Inhibition of neurogenesis by SRp38, a neuroD-regulated RNA-binding protein

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Summary

Although serine-arginine rich (SR) proteins have often been implicated in the positive regulation of splicing, recent studies have shown that one unusual SR protein, SRp38, serves, contrastingly, as a splicing repressor during mitosis and stress response. We have identified a novel developmental role for SRp38 in the regulation of neural differentiation. SRp38 is expressed in the neural plate during embryogenesis and is transcriptionally induced by the neurogenic bHLH protein neuroD. Overexpression of SRp38 inhibits primary neuronal differentiation at a step between *neurogenin* and *neuroD* activity. This repression of neuronal differentiation requires activation of the Notch pathway. Conversely, depletion of SRp38 activity results in a dysregulation of neurogenesis. Finally, SRp38 can interact with the peptidyltransferase center of 28S rRNA, suggesting that SRp38 activity may act, in part, via regulation of ribosome biogenesis or function. Strikingly, recent studies of several cell cycle regulators during primary neurogenesis have also revealed a crucial control step between *neurogenin* and *neuroD*. *SRp38* may mediate one component of this control by maintaining splicing and translational silencing in undifferentiated neural cells.

Key words: SRp38, Neurogenesis, Notch/Delta, Neurogenin, NeuroD, Ribosomal RNA, *Xenopus*

Introduction

During development, the cells of an embryo must rapidly divide and undergo tissue-type specification and physical remodeling. Some aspects of the regulation of gene expression during differentiation, such as the rapid regulation of protein synthesis, are similar to those seen during stress conditions. In response to starvation or heat shock, prokaryotic and eukaryotic organisms have evolved mechanisms to regulate protein synthesis, mainly via modification of ribosome biogenesis and activity (Schultz, 2003; Wada et al., 1995; Yoshida et al., 2002). Examples from morphogenesis and cell differentiation in the development of vertebrate embryos require arrest of the cell cycle. Thus, embryos may have co-opted aspects of the stress response to help maintain the necessary temporal and spatial control over protein synthesis.

In mammals, translational regulation has been shown to be important for the development of the nervous system. Disruption of the basal splicing machinery has been implicated in two common human diseases, retinitis pigmentosa and spinal muscular atrophy (reviewed by Faustino and Cooper, 2003). A number of human neurological diseases have also been linked to misregulation of splicing. For example, nucleotide mutations within the gene *MAPT*, which encodes the neuronal microtubule associated *tau*, result in the selection of an alternative splice site and these mutations have been implicated in Parkinson's disease, frontotemporal dementia and others (Dumanchin et al., 1998). When NOVA-1, a KH domain-containing RNA-binding protein, is mutated in mice, splicing of two neuronal receptors, GlyRα2 and $GABA_A$, is defective (Dumanchin et al., 1998; Jensen et al., 2000). However, despite these studies, our knowledge of the developmental regulation and biological functions of vertebrate RNA binding proteins is limited.

Until recently, SR proteins had been suggested to have two distinct roles during pre-mRNA processing. First, during selection of alternative splice sites, increasing the amount of SR proteins both in vivo and in vitro biases splice site selection towards sub-optimal upstream splice acceptors (Ge and Manley, 1990; Krainer et al., 1990; Sun et al., 1993; Tian and Maniatis, 1993). Second, SR proteins select exonic splicing enhancers in a process requiring sequence specific binding of the RNA recognition motif (RRM) (Graveley, 2000). Recent work suggests that SR proteins may also be important in regulation of mRNA-processing events through translation (Sanford et al., 2004). In general, SR proteins contain two functionally separable domains: one or more RNA-recognition motifs (RRMs); and a serine-arginine rich motif (RS) (Caceres and Krainer, 1993; Tacke and Manley, 1995; Zuo and Manley, 1993). The RRM binds to target RNAs in a sequence-specific manner, while the SR domain interacts with partner proteins, presumably for the recruitment of other splicing machinery (reviewed by Graveley, 2000). Transcription of SR proteins has been shown to be tissue specific and developmentally regulated (Hanamura et al., 1998; Tian and Maniatis, 1993). The proteins themselves are regulated by phosphorylation (Colwill et al., 1996; Wang et al., 1998; Xiao and Manley, 1998). Thus, the

activity of the SR proteins can be controlled rapidly by intracellular and extracellular signals (Du et al., 1998).

The identification of *SRp38* (also known as *NSSR-1*, *TASR-2*, *fusip1* and *SRrp40*) uncovers a surprising new role for SR proteins in splicing regulation (Clinton et al., 2002; Komatsu et al., 1999; Shin and Manley, 2002; Yang et al., 2000; Yang et al., 1998). Unlike the other SR proteins identified, *SRp38* cannot activate splicing; in fact, it is a potent repressor of splicing when dephosphorylated. SRp38 is specifically dephosphorylated during mitosis and heat shock and its activity is required for both mitotic and stress-related splicing repression (Shin et al., 2004; Shin and Manley, 2002).

SRp38 was first identified in a yeast two-hybrid screen for proteins interacting with *TLS/FUS* (Yang et al., 2000; Yang et al., 1998). *TLS/FUS* (translocated in liposarcoma/fusion protein) is a DNA/RNA-binding protein implicated in the most common chromosomal translocation in liposarcomas. It has been shown to bind both RNA pol II and splicing factors, suggesting both a link between transcription and splicing and a potential role in aberrant splicing during carcinogenesis. Yang and colleagues later showed that the two TASR isoforms were generated by alternative splicing (Clinton et al., 2002). Mouse *NSSR-1* (long) and *-2* (short) (*Fusip1* – Mouse Genome Informatics) were discovered in a search for SR proteins in a neural specific cDNA library (Komatsu et al., 1999). A third group found *SRrp40* in a database search for SR proteins (Cowper et al., 2001). Finally, Shin and Manley identified *SRp38* in a yeast two-hybrid screen with the human splicing regulators *Tra2α* and *Tra2β* (Shin and Manley, 2002). All of these groups had observed the inhibitory splicing abilities of *SRp38* but Shin and Manley found that *SRp38* is specifically activated by dephosphorylation during mitosis and heat shock (Shin et al., 2004). They also observed that SRp38 is required for the inhibition of pre-mRNA splicing that occurs in mitotic cell extracts. Dephosphorylation of SRp38 might result in weakened interactions with other SR proteins; however, SRp38 is likely to also bind target RNAs in a sequence-specific fashion, as Shin and Manley were able to identify a high-affinity target sequence by SELEX and use this sequence to deplete SRp38 from mitotic extracts.

Despite this progress in understanding the biochemical regulation and activity of SRp38, many questions remain. In this study, we characterize a novel mechanism underlying the control of neurogenesis. Primary neuronal differentiation in *Xenopus* is governed by a sequential cascade of basic helix-loop-helix (bHLH) transcription factors beginning with *neurogenin*, a vertebrate homolog of *Drosophila atonal*. *neurogenin* activates transcription of a series of bHLH factors including *neuroD*. This proneural pathway results in the expression of markers of neuronal differentiation (Kintner, 2002; Ma et al., 1996). Using an expression screening approach, we identified *Xenopus SRp38* as a modulator of neurogenesis (Grammer et al., 2000). We found that *SRp38* is expressed in the neural plate of the *Xenopus* embryo at the time of primary neurogenesis and is itself induced by *neuroD*. *SRp38* regulates neurogenesis at a crucial step downstream of neurogenin activity and this regulation is Notch dependent. Depletion of SRp38 activity results in a context-dependent increase in neurogenesis, which suggests that SRp38 is a negative feedback regulator that is induced by *neuroD* during neuronal differentiation. Finally, SRp38 interacts with a 289 nucleotide sequence in domain V of the 28s rRNA, which includes the peptidyltransferase domain of 28S ribosomal RNA. This suggests that SRp38 may act by regulation of ribosome biogenesis or function in the developing nervous system.

Materials and methods

Animals and embryo culture

Xenopus laevis embryos were generated and cultured by standard methods (Sive et al., 2000) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

Cloning of SRp38

Xenopus SRp38 (clone #19A5) was originally identified in an expression screen from a *Xenopus laevis* neurula stage (stage 19-22) cDNA library (Grammer et al., 2000; Mariani and Harland, 1998). Single amino acid substitutions, noted in text, were made using DPN mutagenesis (Braman et al., 1996).

