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Notch signaling downstream of *foxD5* promotes neural ectodermal transcription factors that inhibit neural differentiation

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Abstract

We investigated the role of the Notch signaling pathway in regulating several transcription factors that stabilize a neural fate and expand the neural plate. Increased Notch signaling in a neural lineage via a constitutively activated form (NICD) up-regulated *geminin* and *zic2* in a cell autonomous manner, and expanded the neural plate domains of *sox11*, *sox2*, and *sox3*. Loss- and gain-of-function assays show that *foxD5* acts upstream of *notch1* gene expression. Decreasing Notch signaling with an anti-morphic form of a Notch ligand (*X-Delta-1*^{STU}) showed that the *foxD5*-mediated expansion of the *sox* gene neural plate domains requires Notch signaling. However, *geminin* and *zic2* appear to be dually regulated by *foxD5* and Notch1 signaling. These studies demonstrate that: 1) Notch signaling acts downstream of *foxD5* to promote the expression of a subset of neural ectodermal transcription factors; and 2) Notch signaling and the *foxD5* transcriptional pathway together maintain the neural plate in an undifferentiated state.

Introduction

The neural ectoderm (NE) forms on the dorsal side of the *Xenopus* embryo in response to factors secreted from the blastula Chordin- and Noggin-expressing (BCNE) signaling center and the Organizer (De Robertis and Kuroda, 2004; Levine and Brivanlou, 2007). By gastrula stages, the NE expresses a large number of transcription factors whose expression domains widely overlap, and which coordinately promote a neural fate, expand the neural plate and regulate the onset of neural differentiation (Fig. 1; Sasai, 1998; Moody and Je, 2002). These include: 1) *foxD5*, a forkhead/winged helix gene (Sölter et al., 1999; Fetka et al., 2000; Sullivan et al., 2001); 2) *geminin* (*gem*), which interacts with the SWI/SNF complex (Kroll et al., 1998; Seo and Kroll, 2006); 3) the high-mobility group (HMG)-box genes *sox2*, *sox3*, *soxD* and *sox11* (Uwanogho et al., 1995; Mizuseki et al., 1998a, b; Kishi et al., 2000; Hyodo-Miura et al., 2002; Wegner and Stolt, 2005; Dee et al., 2008); 4) the zinc-finger genes *zic1*, *zic2* and *zic3* (Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998a; Nakata et al., 1997, 1998); and 5) the Iroquois genes *Xiro1*, *Xiro2*, and *Xiro3* (Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998, 2001). However, the interactions between these several genes during the establishment of the neural plate are largely unknown. Recently, we proposed a regulatory network, based on loss- and gain-of-function assays, that places *foxD5* in an upstream position. It appears to directly regulate *gem*, *sox11* and *zic2*, which together regulate the expression patterns of the other NE transcription factors (Fig. 1; Yan et al., 2009). This model, however, does not account for signaling pathways that might mediate the interactions.

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Notch signaling, which is highly conserved from *Drosophila* to humans, plays essential roles in many processes during development including neurogenesis (Artavanis-Tsakonas et al., 1999; Lai, 2004; Bray, 2006; Chitnis, 2007). When the Notch transmembrane receptor on the cell surface is bound by DSL (Delta/ Serrate/ Lag-2) ligands on the neighboring cells, the Notch intracellular domain (NICD) is cleaved, released into the cytoplasm and translocated into the nucleus. There, it combines with its transcriptional cofactor CSL (CBF1/ Suppressor of Hairless/ Lag-1) to activate downstream target genes, mainly members of the HES (Hairy/ Enhancer of Split) family of basic helix-loop-helix (bHLH) transcriptional regulators (Mumm and Kopan, 2000; Weinmaster, 2000). During early vertebrate development, Notch is expressed broadly throughout the neural plate, and its signaling pathway plays an essential role in maintaining the neural progenitor state and regulating the diversification of cell fate (Yoon and Gaiano, 2005; Louvi and Artavanis-Tsakonas, 2006; Chitnis, 2007). The classical view is that increasing Notch signaling within a cell up-regulates HES genes that subsequently repress neural differentiation bHLH genes (such as *ngn* and *neuroD*) and Notch DSL ligand genes.

The preponderance of work on Notch signaling during neural development focuses on the onset of neurogenesis, when progenitor cells become post-mitotic and acquire a particular neural cell fate. For example, at *Xenopus* and zebrafish neurula stages, increasing Notch signaling by expressing a constitutively activating form of Notch1 prevents expression of the neural differentiation genes and formation of primary neurons, whereas decreasing Notch signaling by interfering with Delta promotes neurogenesis (Chitnis et al., 1995; Itoh et al., 2003). However, Notch signaling also affects the size of the NE during *Xenopus* gastrulation (Coffman et al., 1993). Because Notch is expressed throughout the neural plate at a similar time as the NE transcription factors (Coffman et al., 1990, 1993), and several of those factors both expand the neural plate and inhibit neural differentiation (Fig. 1), we investigated whether Notch signaling has a role in regulating the expression of these genes. We show that: 1) *foxD5* acts upstream of *notch1*; 2) increasing Notch signaling phenocopies the effects of *foxD5* on *gem*, *sox11*, *sox2*, *sox3* and *zic2*, but it does not alter the expression of NE genes that are inhibited by *foxD5* (*soxD*, *zic1*, *zic3*, *Xiro1-3*); and 3) Notch signaling is required for the *foxD5*-mediated expansion of *sox2*, *sox3* and *sox11* expression domains but not for the up-regulation of *gem* and *zic2*. These studies demonstrate that: 1) Notch signaling acts downstream of *foxD5* to promote the expression of a subset of NE transcription factors; and 2) the Notch signaling pathway and the *foxD5* transcriptional pathway together maintain the neural plate in an undifferentiated state.

