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The homeodomain-containing gene *Xdbx* inhibits neuronal differentiation in the developing embryo

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SUMMARY

The development of the vertebrate nervous system depends upon striking a balance between differentiating neurons and neural progenitors in the early embryo. Our findings suggest that the homeodomain-containing gene *Xdbx* regulates this balance by maintaining neural progenitor populations within specific regions of the neuroectoderm. In posterior regions of the *Xenopus* embryo, *Xdbx* is expressed in a bilaterally symmetric stripe that lies at the middle of the mediolateral axis of the neural plate. This stripe of *Xdbx* expression overlaps the expression domain of the proneural basic/helix-loop-helix-containing gene, *Xash3*, and is juxtaposed to the expression domains of *Xenopus Neurogenin related 1* and *N-tubulin*, markers of early neurogenesis in the embryo. *Xdbx* overexpression inhibits neuronal differentiation in the embryo and when co-injected with *Xash3*, *Xdbx* inhibits the ability of *Xash3* to induce ectopic neurogenesis. One role of *Xdbx* during normal development may therefore be to restrict spatially neuronal differentiation within the neural plate, possibly by altering the neuronal differentiation function of *Xash3*.

Key words: Homeodomain, Neurogenesis, Neural progenitors, *Xenopus*, *Xdbx*

INTRODUCTION

Neural progenitors substantially outnumber differentiating neurons at early stages of vertebrate nervous system development. The eventual fate of these progenitors is in part linked to the timing of their differentiation as neuronal subtypes are generated at specific times during development. The temporal regulation of neuronal differentiation and the maintenance of a pool of neural progenitors therefore insure that the diversity of neuronal cell fates is generated during nervous system development. The *Xenopus* embryo provides a useful system for studying the factors that maintain this balance as undifferentiated progenitors and differentiating neurons are maintained in a stereotypic, regionalized pattern within the neural plate (Hartenstein, 1989, 1993). Differentiating neurons in posterior regions of the embryo form three bilaterally symmetric columns with respect to the mediolateral axis of the neural plate. These neurons, termed primary neurons, are derived exclusively from progenitors in the deep layer of the neural plate (Hartenstein, 1989). Primary neurons are bounded by domains of regionally distinct, undifferentiated neural progenitors that lie in both the superficial and deep layers of the neural plate. The specific neuronal and non-neuronal fates of these progenitors is not fully established, although maintenance of these progenitors is in part required to fuel subsequent waves of differentiation in the embryo, eventually replacing most primary neurons and contributing the majority of neurons and glia to the mature nervous system.

A number of genes are expressed broadly within the neuroectoderm that have the potential to induce ectopic neuronal differentiation when overexpressed. These positive effectors include the zinc finger transcription factor genes, *Gli2*, *Gli3* and *ZicR1* (Brewster et al., 1998; Lee et al., 1997; Marine et al., 1997; Mizuseki et al., 1998a), the HMG domain-containing gene, *SoxD* (Mizuseki et al., 1998b), and the homeodomain-containing gene, *Xiro1* (Gomez-Skarmeta et al., 1998). What negative regulators counter the effects of these genes to maintain progenitor domains in the embryo? The transmembrane receptor, *X-Notch1*, may play a role in this process. *X-Notch1* is expressed throughout the neural plate (Coffman et al., 1990) and, in contrast to positive effectors, the overexpression of either a constitutively active form of *X-Notch1* (Chitnis et al., 1995; Coffman et al., 1993) or downstream effectors of the *Notch* signaling pathway (Marcu et al., 1998; Wettstein et al., 1997) leads to an expansion of the neural progenitor domain within the embryo and the inhibition of neuronal differentiation.

The integration of *X-Notch1* function with positive effectors to retain a balance between neurons and progenitors has been most clearly defined within the early neuronal precursors that give rise to primary neurons (for review, Chitnis, 1999). These neuronal precursors are first marked by expression of the basic/helix-loop-helix (bHLH)-containing transcription factor gene, *Xenopus Neurogenin related 1* (*X-Ngnr1*; Ma et al., 1996). *X-Ngnr1* induces neuronal differentiation when overexpressed in neural precursors (Ma et al., 1996). However, the neuronal differentiation function of *X-Ngnr1* is limited by lateral inhibition (Ma et al., 1996) mediated by *X-Notch1* and

its ligand, *X-Delta1* (Chitnis et al., 1995). Only a subset of *X-Ngnr1*-positive precursors escapes *X-Notch1* inhibition and differentiates to primary neurons marked by expression of *N-tubulin* and other markers of neuronal differentiation (Oschwald et al., 1991). The escape from lateral inhibition is mediated by the induction of a number of other positive effectors including the HLH-containing gene, *Xcoe-2* (Dubois et al., 1998), the zinc finger-containing gene, *X-MyT1* (Bellefroid et al., 1996) and the bHLH-containing gene, *NeuroD* (Chitnis and Kintner, 1996; Lee et al., 1995). The maintenance of progenitor populations within early domains of neurogenesis is therefore a finely tuned process, requiring the combined action of positive effectors, including *X-Ngnr1*, and negative effectors, such as *X-Notch1*.

Regionalized *X-Ngnr1* expression defines domains of early neurogenesis. Are there negative effectors that similarly define neural progenitor domains in the embryo? The homeodomain-containing gene *Xiro3* is expressed at highest levels in neural progenitors and when overexpressed, *Xiro3* inhibits neuronal differentiation in the embryo (Bellefroid et al., 1998). The *Xenopus* zinc finger transcription factor gene *Zic2* may also play a role in patterning neuronal differentiation in the early embryo. *Zic2* expression defines neural progenitor domains in the embryo and is expressed at highest levels in the lateral neural plate (Brewster et al., 1998). When overexpressed in the early embryo, *Zic2* inhibits the expression of *N-tubulin* and other neuronal differentiation markers (Brewster et al., 1998). At the molecular level, Zic2 function may be integrated with positive regulators of neurogenesis by direct competition with Gli2 and Gli3 for DNA-binding sites (Aruga et al., 1994; Brewster et al., 1998). In addition to its ability to suppress neurogenesis within the embryo, *Zic2* overexpression also results in an expansion of neural crest (Brewster et al., 1998). These findings suggest that multiple pathways converge to limit neuronal differentiation in the developing nervous system.

In order to further examine the molecular interactions that govern the patterning of regionalized neural progenitor populations, we searched for genes expressed specifically within the boundary progenitor domain at the middle of the mediolateral axis. This domain is defined at gastrula stages of development by expression of the bHLH-containing gene, *Xash3* (Turner and Weintraub, 1994; Zimmerman et al., 1993). *Xash3* has opposing dose-dependent effects when overexpressed in the embryo. At low doses, *Xash3* induces ectopic neurogenesis in a majority of embryos while at high doses the effect of *Xash3* overexpression is to inhibit neuronal differentiation (Chitnis and Kintner, 1996; Ferreiro et al., 1994). The high-dose effect of *Xash3* to block neuronal differentiation results from direct activation of the *Notch* lateral inhibition pathway via *X-Delta1* induction (Chitnis and Kintner, 1996). Although the precise role of *Xash3* in the embryo remains uncertain, the fact that early *Xash3* expression is not linked to sites of early neurogenesis in the neural plate suggests that the neuronal differentiation capacity of *Xash3* within the midpoint progenitor population is inhibited through interaction with molecules such as *Notch* that repress neurogenesis.