Synthesis and injection of mRNA

Synthetic capped mRNA was generated using the mMessage mMachine Kit (Ambion). RNA was precipitated first with one half volume of 6M LiCl, washed in 80% ethanol, resuspended in 100 μl DEPC-treated water, reprecipitated with ammonium acetate and washed again in 80% ethanol to ensure removal of all LiCl. All synthetic mRNA was quantified using incorporation of trace amounts of α^{32}P-UTP and master stocks were stored at a concentration of 1 μg/μl at –80° until further dilution. Each mRNA (1 μg) was analyzed by agarose gel electrophoresis for quality and quantity assurance. In general, RNAs were then diluted in DEPC-treated water and injected in 5 nl volumes at the one- to four-cell stages.

Ectodermal 'animal cap' explants and RT-PCR

For ectodermal explants, mRNA was injected into the animal hemisphere at the one-cell stage. Embryos were then aged to blastula stages (stage 9) either at 25°C (~5 hours) or at 12°C (overnight). Animal caps (400 μm) were cut from devitellinized embryos using eyebrow knives (courtesy of Dale Frank) or from non-devitellinized embryos using the Gastromaster (Xenotek Engineering). Explants and untreated stage control embryos were then cultured in 75% NAM (+gentamycin 500 μg/ml) until indicated stages and harvested for reverse transcriptase-polymerase chain reaction (RT-PCR) or mRNA in situ hybridization (described below). For RT-PCR analysis, RNA was extracted and RT-PCR performed as described (Wilson and Melton, 1994). All PCRs were performed at 25 cycles, except EF1α and MA at 21 cycles, Sox3 at 23 cycles and S11 at 18 cycles. The following RT-PCR primer pairs were used.

EF1α: cagattggtgctggatatgcac and tgccttgatgactcctag
muscle actin (ma): gctgacagaatgcagaag and ttgcttggaggagtgtgt
NCAM: cacagttccaccaaatgc and ggaatcaagcggtacaga
nrp1: gggtttcttggaacaagc and actgtgcaggaacacaag
β-tubulin (non-sp): taactgggccaaaggtca and catgattcggtctgggta
synaptobrevin II: atttgtctgtgcgcaggt and tttaagccactccctgct
neuroD: cccatgtattccacgtca and gcaggatagtgcatagtg
β-tub (neuronal): cttccgtggaagaatgtc and gagcctttgtcatcaagc
delta-1: tctggcttcaactgtgag and aacctcgtgcacattgac
XVex-1: gaggaaacacaaagttgaag and gcaggaaccaccattaag
Sox2: caaccagaggatggacacttatgc and tggattccgacttgactaccgag
Sox3: aaccctatgatgacctctgccc and tttgaagtgaagggtcgctggc
S11: gcaggaggtgtcagaaaagttacc and tctcacgacggtctaaacccag

Morpholino oligonucleotides

SRp38 was BLASTed against the NCBI expressed sequence tag (EST) database. ESTs were downloaded and aligned in ClustalX to determine sequence similarity, in particular in the 5′UTR and at the

start codon. This allowed us to identify paralogous genes (owing to the presumed pseudotetraploidy of *X. laevis*) and design oligonucleotides targeted towards one or both copies of the genes. The following sequences were used to block translation of SRp38: AMO1, 5′-GCG GCC TTG AAT AGC GAG ACA TCC T-3′; AMO2, 5′-CAA GCG CCA CAC TTC GAC AAC AAT A-3′. The control oligonucleotide is 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′. All morpholino oligonucleotides were ordered from GeneTools, and resuspended at 1 mM concentrations in DEPC-treated 0.1×MR. AMOs were further diluted in DEPC-treated water at the concentrations indicated. Both AMO1 and AMO2 inhibited in vitro translation of the original *SRp38* cDNA but not that of a Myc-tagged *SRp38* that lacked the 5′UTR. Both AMOs also inhibited activity of SRp38 when co-injected in vivo.

Whole-mount RNA in situ hybridization and immunohistochemistry

Embryos were fixed for 1 hour in MEMFA, dehydrated in methanol and stored at –20°C until further processing. In some cases, the vitelline membrane was removed before fixation using watchmaker's forceps.

RNA in situ hybridization was performed using a multibasket technique previously described (Sive et al., 2000) with the following modifications. In situs were developed using BM Purple (Boehringer Mannheim). For certain probes (*neuroD*), in situs were developed using 0.45 μl NBT (stock 75 mg/ml in 70%DMF) and 3.5 μl BCIP (stock 50 mg/ml in 100% DMF) per ml of AP buffer (Lee et al., 1995). All in situs were postfixed in Bouin's Fix. Embryos were rinsed in 1×PBS-0.1%Tween or TE-buffered 70% ethanol then bleached in 0.5×SSC, 5% formamide and 1%H_2O_2.

For antibody staining, embryos were aged to the indicated stages, dissected out of their vitelline membranes, fixed for 45 minutes in 1× MEMFA and dehydrated in methanol. Embryos were rehydrated in a stepwise fashion and subsequently washed in 1× PBS, 1×PBS + 0.1% Triton X-100 (PBS-Tr) and preblocked for 1 hour in 1×PBSTr+10% heat inactivated goat serum. Primary antibodies were then added at the indicated dilutions and incubated overnight at 4°C. Embryos were washed several times in PBS-Tr and then incubated with secondary antibodies, again as indicated for 1 hour at room temperature. Samples were then washed in PBS-Tr repeatedly for at least 5 hours at room temperature. HRP activity was revealed using H_2O_2 and diaminobenzidine (DAB) as the histochemical substrate.

TUNEL staining

To assess the amount of apoptosis in the embryo, we used the TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) technique to label dying cells in situ. Embryos were analyzed essentially as described by Hensey and Gautier (Hensey and Gautier, 1997). The anti-digoxigenin antibody incubation was carried out in 2% BM Block (Boehringer Mannheim) in 1× MAB and all subsequent steps were performed essentially as those for in situ hybridization (above).

RNA immunoprecipitation

In an initial pilot experiment, 300 embryos each were untreated or injected with 250 pg of synthetic flag-SRp38 mRNA or flag-SRp38* at the one- to two-cell stage and aged to neurula stages. Co-immunoprecipitated RNAs from the pilot experiment were radiolabelled using T4 RNA ligase for analysis (see below). Subsequently, in two independent experiments, 1000 embryos in each group were treated as above. In each experiment, all three sets of embryos were lysed in 10 μl per embryo of homogenization buffer (HB: 15 mM HEPES pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 44 mM sucrose) with the addition of 3 mM Na_2VO_4, 1 mM DTT, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 4 μg/ml pepstatin, 0.75 mM PMSF and 1× vanadyl ribonucleoside complex (Gibco). Lysates were spun for 10 minutes at 4°C, 20,817 ***g*** and supernatants removed in order to separate yolk. Anti-Flag antibody M2 (Sigma) was then added to a final concentration of 2.8 ng/μl and incubated for 1 hour at 4°C. Protein A Sepharose beads which had been equilibrated in HB (300 μl each sample) were then added and again incubated for one hour at 4°C. Samples were centrifuged for 15 minutes at 106 ***g*** at 4°C and washed three times in HB. To elute the bound protein, beads were incubated three times for 1 hour each in 1 ml of HB containing 30 μl of 5 μg/μl Flag peptide (Sigma). A final elution was carried out overnight at 4°C. All the supernatants were phenol:chloroform extracted and remaining nucleic acids were precipitated in the presence of isopropanol, NH_4OAc and glycogen. Aliquots were removed at each step and saved for protein gel analysis. RNAs co-immunoprecipitated in these experiments were cloned for subtractive hybridization.

cDNA synthesis and subtraction of immunoprecipitated RNAs

RNAs were immunoprecipitated in two independent experiments using the SMART PCR cDNA synthesis kit from Clontech. Efficiency of first and second strand synthesis of cDNA was monitored by α^{32}P-dCTP according to standard protocols. Subtractive hybridization was performed essentially as described by Diatchenko et al. (Diatchenko et al., 1999) using Flag-SRp38* (mutant RNP) as driver and Flag-SRp38 as tester. The product of the subtractive hybridization was then amplified by suppression PCR (Clontech).

Luciferase assays and ^{35}S-methionine incorporation

At the one-cell stage, embryos were uninjected, injected with SRp38 or mutant SRp38. Embryos were allowed to divide and each set of embryos was subsequently injected with luciferase DNA. Embryos were then cultured and harvested for luciferase readings at stage 21.

For ^{35}S-methionine incorporation, embryos were injected at one cell stage with 250 pg luciferase, SRp38 or SRp38* mRNA. At stage 17, ^{35}S-methionine was added to the culture media. Embryos were then lysed, proteins were precipitated and incorporated ^{35}S-methionine was counted.