Results

Our previous work shows that *foxD5* is required for the normal expression of *gem*, *sox2*, *sox3*, *sox11*, *soxD*, *zic1-3* and *Xiro1-3* in the nascent neural ectoderm (NE), and that increased levels of *foxD5* induce *gem*, *sox11*, and *zic2*, expand the *sox2* and *sox3* NE domains and repress *zic1*, *zic3* and *Xiro1-3* (Fig. 1; Yan et al., 2009). To assess whether Notch signaling is involved in the regulation of any of these effects, we tested whether: 1) *foxD5* acts upstream of the *notch1* gene; 2) increased Notch signaling phenocopies *foxD5*-mediated effects on the NE genes that inhibit neural differentiation; and 3) decreased Notch signaling interferes with *foxD5* regulation of these NE genes.

foxD5 acts upstream of notch1 gene expression

Endogenous *foxD5* and *notch1* mRNAs both are expressed diffusely throughout the early NE (Coffman et al., 1990,1993;Sullivan et al., 2001). To assess whether *foxD5* regulates *notch1* expression, we microinjected into the precursor blastomere of the neural ectoderm (D11) either *foxD5* anti-sense morpholino oligonucleotides (*foxD5*-MOs) to locally decrease FoxD5 levels, or *foxD5* mRNA to locally increase them. Reducing endogenous *foxD5* levels

with *foxD5*-MOs blocked *notch1* expression in the neural plate, whereas increased *foxD5* significantly expanded the neural plate expression domain of *notch1* (Fig. 2). The level of *notch1* expression in the *foxD5*-expressing cells in the neural plate (tagged red by a βGal lineage tracer) was not increased above that of neighboring cells. Furthermore, expression of *foxD5* in the epidermis adjacent to the neural plate or in a ventral epidermis precursor blastomere (V11) did not induce ectopic *notch1* (Fig. 2B). These results demonstrate that *foxD5*: 1) is required for *notch1* expression in the neural plate; 2) expands the *notch1* domain in the neural plate in a non-cell autonomous manner; and 3) is not sufficient to activate ectopic *notch1* transcription in the dorsal or ventral epidermis. Finally, expression of the constitutively signaling Notch1 construct (NICD) did not alter the expression of *foxD5* in the neural ectoderm nor induce it in the ventral epidermis (Fig. 3A). Together, these results indicate that *foxD5* acts upstream of *notch1* during the formation of the neural ectoderm, but does not activate it in the epidermis.

Notch signaling up-regulates a subset of NE transcription factors

Because Notch signaling is proposed to expand an undifferentiated precursor population in the neural plate independent of regulating the cell cycle (Coffman et al., 1993), we tested whether expression of NICD would up-regulate those NE transcription factors, previously reported to also expand the NE, that function downstream of *foxD5* (Fig. 1). First, expression of NICD in a single neural plate precursor did not alter the expression levels of *soxD, zic1, zic3* or *Xiro1-3* (Fig. 3A); this result is consistent with previous reports that these genes promote the onset of neural differentiation and are inhibited by *foxD5* (Sasai 1998; Moody and Je, 2002; Yan et al., 2009). Second, *gem* and *zic2*, both of which maintain NE cells in an undifferentiated state (Brewster et al., 1998; Kroll et al., 1998), were up-regulated in the neural plate at high frequency, and their expression was elevated above endogenous levels in a cell autonomous manner (Fig. 3). Third, the neural plate expression domains of *sox11*, *sox2* and *sox3* were expanded, but their expression was not elevated above endogenous levels (Fig. 3); further, the expansion of the neural plate extended beyond the NICD-expressing clone indicating involvement of cell-to-cell signaling. The effects of increased Notch signaling on the NE genes partially phenocopied those of *foxD5* (Sullivan et al., 2001; Yan et al., 2009): those genes expanded by *foxD5* were also expanded by NICD, and two genes up-regulated cell autonomously by *foxD5* (*gem*, *zic2*) were also up-regulated cell-autonomously by NICD in the neural plate. However, some effects of increased Notch signaling were not the same as those of *foxD5*. First, those genes inhibited by *foxD5* (*soxD, zic1-3, Xiro1-3*) were unaffected by NICD. Second, the expression of *sox11* was increased by both genes but not in the same way: *foxD5* both up-regulated *sox11* levels cell-autonomously in the neural plate and expanded its neural plate domain, whereas NICD only expanded the *sox11* neural plate domain.

We also tested whether NICD can induce ectopic neural tissue by injecting the mRNA into a ventral epidermis precursor blastomere (V11). Consistent with a report that a different form of activated Notch does not convert epidermis to neural tissue based on a histological analysis (Coffman et al., 1993), we observed that of all the NE transcription factors tested, only *gem* was ectopically induced (Fig. 3). This is in contrast to *foxD5*, which ectopically induces *gem*, *zic2*, *sox11*, and *sox3* at high frequency, and *sox2*, *zic1* and *Xiro2* at low frequency (Yan et al., 2009). Taken together, these results indicate that Notch signaling acts downstream of *foxD5* to up-regulate or expand the expression of a subset of transcription factors in the NE, but unlike *foxD5*, it does not convert non-neural ectoderm to a neural fate.

Notch signaling is required for increased expression of some NE transcription factors

To determine whether Notch signaling is required for the increased expression of *gem*, *zic2*, *sox11, sox2* or *sox3*, a dominant-interfering form of the ligand *X-Delta-1* (*X-Delta-1*^{STU};

Chitnis et al., 1995) was expressed in the neural precursor blastomere. This construct, which lacks the intracellular domain (ICD), is thought to prevent Notch signaling by preventing the cisendocytosis of endogenous Delta-1 and being unable to promote the Notch1 intracellular cleavage that releases the active NICD fragment (reviewed in Kiyota and Kinoshita, 2004). This construct has been used extensively to show that it blocks: 1) the ability of NICD to activate neuronal differentiation genes (Chitnis et al., 1995; Chitnis and Kintner, 1996; Ma et al., 1996; Chalmers et al., 2002); 2) lateral inhibition (Papalopulu and Kintner, 1996); 3) formation of ciliated epidermal cells (Deblandre et al., 1999); and 4) NICD expansion of the neural crest domain (Glavic et al., 2004; Kuriyama et al., 2006). Herein we show that *X-Delta-1*^{STU} inhibits the expression of Hes1, a direct transcriptional target of NICD (Fig. 4B, 83.3%, n=36), thus demonstrating its efficacy in blocking Notch signaling in our assay. In contrast to NICD, *X-Delta-1*^{STU} significantly decreased the expression of *sox11, sox2* and *sox3* (Fig. 4). *sox11* expression was suppressed in the *X-Delta-1*^{STU} –expressing cells in a cell autonomous manner without affecting the width of its neural plate domain. *sox2* and *sox3* expression also were suppressed within the clone at low frequencies (17%, 14%, respectively), but most notably the neural plate domain was smaller on the injected side (Fig. 4). We next co-expressed *foxD5* and *X-Delta-1*^{STU} mRNAs to test whether inhibition of Notch signaling blocked the effects of *foxD5* on these genes. *X-Delta-1*^{STU} inhibited the *foxD5*-mediated expansion of the *sox11, sox2* and *sox3* neural plate domains (Fig. 5). These results indicate that the expansion of the neural plate domains of the *sox* genes by *foxD5* requires Notch signaling. Interestingly, the cell-autonomous up-regulation of *sox11* by *foxD5* is independent of Notch signaling.