In the current study, we describe the isolation and characterization of *Xdbx*, a *Xenopus* homolog of murine *Dbx* (Lu et al., 1992). *Xdbx* is first expressed at the midpoint of the mediolateral axis of the neural plate and is subsequently expressed at the middle of the dorsoventral axis of the neural tube, an expression pattern overlapping that of *Xash3*. *Xdbx* overexpression specifically inhibits neuronal differentiation within the neural plate and, in co-injection experiments, *Xdbx* inhibits the ability of *Xash3* to promote ectopic neuronal differentiation. When taken together, our results suggest that *Xdbx* may function within the midpoint progenitor population to inhibit neuronal differentiation, possibly through modulating the function of *Xash3*.

MATERIALS AND METHODS

Molecular cloning of the *Xdbx* gene

A probe containing the homeodomain region of the chicken *ChoxE* gene (Rangini et al., 1991) was used at low stringency to screen a *Xenopus* stage 17 cDNA library (Kintner and Melton, 1987). A 2.1 kb. cDNA clone (*Xdbx*) was isolated in a screen of approximately 10^6 plaques. The putative protein-coding region contained within this clone was sequenced in its entirety.

In situ hybridization analyses

The *Xdbx* clone was linearized with *Bgl*II and transcribed with T7 to generate a 1.6 kb antisense probe for *Xdbx*. Antisense probes for *X-HES-5* and *Hairy-2A* were generated by digesting with *Bam*HI and transcribing with T3 and T7, respectively. Probes for *N-tubulin*, *NeuroD*, *X-Delta1*, *N-CAM*, *F-cadherin*, *Xash3*, *En2*, *Krox20* and *X-Ngnr1* have all been described previously (Bradley et al., 1993; Chitnis et al., 1995; Espeseth et al., 1995; Hemmati-Brivanlou et al., 1991; Krieg et al., 1989; Lee et al., 1995; Ma et al., 1996; Richter et al., 1988; Zimmerman et al., 1993).

Whole-mount in situ hybridization analyses were performed using previously described techniques (Harland, 1991; Hemmati-Brivanlou et al., 1990). For double in situ hybridizations, embryos were simultaneously hybridized with a fluorescein-substituted *Xdbx* probe and either a digoxigenin-substituted *X-Ngnr1*, *N-tubulin*, *Xash3*, *Krox20* or *En2* probe. Following overnight incubation with anti-fluorescein antibody at a dilution of 1:2000, embryos were washed in MAB (100 mM maleic acid/150 mM NaCl, pH 7.5) and developed using a magenta phos (3.5 ul of 50 mg/ml solution/ml)/red tetrazolium (4.5 ul of 75 mg/ml solution/ml) chromagen solution. After the reaction had developed to the correct intensity, embryos were washed in MAB and incubated in 0.1 M EDTA/MAB for 20 minutes at 65°C. Embryos were then washed twice for 10 minutes at room temperature in methanol followed by three 10 minute washes in MAB. Embryos were incubated overnight in anti-digoxigenin antibody at a dilution of 1:1000. After washing in MAB, embryos were developed with BCIP (3.5 ul of 50 mg/ml solution/ml) at 37°C. Selected embryos were dehydrated and mounted in paraplast for sectioning.

Construction of *Xdbx* and *GR-Xash3* constructs for injection

All constructs for injection were subcloned into the CS2 derivative, CS2 NLS MT (Rupp et al., 1994; Turner and Weintraub, 1994), which contains an SV40 T antigen nuclear localization sequence (NLS) as well as sequences encoding 6 myc epitope tags (MT). All constructs were sequenced to insure correct protein-coding joins. Two *Xdbx* expression constructs (*Xdbxmet* and *Xdbxvmet*) were generated that contained the full-length protein-coding domain of *Xdbx* but the proteins differed in their N-terminal amino acids. These constructs yielded indistinguishable results in overexpression assays. The *Xdbxmet* construct includes 60 bp of 5′ *Xdbx* flanking sequence in addition to the normal *Xdbx* start codon (see Fig. 1A). The *Xdbxvmet* construct includes the amino acids MetGlyGlyGly N-terminal to the start site of *Xdbx*. Both derivatives were subcloned in frame with the downstream NLS and MT region of the CS2 vector. A homeodomain alone (*XdbxHD*) and a homeodomain deletion (*Xdbx∆HD*) construct

were also generated. *XdbxHD* contains the homeodomain coding region of *Xdbx* (GlyMet…SerLys) linked to two N-terminal Met codons. The *XdbxΔHD* construct is a variant of *Xdbxvmet* in which the homeodomain-coding region (GlyMet…SerLys) has been replaced by sequences encoding the amino acids GlyGlyGly. The *VP16Xdbx* construct includes sequences encoding the amino acids MetGlyGlyGly N-terminal to sequences encoding the 78 C-terminal amino acids of herpes simplex virus VP16 (AlaPro…GlyGly) which are in turn linked to downstream sequences encoding the homeodomain of *Xdbx*. The *En^RXdbx* construct links sequences encoding the 298 N-terminal amino acids of *Drosophila Engrailed* (MetAla…LeuGly) to the *Xdbx* homeodomain region.

The *Xash3* expression construct *GR-Xash3* links the protein-coding region of *Xash3* to sequences encoding the ligand-binding domain of the *Glucocorticoid Receptor* gene. Sequences encoding the glucocorticoid ligand-binding domain (aa 512-777; Hollenberg et al., 1985) were subcloned in frame with the downstream NLS and MT region of CS2. The *Xash3* protein-coding domain (Zimmerman et al., 1993) was subcloned in frame with all of these regions, yielding a fusion protein consisting of GR-NLS-MT-Xash3. In this construct, *Xash3* function is hormone regulated in a manner similar to that previously reported for the related bHLH-containing gene, *myoD* (Kolm and Sive, 1995).

Preparation and injection of mRNA

The *Xdbxmet*, *Xdbxvmet*, *XdbxHD*, *XdbxΔHD*, *En^RXdbx* and *VP16Xdbx* mRNAs were prepared for injection by linearizing with *Not*I and transcribing with SP6. *X-Ngnr1, X-Notch1/ICD, X-Delta1^{STU}, lacZ* mRNAs were prepared for injection as previously described (Chitnis et al., 1995; Chitnis and Kintner, 1996; Ma et al., 1996; Vize et al., 1991) All mRNAs were synthesized using recommended protocols (Ambion). Yields were calculated based on incorporation of a radioactive tracer nucleotide. Embryos from either wild-type or albino *Xenopus laevis* were injected in a single blastomere at the 2-cell stage of development. In panels shown, 100-200 pg of the *Xdbxmet* or *Xdbxvmet* mRNA, 1 ng of *XdbxHD*, *XdbxΔHD*, *En^RXdbx* or *VP16Xdbx*, 1 ng of *GR-Xash3*, 1 ng of *X-delta1^{STU}* and 1 ng of *X-Notch1/ICD* were injected in a volume of either 5 or 10 nl. 100 pg of *lacZ* mRNA was included in each injection. Injection of lower doses of *Xdbx* (10 or 1 pg) showed a similar phenotype but in a lower percentage of embryos. For *GR-Xash3*, *GR-Xash3/X-Delta1^{STU}*, *GR-Xash3/Xdbxvmet* and *GR-Xash3/X-Delta1^{STU}/Xdbxvmet* injections, embryos were incubated in 10^{-5} M dexamethasone after stage 7 in order to activate the GR-Xash3 fusion protein. In the absence of dexamethasone, ectopic *N-tubulin* expression was not observed in *GR-Xash3*-injected embryos. Exposure of uninjected embryos to dexamethasone had no effect on patterns of *N-tubulin* expression. Staged embryos were fixed for 1 hour in MEMFA. For in situ hybridization, embryos were first analyzed for expression of co-injected β-gal before transfer to methanol and storage at −20°C.