Results

Identification and expression of Xenopus SRp38

Functional screens using the *Xenopus laevis* embryo have been remarkably successful in the identification of new molecules active in development. Injection of DNA, mRNA and proteins into developing embryos allows overexpression of genes, while well-established fate maps allow treatments to be targeted to specific cell types (Dale and Slack, 1987). Using a simple overexpression screen, we identified *SRp38* (clone #19A5) for its ability to perturb neural development (Grammer et al., 2000).

Sequencing of the clone 19A5 revealed an open reading frame of 239 amino acids (Fig. 1A). Analyses of the sequence by BLAST search showed that the RNA-binding domain of 19A5 is 87% identical to that of mouse *SRp38*. The serine-arginine rich (RS) domain is a region of low complexity and identity drops off considerably (Fig. 1B).

Using whole-mount in situ hybridization, we found that during neurulation SRp38 is expressed diffusely throughout the neural plate (Fig. 1C,D). At later stages, SRp38 is expressed in the anterior of the embryo, throughout the neural tube, eyes, branchial arches and surrounding the otic vesicle (Fig. 1E,F).

SRp38 can inhibit differentiation during early germ layer formation

SRp38 was identified from two effects on development: induction of ectopic pigmentation and disruption of the general

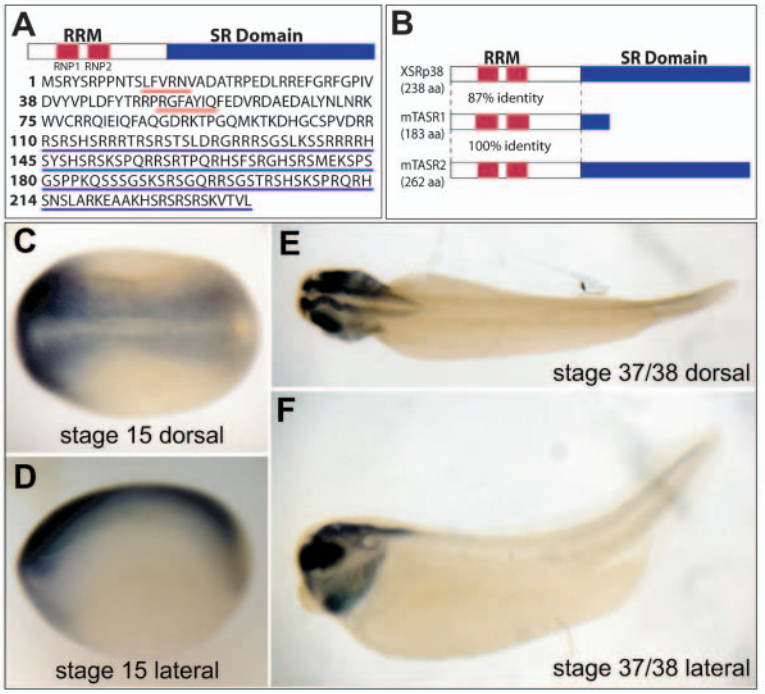

Fig. 1. Sequence and expression pattern of SRp38. (A) Schematic diagram of SRp38. SRp38 is a 238 amino acid protein with an N-terminal RNA recognition motif (RRM) containing two conserved ribonucleoprotein (RNP) domains and a C-terminal serine-arginine rich (SR) domain. RNPs are underlined in red and SR domain is underlined in blue. (B) The RRM of SRp38 is 87% identical to that of the mouse SRp38 isoforms (TASR1 and TASR2). The SR domain is low complexity and the homology drops considerably in this region. (C-F) Expression pattern of SRp38. (C,E) Dorsal view. (D,F) Lateral views. (C,D) At stage 15, SRp38 is expressed diffusely throughout the neural plate. (E,F) At later stages, SRp38 is expressed in the anterior of the embryo, throughout the neural tube, eyes and branchial arches, and surrounding the otic vesicle.

neural marker *nrp1* (Fig. 2A) (Grammer et al., 2000). Closer analysis revealed that differences in injection site could account for these different activities: overexpression of SRp38 in the animal hemisphere at an early stage (targeting the neurectoderm) resulted in the loss of *nrp1* expression at neurula stages (Fig. 2A). Injection into the marginal zone (targeting presumptive mesoderm) resulted in patches of ectopic pigmentation (Grammer et al., 2000). Morphologically, the ectopic pigmentation appeared to result from an accumulation of cells in the mesodermal tissue, rather than excess melanin synthesis. We also found that mouse and human SRp38 recapitulates these activities (data not shown).

To determine what kind of tissue resulted from ectopic *SRp38* expression, we analyzed the expression of a number of tissue-specific mRNAs by in situ hybridization (Table 1). SRp38 mRNA was injected into the animal hemisphere to

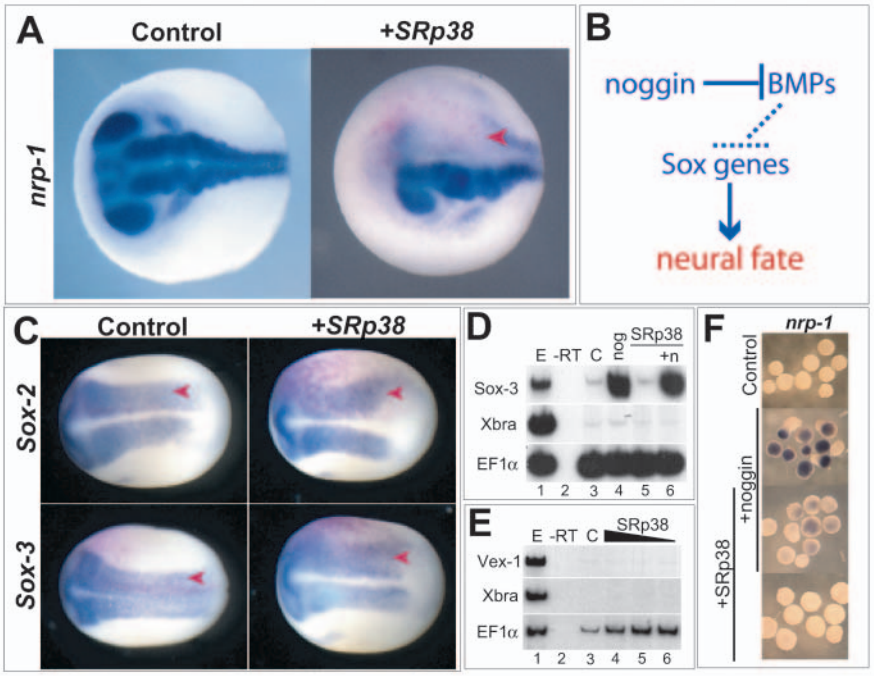

Fig. 2. SRp38 inhibits neural development but not initial induction of neural tissues. (A) In situ hybridization for neural specific *nrp1*, stage 21. Control embryo shows staining in the neural tube and eyes. Embryo injected in one cell at the two-cell stage with 500 pg *SRp38* (right). *Nrp-1* expression is lost at the site of injection (red arrowhead). (B) Simplified schematic of neural induction. Inhibition of bone morphogenetic protein (BMP) signaling by BMP antagonists, such as noggin, allows expression of Sox genes (*Sox2* and *Sox3*) which then induce neural fates. (C) *SRp38* does not block expression of *Sox3* or *Sox2* in whole embryos. Dorsal view of control embryos (left column) expressing *Sox2* and *Sox3* in the neural plate. Embryos injected in one cell at the two cell stage with 500 pg *SRp38* (right column) also express *Sox2* and *Sox3* normally in the neural plate. Injected sides marked with red arrowhead. Lineage tracer in pink. (D) Ectodermal explant RT-PCR, stage 10.5. *Noggin* mRNA injection induces robust expression of *Sox3* (lane 4). *SRp38* co-expression is unable to block *Sox3* expression (lane 6). (E) Ectodermal explant RT-PCR, stage 10.5. *SRp38* mRNA injection does not induce expression of the BMP target *Vex1*. Embryos were injected with 500 pg (lane 4), 250 pg (lane 5) or 125 pg (lane 6). *EF1α* is a loading control and *Xbra* controls for mesodermal contamination. (F) In situ hybridization for *nrp1* (neural). *Noggin* injected animal caps express *nrp1* and co-injection of *SRp38* prevents *nrp1* expression.

investigate effects on neural tissue or neural crest, whereas marginal zone or vegetal hemisphere injections assessed the effects on mesodermal and endodermal tissues. In each case we found that early markers of different tissue types were expressed, but later differentiation markers were not expressed. For example, injections targeted toward the neural tissue did not disrupt the markers of neural 'competence' (*Sox2* and *Sox3*) (Fig. 2C) but did disrupt expression of 'differentiation' genes such as *Krox20, neurogenin, neuroD* (rhombomeres 3 and 5, and neural crest; Table 1; data not shown) and *nrp1* (Fig. 2A).

