mechanism by which SoxB1 proteins maintains progenitor cell properties is independent of Notch signaling.

To corroborate the genetic data, we blocked Notch signaling using the gamma-secretase inhibitor DAPT, which blocks the final ligand-induced cleavage of Notch and thus the release of NICD (Cheng et al., 2003; Dovey et al., 2001; Sastre et al., 2001). Neural tissue explants, isolated from the caudal part of HH stage 11 chick spinal cords, were cultured in vitro with or without DAPT. After 35 hours of culture without DAPT, the majority of the cells were still in the cell cycle, and only a minority of the cells expressed the neuronal markers Tuj1 or NeuN (Fig. 2M,O,P). By contrast, the presence of DAPT, in a concentration-dependent fashion, induced cell cycle exit, downregulation of progenitor identities and upregulation of neuronal markers (Fig. 2M,O,P). Interestingly, neural cells transfected with a *Sox3* expression vector prior to the in vitro culture failed to exit the cell cycle and upregulate neuronal markers, even when cultured for 35 hours in the presence of 6 μM DAPT (Fig. 2N-P). Thus, by inhibiting the proteolytic processing of the Notch receptor and the liberation of its intracellular domain, DAPT caused neural cells to leave the undifferentiated progenitor state and commit to neuronal differentiation. Misexpression of Sox3 blocked cells to commit to neuronal differentiation even in the absence of Notch receptor signaling, strengthening the notion that the mechanisms by which Notch signaling and SoxB1 transcription factors regulate neurogenesis are distinct.

Notch, but not SoxB1 activity, represses proneural bHLH and E-protein expression

As both Notch signaling and SoxB1 transcription factors counteract neurogenesis by regulating proneural activity (Bylund et al., 2003; Ross et al., 2003), we next determined how Notch and Sox3 proteins affected the expression of the proneural factors Ngn2 and Cash1, and the genes encoding the E-proteins E47 and Tcf12. Forty-two hours after *NICD* electroporation, both Ngn2 and *Cash1* were significantly downregulated (Fig. 3A; and data not shown), whereas these genes were induced already 10 hours after misexpression of dnCSL or NICD$^{\Delta CT}$-EnR (Fig. 3C; and data not shown). Interestingly, the expression profiles of *E47* and *TCF12* mimicked those of the proneural genes, and were down- and upregulated in response to NICD and dnCSL misexpression, respectively (Fig. 3B,D; and data not shown). By contrast, overexpression of Sox3 did not block the expression of proneural or E-protein-coding genes (Fig. 3E,F). Hence, the expression of proneural bHLH proteins and E-proteins was efficiently downregulated by active Notch signaling, but not by Sox3, indicating that Notch and SoxB1 proteins use different strategies to control the activity of proneural bHLH factors and E-proteins.

Combined expression of Ngn2 and E47 rescues Notch-induced block of neuronal differentiation

The finding that Notch suppresses the expression of both proneural and E-protein genes evokes the question is this sufficient for Notch to counteract neuronal differentiation? To examine this issue, NICD was misexpressed alone (Fig. 4A-C) or together with either Ngn2 (Fig. 4D-F) or E47 (Fig. 4G-I), or together with both Ngn2 and E47 (Fig. 4J-L). When misexpressed alone, Ngn2, and to some extent also E47, promoted cells to migrate laterally from the ventricular zone, downregulate progenitor features and upregulate the expression of neuronal markers (see Fig. S5A-F in the supplementary material). The capacity of *Ngn2* or *E47* to promote neurogenesis individually was, however, efficiently blocked by co-electroporated *NICD* (Fig. 4A-I,T) and the cells were independent of the amounts of transfected Ngn2 or E47 expression vectors (0.7 or 1.5 μg/μl) maintained as self-renewing and Sox3^{+} progenitor cells (Fig. 4A,B,D,E,G,H) that failed to upregulate the expression of neuronal markers (Fig. 4C,F,I,T). By contrast, the combined expression of Ngn2 and E47 efficiently rescued the NICD-induced block of neurogenesis, and the transfected cells strongly downregulated progenitor characters and instead upregulated the