RESULTS

Isolation of the *Xenopus Dbx* gene

The murine *Dbx* gene (Lu et al., 1992) is a member of the *hlx* family of genes encoding homeodomain-containing transcription factors. We have isolated the *Xenopus* homolog of this gene in a low-stringency screen of a *Xenopus* neurula stage cDNA library. *Xdbx* is homologous to *Dbx* throughout its protein-coding domain with an overall homology of greater than 73% (Fig. 1A; Lu et al., 1992). Within the homeodomain region, homology between *Dbx* and *Xdbx* approaches 97% (Fig. 1B) while the homology in non-homeodomain regions is approximately 68% (Fig. 1A; Lu et al., 1992). *Xdbx* also shares high homology with the homeodomain region of the zebrafish *hlx1* gene (Fig. 1B; Fjose et al., 1994), the only domain of *hlx1* for which published sequence is available. In contrast to these extensive homologies, the homology between *Xdbx* and other members of the *hlx* class of homeodomain genes is specific to the homeodomain region with no regions of homology apparent within other protein domains (Fig. 1B and data not shown). In summary, these homology comparisons suggest that *Xdbx*, *Dbx* and possibly zebrafish *hlx1* represent evolutionarily conserved orthologs.

Expression of *Xdbx* during *Xenopus* development

The expression of *Xdbx* during *Xenopus* development was characterized using whole-mount in situ hybridization techniques. The expression of *Xdbx* appears neural specific. *Xdbx* expression is first detected in neural plate stage embryos within two domains (Fig. 2A). In anterior regions of the embryo, *Xdbx* expression is detected within a zone at the midline of the neural plate (Fig. 2A, arrowhead, C) while, in posterior regions, *Xdbx* is expressed in a bilaterally symmetric stripe that lies at the middle of the mediolateral axis (Fig. 2A and B, arrow). This pattern of *Xdbx* expression is maintained throughout neural fold stages of development.

Following neural tube closure, *Xdbx* is maintained in posterior regions of the embryo at the midpoint of the dorsoventral axis of the neural tube (Fig. 2D, arrow, E). The expression domain apparent at the anterior midline of the neural plate (Fig. 2A, arrowhead), the presumptive ventral region of the neural tube, is not detectable following neural tube closure. At stages intermediate to stage 20 and stage 28, an anteroposterior striped pattern of *Xdbx* appears within anterior regions of the embryo (data not shown). By stage 28, these stripes have coalesced to yield a uniform zone of *Xdbx* expression (Fig. 2F). In anterior regions, *Xdbx* expression is largely specific to the dorsal neural tube (Fig. 2G). Expression in more posterior regions of the embryo remains specific to the midpoint of the dorsoventral axis (Fig. 2H). This midpoint expression is restricted to the ventricular zone suggesting that *Xdbx* expression is specific to mitotic progenitors within the developing nervous system (Fig. 2H).

Mapping *Xdbx* expression with respect to the anteroposterior and mediolateral axes

In order to gain a more detailed view of the pattern of *Xdbx* expression in early neural development, we compared *Xdbx* expression to that of other regionalized neural markers. The *Xdbx* expression boundaries in anterior regions of the developing nervous system were mapped relative to *Krox20* and *Engrailed 2* (*En2*) using double-label, whole-mount in situ hybridization techniques. *En2* is expressed specifically at the midbrain-hindbrain boundary in the developing nervous system (Hemmati-Brivanlou et al., 1991). In embryos double-labeled with *En2* and *Xdbx*, the anterior domain of *Xdbx* expression lies immediately rostral to the *En2* domain (Fig. 3A, *Xdbx*, arrowhead; *En2*, asterisk).

The posterior striped domain of *Xdbx* expression was compared to that of *Krox20*, a gene expressed specifically within rhombomeres 3 and 5 of the developing hindbrain (Bradley et al., 1993). The anterior limit of the striped domain overlaps the rhombomere 5 domain of *Krox20* expression indicating that the bilaterally symmetric stripe of *Xdbx*

expression is specific to the posterior hindbrain and spinal cord (Fig. 3B, *Xdbx*, arrow; *Krox20*, asterisks).

The posterior domain of *Xdbx* expression was also mapped relative to the mediolateral domains of *Xash3*, *X-Ngnr1* and *N-tubulin* expression. *Xash3* is expressed at the apparent midpoint of the mediolateral axis commencing at mid-gastrula stages of development (Zimmerman et al., 1993). Comparison of *Xdbx* and *Xash3* expression indicates that *Xdbx* overlaps the *Xash3* expression domain at the middle of the mediolateral axis although *Xash3* expression extends to a more medial region of the axis (Fig. 3C,E, *Xdbx*, arrow; *Xash3*, gray arrow; superficial light blue staining in E is background reactivity resulting from prolonged chromagenic incubation). In contrast, comparison of *Xdbx* expression to that of *X-Ngnr1* and *N-tubulin* at the neural plate stage of development indicates juxtaposed patterns of expression. *X-Ngnr1* is expressed in three symmetric domains of neuronal precursors that correlate with sites of primary neurogenesis (Ma et al., 1996). *N-tubulin* is expressed subsequent to *X-Ngnr1* within these domains and is specific to differentiating primary neurons (Oschwald et al., 1991; Richter et al., 1988). Comparison of *Xdbx* expression to that of *X-Ngnr1* and *N-tubulin* indicates that, within the posterior neural plate, *Xdbx* expression juxtaposes the two most medial zones of *X-Ngnr1* and *N-tubulin* expression (Fig. 3D,F,G; *Xdbx*, arrow; *X-Ngnr1* and *N-tub*, asterisks). Our findings suggest that *Xdbx* is not expressed within primary neurons or their precursors but rather is expressed together with *Xash3* in neural progenitors within the neural plate.

Negative crossregulation of *Xdbx* and *X-Ngnr1* expression

The finding that *Xdbx* expression forms a boundary between domains of *X-Ngnr1* expression suggested that these genes might negatively regulate each other at a transcriptional level. We therefore examined the expression of *X-Ngnr1* in embryos injected with *Xdbx* as well as the expression of *Xdbx* in embryos injected with *X-Ngnr1*. In both sets of injected embryos, there was a reciprocal relationship between the expression of the *Xdbx* and *X-Ngnr1* genes. In embryos overexpressing *Xdbx*, *X-Ngnr1* expression was downregulated (30/40 embryos, Fig. 3H) while, in embryos overexpressing *X-Ngnr1*, endogenous *Xdbx* expression was downregulated (8/11 embryos, Fig. 3I).

A.