Unexpectedly, *X-Delta-1*^{STU} cell autonomously up-regulated the expression of *gem* and *zic2* in the neural plate (Fig. 4), at frequencies very similar to the effects of NICD and *foxD5* (Fig. 3; Yan et al., 2009). Co-expression of both *foxD5* and *X-Delta-1*^{STU} mRNAs did not alter these phenotypes, indicating that the *foxD5* effects on *gem* and *zic2* do not require Notch signaling in the same cell. Thus, *gem* and *zic2* appear to be dually regulated by *foxD5* and Notch signaling (Fig. 6). It is seemingly contradictory that *gem* and *zic2* can be up-regulated by both increasing (by NICD) and decreasing (by *X-Delta-1*^{STU}*)* Notch signaling within the same cell. Although we do not know the mechanism by which *X-Delta-1*^{STU} promotes *gem* and *zic2* expression, previous studies showed that Delta1 and Serrate1 are both expressed and available in their proteolytically cleaved, active forms (the ICDs) in the neural ectoderm, and both induce Notch target genes (Kiyota et al., 2001;Wettstein et al., 1997;Kiyota and Kinoshita, 2004). Furthermore, while the ICD-truncated forms of Serrate1 and Delta1 both cause increased numbers of primary neurons, they have distinctly different activities. The Serrate ICD acts in both the canonical Notch pathway and in an independent manner to activate genes that repress neuronal differentiation, and it can rescue the neurogenic effect of *X-Delta-1*^{STU} (Kiyota and Kinoshita, 2004). Therefore, we predict that in our assay Serrate activity is intact in *X-Delta-1*^{STU} —expressing cells, leading to induction of *gem* and *zic2* expression. Alternatively, the *X-Delta-1*^{STU} —expressing cells may lack a recently described interaction with the TGF-β/Smad signaling pathway (Hiratochi et al., 2007) that would normally repress *gem* and *zic2*. While our data indicate that *gem* and *zic2* are up-regulated by both the NICD pathway and a pathway that is revealed in the absence of Delta1 function, it will be important to identify this additional pathway in order to understand the full scope of Notch signaling in the regulation of NE genes.

Discussion

During embryogenesis, Notch signaling is required for the maintenance and expansion of the neural stem and progenitor cells, and it inhibits neurogenesis by activating Hes1 and Hes5, the homologues of *Drosophila hairy* and *Enhancer of split*, which in turn repress the expression of the bHLH neural differentiation genes (Jarriault et al., 1995; Kageyama and

Nakanishi, 1997; Lai, 2004; Chiba, 2006; Louvi and Artavanis-Tsakonas, 2006; Chitnis, 2007). Previous studies identified a large number of transcription factors that maintain neural stem and progenitor cells, but the integration of Notch signaling with the regulation of these factors is mostly unknown. In this study, we show that Notch signaling acts downstream of *foxD5* to affect a subset of NE transcription factors that promote an undifferentiated neural state. Notch signaling is required for the expanded neural plate expression domains of *sox11*, *sox2* and *sox3*, and it up-regulates *gem* and *zic2* expression in the neural plate (Fig. 6).

Notch signaling expands the neural plate domains of sox genes downstream of foxD5

sox2 and *sox3*, which belong to the SoxB1 subgroup of HMG-box transcription factors, are highly expressed in the NE and in the endogenous neural stem cells in the adult central nervous system. Functionally, they both maintain neural stem and progenitor cells and inhibit neuronal differentiation (Avilion et al., 2003; Bylund et al., 2003; Graham et al., 2003; Ferri et al., 2004; Pevny and Placzek, 2005; Wegner and Stolt, 2005; Wang et al., 2006; Cavallaro et al., 2008; Dee et al., 2008; Kim et al., 2008). There are some conflicting data regarding how Notch signaling and the functions of SoxB1 genes interface. Some reports indicate that Sox2 acts upstream of Notch signaling because over-expressed Sox2 up-regulates the expression of Notch1, Notch ligands and its downstream target genes (Bani-Yaghoub et al., 2006). Moreover, Sox2 can directly regulate *notch1* gene expression by binding to its 13th intron, which has a biological function in retinal neural progenitors (Taranova et al., 2006). However, there also is evidence that Sox2 responds to Notch signaling: 1) as we also report herein, expression of NICD expands the *sox2* expression domain in the neural plate (Glavic et al., 2004); and 2) in avian neural crest cells, *sox2* inhibition of differentiation is placed downstream of Notch signaling (Wakamatsu et al., 2004). Finally, some studies indicate that Notch signaling and SoxB1 work in parallel to inhibit neuronal differentiation (Bylund et al., 2003; Holmberg et al., 2008). Our studies provide an additional step in the pathway by showing that the expansion of *sox2* and *sox3* achieved by *foxD5* during neural plate formation requires Notch signaling (Fig. 6).

sox11, a member of the SoxC subgroup, also is strongly expressed in the NE and functions in both neural induction and neural differentiation (Uwanogho et al., 1995; Hargrave et al., 1997; Hyodo-Miura et al., 2002; Bergsland et al., 2006). Although there is evidence that *sox11* is involved in neuronal differentiation downstream of the bHLH proneural genes, our previous work indicates that in the embryonic NE *sox11* functions in tandem with *gem* and *zic2* to expand the neural plate expression of *sox2* and *sox3* (Fig. 1; Yan et al., 2009). To our knowledge there has not been a previous study of the interface between Notch signaling and *sox11*. Herein we show that Notch signaling is required for the *foxD5*-mediated expansion of *sox11* neural plate expression, but it does not up-regulate the levels of *sox11* in the NE.