Because *SRp38* is expressed predominantly in the neural plate during embryogenesis, we pursued its role in the context of neural induction and patterning. Using explants, we tested the hypothesis that neural induction occurs but SRp38 blocks further differentiation. When the ectoderm (the 'animal cap') of blastula-stage embryos is explanted and cultured in a simple salt solution, it will differentiate into epidermis. Addition of the bone morphogenetic protein (BMP) antagonist noggin results in the expression of markers of the early neural plate (such as *Sox3*) and of the differentiating nervous system (such as *nrp1*) (Knecht et al., 1995; Lamb et al., 1993; Smith and Harland, 1992) as outlined in Fig. 2B. We found that co-expression of *noggin* and *SRp38* mRNA in animal caps resulted in robust expression of *Sox3* but not *nrp1* (Fig. 2D,F). This result confirmed that initial induction of the neural plate was undisturbed but that later differentiation was inhibited (Fig. 2). This block in neural differentiation was unlikely to be the result of BMP activation because overexpression of *SRp38* in explants never elicited expression of *Vex1* (Fig. 2E), a direct target of BMP signals (Shapira et al., 1999). Similar results were seen with experiments targeted towards mesodermal and endodermal tissues; ectopic expression of *SRp38* did not disrupt early mesodermal 'competence' genes (*Bix1* and *Bix4*; Fig. 3A; data not shown) but did disrupt expression of later genes such as *Brachyury* and *Wnt8* (Fig. 3B,C). Further analysis at tadpole stages shows persistent disruption in *MyoD* (Fig. 3D), *α-globin* (Fig. 3E) and the notochord antigen

Table 1. Analysis of gene expression in SRp38-injected tissues

Tissue	Early	Expression	Late markers	Expression
Neural	Sox2	+	Nrp1 (general neural)	–
	Sox3	+	Otx2 (eyes, forebrain	–
			Krox20 (rhomb3/5, crest)	–
			HoxB9 (spinal cord)	–
			Xash3	–
			Neurogenin	–
			NeuroD	–
			Neuronal β-tubulin (neurons)	–
Neural crest			Slug	–
			Twist	–
Mesoderm	Bix1	+	Goosecoid (dorsal mesoderm)	–
	Bix4	+	Brachyury (mesoderm)	–
			Wnt8 (ventral mesoderm)	–
			MyoD (somites)	–
			Pax8 (otic vesicle, pronephros)	–
			Tor70 (notochord)	–
			α-globin (ventral blood islets)	–
Endoderm	VegT	+		
	Sox17b	+	Sox17b (endoderm)	–

Summary analysis of genes affected by ectopic expression of SRp38. Genes are divided into tissue type and a note made of whether these genes mark differentiated or undifferentiated tissues. In each tissue type it appears that early markers of induction are expressed as expected (+) but later differentiation markers are not expressed (–).

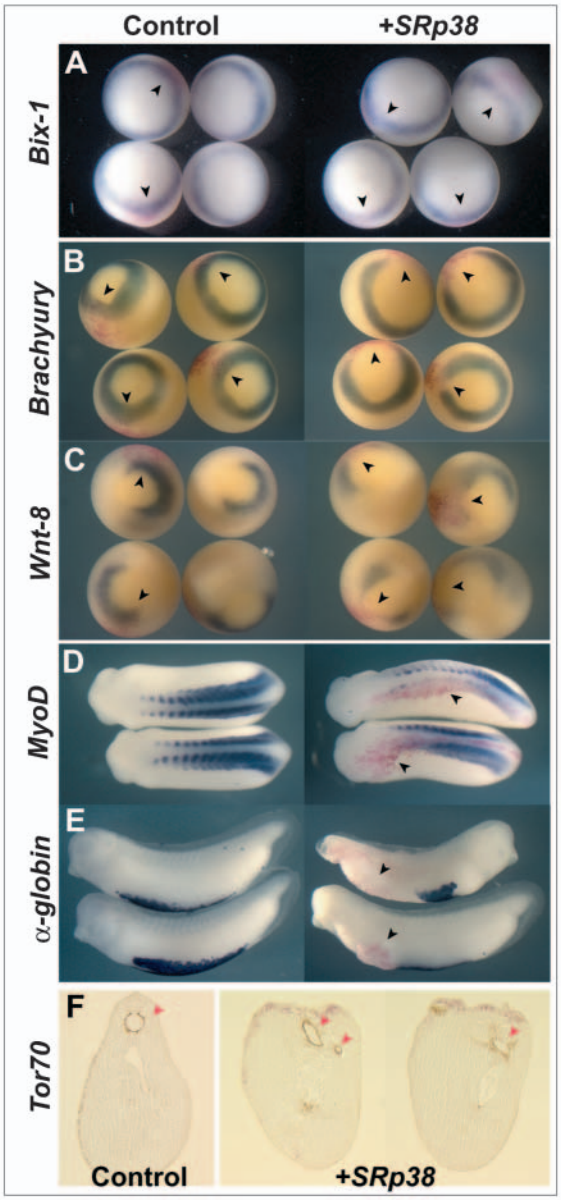

Fig. 3. SRp38 can inhibit mesodermal differentiation. Control embryos on left. SRp38 injection (250 pg each) on right. Some embryos are also stained for *lacZ* activity (in red) as a lineage tracer. All injection sites are marked with black arrowhead. (A-C) Early mesoderm injections were targeted toward ventral domains. In situ hybridization for *Bix1*, stage 9.5 (A); *brachyury*, stage 11 (B); and *wnt8*, stage 11 (C). SRp38 injection (250 pg each, right) does not inhibit expression of *Bix1* but does inhibit expression of *Brachyury* and *wnt8.* (D) In situ hybridization for *myoD,* stage 25. Dorsal injection of SRp38 (250 pg each, right) inhibits expression of *myoD* at the site of injection (marked by red tracer). (E) In situ hybridization for *α-globin,* stage 25. Ventral injection of SRp38 (250 pg each, right) inhibits expression of *α-globin* at the site of injection. (F) *Tor70* antibody reveals disrupted notochord development in embryos injected dorsally with SRp38 (two sections on right, notochord indicated by red arrowhead).

recognized by Tor70 (Fig. 3F). Our interpretation of these results is that excess SRp38 does not disrupt the initial specification of the germ layers. For example, the expression of *Sox2* and *Sox3* shows that initial neural induction has occurred, and the neural plate is morphologically present, but that further differentiation does not occur. This prompted us to determine what step in neural differentiation was blocked by *SRp38* overexpression.

SRp38 blocks the activity of neurogenin but not that of neuroD

The differentiation of primary neurons is governed in part by the sequential expression of proneural genes, basic helix-loop-helix (bHLH) transcription factors that were first identified in *Drosophila*. Early expression of these bHLH factors define groups of cells that are competent to become neuronal precursors (Lee et al., 1995; Ma et al., 1996). Following the formation of the neural plate, sites of neurogenesis are marked by expression of the *atonal* homolog *neurogenin. neurogenin* in turn activates transcription of several downstream genes, including *neuroD* (Ma et al., 1996; Perron et al., 1999). *neuroD* then induces genes characteristic of neuronal differentiation (Lee et al., 1995) (Fig. 4D). Concurrent with activation of this transcriptional cascade, individual cells are selected to become neurons from among a field of initially equivalent cells. This process, called lateral inhibition, is dictated by relative levels of Delta ligand and Notch receptor (Chitnis et al., 1995; Chitnis and Kintner, 1996). Cells with higher levels of Delta differentiate into neurons, while cells with higher levels of Notch remain undifferentiated, contributing to a persistent pool of neural precursors. Neuronal specification ultimately results in exit from the cell cycle and expression of definitive neuronal markers, including *neuronal β-tubulin* and *synaptobrevin II* (*VAMP2*) (reviewed by Bertrand et al., 2002).