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ATG ATG TTC CCA AGC CTC TTA GCT CCC CCT GCG GTG TAC CCC AAT CTA CTG AGA CCC ACC  60
M   M   F   P   S   L   L   A   P   P   A   V   Y   P   N   L   L   R   P   T    20

CCG ACT CTG ACG CTG CCA CAG TCT ATC CAG ACT GCC CTG TCC AAC CAC ACC AGC TTT TTA 120
P   T   L   T   L   P   Q   S   I   Q   T   A   L   S   N   H   T   S   F   L    40

ATA GAA GAC TTG ATC AGG ATC AGC CGA CCA GCA GGA TTC CTG CCC AGG GCT GTC CCA CCT 180
I   E   D   L   I   R   I   S   R   P   A   G   F   L   P   R   A   V   P   P    60

CCC AGC ATG TCC CCT CCA ACC TCT GAA AGT CCC AAC TGC ATG AGC GAG ACA TCA GAC TTG 240
P   S   M   S   P   P   T   S   E   S   P   N   C   M   S   E   T   S   D   L    80

GCA AGA AGA GAG GGT CCA AAC CAG ACT TCA ATT TCT TCC AAC AAC AGT TCC CCT TTT CTG 300
A   R   R   E   G   P   N   Q   T   S   I   S   S   N   N   S   S   P   F   L   100

AAG TTT GGA GTC AAT GCA ATC CTC TCC TCC AGC CCT AGG ACA GAG TCG GCA CAA GTC TTG 360
K   F   G   V   N   A   I   L   S   S   S   P   R   T   E   S   A   Q   V   L   120

CTC CCC AGC GCC CAT CCA AAG CCC TTC ACC TTC CCT TAC TTT GAA GGA TCC TTC CAG CCT 420
L   P   S   A   H   P   K   P   F   T   F   P   Y   F   E   G   S   F   Q   P   140

TTC ATT AGA TCT TCC TAT TTC CCA GCT TCC TCT TCG GTG GTG CCC ATC CCC GGC ACA TTC 480
F   I   R   S   S   Y   F   P   A   S   S   S   V   V   P   I   P   G   T   F   160

TCC TGG CCC CTG GTC GCT CGA GGG AAG CCC CGC CGG GGC ATG TTG CGC AGA GCC GTG TTC 540
S   W   P   L   V   A   R   G   K   P   R   R   G   M   L   R   R   A   V   F   180

TCA GAT GTC CAA CGC AAA GCT CTA GAG AAG ATG TTC CAG AAG CAG AAA TAT ATC AGC AAA 600
S   D   V   Q   R   K   A   L   E   K   M   F   Q   K   Q   K   Y   I   S   K   200

CCG GAC AGA AAG AAA CTG GCT GGG AAG CTG GGG CTC AAA GAC TCT CAG GTA AAA ATT TGG 660
P   D   R   K   K   L   A   G   K   L   G   L   K   D   S   Q   V   K   I   W   220

TTC CAG AAT CGG CGA ATG AAG TGG CGA AAC TCC AAG GAA CGG GAA TTG CTG TCC TCC GGA 720
F   Q   N   R   R   M   K   W   R   N   S   K   E   R   E   L   L   S   S   G   240

GGG TGC AGG GAG CAG ACA CTA CCC ACC AAA TTT AAC CCC CAC CCA GAC CTC AGT GAC GTG 780
G   C   R   E   Q   T   L   P   T   K   F   N   P   H   P   D   L   S   D   V   260

AGC AAG AAA TCC TCG GGG GAG GGA GAG GAG GAG CCA TTG TGC CCA GGA AAC AGC CCC GCC 840
S   K   K   S   S   G   E   G   E   E   E   P   L   C   P   G   N   S   P   A   280

CAC GCC TTG CCT TAT CAA TGC CCA GAG CAC CAT TTA AGA CTT GAT ACC CAG CTT CCT TCC 900
H   A   L   P   Y   Q   C   P   E   H   H   L   R   L   D   T   Q   L   P   S   300

TCC CCT TTT AAC TCC AGC AGT GCC AGT AAA CCC TCA GAC TTC TCA GAC TCA GAG GAA GAG 960
S   P   F   N   S   S   S   A   S   K   P   S   D   F   S   D   S   E   E   E   320

GGA GGG GAA CAG GAA GAA GAG ATC ACA GTC TCC TAA                                 996
G   G   E   Q   E   E   E   I   T   V   S   *                                   332
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B.

Gene	10	20	30	40	50	60	Identity
Xdbx	GMLRRAVFSD	VQRKALEKMF	QKQKYISKPD	RKKLAGKLGL	KDSQVKIWFQ	NRRMKWRNSK	100%
hlx1	----------	----------	----------	-----A----	----------	----------	98%
Dbx	----------	--------T-	----------	-----S----	----------	----------	97%
CHoxE	-I-------E	D---------	--------T-	-----IN---	-E--------	----------	88%
Dbx2	-I-------E	E---------	--------T-	-R---VS---	-E--------	----------	87%
Hlx	RSWS-----N	L---G---R-	EI---VT---	--Q--AM---	T-A---V---	-------H--	68%
H2.0	RSWS-----N	L---G--IQ-	-Q----T---	-R---AR-N-	T-A---V---	-------HTR	65%

Fig. 1. *Xdbx* encodes an *hlx* class homeodomain-containing protein. (A) The *Xdbx* sequence and putative protein-coding domain are shown. The homeodomain region is underlined. The *Xdbx* sequence is available through GenBank (accession number AF253504). (B) Comparison of the *Xdbx* homeodomain to other members of the *hlx* homeodomain family. Dashes indicate conserved residues. The genes listed include zebrafish *hlx*1 (Fjose et al., 1994), murine *Dbx* (Lu et al., 1992), chick *ChoxE* (Rangini et al., 1991), murine *Dbx2* (Shoji et al., 1996), murine *Hlx* (Allen et al., 1991) and *Drosophila H2.0* (Barad et al., 1988).

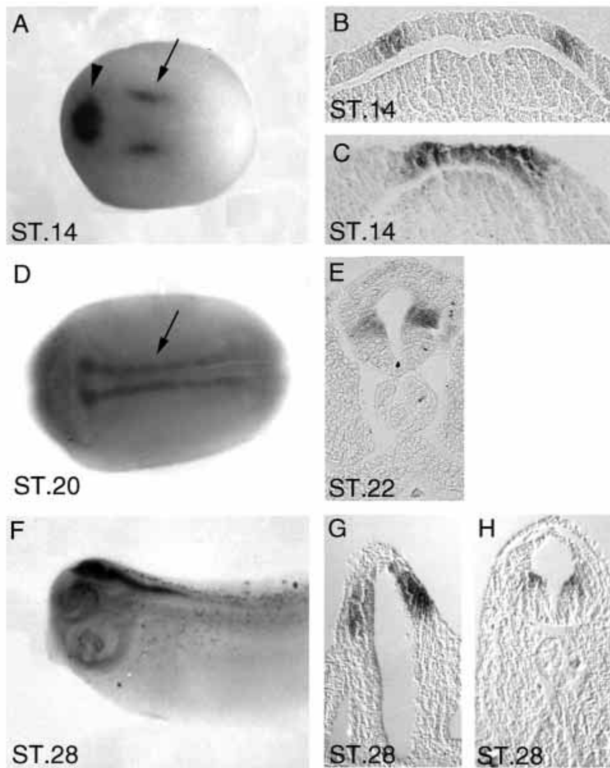

Fig. 2. *Xdbx* expression during *Xenopus* development. Whole-mount in situ hybridization of *Xdbx* at stage 14 (A), stage 20 (D) and stage 28 (F). The expression domain at the midline of the axis in anterior regions is indicated by an arrowhead (A). Cross sections of this region are shown in C. The expression in a bilaterally symmetric stripe in posterior regions is indicated by an arrow (A,D). Cross sections within this region of the embryo reveal *Xdbx* expression at the midpoint of the mediolateral axis of the neural plate (B) and subsequently at the midpoint of the dorsoventral axis of the neural tube (E). At later stages of development (stage 28, F), *Xdbx* expression is prominent in anterior regions of the embryo. Cross sections indicate that *Xdbx* expression is dorsally restricted within anterior regions of this domain (G) but remains specific to the midpoint of the dorsoventral axis within the posterior hindbrain (H). Note that within this region *Xdbx* expression is specific to cells at the ventricular surface.