Notch signaling up-regulates some NE transcription factors in parallel with foxD5

foxD5, *gem* and *zic2* are well characterized as inhibitors of neural differentiation. *foxD5* expands the neural plate and inhibits the expression of markers of axial differentiation in the NE (*en2*, *Krox20*) and of neural differentiation (*ngnr1*, *neuroD*, *n-tubulin*), leading to the interpretation that it functions to maintain the NE in an immature state (Sullivan et al., 2001). This is supported by the observations that *foxD5* transcriptionally activates *gem* and *zic2* and transcriptionally represses NE genes that promote neural differentiation (Yan et al., 2009). *gem* expands the neural plate, is expressed in neural stem cells and antagonizes Brg1, a protein that is required for activation of the bHLH neural differentiation genes (Kroll et al., 1998; Seo et al., 2005; Seo and Kroll 2006; Spella et al., 2007). *zic2* expands the neural plate, is expressed in neural progenitor cells and inhibits the onset of neurogenesis (Brewster et al., 1998; Nakata et al., 1998; Aruga, 2004; Merzdorf, 2007).

The role of Notch signaling in the expression of these three NE genes has not been previously explored. We show by loss- and gain-of-function assays that *notch1* expression is downstream of *foxD5*. Interestingly, strong Notch signaling, accomplished by expressing NICD, up-regulated both *gem* and *zic2* in the neural ectoderm. Thus, in addition to being direct transcriptional targets of *foxD5* (Yan et al., 2009), these genes appear to be either independently or co-regulated by Notch signaling. An important future goal will be to discern between these two possibilities. The fact that *X-Delta-1*^{STU} also up-regulates *gem* and *zic2* suggests that these two genes may be additionally regulated by another Notch ligand, such as Serrate, or another interacting pathway. It will be important to elucidate the involvement of the different Notch ligands in this signaling pathway (e.g., D'Souza et al., 2008).

In conclusion, elucidating the interactions between Notch signaling and the transcription factors involved in neural differentiation have been very important for understanding how this pathway regulates neuronal fate acquisition. We show that Notch signaling also interfaces with a subset of transcription factors that regulate the expansion of the neural plate and prevent neural differentiation. Further study of the role of Notch signaling in the earliest steps of neural fate acquisition should provide insights into the molecular mechanisms controlling the expansion of neural stem and progenitor cells (Gaulden and Reiter, 2008).

Experimental procedures

Embryo manipulation and mRNA injection

Fertilized *Xenopus laevis* eggs were obtained by gonadotropin-induced natural mating of adult frogs and prepared for injection as described (Moody, 2000). mRNAs for injection were synthesized by in vitro transcription with Ambion mMessage mMachine kits (Austin, TX, USA). The mRNAs of *foxD5* (150pg, Sullivan et al., 2001), an activated form of *notch1* (XNICD, 500pg, Coffman et al., 1993) or *X-Delta-1*^{STU}, a truncated ligand lacking the intracellular domain (500pg, Chitnis et al., 1995) were mixed with *β-gal* mRNA (100pg; as a lineage tracer) and microinjected into either a dorsal animal (D11; neural plate precursor) or a ventral animal (V11; ventral epidermis precursor) blastomere at the 16-cell stage (Moody, 1987).

Morpholino antisense oligonucleotides

Two *foxD5* morpholino antisense oligonucleotides (*foxD5*-MO) were synthesized to recognize the translational start site of all three *foxD5* paralogues found in *Xenopus laevis* (5′-CAGACTCCTGGCTAAAGCTCATTGT-3′; 5′-TATACTCTGATGCTGGGTTTGTAGC-3′) (GeneTools, Philomath, OR). An equimolar mixture of the two *foxD5*-MOs was microinjected (16ng) into blastomere D11. *foxD5*-MO knock-down efficacy was assessed previously (Yan et al., 2009).

Whole mount in situ hybridization

Embryos were fixed and processed for whole mount in situ hybridization according to standard protocols (Sive et al., 2000) using digoxigenin-labeled RNA probes as following: *foxD5* (Sullivan et al., 2001), *geminin* (Kroll et al., 1998), *notch1* (Coffman et al., 1993), *hes1* (Open BioSystems, BC041261) *sox2* (Mizuseki et al., 1998a), *sox3* (Penzel et al., 1997), *sox11* (Hiraoka et al., 1997), *soxD* (Mizuseki et al., 1998b), *Xiro1* (Gomez-Skarmeta et al., 2001), *Xiro2* (Gomez-Skarmeta et al., 1998), *Xiro3* (Bellefroid et al., 1998), *zic1* (Mizuseki et al., 1998a), *zic2* (Brewster et al., 1998) and *zic3* (Nakata et al., 1997). Embryos were analyzed for whether the expression domain was expanded or decreased in size

compared to the uninjected side of the same embryo and for changes in staining intensity compared to adjacent uninjected cells.