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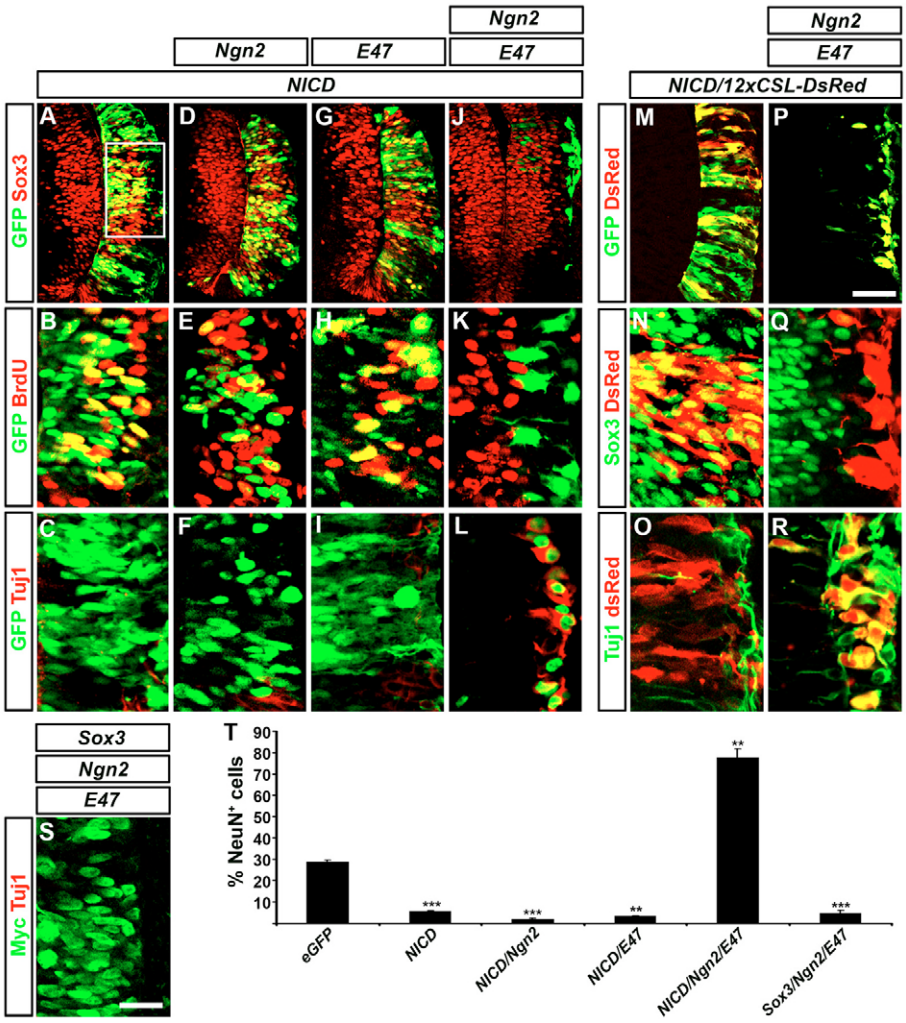

Fig. 4. Ngn2 and E47 can rescue NICD induced block of neurogenesis. (**A-C**,T) Cells transfected with *NICD* (0.7 μg/μl) for 42 hours were Sox3$^+$ (A) and incorporated BrdU (B), but had failed to upregulate the expression of the neuronal markers NeuN and Tuj1 (C,T). (**D-I**,T) Co-transfection with either *Ngn2* or *E47* (0.7 μg/μl) did not block the capacity of *NICD* to maintain cells in an undifferentiated and self-renewing state. (**J-L**,T) Forty-two hours after electroporation, neural cells co-transfected with *Ngn2*, *E47* and *NICD* (0.7 μg/μl of each expression vector) had downregulated Sox3 (J), exited the cell cycle (K) and upregulated the expression of neuronal markers (L,T). (**M**) The Notch responsive reporter construct, *12xCSL-DsRed*, was highly activated in *NICD*-transfected cells. (**N**,**O**) These cells were maintained in a Sox3-expressing state (N) and failed to upregulate the expression of Tuj1 (O). (**P-R**) In cells co-transfected with *NICD/NGN2/E47* the *12xCSL-DsRed* reporter construct was expressed in cells located in the marginal zone (P) that had downregulated Sox3 (Q) and instead upregulated the expression of Tuj1 (R). (**S**) Misexpression of Sox3 efficiently blocked the generation of neurons, even when co-transfected with high levels of *Ngn2* and *E47*. (**T**) Quantification of the number of electroporated cells expressing the neuronal marker NeuN. The white square in A indicates regions analyzed. The figures in T are represented as percentage of electroporated cells expressing NeuN, mean±s.e.m. $^{**}P<0.01$, $^{***}P<0.001$, relative to *EGFP*-transfected control cells, Student's *t*-test. Scale bar: 40 μm in P; 10 μm in S.

expression of pan-neuronal proteins (Fig. 4J-L,T). Thus, the combination of the proneural protein Ngn2 and the E-protein E47 is sufficient to rescue the block of neurogenesis induced by NICD overexpression.

To exclude the possibility that the presence of misexpressed Ngn2 and E47 interfered with Notch-mediated downstream signaling, we monitored the activity of a 12XCSL-DsRed reporter construct, which reflects NICD-induced CSL activity (Hansson et al., 2006). When misexpressed alone, the reporter activity of 12xCSL-DsRed was low or undetectable (data not shown), in keeping with Hansson et al. (Hansson et al., 2006). However, in the presence of either co-electroporated *NICD* or *NICD/NGN2/E47* both progenitor cells and post-mitotic neurons expressed high levels of DsRed (Fig. 4M-R), indicating that neither Ngn2 nor E47 inhibits NICD-mediated downstream signaling.

As the combined expression of E47 and Ngn2 promoted neurogenesis also in the presence of NICD overexpression, we next examined whether these bHLH factors also could rescue the Sox3-induced block in neuronal differentiation. In contrast to Notch, Sox3 completely blocked neuronal differentiation both when misexpressed with Ngn2 or E47 alone, or in combination with both Ngn2 and E47 (Fig. 4S,T). Together these data indicate that Notch-signaling maintains neural cells in an undifferentiated state by repressing the expression of proneural bHLH and E-proteins, whereas SoxB1 proteins can suppress the progression of neurogenesis by blocking the capacity of proneural bHLH and E-proteins to promote neurogenesis.