Effects of *Xdbx* overexpression on patterns of neurogenesis within the neural plate

Our finding that *Xdbx* is expressed in neural progenitors and that *Xdbx* overexpression downregulates expression of *X-Ngnr1*, a positive effector of neurogenesis, suggested that *Xdbx* might play the opposing role of inhibiting neurogenesis within neural progenitors. Embryos injected with *Xdbx* were therefore characterized for expression of the neuronal differentiation marker *N-tubulin,* which marks zones of primary neurogenesis in the embryo (Oschwald et al., 1991; Richter et al., 1988). In response to *Xdbx* overexpression, *N-tubulin* expression was downregulated at the neural plate stage of development (44/51 embryos; Fig. 4A).

Additional markers of neuronal differentiation were also investigated in *Xdbx*-injected embryos. Expression of the bHLH-containing gene *NeuroD* and the neurogenic gene *X-Delta1* precede and then overlap domains of *N-tubulin* expression during normal development (Chitnis et al., 1995; Lee et al., 1995). In embryos overexpressing *Xdbx*, *NeuroD* and *X-Delta1* expression was downregulated (*NeuroD*, 5/5 embryos; *X-Delta1*, 6/9 embryos; Fig. 4B and data not shown). As *N-tubulin*, *NeuroD* and *X-Delta1* expression is positively correlated with neuronal differentiation during normal development, these data support an opposing role for *Xdbx* in inhibiting neurogenesis within the developing embryo.

The homeodomain region of *Xdbx* is sufficient for function

In order to investigate the functional domains of the Xdbx protein, we constructed two variants of *Xdbx*, *XdbxHD* and *XdbxΔHD*, and tested their function in *Xenopus* embryos. *XdbxHD* encodes only the homeodomain region of the Xdbx protein and *XdbxΔHD* contains a specific deletion of the same homeodomain region. In response to *XdbxHD* overexpression, *N-tubulin* expression was downregulated at the neural plate stage (16/35 embryos; Fig. 4C). In contrast, embryos overexpressing *XdbxΔHD* showed normal patterns of N-tubulin expression (17/19 embryos; data not shown). These findings suggest that the homeodomain region of Xdbx is both required and sufficient for inhibiting neuronal differentiation in the embryo. However, the inhibition observed following *XdbxHD* overexpression was not as efficient as that observed following overexpression of the full-length *Xdbx* protein-coding domain (compare 45% for *XdbxHD* versus 86% for *Xdbx*) suggesting that other regions of the protein cooperate to enhance the function of the homeodomain region.

In order to assess whether the inhibitory effects of *Xdbx* on neuronal differentiation were mediated either via transcriptional activation or repression, we linked either a heterologous transcriptional repressor (En^R; Han and Manley, 1993; Jaynes and O'Farrell, 1991; Smith and Jaynes, 1996) or a heterologous transcriptional activator (VP16; Sadowski et al., 1988; Treizenberg et al., 1988) to the homeodomain region of the *Xdbx* protein-coding domain and tested function in overexpression assays. The overexpression of either *VP16Xdbx* or *En^RXdbx* in developing embryos resulted in the inhibition of *N-tubulin* expression (*VP16Xdbx*, 20/37 embryos; *En^RXdbx,* 11/26 embryos; data not shown). These results suggest that the function of the *Xdbx* homeodomain is dominant to heterologous repressor or activator regions.

Xdbx overexpression does not disrupt the early regionalization of the neuroectoderm

Xdbx overexpression inhibits a number of neuronal differentiation markers including *N-tubulin*, *X-Ngnr1* and *NeuroD*. We next investigated the expression of neural markers not specifically associated with differentiating neurons in order to determine the range of *Xdbx* function. *N-CAM* is expressed in both progenitor and differentiated neuron populations (Krieg et al., 1989). In contrast to neuronal differentiation markers, expression of *N-CAM* was not inhibited in embryos overexpressing *Xdbx* (10/11 embryos; Fig. 4D). Some embryos exhibited lower levels of *N-CAM* expression but, unlike the complete absence of *N-tubulin* expression in response to *Xdbx* overexpression, *N-CAM* expression always remained detectable (Fig. 4D). In addition, the overexpression of *Xdbx* resulted in a lateral expansion of the *N-CAM*-positive domain in a minority of embryos (3/11 embryos; data not shown).

We also examined the expression of *Xash3* and *F-cadherin*,

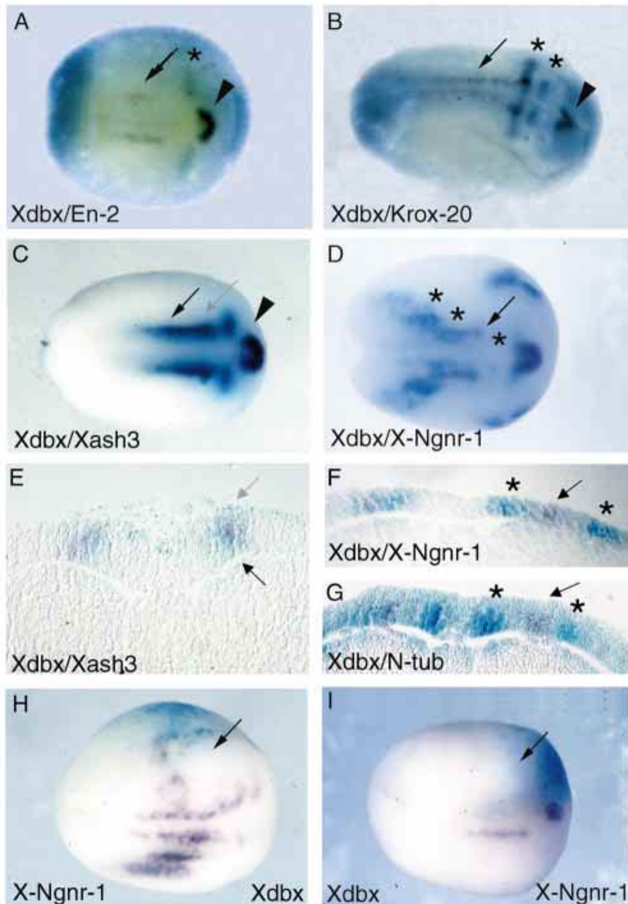

Fig. 3. *Xdbx* expression is restricted with regards to the mediolateral and anteroposterior axes of the neural plate. (A) Comparison of the anterior domain of *Xdbx* expression (arrowhead) to that of *En-2* (asterisk) in neural plate stage embryos. (B) Comparison of *Xdbx* expression (arrow and arrowhead) to that of *Krox20* (asterisks) in neurula stage embryos. (C,D) Comparison of *Xdbx* (arrow and arrowhead), *Xash3* (C, gray arrow) and *X-Ngnr1* (D, asterisks) expression in neural plate stage embryos. (E-G) Cross-sections of embryo co-hybridized with *Xdbx* (black arrow) and *Xash3* (E, gray arrow), *X-Ngnr1* (F, asterisks) and *N-tubulin* (G, asterisks) are shown. *Xdbx* expression is marked by a red precipitate and expression of the other genes is marked by a light-blue precipitate. Scattered light-blue precipitate at surface in E is background BCIP reactivity resulting from prolonged chromagenic incubation (approximately 72 hours). (H) Effects of *Xdbx* overexpression on *X-Ngnr1* expression. (I) Effects of *X-Ngnr1* overexpression on *Xdbx* expression. (H,I) Arrow indicates injected side of the embryo. Light-blue staining represents expression of co-injected β-gal lineage tracer.