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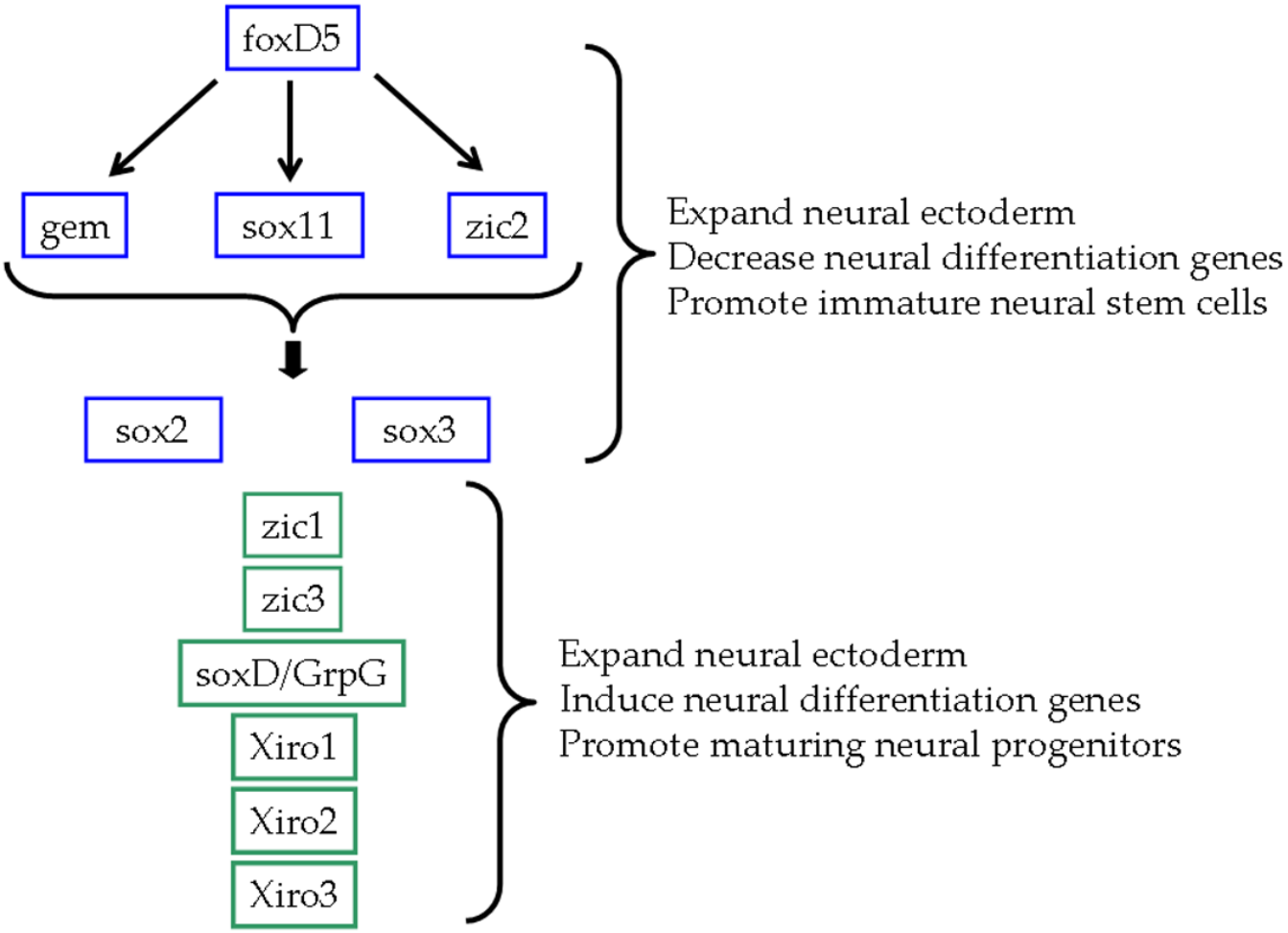

Figure 1.

Previous studies indicate that *foxD5* acts upstream of the other neural ectodermal transcription factors that expand the neural plate. Some of these decrease neural differentiation genes (blue boxes) and some promote the onset of neural differentiation (green boxes). *foxD5* directly activates the transcription of *gem*, *sox11* and *zic2*, and these three genes coordinately regulate the remaining NE transcription factors. (Based on Yan et al., 2009)

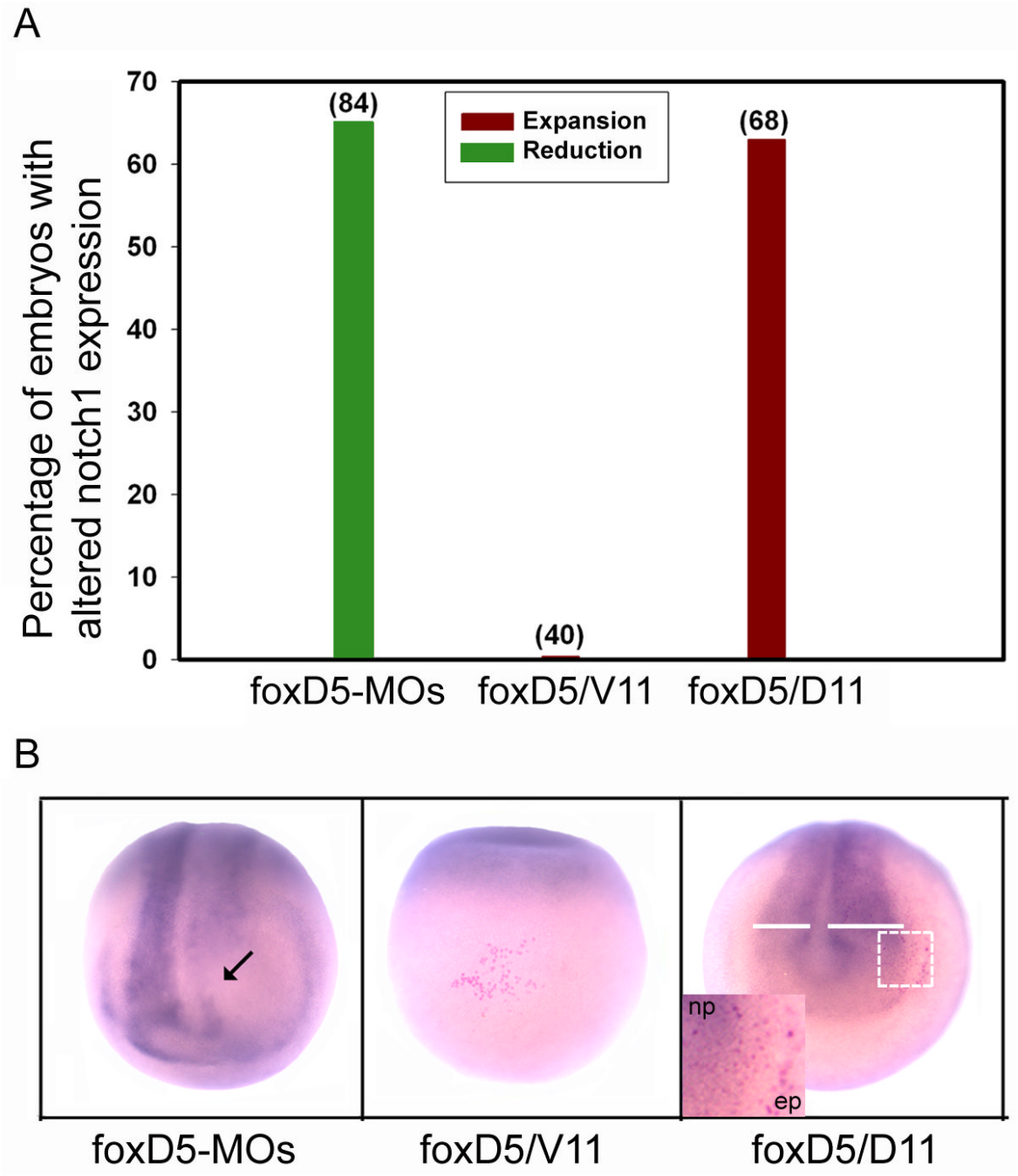

Figure 2.