In order to place *SRp38* within the context of neural development and differentiation, we overexpressed members of the neurogenic cascade in the presence or absence of *SRp38* (Fig. 4). In whole embryos and explants, ectopic expression of neurogenin induces expression of *neuroD, neural cell adhesion marker* (*NCAM*), *neuronal β-tubulin* and *synaptobrevin II* (*VAMP2*) (Fig. 4A; Fig. 4B, lane 4). Co-expression with SRp38 inhibits neurogenin induction of *neuroD* and other target genes (Fig. 4A; Fig. 4B, lane 5).

We then overexpressed *SRp38* in the presence and absence of *neuroD* mRNA. *SRp38* was unable to block the induction of *synaptobrevin II* by neuroD. Injection of *neuroD* mRNA induces ectopic expression of *synaptobrevin II* (Fig. 4C), while co-expression of *SRp38* with *neuroD* does not inhibit neuroD induction of *synaptobrevin II* (Fig. 4C). Therefore, SRp38 was able to block the activity of neurogenin (Fig. 4A,B) but not that of neuroD (Fig. 4C), suggesting that neuroD is able to act downstream of SRp38.

Activation of Notch Signaling in tissues expressing SRp38

This activity seen above mimics activation of the Notch signaling pathway, which also blocks the ability of *neurogenin* but not *neuroD* to induce *neuronal β-tubulin* (schematic in Fig. 4D) (Ma et al., 1998; Olson et al., 1998). To test whether SRp38 could be activating Notch signaling, we examined its

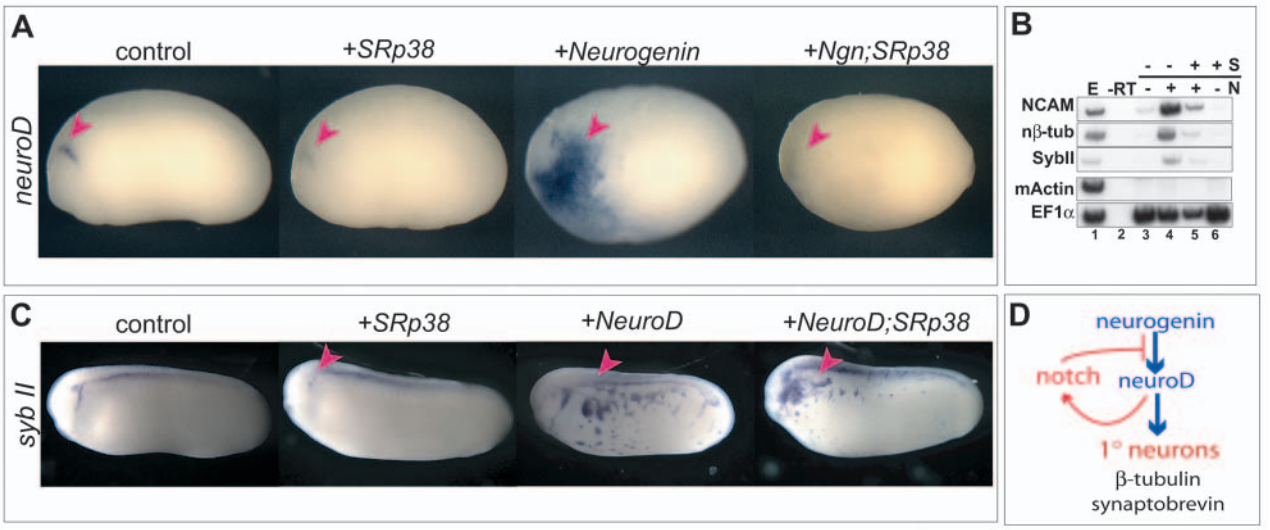

Fig. 4. SRp38 inhibits the activity of neurogenin but not that of neuroD. (A) SRp38 blocks neurogenin induction of neuroD. Stage 15 lateral view, *neuroD* in situ hybridization. Control embryo: normal expression pattern of *neuroD* (in trigeminal ganglia indicated by red arrowhead). Expression of 500 pg SRp38 inhibits *neuroD* expression. However, expression of 250 pg of *neurogenin* induces ectopic expression of *neuroD*, while co-expression of SRp38 with neurogenin inhibits induction of *neuroD* (right embryo). (B) Ectodermal explant RT-PCR. SRp38 blocks neurogenin induction of *NCAM*, *neuronal β-tubulin*, *synaptobrevin II* and *neuroD.* Analysis of animal caps expressing either 500 pg of *SRp38* and/or 250 pg *neurogenin* as indicated. *EF1α* is a loading control and *muscle actin* (mActin) controls for mesodermal contamination. (C) SRp38 does not block neuroD induction of *synaptobrevin II* (*sybII*). Stage 25 embryos stained for *sybII*. Control embryo: normal expression pattern of *sybII* in trigeminal ganglia and neurons. Expression of 500 pg SRp38 inhibits *sybII* expression. Ectopic expression of 250pg of neuroD induces ectopic expression of for *sybII*, while co-expression of SRp38 with neuroD does not inhibit neuroD induction of *sybII*. (D) Simplified schematic of neurogenic cascade. In the neural plate, neurogenin activates transcription of several downstream genes, including *neuroD*. neuroD then induces genes characteristic of neuronal differentiation such as *neuronal β-tubulin* and *synaptobrevin*. Notch signaling can inhibit the neurogenic effects of neurogenin but not neuroD.

effect on lateral inhibition of the ciliated epidermis. Targeting the mRNA to the ventral epidermis of the embryo resulted in fewer ciliated epidermal cells (revealed by non-specific *β-tubulin expression*; Fig. 5A, right embryo), a hallmark of increased Notch signaling (Deblandre et al., 1999). Targeting the mRNA to the neural plate resulted in the loss of primary neurons (marked by *neural specific β-tubulin*, Fig. 5A, center embryo), again similar to ectopic Notch activation (Chitnis et al., 1995).

To our surprise, we found that although *SRp38* inhibited the induction by neurogenin of *neuroD*, *NCAM* (neural cell adhesion marker), *neuronal β-tubulin* and *synaptobrevin II* (Fig. 4B), it did not inhibit neurogenin induction of *Delta*, which encodes a ligand for the Notch signaling pathway (data not shown). In fact, we found that *SRp38* could itself induce expression of *Delta* in embryos and explants (Fig. 5C,D), suggesting that *SRp38* acts in part via activation of Notch.

We also determined that Srp38 caused ectopic expression of *Id3* mRNAs (Fig. 5E), and activation of *esr-1* transcription in animal caps (data not shown). Both *Id3* and *esr1* are direct targets of Notch signaling and expression of *Id3* is often correlated with proliferating, undifferentiated cell types (Reynaud-Deonauth et al., 2002; Wettstein et al., 1997). Id genes have been shown to inhibit differentiation by binding to and inhibiting the transcriptional activities of bHLH proteins. *Id* genes are spliced and *Xenopus* Id3 has been shown to be cytoplasmically polyadenylated, suggesting that *Id* genes undergo post-transcriptional regulation (Afouda et al., 1999). Using northern blotting, we looked for changes in the mRNA of *Id2*, *Id3* and *Delta* in samples of tissues overexpressing SRp38. We analyzed total extracted RNA and polyA+ selected RNA from whole embryos but found no change in the size or polyadenylation of these genes (data not shown). We also examined the splicing status of Id2 and Id3 by designing intron-spanning primers, but found no changes in the splicing of these genes (Fig. 5F; data not shown).

Inhibition of SRp38 translation using antisense morpholino oligonucleotides

To study the requirement for *SRp38* activity during neurogenesis, we used two different methods to inhibit *SRp38* function. First, we designed two antisense morpholino oligonucleotides (AMO) targeting the *SRp38* 5′UTR and start codon (AMO1 and AMO2 see Materials and methods). Antisense morpholino oligonucleotides bind to target RNAs in a sequence-specific fashion and prevent translation (Heasman et al., 2000). Both AMOs inhibited the activity of *SRp38* in vivo when co-injected with exogenous mRNA and both AMOs inhibited translation of *SRp38* in vitro (data not shown). All loss-of-function experiments were initially performed with AMO1 at a dose of 80 ng per embryo (control embryos were injected with a control oligonucleotide, see Materials and methods). Results were then independently confirmed using AMO2 (80 ng). Lower doses of the individual AMOs had only subtle effects. We then found that a mixture of AMO1 and AMO2 (20 ng to 40 ng each) was most effective. All experiments pictured used a combination of the two AMOs.