two markers expressed with *Xdbx* within the midpoint neural progenitor population. Both *Xash3* and *F-cadherin* initiate expression at gastrula stages of development, prior to the onset of *Xdbx* expression in the embryo (Espeseth et al., 1995; Zimmerman et al., 1993). In embryos overexpressing *Xdbx*, normal expression of *Xash3* and *F-cadherin* was noted in a majority of embryos (*Xash3*, 7/10 embryos; *F-cadherin*, 20/20 embryo, Fig. 4E,F). Taken together, these overexpression results suggest that *Xdbx* does not affect the early regionalization of the neuroectoderm but instead specifically inhibits neuronal differentiation within the developing neural plate.

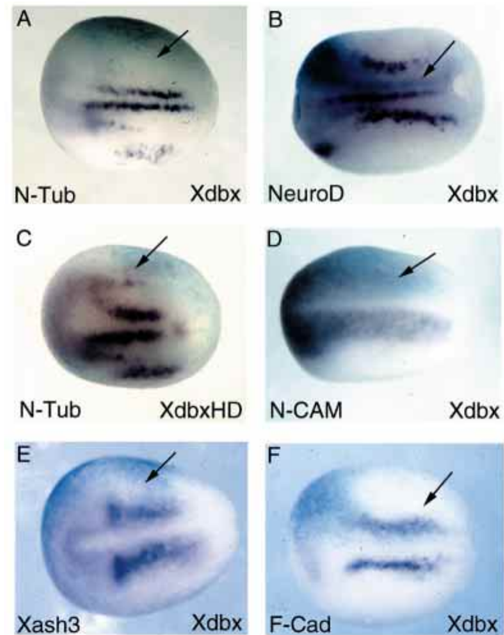

Fig. 4. *Xdbx* overexpression inhibits neuronal differentiation but not neural patterning. The effects of *Xdbx* overexpression on the expression of *N-tubulin* (A), *NeuroD* (B), *N-CAM* (D), *Xash3* (E) and *F-cadherin* (F) are shown. (C) The effect of *XdbxHD* overexpression on the expression of *N-tubulin*. In all panels, the injected side of the embryo is indicated by an arrow. Light-blue staining represents expression of the co-injected β-gal lineage tracer.

Xdbx function appears independent of *X-Notch1 and Xiro3* activity

The inhibition of neuronal differentiation markers in response to *Xdbx* overexpression is similar to the previously characterized phenotype in embryos in which the *X-Notch1* signaling cascade has been activated (Chitnis et al., 1995; Coffman et al., 1993). We therefore examined the effects of activated *Notch* signaling on *Xdbx* expression. Although *Xdbx* and *X-Notch1* have a similar overexpression phenotype, *X-Notch1* signaling does not activate *Xdbx* expression. In fact, *Xdbx* expression was inhibited in embryos that overexpressed a constitutively active form of the *X-Notch1* receptor (8/10 embryos; Fig. 5A). We also examined *Xdbx* expression in embryos in which the *Notch* signaling cascade was disrupted via overexpression of a dominant negative variant of *X-Delta1*, *X-Delta1^{STU}*. In embryos overexpressing *X-Delta1^{STU}* an increased density of neuronal differentiation as marked by *N-tubulin* expression was noted in domains of primary neurogenesis as previously reported (Chitnis et al., 1995 and data not shown). Normal patterns of *Xdbx* expression were observed in these embryos (5/5 embryos; Fig. 5B).

We next examined downstream mediators of the *Notch* pathway in embryos overexpressing *Xdbx*, including *Hairy-2A* and *X-HES-5*. The *Hairy-2A* gene is normally expressed at the boundaries of the neural plate (Turner and Weintraub, 1994) while *X-HES-5* is expressed in bilaterally symmetric stripes along the mediolateral axis of the neuroectoderm that apparently overlap domains of *X-Delta1* expression (P. Wilson and K. Z., unpublished data). In *Xdbx*-injected embryos, neither *Hairy-2A* nor *X-HES-5* expression was positively

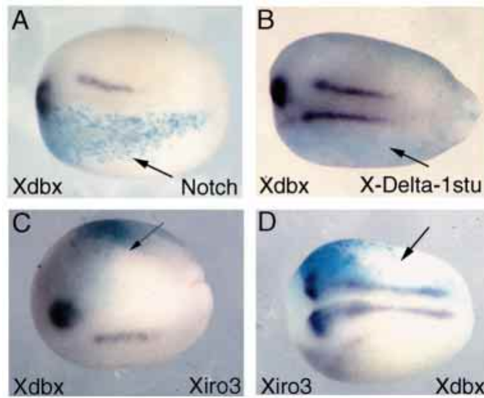

Fig. 5. *Xdbx* is not positively linked to either *X-Notch1* or *Xiro3*. The effects of *X-Notch1/ICD* (A), *X-Delta1^{STU}* (B) and *Xiro3* (C) overexpression on *Xdbx* expression are shown. *Xiro3* expression in embryos overexpressing *Xdbx* (D). In all panels, the injected side of the embryo is indicated by an arrow. Light-blue staining represents expression of the co-injected β-gal lineage tracer.

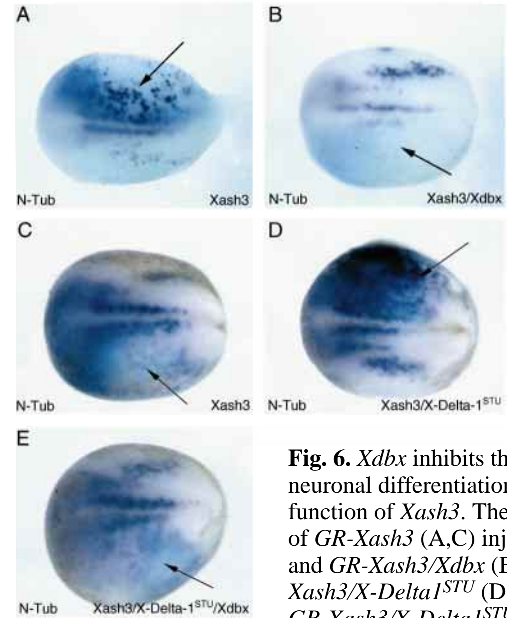

Fig. 6. *Xdbx* inhibits the neuronal differentiation function of *Xash3*. The effects of *GR-Xash3* (A,C) injection and *GR-Xash3/Xdbx* (B), *GR-Xash3/X-Delta1^{STU}* (D) and *GR-Xash3/X-Delta1^{STU}/Xdbx* (E) co-injections on the expression of *N-tubulin* are shown. See Fig. 4 (A) for effects of *Xdbx* alone on *N-tubulin* expression. Arrows indicate injected side of embryo and light blue staining represents expression of the co-injected β-gal lineage tracer.

regulated (0/8 and 0/5 embryos respectively; data not shown). These combined results suggest that *Xdbx* does not function as either an upstream or downstream positive transcriptional effector of the *X-Notch1* pathway.