foxD5 acts upstream of *notch1*. A. Percentage of the embryos with altered *notch1* expression after injection of *foxD5*-MOs in a neural plate precursor, or injection of *foxD5* mRNA in either a ventral epidermis precursor (V11) or a neural plate precursor (D11). Numbers in parentheses indicate sample sizes. B. Embryos at neural plate stages processed for in situ hybridization detection of *notch1* mRNA. Local depletion of FoxD5 by *foxD5*-MOs causes a loss of *notch1* expression (arrow). Locally increasing FoxD5 by mRNA injection in the ventral epidermis (red cells) does not induce ectopic *notch1* expression (middle panel), but doing so in the neural plate (right panel) expands the *notch1* expression domain (compare white bars on injected [right] versus uninjected [left] sides). The inset shows that the *foxD5*-

expressing cells (red nuclei) do not express *notch1* at levels greater than surrounding cells in the neural plate (np), nor induce it in the adjacent dorsal epidermis (ep).

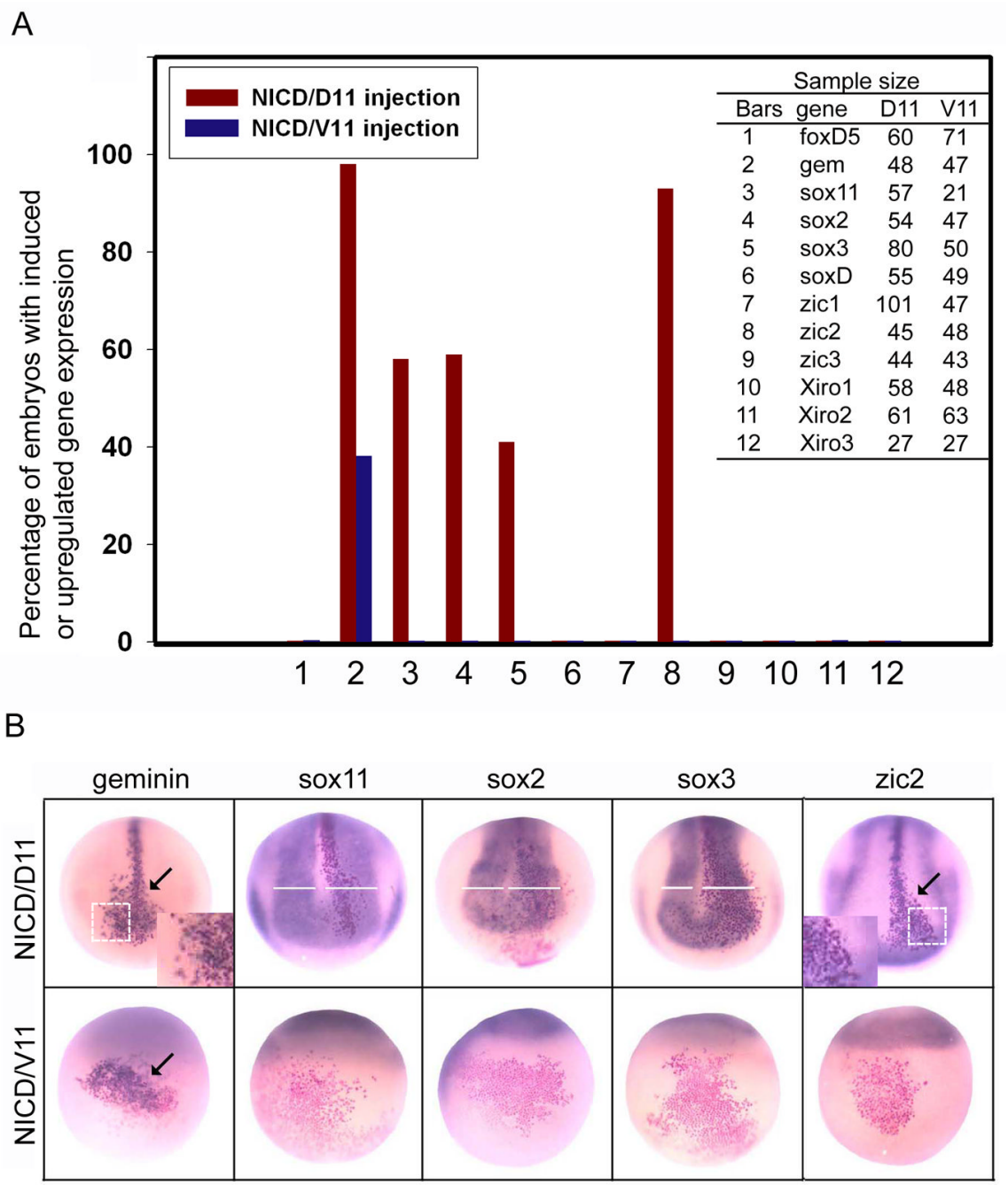

Figure 3.

Effects of increased Notch1 signaling on the NE genes. A. Percentage of embryos in which NICD, expressed in either the neural plate (D11) or ventral epidermis (V11) lineages, altered the expression of twelve NE genes. The table to the right reports the numbers of embryos examined for each experiment. B. Examples of the in situ hybridization assays for those NE genes altered by NICD expressed in the indicated lineages. *geminin* and *zic2* are up-regulated in a cell autonomous manner (arrows); insets show that NICD-expressing cells (red nuclei) express elevated levels of the respective genes compared to adjacent cells. NICD causes the expansion of the neural plate domains of the three *sox* genes (compare white bars on injected [right] versus uninjected [left] sides). NICD induces the ectopic

expression of *geminin* in the ventral epidermis (arrow), but does not induce any of the other NE genes in this tissue (red cells; see also 3A).