Notch controls E47 expression in a Hes-independent manner

As Hes genes are key components of the Notch downstream response, we next asked if the Notch-mediated repression of *Ngn2* and *E47* expression is achieved through the regulation of *Hes* gene activity. To answer this question, we first examined the expression of *Hes1* and *Hes5* in *NICD* electroporated neural cells. Misexpression of NICD for 24 hours strongly upregulated the expression of both *Hes1* and *Hes5* in the neural tube (Fig. 5A,B), whereas misexpression of dnCSL or NICD$^{\Delta CT}$-EnR decreased the levels of both *Hes1* and *Hes5* (Fig. 5C,D; and data not shown). To determine whether Hes proteins could substitute for Notch in this regard, we transfected neural cells with a *Hes5-IRES-EGFP* expression vector (*Hes5*). Hes5 misexpression efficiently reduced the amount of Ngn2$^+$ cells (Fig. 5E,G) and also suppressed the generation of Tuj1$^+$ neurons (Fig. 5H,I). Interestingly, however, Hes5 did not alter the expression of *E47* (Fig. 5F). Similar results were obtained with Hes1 or the combined misexpression of Hes1 and Hes5 (see Fig. S6 in the supplementary material). The finding

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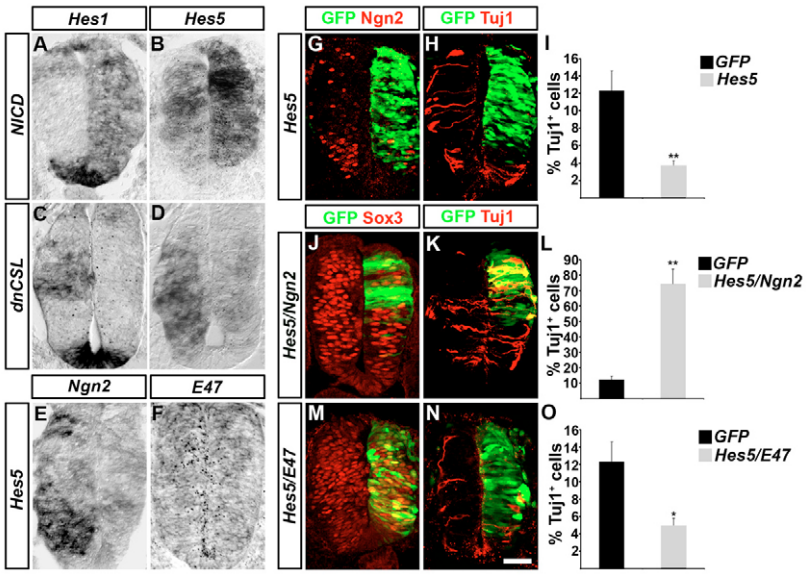

Fig. 5. Hes repress proneural proteins, but not E-protein expression. (**A-D**) Electroporation of *NICD* induced *Hes1* (A) and *Hes5* (B) expression. Misexpression of dnCSL downregulated *Hes1* and *Hes5* (C,D). (**E**,**F**) Misexpression of Hes5 efficiently attenuated *Ngn2* expression (E) but had no effect on *E47* transcription (F). (**G-I**) Misexpression of Hes5 suppressed Ngn2 (G) and Tuj1 expression (H,I). (**J-O**) Co-transfection of *Ngn2* and *Hes5* promoted electroporated cells to differentiate into post-mitotic neurons (J-L), whereas misexpression of Hes5 in combination with E47 blocked neuronal differentiation (M-O). Embryos were analyzed 24 hours after electroporation. Black and white representation of G,H,J,K,M,N are shown in Fig. S7 (see supplementary material). Data in I,L,O are represented as percentage of electroporated cells expressing Tuj1, mean±s.e.m. **P*<0.05, ***P*<0.01, relative to *EGFP*-transfected control cells, Student's *t*-test. Scale bar: 50 μm.

that Hes5 reduced expression of Ngn2 but not that of *E47* prompted us to determine capacity of Ngn2 or E47 to rescue Hes5-mediated repression of neurogenesis. Cells transfected with *Hes5* differentiated efficiently into neurons when co-transfected with *Ngn2* (Fig. 5J-L). By contrast, neural cells transfected with *Hes5* in the combination with *E47* failed to upregulate the expression of Tuj1 and instead remained as Sox3^{+} progenitor cells (Fig. 5M-O). Similar results were obtained with *E47* co-transfected with *Hes1* (see Fig. S6 in the supplementary material). Hence, Notch signaling appears to control neurogenesis via the combined Hes-dependent downregulation of Ngn2 and the Hes-independent downregulation of E-proteins.