We found that reduction of *SRp38* translation in vivo using a cocktail of AMOs (40 ng each) did not perturb expression of *neuronal β-tubulin* at early stages (Fig. 6A, upper right). We hypothesized that *SRp38* acts in parallel with other mechanisms to regulate the amount of neurogenesis in vivo and, thus, chose to examine loss of *SRp38* in sensitized assays. Expression of $Delta^{stu}$ in the embryo results in increased neurogenesis (Chitnis et al., 1995) (Fig. 6A, lower left). When we depleted *SRp38* from embryos concurrently expressing $Delta^{stu}$ we found that these embryos had an increase in

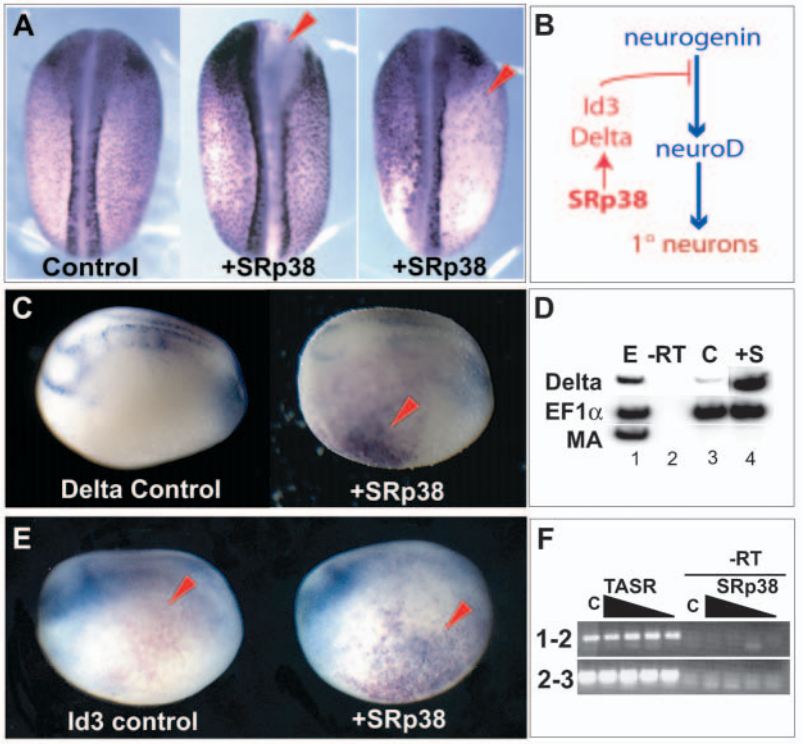

Fig. 5. SRp38 inhibits neurogenesis and induces Delta/Id3. (A) In situ hybridization for non-specific *β-tubulin* stains the ciliated epidermis and neurons. Left, control embryo. Middle, injection of 500 pg SRp38 targeted to the neural plate results in a loss of *neuronal β-tubulin*. Right, overexpression of 500 pg SRp38 in the epidermis leads to a decrease in ciliated epidermal cells. Both of these phenotypes are symptomatic of Notch activation. Injection sites marked by red arrowhead. (B) Schematic model of SRp38 inhibition of neurogenesis. SRp38 inhibition of neurogenin activity may act via Delta and Id3. (C) Lateral views of stage 18 embryos stained for *Delta*. Left: control embryo. Right: injection of 500 pg of SRp38 induces robust expression of *Delta* (red arrowhead). (D) RT-PCR analysis of animal caps expressing 500 pg SRp38 (lane 4). (Uninjected control: C, lane 3.) SRp38 induces ectopic expression of Delta, lane 4. *EF1α* is a loading control and *muscle actin* (MA) controls for mesodermal contamination. (E) Lateral views of stage 18 embryos stained for *Id3*. Left: control embryo. Right: injection of 500 pg of SRp38 induces expression of *Id3* (red arrowhead). (F) RT-PCR analysis of Id3 splicing. Primers were designed to span exons 1-2 or exons 2-3. Embryos treated with decreasing doses of SRp38 (500 pg to 100 pg) were analyzed for changes in the amount of spliced products. No discernible changes were found.

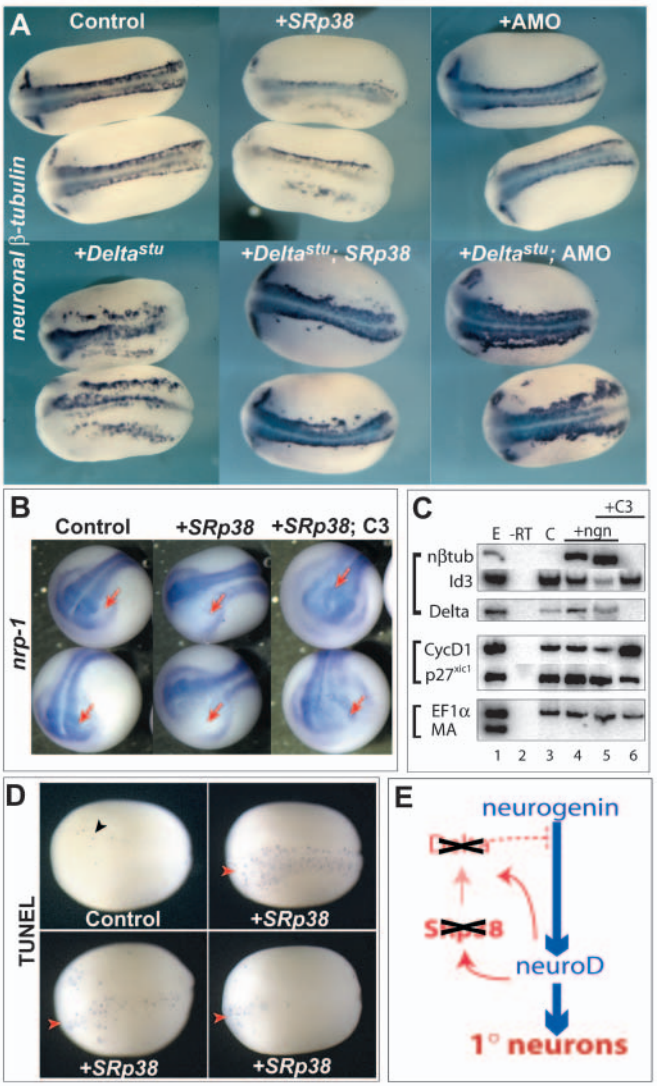

Fig. 6. SRp38 is required for regulated neurogenesis. (A) Dorsal views, in situ hybridization for *neuronal specific β-tubulin*. Top, left to right: control embryos show expression of *β-tubulin* in neurons and trigeminal ganglia; *SRp38*-injected embryos show a loss of *β-tubulin*-expressing cells; injection of antisense morpholino oligonucleotides (AMOs) does not perturb expression of *β-tubulin*. Bottom, left to right: inhibitory Delta (Delta^{stu}) injection results in increased and disorganized expression of *β-tubulin*; co-expression of Delta^{stu} and SRp38 results in almost normal embryos (compare with +SRp38 above); co-injection of Delta^{stu} and AMOs results in increased expression of *β-tubulin* (compare with +AMO embryo above and to controls). (B) In situ hybridization for *nrp1*, dorsoanterior view, stage 17. Left: control embryos show *nrp1* staining in the neural plate and eye primordia. Middle: SRp38 overexpression inhibits expression of *nrp1*, red arrows. Right: co-injection of SRp38 with the function-blocking sequence C3 rescues expression of *nrp1*, red arrows. (C) RT-PCR analysis on animal cap ectodermal explants. Expression of 100 pg of neurogenin in the animal cap results in expression of *neuronal β-tubulin* and a mild decrease in *Id3* (lane 3). Co-expression of neurogenin and 5ng of C3 results in a greater increase of *neuronal β-tubulin* and concomitant decrease in *Id3* (compare control lane 5 with lane 3). C3 alone (lane 6, 5 ng) results in complete loss of *Delta* and no effect on Id3 expression. *CyclinD1* and *p27^{xic1}* expression are unchanged in neuralized explants upon addition of C3 (compare lane 5 to lane 4). (D) TUNEL staining indicates apoptotic cells. Dorsal views of stage 15 embryos. Control embryos show very few TUNEL-positive cells (black arrowhead), while embryos injected with 250 pg of SRp38 RNA in one cell at the two-cell stage show a variable, though clear increase, in the number of TUNEL-positive cells (red arrowheads). (E) Primary neurogenesis is increased in the absence of SRp38 and Delta function.

primary neurons above the levels induced by *Delta^{stu}* alone (Fig. 6A, lower right).