The homeodomain-containing gene *Xiro3* is expressed at the midpoint of the mediolateral axis of neural plate stage embryos and *Xiro3* overexpression blocks neuronal differentiation in the embryo (Bellefroid et al., 1998). In order to examine whether *Xdbx* and *Xiro3* function within the same transcriptional pathway, we examined both the regulation of *Xdbx* expression in *Xiro3*-injected embryos and *Xiro3* expression in *Xdbx*-injected embryos. In embryos overexpressing *Xiro3*, *Xdbx* expression was downregulated (9/10 embryos; Fig. 5C). In embryos overexpressing *Xdbx*, *Xiro3* expression was either normal or downregulated in the majority of embryos (normal, 5/12 embryos; downregulated 5/12 embryos; Fig. 5D and data not shown). In a subset of embryos, the expression domain of *Xiro3* was broadened in response to *Xdbx* overexpression (2/12 embryos and data not shown). The downregulation of *Xdbx* expression in response to *Xiro3* and the mixed *Xiro3* expression phenotype that we observe following *Xdbx* overexpression suggests that the common ability of these molecules to inhibit *N-tubulin* expression is not mediated through positive transcriptional crossregulation between *Xdbx* and *Xiro3*.

Xdbx inhibits *Xash3* function

The ability of *Xdbx* to inhibit neurogenesis when overexpressed is correlated with its normal expression in the midpoint neural progenitor domain. This domain also expresses *Xash3*, a bHLH-containing transcription factor that when overexpressed can drive neurogenesis in the embryo in a dose-dependent manner (Chitnis and Kintner, 1996; Ferreiro et al., 1994). In order to investigate the ability of *Xdbx* to modulate the neuronal differentiation function of *Xash3*, we assayed their integrated effects on neuronal differentiation. For these experiments, we used a *Xash3* fusion construct that linked the *Xash3* protein-coding domain to the ligand-binding domain of the *Glucocorticoid Receptor* gene (Hollenberg et al., 1985). In this construct, Xash3 function is dependent upon hormone addition and thus all embryos shown were incubated in dexamethasone (see Materials and Methods). *GR-Xash3* overexpression led to ectopic neurogenesis (12/28 embryos; Fig. 6A) in a number of embryos as previously reported for *Xash3* (Chitnis and Kintner, 1996). However, when *GR-Xash3* and *Xdbx* were co-injected into the embryo, no ectopic neurogenesis was observed (0/21 embryos; Fig. 6B). In addition, normal patterns of primary neurogenesis were disrupted in *GR-Xash3*/*Xdbx*-injected embryos (19/21 embryos; Fig. 6B).

In a subset of embryos, the effect of *GR-Xash3* overexpression was to inhibit rather than to promote neuronal differentiation (8/17 embryos decrease *N-tubulin*; 6/17 embryos increase; Fig. 6A,C) suggesting the possibility that *Xdbx* functioned in cooperation with *Xash3* to inhibit neuronal differentiation. The ability of *Xash3* to limit neurogenesis in the embryo depends upon *X-Delta1*-mediated activation of the *Notch* pathway (Chitnis and Kintner, 1996). The inhibition of *X-Delta1* function in *Xash3*-expressing embryos by co-injection of the dominant negative *Delt*a variant *X-Delta1^{STU}* relieves inhibition and results in a dense pattern of ectopic neurogenesis in the majority of embryos (Chitnis and Kintner, 1996; 12/18 embryos increase *N-tubulin*; 2/18 embryos decrease *N-tubulin*; Fig. 6D). In contrast, the co-injection of *Xdbx* together with *GR-Xash3* and *X-Delta1^{STU}* results in the inhibition of *N-tubulin* expression in the majority of embryos (11/17 embryos decrease *N-tubulin*; 2/17 increase *N-tubulin*; Fig. 6E). Thus *Xdbx* does not positively cooperate with *Xash3* via *Notch* activation to inhibit neurogenesis but rather appears to activate an independent pathway to alter the neuronal differentiation function of *Xash3*.

DISCUSSION

The homeobox motif is common to a large number of genes

that play important roles during vertebrate development (Gehring et al., 1994). Our current studies have focused on a single, evolutionarily conserved member of the *hlx* class of homeodomain-containing genes, *Xdbx*. The expression of *Xdbx* is restricted with respect to both the anteroposterior and mediolateral axes of the neural plate, indicating a regionally specific role in nervous system development. Overexpression studies in *Xenopus* embryos indicate that *Xdbx* inhibits neuronal differentiation suggesting that it may function to restrict neurogenesis temporally and spatially within the developing nervous system.

Pattern of *Xdbx* expression during development

Expression of the *Xdbx* gene initiates at the neural plate stage of development and appears neural specific. At early stages, *Xdbx* expression is regionally restricted both within anterior and posterior domains of the embryo. Within anterior regions, *Xdbx* is expressed at the midline of the mediolateral axis of the neuroectoderm in a zone immediately adjacent to the domain of *En2* expression. Based on the fate map of the *Xenopus* neural plate (Eagleson and Harris, 1990), this region will later give rise to diencephalic derivatives but as *Xdbx* expression is transient within this zone the more specific fate of these progenitors remains unknown.

Xdbx is also expressed in longitudinal stripes that first define the middle of the mediolateral axis of the neural plate and subsequently define the midpoint of the dorsoventral axis of the neural tube. This regional expression is shared with murine *Dbx* and zebrafish *hlx1* (Fjose et al., 1994; Lu et al., 1992) as well as with more divergent members of this family, including murine *Dbx2* and chicken *ChoxE* (Rangini et al., 1991; Shoji et al., 1996). The transient expression of *Xdbx* within ventricular zone progenitors in the frog precludes tracking the differentiated fate of the *Xdbx*-expressing progenitors. However, recent work in both mouse and chick suggests that *Dbx1* and *Dbx2* progenitors are bound for an interneuron fate (Matise et al., 1999; Pierani et al., 1999). Expression studies in the chick spinal cord indicate that the Dbx1 protein is transiently co-localized with two differentiated markers of V0 interneurons, *Lim1/2* and *Evx1/2* (Pierani et al., 1999). Moreover, the downregulation of *Dbx1* expression is correlated with a loss of V0 interneurons while downregulation of *Dbx2* expression correlates with a loss of the V1 interneuron population (Pierani et al., 1999).

The role of the *Xdbx* homeodomain region

The homeodomain region of *Xdbx* is both required and sufficient to inhibit *N-tubulin* expression when overexpressed in the embryo indicating the importance of this domain in *Xdbx* function. The homeodomain region facilitates the DNA-binding properties of the homeodomain class of proteins (for review, Gehring et al., 1994). Recent studies have shown that the DNA target specificities of homeodomain-containing proteins can be altered by protein-protein interactions (Mann and Affolter, 1998; Wilson and Desplan, 1995). Sites of protein-protein interaction have been mapped both within homeodomain as well as non-homeodomain regions of proteins. In addition, these interactions occur both with other homeodomain-containing proteins (for review, Mann and Affolter, 1998; Wilson and Desplan, 1995) as well as with other classes of transcription factors, including zinc finger and bHLH-containing proteins (Johnson et al., 1997; Lee et al., 1998). The dominance of the Xdbx homeodomain to both a heterologous activator (VP16) as well as a heterologous repressor (En^R) that we observe in overexpression assays might be explained by interactions mediated by the homeodomain with additional dominantly acting transcriptional regulatory proteins. The activity of the homeodomain region of Xdbx in functional assays, albeit lower than the activity of the wild-type protein, therefore provides a starting point for further study of both the Xdbx-DNA and/or Xdbx-protein interactions that govern functional activity.