A dominant active version of Hes induces Ngn2 but not E47 expression

To further address the mechanistic role of Hes proteins both during neurogenesis and in the Notch-mediated repression of proneural proteins and E-proteins, we next explored the effects of a dominant active form of Hes5. This construct was generated by replacing the Groucho-interacting C-terminal WRPW-motif with the transactivation domain of the viral protein VP16 (*Hes5*$^{\Delta ct}$*-VP16*) (Berk et al., 1998). In order to verify its function as a dominant active version, 293 HEK cells were transfected with expression vectors encoding Hes5$^{\Delta ct}$-VP16, full-length Hes5 or NICD together with a Luc reporter, containing a 1200 bp upstream region (–1 to –1200) of the mouse *Ngn2* gene (*Ngn2*$^{-1200bp}$*-Luc*). Both NICD and Hes5 repressed the *Ngn2*$^{-1200bp}$*-Luc* reporter, whereas the activity of this reporter was upregulated by Hes5$^{\Delta ct}$-VP16 (Fig. 6A). Furthermore, electroporation of *Hes5*$^{\Delta ct}$*-VP16* in the neural tube rapidly induced cells to exit the cell cycle and upregulate the expression of *Ngn2* (Fig. 6B,C) and the neuronal marker Tuj1 (Fig. 6E). However, misexpression of Hes5$^{\Delta ct}$-VP16 did not induce the expression of *E47* (Fig. 6D). Thus, a dominant active version of Hes5 promotes neurogenesis and induces the expression of *Ngn2*, but not that of *E47*.

Misexpression of NICD suppressed the expression of both *Ngn2* and *E47*, whereas Hes5$^{\Delta ct}$-VP16 only upregulated *Ngn2* expression (Fig. 6C,D). These findings strengthen the notion that Notch represses the expression of E-proteins in a Hes-independent manner. To further examine this issue, we next analyzed the ability of Hes5$^{\Delta ct}$-VP16 to rescue NICD-induced block of neurogenesis. In line with the previous findings, co-electroporation of *Hes5*$^{\Delta ct}$*-VP16* and *NICD* did not promote transfected cells to commit to neurogenesis (Fig. 6F,G,H). Neither did the combined misexpression of Hes5$^{\Delta ct}$-VP16, NICD and Ngn2 result in an upregulation of neuronal markers (Fig. 6F,I). By contrast, co-electroporation of *Hes5*$^{\Delta ct}$*-VP16* and *NICD* together with *E47* caused many cells to upregulate the expression of neuronal markers, 42 hours after transfection (Fig. 6F,J). Together, these findings strongly argue that Notch maintains neural cells in an undifferentiated state both by suppressing the expression of E-proteins and by activating the expression of Hes proteins that, in turn, specifically repress proneural bHLH gene expression (Fig. 6K).

DISCUSSION

In this report, we have addressed the functional relationship between Notch signaling and SoxB1 transcription factors in the regulation of vertebrate neurogenesis. Although elevated expression levels of either Notch or Sox3 leads to a block of neuronal differentiation, we demonstrate that they regulate neurogenesis by distinct mechanisms. In Fig. 6K, we propose a model for the distinct role of Notch and SoxB1. This model receives from the findings that overexpression of Sox3 maintains neural cells as precursors even in the absence of Notch signaling, whereas NICD requires SoxB1 activity to maintain neural cells in an undifferentiated state. Furthermore, although both Notch signaling and SoxB1 proteins suppress the activity of proneural proteins, they do so at distinct regulatory levels. Although Notch signaling represses the transcription of proneural bHLH and E-proteins, SoxB1 proteins suppress their ability to promote progenitor cells to commit to a neurogenic program. Consequently, the concomitant expression of Ngn2 and E47 could rescue NICD-, but not Sox3-, induced block of neurogenesis.

Whether Sox3 suppresses the neurogenic activity of Ngn2 and E47 through a direct block of E47/Ngn2-protein activities or induces a molecular environment in which E47 and Ngn2 proteins are unable to promote neuronal differentiation is currently unknown. We favor the latter idea as Sox3 failed to block Ngn2/E47 proteins from transactivating the E-box containing NeuroD promoter (Huang et al., 2000) in vitro (data not shown). Furthermore, the Sox3-mediated block of neuronal differentiation could not be counteracted by high

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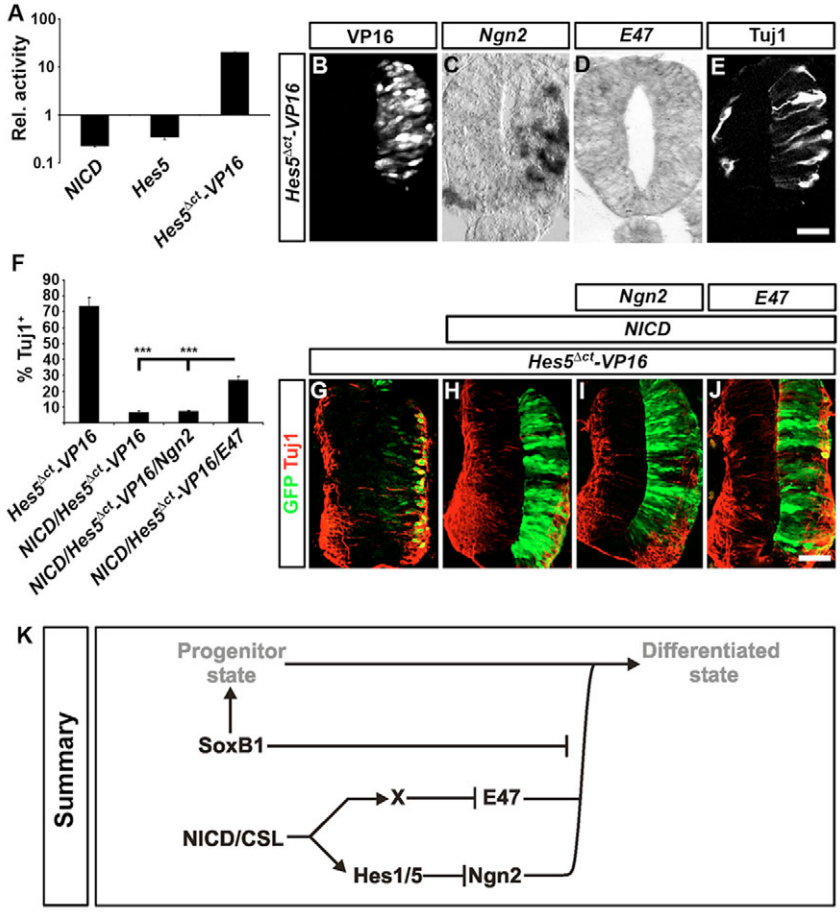