Thus, increasing SRp38 results in decreased neurogenesis (Fig. 6A, upper center), consistent with an increase in Notch signaling, while removing SRp38 and Delta function (using Delta^{stu}) result in increased neurogenesis (6A, lower right, schematized in Fig. 6E). This is consistent with our hypothesis that *SRp38* acts as a feedback mechanism to inhibit excess neurogenesis.

Inhibition of SRp38 activity using a function blocking consensus binding motif

Next, we took advantage of an *SRp38* consensus binding motif (C3) to inhibit *SRp38* function in vivo. This sequence has been shown to deplete SRp38 protein from cell extracts (Shin and Manley, 2002). We predicted that *Xenopus* SRp38 would also be able to bind specifically to the consensus motif and that excess C3 might block SRp38 activity (mouse and human *SRp38* recapitulate the activity of *Xenopus SRp38* in our assays, not shown). We found that co-injection of capped RNA from this construct is sufficient to inhibit the differentiation-blocking activity of injected *SRp38* in vivo (Fig. 6B) in a dose-dependent fashion. Injection of *SRp38* inhibits expression of the neural marker *nrp1* (Fig. 6B; Fig. 2B). When 5 ng of C3 was co-injected with 250 pg of *SRp38*, *nrp1* expression was rescued in 100% (22/22) of the embryos (Fig. 6B). Lower doses of C3 gave less penetrant phenotypes [3.75 ng, 91% (20/22) rescue; 2.5 ng, 87% (20/23) rescue; 1.25 ng, 64% (16/25) rescue], while injection of other capped mRNA sequences did not inhibit *SRp38* activity (data not shown).

We then used the C3 sequence to analyze the requirement for endogenous SRp38 activity during neurogenesis. Using RT-PCR on ectodermal explants, we found that depletion of SRp38 activity in conjunction with ectopic expression of neurogenin resulted in an increase in *neuronal β-tubulin* expression above that normally induced by neurogenin alone (compare lanes 5 and 4 in Fig. 6C). Conversely, we saw a comparable decrease in *Id3* mRNA, marking an equivalent reduction in undifferentiated cells (again, compare lanes 5 and 4 in Fig. 6C). Thus, although reduction of SRp38 does not affect primary neurogenesis in vivo, there is a clear role for *SRp38* in regulating levels of neurogenesis in response to changing levels of *Delta^{stu}* or *neurogenin*. These data is consistent with a role for SRp38 as part of an inhibitory feedback mechanism during neuronal differentiation.

Effects of SRp38 on proliferation and cell death

Because SRp38 acts as a splicing repressor during mitosis (Shin and Manley, 2002), we considered the possibility that the phenotype we saw was due to selective repression of splicing within the affected cells. This splicing repression might prevent cells from exiting the cell cycle and differentiating;

alternatively, splicing repression might induce the block to differentiation characteristic of mitotically active cells without triggering proliferation. The process of neurogenesis is particularly sensitive to this effect, as it is crucial that cells be able to exit the cell cycle in order to become neurons (Vernon et al., 2003).

Thus, cells expressing SRp38 might have several alternative fates. As these cells may not properly splice (and subsequently translate) appropriate genes, they might remain in an undifferentiated state or undergo apoptosis. Another possibility is that there would be an increase in mitotic cells, because splicing and translational silencing are characteristic of mitosis.

In order to determine whether SRp38-expressing cells undergo mitosis, we used an anti-phosphorylated Histone H3 (anti-pH3) antibody that specifically marks mitotic cells. We saw no change in αnti-pH3 staining in SRp38-injected embryos when compared with controls (data not shown). Because perturbations in the cell cycle can often induce apoptosis (Gartel and Tyner, 2002), we also analyzed injected embryos by TUNEL (TdT-mediated dUTP-digoxigenin nick end labeling) to assess the amount of programmed cell death. There was a clear, though variable, increase in the number of TUNEL-positive cells in *SRp38*-injected embryos (see Fig. 6D); however, most cells expressing *SRp38* were not TUNEL positive (data not shown), suggesting that increased apoptosis in these tissues might be secondary to the inability to differentiate. Finally, we saw no change in the expression of the cell cycle regulators $p27^{xic1}$ (Vernon et al., 2003) or Cyclin D1 (Ratineau et al., 2002) in neuralized tissues treated with C3 (Fig. 6C, compare lane 5 with lane 4).

SRp38 is upregulated by neuroD

If SRp38 is indeed a component of a mechanism to limit neuronal differentiation, we would expect it to be regulated by the neurogenic genes (schematic, Fig. 7D). Consistent with this, we found that increasing amounts of neuroD resulted in increased amounts of *SRp38* in the neural plate (Fig. 7A,C) and in ectodermal explants (Fig. 7B). Thus, neuroD, a transcription factor that acts at the end of the neurogenic cascade, induces expression of *SRp38*. *SRp38* then inhibits upstream transcription factors such as *neurogenin* (Fig. 4), preventing excessive neurogenesis. Similarly, *neurogenin* and *neuroD* induce expression of *Delta* and this activation is also thought to limit the amount of neurogenesis in the embryo (Ma et al., 1998) (data not shown).

Targets of SRp38

Although these results place SRp38 firmly as a component of the machinery that limits neurogenesis, we sought to identify the molecular targets of SRp38. Based on its position in the Notch cascade, conceivable targets were Notch itself and the Notch targets *Id3* and *Delta*. In light of the activity of SRp38 as a splicing repressor, however, it seems unlikely that SRp38 is directly binding to and regulating the splicing of *Id3*, *Delta* or *Notch*. Examination of these RNA populations confirmed that there were no discernible changes (Fig. 5F; data not shown).

We then took a broader biochemical approach towards the identification of SRp38 targets. Flag-tagged wild-type SRp38 or a crippled RNA-binding mutant SRp38 were expressed in

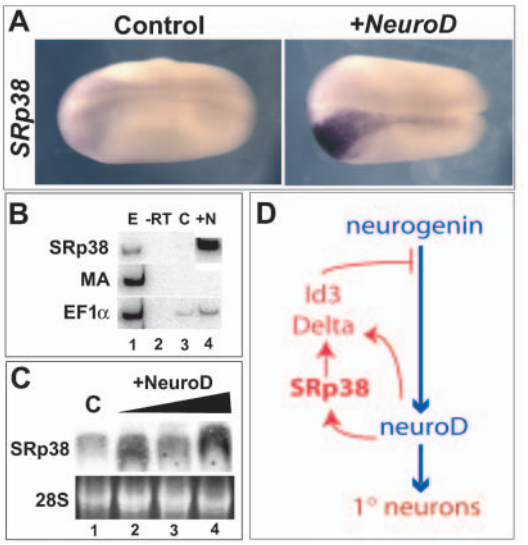

Fig. 7. SRp38 is induced by *neuroD*. (A) Dorsal views of *SRp38* expression are pictured at stage 21. Left: control. Right: injection of 250 pg of neuroD into one cell at two cell-stage induces robust expression of *SRp38*. (B) RT-PCR on ectodermal explants. Injection of 250 pg of neuroD (+N) induces expression of SRp38. *EF1α* is a loading control and *muscle actin* (MA) controls for mesodermal contamination. (C) SRp38 northern blot on total RNA in embryos overexpressing increasing doses of neuroD (125 pg to 500 pg) results in increasing amounts of SRp38 RNA. (D) SRp38 is itself regulated by neuroD, suggesting it is a component of a neuroD-induced mechanism serving to limit neurogenesis.

embryos and used to immunoprecipitate associated RNAs. In the SRp38 mutant (SRp38*), all four conserved phenylalanines and tyrosines in the RNA-binding domain were converted to alanines, changes that should abrogate sequence-specific binding (Fig. 8A). To identify targets, embryos were injected with Flag-SRp38 or Flag-SRp38* and allowed to develop to mid-neurula stages. Embryos were then lysed, Flag-SRp38 was immunoprecipitated with an anti-Flag antibody (Sigma, M2) and eluted with excess Flag epitope. The co-immunoprecipitated RNAs were then extracted, and the population precipitated with SRp38 was subtracted with the control population (SRp38*) prior to being cloned.

We sequenced 120 RNAs cloned using the subtraction technique. A number of the immunoprecipitated RNAs (47/120), contained a 289 nucleotide sequence (S11) (Fig. 8C) that maps to domain V of the 28S ribosomal RNA (Fig. 8D). This region includes the peptidyltransferase center and borders on the binding site for EF-G (EF-2) (reviewed by Rodnina and Wintermeyer, 2003). This region of the ribosome is strictly conserved from *E. coli* to humans and is required for translocation of both the tRNA and mRNA after peptide bond synthesis.