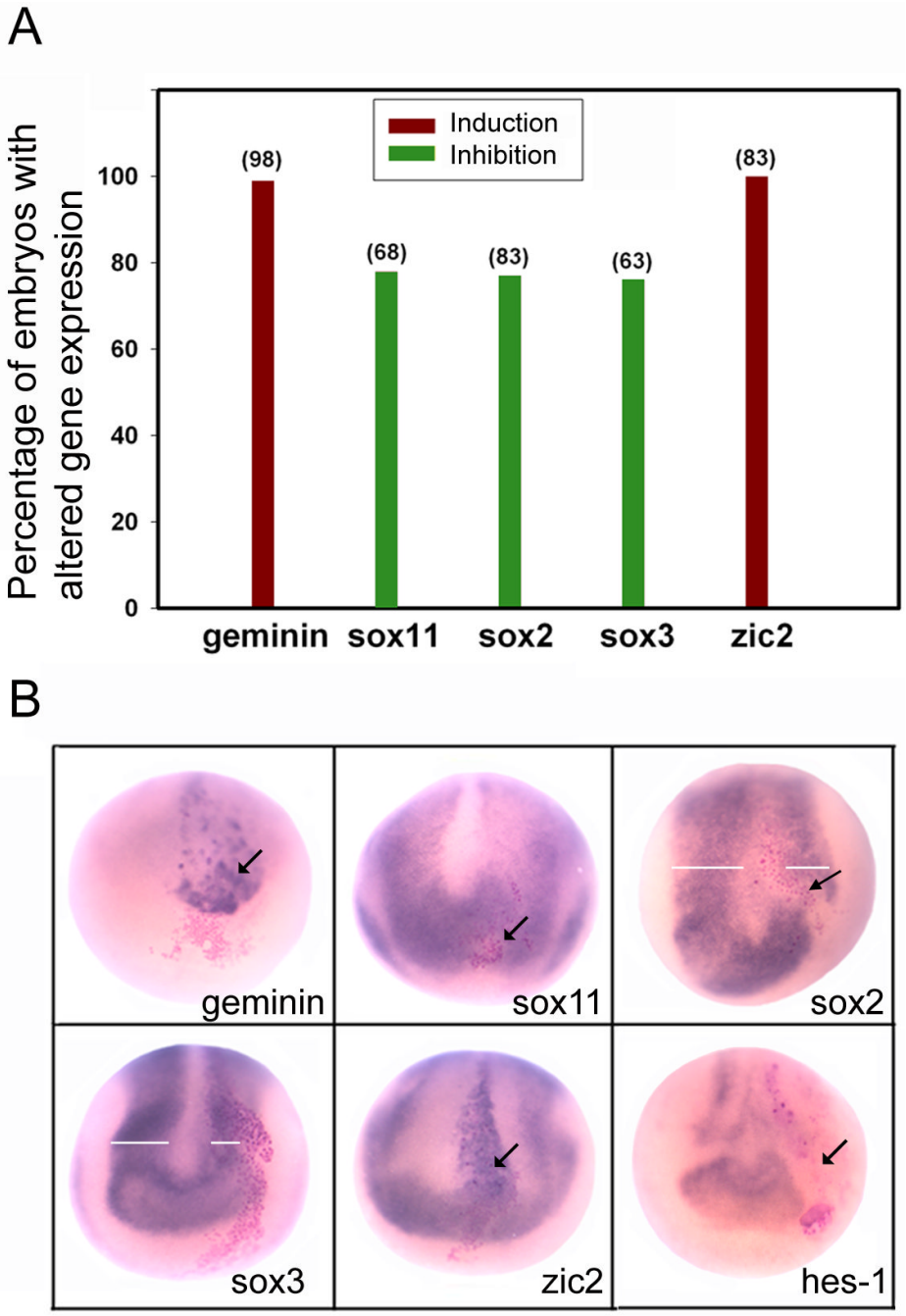

Figure 4.

Decreased Notch signaling reduces the expression of the *sox* genes. A. Percentage of the embryos with altered NE gene expression after injection of *X-Delta-1*^{STU} mRNA in the D11 lineage. The numbers in parentheses indicate sample sizes. B. Examples of the in situ hybridization assays showing cell autonomous up-regulation of *geminin* and *zic2* (arrows) in the neural plate, cell autonomous repression of *sox11*, *sox2* and *hes1* (arrows) and reduction in width of the *sox2*- and *sox3*-expression domains in the neural plate (white bars).