Fig. 6. A dominant-active version of Hes5 upregulates *Ngn2* but not *E47* expression. (**A**) NICD and Hes5 repressed the activity of the Ngn2 reporter construct in 293 HEK cells, whereas this reporter was activated by Hes5$^{\Delta ct}$-VP16. Data are represented as a logarithmic scale where mock is set to one. (**B-E**) Within 5 hours, *Hes5$^{\Delta ct}$-VP16* transfected cells had upregulated the expression of *Ngn2* (B,C) but not that of *E47* (D); ectopic expression of neuronal markers could be detected 12 hours after transfection (E). (**F**) Quantification of electroporated cells (GFP^{+}) in G-J expressing Tuj1. Data are represented as mean±s.e.m. ***P<0.001, relative to *NICD* electroporated cells, Student's t-test. (**G**) Forty-two hours after electroporation, a majority of cells expressing Hes5$^{\Delta ct}$-VP16 was terminally differentiated and expressed Tuj1. (**H**) Cells co-transfected with *NICD* together with *Hes5$^{\Delta ct}$-VP16* cells remained undifferentiated. (**I**) Similarly, cells co-transfected with Ngn2, NICD and Hes5$^{\Delta ct}$-VP16 remained undifferentiated and failed to upregulate the expression of Tuj1. (**J**) Misexpression of E47, Hes5$^{\Delta ct}$-VP16 and NICD promoted cells to commit to neurogenesis. (**K**) Proposed molecular pathway regulating neurogenesis in the vertebrate CNS. The proneural bHLH protein Ngn2 acts together with the E-protein E47 to drive the differentiation of neural progenitor cells by promoting cell cycle exit and the upregulation of neuronal protein expression. Notch signaling maintains neural cells in an undifferentiated state via the activation of CSL. Activated CSL is, in turn, inducing the expression of Hes1/5 and an alternative repressor (designated X), which subsequently represses the expression of Ngn2 and E47, respectively. SoxB1 transcription factors are according to this model, maintaining progenitor cells in an undifferentiated state by activating the expression of progenitor features and, in addition, blocking the activity of Ngn2 and E47. Scale bars: 20 μm in D; 50 μm in G.

levels of misexpressed Ngn2 and E47. In addition, blockage of Notch signaling, which induces high levels of Ngn2 and E47 expression, failed to rescue Sox3-mediated inhibition of neurogenesis. Thus, overexpression of Ngn2 and E47 is unable to rescue the block of neuronal differentiation mediated by high levels of Sox3, suggesting that Sox3 also maintains neural cells in an undifferentiated state by a Ngn2/E47 independent mechanism. It should be noted, however, that the gamma-secretase inhibitor has previously been reported to cause a subset of cultured Sox2 transduced neural cells to upregulate the expression of the pan-neuronal marker Map2 (Bani-Yaghoub et al., 2006). One possible explanation for these discrepancies is that elevated expression of proneural proteins and E-proteins induced by blocked Notch signaling only can be counteracted by SoxB1 proteins misexpressed at sufficient levels. Nevertheless, the mechanism by SoxB1 proteins maintain neural cells in an undifferentiated state remains to be elucidated, but it is of note that binding sites for SoxB1 proteins are frequently found in regulatory enhancer regions of genes expressed in neural progenitor cells (Bailey et al., 2006), suggesting a broader repertoire of SoxB1 downstream genes. Interestingly, misexpression of a dominant-negative version of Sox3 (*HMG^{Sox3}-EnR*) or Sox21, which represses genes normally activated by SoxB1 proteins (Bylund et al., 2003; Graham et al., 2003; Sandberg et al., 2005), caused neural cells to differentiate even in the presence of high levels of Notch signaling. One interpretation of these results is that Notch has a more defined role in balancing the maintenance versus differentiation of neural cells, by predominantly acting on Ngn2 and E47 expression, whereas SoxB1 proteins control the progenitor state in a wider context (Fig. 6K).