Xdbx may act to refine patterns of neurogenesis within the neural plate

The spatially restricted expression of the proneural bHLH gene *X-Ngnr1* is important for the stereotypic pattern of primary neurogenesis during normal development as *X-Ngnr1* misexpression drives neurogenesis throughout the neural plate (Ma et al., 1996). In contrast, *Xdbx* overexpression has the opposite effect of suppressing neurogenesis throughout the neural plate. We have noted a reciprocal transcriptional relationship between the expression of these genes. *X-Ngnr1* and *Xdbx* are normally expressed in juxtaposed domains within the neural plate and our findings indicate that the overexpression of one gene has a negative effect upon expression of the other. This negative reciprocal transcriptional regulation may shape the expression borders of each gene during normal development and the negative regulation of *X-Ngnr1* expression by *Xdbx* may in turn play a role in inhibiting neurogenesis within the midpoint progenitor population at early stages of nervous system development.

Xdbx expression is also downregulated by the overexpression of *Notch* and *Xiro3*, genes that inhibit rather than promote neuronal differentiation in the embryo. Why is *Xdbx* downregulated both by genes that promote as well as inhibit neuronal differentiation? As *X-Ngnr1* and *Xiro3* induce *X-Delta1* (Bellefroid et al., 1998; Ma et al., 1996) expression, it is possible that the downregulation of *Xdbx* in embryos overexpressing *X-Ngnr1* and *Xiro3* is an indirect consequence of *Notch* activation. Alternatively, the downregulation of *Xdbx* in response to both genes that promote an early neural progenitor state as well in response to genes that promote neuronal differentiation may reflect the transient expression pattern of *Xdbx* during normal development. *Xdbx* is not expressed in differentiated neurons and in neural progenitors, *Xdbx* expression initiates subsequent to *Xash3* and *F-cadherin* (Espeseth et al., 1995; Zimmerman et al., 1993), suggesting the possibility that *Xdbx* expression specifically marks an intermediate stage in the differentiation of these progenitors.

What role does *Xdbx* play in molecular interactions at the midpoint of the mediolateral axis?

One of the earliest markers of the midpoint of the neuroectoderm is the proneural bHLH gene, *Xash3* (Zimmerman et al., 1993). Overexpression studies indicate that *Xash3* can act as a dose-dependent positive effector of neurogenesis (Chitnis and Kintner, 1996). The eventual neuronal or non-neuronal fate of *Xash3*-expressing progenitors is not known in the embryo; however, it is clear that neuronal differentiation is not observed in domains of *Xash3* expression at early stages of development. What factors modulate *Xash3*

function to inhibit neuronal differentiation? Activated *X-Notch1* signaling may play a role in shaping *Xash3* function during development. Co-injection experiments indicate that *Xash3*-expressing progenitors remain responsive to activated *Notch* signaling (Chitnis and Kintner, 1996) and overexpression experiments indicate that *Xash3* induces expression of the *Notch* ligand, *X-Delta1*. As activated *X-Notch1* results in the inhibition of neuronal differentiation (Chitnis et al., 1995), the responsiveness of *Xash3*-expressing cells to *Notch* may play a role in inhibiting the differentiation of *Xash3*-positive progenitors.

Our current analyses suggest that *Xdbx* may play a distinct role in controlling *Xash3* function within the midpoint progenitors of the posterior neural plate. Overexpression of *Xdbx* does not downregulate the expression of *Xash3* and thus *Xdbx* does not inhibit *Xash3* at a transcriptional level. However, *Xdbx* is capable of altering the neuronal differentiation function of *Xash3*. When co-injected into the embryo, *Xdbx* inhibits the ectopic neuronal differentiation that results when *Xash3* is injected alone. Given the ability of *Xash3* to both promote and inhibit neuronal differentiation when overexpressed, this alteration in the neuronal differentiation function of *Xash3* could result either from an inhibition or an enhancement of *Xash3* function. We favor the hypothesis that *Xdbx* has a negative rather than a cooperative effect on *Xash3* function based on our finding that *Xdbx* function is independent of lateral inhibition, the mechanism responsible for the high dose, inhibitory effects of *Xash3* on neuronal differentiation.

The overlap in *Xash3* and *Xdbx* expression is not complete even at the midpoint of the axis suggesting that these genes may interact with additional genes in other regions of the embryo. In this regard, recent characterization of *Dbx1* and *Dbx2* expression in chick indicates that *Dbx2* is expressed in a broader domain with respect to the dorsoventral axis of the spinal cord than is *Dbx1* (Pierani et al., 1999). The isolation of additional *Dbx* homologs in *Xenopus* may therefore identify a related gene that more completely overlaps the *Xash3* expression domain, particularly at the midpoint of the mediolateral axis of the developing neural plate.

What is the mechanism by which *Xdbx* mediates altered *Xash3* function? Our current analyses indicate that *Xdbx* overexpression inhibits the expression of *X-Ngnr1* and *NeuroD*, genes with direct links to neuronal differentiation in the early embryo. Thus the ability of *Xdbx* to alter *Xash3* function may depend upon its ability to either directly or indirectly inhibit the expression of *X-Ngnr1* and/or *NeuroD* within zones of *Xash3* expression. *Xash3* overexpression induces the ectopic expression of *NeuroD* within neural and non-neural ectodermal progenitors (Kanekar et al., 1997) and thus *NeuroD* appears to be a downstream target of *Xash3*. The fact that *Xash3* and *NeuroD* are not normally co-expressed in neural plate progenitors therefore suggests that functions such as those described for *Xdbx* limit *Xash3* target regulation at early stages of normal development. The regulation of *Xash3* function by *Xdbx* could result either from competition for transcriptional targets, activation of reciprocal targets and/or direct protein-protein interactions such as those outlined above for homeodomain-containing proteins.

Conclusions

Although a subset of neurons differentiate at the neural plate stage of development, the majority of neurons and glia of the mature *Xenopus* nervous system derive at later time points from the neural progenitor populations of the neural plate stage embryo. The integration of positive and negative inducers likely creates a balance between neuronal differentiation and progenitor cell maintenance throughout the neural plate. Recently several pairings of positive and negative inducers have been identified within vertebrate embryos, including *Gli/Zic2* (Brewster et al., 1998), *Xash3/Notch* (Chitnis and Kintner, 1996) and *X-Ngnr1/Notch* (Ma et al., 1996). In these pairings, one gene is widely expressed (*Gli* and *Notch*; Brewster et al., 1998; Coffman et al., 1990; Marine et al., 1997) while the other is expressed specifically within neural progenitors (*Zic2* and *Xash3*; Brewster et al., 1998; Zimmerman et al., 1993). *Xdbx* and *Xash3* therefore represent a novel pairing in that each gene is expressed specifically within a domain of neural progenitors. The integrated functions of regionalized pairs such as *Xash3* and *Xdbx* may complement previously defined mechanisms and represent another level of control in the maintenance of progenitor populations in the early embryo. The maintenance of neural progenitors for later differentiation therefore appears to depend on the additive effects of a number of independent molecular pathways.

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