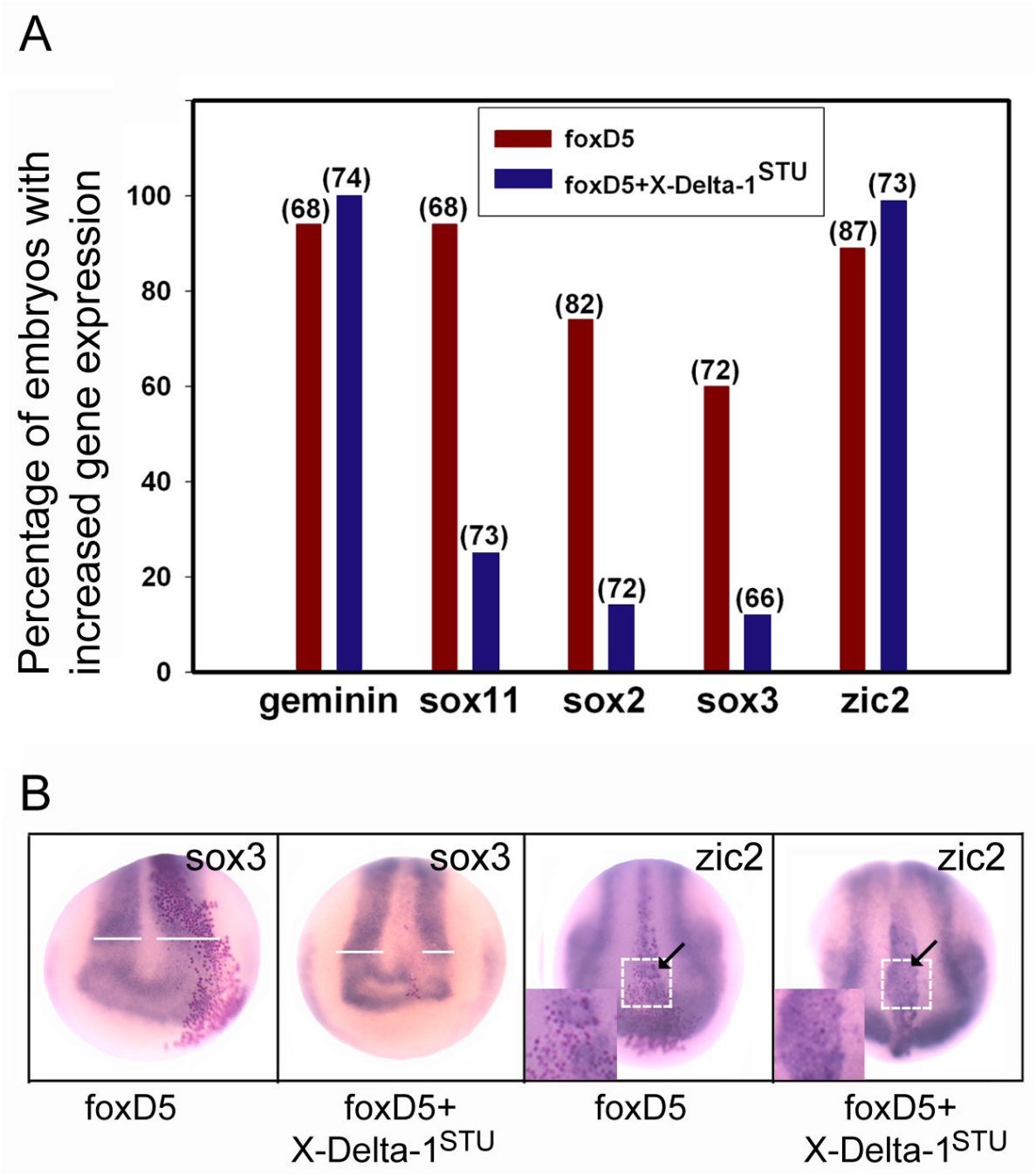

Figure 5.

Decreased Notch signaling reverses *foxD5*-mediated effects on *sox* genes. A. Either *foxD5* mRNA alone or *foxD5* plus *X-Delta-1*^{STU} mRNAs were injected into the D11 lineage. The numbers in parentheses indicate sample sizes. *X-Delta-1*^{STU} interfered with *foxD5*-mediated expansion of the *sox* gene neural plate domains. The up-regulation of *geminin* and *zic2* were not affected. B. Examples of the in situ hybridization assays showing reversal of *foxD5* expansion of the *sox3* domain and lack of reversal of the up-regulation of *zic2* in the neural plate. *zic2* expression is normally absent from the midline of the st14 neural plate (Brewster et al., 1998). When *foxD5* is expressed in the midline by injecting mRNA into blastomere D11, *zic2* is ectopically expressed (arrow). This ectopic expression is also observed when *X-*

Delta-1^{STU} is co-expressed (arrow). Insets show the effect is cell autonomous; nearly all of the mRNA-expressing cells (red nuclei) show ectopic *zic2* expression in both samples.

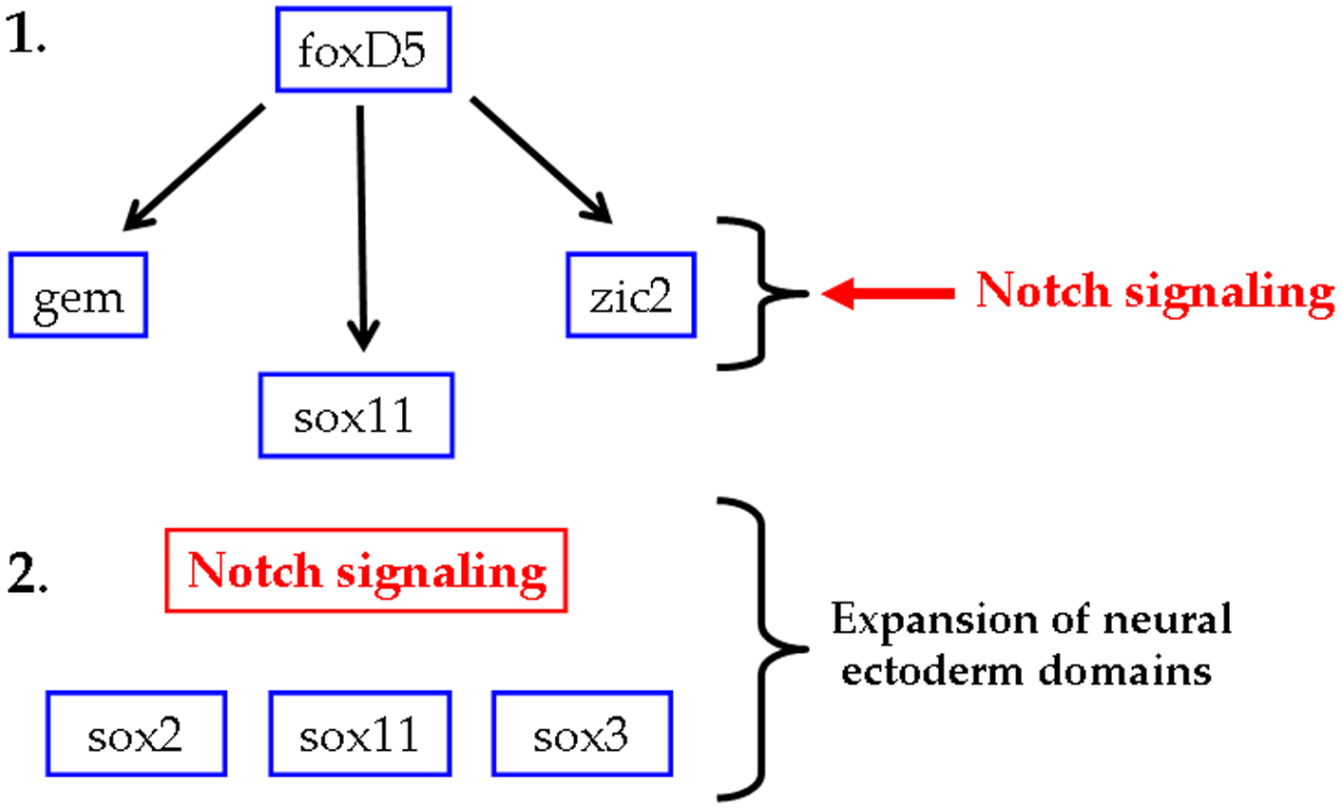

Figure 6.

Notch signaling impacts the expression of NE transcription factors in two ways. 1. *foxD5* directly up-regulates the expression of *geminin* (*gem*), *sox11* and *zic2* in both the neural plate and the ventral epidermis. Notch signaling, acting downstream of *foxD5*, co-regulates *gem* and *zic2* expression levels, but only in the neural plate; it does not up-regulate *sox11* expression levels. 2. Downstream of *foxD5*, Notch signaling is required for the expansion of the neural plate domains of the *sox* genes.