We demonstrate that the regulation of proneural bHLH and E-protein expression by Notch is accomplished by two distinct mechanisms, which differ in their requirements for Hes transcription

factors. Our data show, in accordance with previous findings, that Ngn2 expression is repressed through the activation of Hes proteins (Yoon and Gaiano, 2005). By contrast, the Notch-mediated repression of *E47* transcription is mediated by a Hes-independent mechanism, as supported by our findings that neither Hes5 nor its dominant active version (Hes5$^{\Delta ct}$-VP16) altered the expression levels of *E47* or *Tcf12*. Moreover, the capacity of Hes5 to block neurogenesis could be counteracted by Ngn2 alone. As misexpression of a dominant-negative version of CSL (dnCSL) upregulated both the expression of Ngn2 and *E47* and promoted neurogenesis, we interpret this to suggest that the repression of both Ngn2 and E47 by Notch is mediated by the activation of CSL. As Notch activation in most situations converts CSL from a repressor to an activator, it may be difficult to envisage *E47* as a direct Notch-ICD/CSL downstream gene. The identification of an increasing number of Notch downstream genes, in addition to *Hes* and *Hey* (Hurlbut et al., 2007; Iso et al., 2003), may, however, suggest that an alternative transcriptional repressor is induced, which in turn suppresses *E47*.

We have demonstrated that the control of E-protein expression is a vital mechanism by which Notch regulates the progression of neuronal differentiation. The role of E-proteins in progenitor cells has previously been studied in muscle cell differentiation, where E47 was demonstrated to heterodimerize with the bHLH protein MyoD (Lassar et al., 1991). This interaction was shown to be strictly required for the ability of MyoD to bind DNA and promote muscle cell differentiation. Similar requirements for E-proteins have been suggested for proneural proteins during neurogenesis. Proneural proteins can physically interact and bind DNA together with E47 (Johnson et al., 1992a; Johnson et al., 1992b; Wang et al., 2006), but the functional role of E47 in the regulation of neurogenesis has not been thoroughly examined. Our data provide evidence for the regulation of E-protein expression as an important mechanism by which Notch signaling controls neural progenitor cell differentiation. The regulatory relationship between Notch and E-proteins is further underscored by observations in early hematopoiesis, in which Notch promotes differentiation towards the T-cell linage at the expense of B-cell differentiation. In this process, instead of being transcriptionally controlled, *E47* is rapidly ubiquitinated in a Notch-dependent manner and proteasomally degraded (Nie et al., 2003; Ordentlich et al., 1998). Thus, depending on the cellular context, Notch can operate both transcriptionally and post-transcriptionally to control the presence of E-proteins.

Although Hes1 and Hes5 proteins are transcriptional repressors, a number of studies based on in vitro experiments have emphasized their ability to form non-functional heterodimers with proneural proteins and E-proteins (Akazawa et al., 1992). These findings stress the capacity of Hes proteins to regulate the activity of proneural bHLH and E-proteins also at the post-transcriptional level. In this report, we have shown that misexpression of Hes5 downregulated Ngn2 and that Ngn2 was sufficient to rescue the Hes-mediated block of neuronal differentiation. Furthermore, a dominant-active version of Hes5 (*Hes5$^{\Delta ct}$-VP16*), with its putative protein dimerization domain retained, promoted neural cells to commit to differentiation. Hence, misexpression of Hes5, or derivatives of Hes5, does not block the function of proneural transcription factors or E-proteins. Together, these results argue that post-transcriptional mechanisms are unlikely to be the main mechanisms by which Hes proteins regulate neurogenesis in the developing CNS and indicate that the primary role of Hes proteins during neurogenesis is rather to act as transcriptional repressors.

Our results argue for a model in which SoxB1 transcription factors preserve the undifferentiated state by maintaining the expression of neural progenitor identities and blocking the capacity of proneural proteins to promote neurogenesis. In this model, the main role of Notch is, through the regulation of proneural- and E-protein expression, to ascertain that the correct number of cells initiate neuronal differentiation at a specific stage (Fig. 6K). This is similar to the role of Notch in *Drosophila*, where Notch signaling, via lateral inhibition, sorts out progenitor cells that should remain undifferentiated from those that should commit to differentiation (Chitnis, 1995). Notch, Sox and bHLH proteins are also expressed in muscle and neural crest progenitor populations, and have in these tissues been ascribed similar regulatory roles as in the developing and adult CNS (Beranger et al., 2000; Braun et al., 1990; Cheung and Briscoe, 2003; Delfini et al., 2000; Heeg-Truesdell and LaBonne, 2004; Hirsinger et al., 2001; Hong and Saint-Jeannet, 2005; Lassar et al., 1991; Schmidt et al., 2003). Thus, it is likely that Notch signaling and Sox transcription factors are required for the maintenance and the coordinated differentiation of progenitors also outside the CNS. Interestingly, Notch signaling appears not to be a requirement for maintaining embryonic stem cells in a undifferentiated state, as CSL-deficient ES cells can be preserved in a self-renewing and pluripotent state (Hitoshi et al., 2002; Lowell et al., 2006). Instead ES cells rely on Sox2, which, together with the transcription factors Oct3/4, preserves cells in a self-renewing pluripotent state (Masui et al., 2007; Matoba et al., 2006). Thus, in the absence of a differentiation program, regulated by tissue-specific bHLH factors and E-proteins, Notch signaling is not a prerequisite for maintaining cells in an undifferentiated state.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/10/1843/DC1

References

Akazawa, C., Sasai, Y., Nakanishi, S. and Kageyama, R. (1992). Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. *J. Biol. Chem.* **267**, 21879-21885.

Bailey, P. J., Klos, J. M., Andersson, E., Karlen, M., Kallstrom, M., Ponjavic, J., Muhr, J., Lenhard, B., Sandelin, A. and Ericson, J. (2006). A global genomic transcriptional code associated with CNS-expressed genes. *Exp. Cell Res.* **312**, 3108-3119.

Bani-Yaghoub, M., Tremblay, R. G., Lei, J. X., Zhang, D., Zurakowski, B., Sandhu, J. K., Smith, B., Ribecco-Lutkiewicz, M., Kennedy, J., Walker, P. R. et al. (2006). Role of Sox2 in the development of the mouse neocortex. *Dev. Biol.* **295**, 52-66.

Beatus, P., Lundkvist, J., Oberg, C., Pedersen, K. and Lendahl, U. (2001). The origin of the ankyrin repeat region in Notch intracellular domains is critical for regulation of HES promoter activity. *Mech. Dev.* **104**, 3-20.

Beranger, F., Mejean, C., Moniot, B., Berta, P. and Vandromme, M. (2000). Muscle differentiation is antagonized by SOX15, a new member of the SOX protein family. *J. Biol. Chem.* **275**, 16103-16109.

Bergsland, M., Werme, M., Malewicz, M., Perlmann, T. and Muhr, J. (2006). The establishment of neuronal properties is controlled by Sox4 and Sox11. *Genes Dev.* **20**, 3475-3486.

Berk, A. J., Boyer, T. G., Kapanidis, A. N., Ebright, R. H., Kobayashi, N. N., Horn, P. J., Sullivan, S. M., Koop, R., Surby, M. A. and Triezenberg, S. J. (1998). Mechanisms of viral activators. *Cold Spring Harb. Symp. Quant. Biol.* **63**, 243-252.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.

Braun, T., Winter, B., Bober, E. and Arnold, H. H. (1990). Transcriptional activation domain of the muscle-specific gene-regulatory protein myf5. *Nature* **346**, 663-665.

Bray, S. J. (2006). Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* **7**, 678-689.
Bylund, M., Andersson, E., Novitch, B. G. and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nat. Neurosci.* **6**, 1162-1168.
Castro, D. S., Arvidsson, M., Bondesson Bolin, M. and Perlmann, T. (1999). Activity of the Nurr1 carboxyl-terminal domain depends on cell type and integrity of the activation function 2. *J. Biol. Chem.* **274**, 37483-37490.
Cheng, H. T., Miner, J. H., Lin, M., Tansey, M. G., Roth, K. and Kopan, R. (2003). Gamma-secretase activity is dispensable for mesenchyme-to-epithelium transition but required for podocyte and proximal tubule formation in developing mouse kidney. *Development* **130**, 5031-5042.
Cheung, M. and Briscoe, J. (2003). Neural crest development is regulated by the transcription factor Sox9. *Development* **130**, 5681-5693.
Chitnis, A. B. (1995). The role of Notch in lateral inhibition and cell fate specification. *Mol. Cell. Neurosci.* **6**, 311-321.
Chung, C. N., Hamaguchi, Y., Honjo, T. and Kawaichi, M. (1994). Site-directed mutagenesis study on DNA binding regions of the mouse homologue of Suppressor of Hairless, RBP-J kappa. *Nucleic Acids Res.* **22**, 2938-2944.
Delfini, M. C., Hirsinger, E., Pourquie, O. and Duprez, D. (2000). Delta 1-activated notch inhibits muscle differentiation without affecting Myf5 and Pax3 expression in chick limb myogenesis. *Development* **127**, 5213-5224.
Dovey, H. F., John, V., Anderson, J. P., Chen, L. Z., de Saint Andrieu, P., Fang, L. Y., Freedman, S. B., Folmer, B., Goldbach, E., Holsztynska, E. J. et al. (2001). Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J. Neurochem.* **76**, 173-181.
Fior, R. and Henrique, D. (2005). A novel hes5/hes6 circuitry of negative regulation controls Notch activity during neurogenesis. *Dev. Biol.* **281**, 318-333.
Fischer, A. and Gessler, M. (2007). Delta-Notch-and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. *Nucleic Acids Res.* **35**, 4583-4596.
Graham, V., Khudyakov, J., Ellis, P. and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron* **39**, 749-765.
Guillemot, F. (2007). Spatial and temporal specification of neural fates by transcription factor codes. *Development* **134**, 3771-3780.
Hansson, E. M., Teixeira, A. I., Gustafsson, M. V., Dohda, T., Chapman, G., Meletis, K., Muhr, J. and Lendahl, U. (2006). Recording Notch signaling in real time. *Dev. Neurosci.* **28**, 118-127.
Heeg-Truesdell, E. and LaBonne, C. (2004). A slug, a fox, a pair of sox: transcriptional responses to neural crest inducing signals. *Birth Defects Res. C Embryo Today* **72**, 124-139.
Hirsinger, E., Malapert, P., Dubrulle, J., Delfini, M. C., Duprez, D., Henrique, D., Ish-Horowicz, D. and Pourquie, O. (2001). Notch signalling acts in postmitotic avian myogenic cells to control MyoD activation. *Development* **128**, 107-116.
Hitoshi, S., Alexson, T., Tropepe, V., Donoviel, D., Elia, A. J., Nye, J. S., Conlon, R. A., Mak, T. W., Bernstein, A. and van der Kooy, D. (2002). Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes Dev.* **16**, 846-858.
Hong, C. S. and Saint-Jeannet, J. P. (2005). Sox proteins and neural crest development. *Semin. Cell Dev. Biol.* **16**, 694-703.
Huang, H. P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M. and Tsai, M. J. (2000). Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. *Mol. Cell. Biol.* **20**, 3292-3307.
Hurlbut, G. D., Kankel, M. W., Lake, R. J. and Artavanis-Tsakonas, S. (2007). Crossing paths with Notch in the hyper-network. *Curr. Opin. Cell Biol.* **19**, 166-175.
Iso, T., Kedes, L. and Hamamori, Y. (2003). HES and HERP families: multiple effectors of the Notch signaling pathway. *J. Cell. Physiol.* **194**, 237-255.
Jasoni, C. L., Walker, M. B., Morris, M. D. and Reh, T. A. (1994). A chicken achaete-scute homolog (CASH-1) is expressed in a temporally and spatially discrete manner in the developing nervous system. *Development* **120**, 769-783.
Johnson, J. E., Birren, S. J., Saito, T. and Anderson, D. J. (1992a). DNA binding and transcriptional regulatory activity of mammalian achaete-scute homologous (MASH) proteins revealed by interaction with a muscle-specific enhancer. *Proc. Natl. Acad. Sci. USA* **89**, 3596-3600.
Johnson, J. E., Zimmerman, K., Saito, T. and Anderson, D. J. (1992b). Induction and repression of mammalian achaete-scute homologue (MASH) gene expression during neuronal differentiation of P19 embryonal carcinoma cells. *Development* **114**, 75-87.
Kamachi, Y., Uchikawa, M., Collignon, J., Lovell-Badge, R. and Kondoh, H. (1998). Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction. *Development* **125**, 2521-2532.
Kato, H., Taniguchi, Y., Kurooka, H., Minoguchi, S., Sakai, T., Nomura-Okazaki, S., Tamura, K. and Honjo, T. (1997). Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives. *Development* **124**, 4133-4141.
Kopan, R., Schroeter, E. H., Weintraub, H. and Nye, J. S. (1996). Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. *Proc. Natl. Acad. Sci. USA* **93**, 1683-1688.
Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991). Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* **66**, 305-315.
Louvi, A. and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nat. Rev. Neurosci.* **7**, 93-102.
Lowell, S., Benchoua, A., Heavey, B. and Smith, A. G. (2006). Notch promotes neural lineage entry by pluripotent embryonic stem cells. *PLoS Biol.* **4**, e121.
Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A. A. et al. (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat. Cell Biol.* **9**, 625-635.
Matoba, R., Niwa, H., Masui, S., Ohtsuka, S., Carter, M. G., Sharov, A. A. and Ko, M. S. (2006). Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. *PLoS ONE* **1**, e26.
Nie, L., Xu, M., Vladimirova, A. and Sun, X. H. (2003). Notch-induced E2A ubiquitination and degradation are controlled by MAP kinase activities. *EMBO J.* **22**, 5780-5792.
Niwa, H., Yamamura, K. and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-199.
Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F. and Kageyama, R. (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *EMBO J.* **18**, 2196-2207.
Ordentlich, P., Lin, A., Shen, C. P., Blaumueller, C., Matsuno, K., Artavanis-Tsakonas, S. and Kadesch, T. (1998). Notch inhibition of E47 supports the existence of a novel signaling pathway. *Mol. Cell. Biol.* **18**, 2230-2239.
Perez, S. E., Rebelo, S. and Anderson, D. J. (1999). Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. *Development* **126**, 1715-1728.
Ross, S. E., Greenberg, M. E. and Stiles, C. D. (2003). Basic helix-loop-helix factors in cortical development. *Neuron* **39**, 13-25.
Sandberg, M., Kallstrom, M. and Muhr, J. (2005). Sox21 promotes the progression of vertebrate neurogenesis. *Nat. Neurosci.* **8**, 995-1001.
Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev.* **6**, 2620-2634.
Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condron, M. M., Teplow, D. B. and Haass, C. (2001). Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. *EMBO Rep.* **2**, 835-841.
Schmidt, K., Glaser, G., Wernig, A., Wegner, M. and Rosorius, O. (2003). Sox8 is a specific marker for muscle satellite cells and inhibits myogenesis. *J. Biol. Chem.* **278**, 29769-29775.
Schroeter, E. H., Kisslinger, J. A. and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-386.
Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.
Wang, C., Bian, W., Xia, C., Zhang, T., Guillemot, F. and Jing, N. (2006). Visualization of bHLH transcription factor interactions in living mammalian cell nuclei and developing chicken neural tube by FRET. *Cell Res.* **16**, 585-598.
Wegner, M. and Stolt, C. C. (2005). From stem cells to neurons and glia: a Soxist's view of neural development. *Trends Neurosci.* **28**, 583-588.
Yoon, K. and Gaiano, N. (2005). Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat. Neurosci.* **8**, 709-715.

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