Specific binding of this sequence to SRp38 was confirmed by immunoprecipitation followed by gene-specific RT-PCR (Fig. 8B). Control, Flag-SRp38 or Flag-SRp38*-expressing embryos were lysed and immunoprecipitated with anti-Flag (M2) agarose beads. IP products were then subjected to RT-PCR for the S11 sequence. S11 was not immunoprecipitated in control uninjected embryos (lane 1) but was efficiently

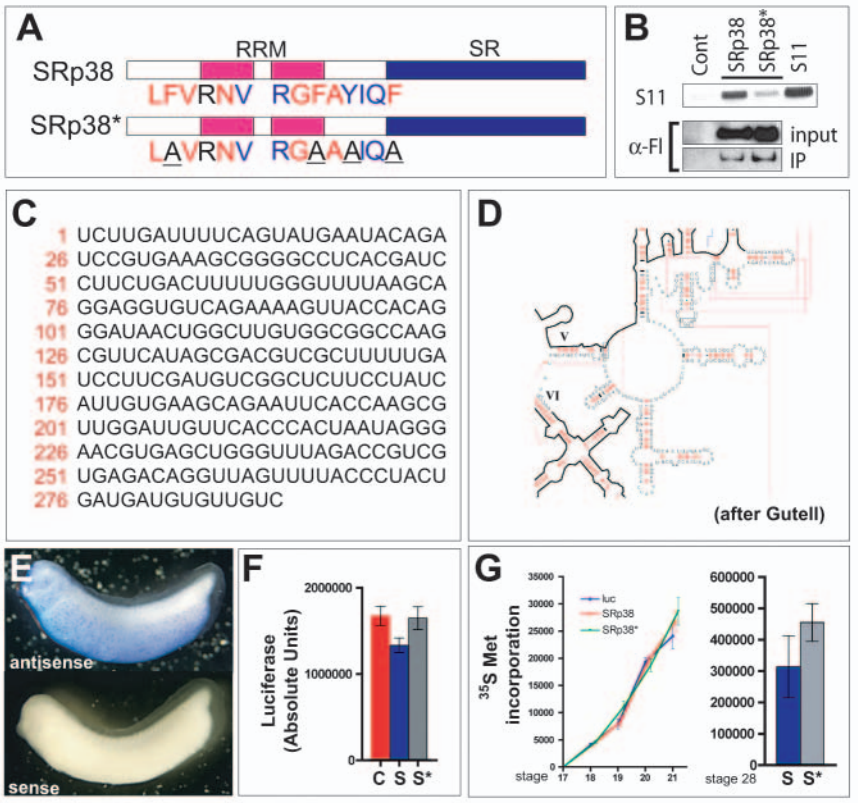

Fig. 8. SRp38 can interact with the peptidyl transferase domain of 28s ribosomal RNA. (A) RNA immunoprecipitation was performed using Flag-tagged wild-type SRp38 or a mutant SRp38 (SRp38*), in which all four conserved phenylalanines and tyrosines in the RNA-binding domain were converted to alanines (underlined), which should abrogate sequence-specific binding. (B) Immunoprecipitation of S11 sequence with SRp38. Control, Flag-SRp38- or Flag-SRp38*-expressing embryos were lysed and immunoprecipitated with anti-Flag (M2) agarose beads. IP products were then subjected to RT-PCR for S11 sequence. S11 was not immunoprecipitated in control uninjected embryos (lane 1) but was efficiently brought down in the wild-type SRp38 injected embryos (lane 2). A significantly smaller amount of S11 was also brought down with mutant SRp38*; this may reflect interactions of S11 with SRp38 partner proteins. A proportion of the samples were also used for western blotting to determine efficiency of initial protein expression (input) and immunoprecipitation (IP). (C) Nucleotide sequence of S11. (D) S11 maps to a portion of Domain V of *Xenopus* 28s ribosomal RNA, modeled after Gutell Lab Comparative RNA Web Site (http://www.rna.icmb.utexas.edu/). (E) S11 is expressed in a punctate pattern in the tadpole. Sense probe was used as a control for background. (F) Embryos injected with SRp38 (S) compared to mutant SRp38 (S*) show some decrease in the activity of a luciferase plasmid reporter. At the one-cell stage, embryos were uninjected (C), injected with SRp38 (S) or mutant SRp38 (S*). Embryos were allowed to divide and each set (C, S, S*) of embryos were injected with 50 pg luciferase DNA. Embryos were cultured and harvested for luciferase readings at stage 21. Error bars represent 95% confidence intervals. (G) ^{35}S-methionine incorporation is mildly decreased in SRp38 (S)-injected embryos. Embryos were injected at one cell stage with 250 pg luciferase, SRp38 or SRp38* mRNA. At stage 17, 0.1 mCi/ml ^{35}S-methionine was added to the culture media. Embryos were subsequently lysed, proteins were acetone precipitated and incorporated ^{35}S-methionine was counted. Left: from stage 17 to 21, there were no significant differences. By stage 28 (right) there was a small difference between SRp38- and SRp38*-injected embryos. Error bars represent 95% confidence intervals.

brought down in the wild-type SRp38 injected embryos (lane 2). A significantly smaller amount of S11 was also brought down with mutant SRp38*, and this may reflect interactions of S11 with SRp38 partner proteins. A proportion of the samples was also used for western blotting to determine efficiency of initial protein expression (input) and immunoprecipitation (IP). Thus, although SRp38 may inhibit splicing, it probably also interacts with the ribosome either during ribosome biogenesis or functionally during ribosome activity. SRp38 activity may halt ribosome function during mitosis, or it could be preserving partially processed pre-rRNA during mitosis.

Based on its interaction with the ribosome, we considered the possibility that SRp38 might influence translation in the embryo. We used two assays to test this possibility: first, we measured the rate of translation of a luciferase DNA reporter (Fig. 8F); and second, we determined the rate of ^{35}S-methionine incorporation in treated embryos (Fig. 8G). Embryos injected with SRp38 show some decrease in the activity of the luciferase plasmid reporter when compared with mutant SRp38* (Fig. 8F). ^{35}S-methionine incorporation was also mildly decreased in SRp38-injected embryos (Fig. 8G). From stage 17 to 21, there were no significant differences. By stage 28 (Fig. 8G, right) there was a small difference between SRp38- and SRp38*-injected embryos.

Discussion

In this study we have characterized a novel biological role for the atypical SR protein SRp38. Our findings indicate that SRp38 is transcriptionally regulated during neurogenesis and plays a specific role in the feedback regulation of neuronal differentiation during early embryogenesis. We have also defined a novel link between post-transcriptional gene regulation and neuroD/Notch control of neurogenic progenitors.

Our studies provide significant insights into the mechanism of SRp38 function, and control of neurogenesis. SRp38 had previously been shown to be a neural-specific splicing repressor (Komatsu et al., 1999) that is activated in response to stress and cell cycle regulation (Shin et al., 2004). However, these previous studies did not place SRp38 within a signaling cascade or identify any of its biological targets. Our findings implicate SRp38 in the regulation of vertebrate neurogenesis, linking it to signaling through both proneural genes and to the Notch/Delta pathway. We show that SRp38 can inhibit neural differentiation when overexpressed in the *Xenopus* embryo but that SRp38 does not affect neural induction or competence (Fig. 2). Furthermore, SRp38 inhibits the neurogenic activity of neurogenin but not that of neuroD (Fig. 4). SRp38 inhibition of primary neurogenesis requires active Notch signaling (Fig. 6). Finally, neuroD induces *SRp38* expression, perhaps as a

negative feedback mechanism to limit neurogenesis in vivo (Fig. 7).

Notch is thought to maintain neural progenitor cells in an undifferentiated state. Activation of Notch signaling during early neural patterning blocks differentiation with a resulting decrease in neurogenesis, whereas in the mouse, depletion of *Notch1* in the mid-hindbrain boundary results in the premature onset of neurogenesis (Ahmad et al., 1997; Austin et al., 1995; Chitnis et al., 1995; Coffman et al., 1993; Lutolf et al., 2002). The findings that SRp38 acts in the context of Notch signaling (Fig. 5) and that active Notch signaling is required for inhibition of neurogenesis (Fig. 6) suggest that a combination of SRp38 and Notch signaling serve as a bridge between cell cycle regulation and cell fates (Campos et al., 2002; Cereseto and Tsai, 2000; Ohnuma et al., 2002; Ohnuma et al., 1999).

The control of proliferation and cell cycle progression is crucial for the correct determination of the nervous system. Negative regulators of the cell cycle, such as $p27^{XIC1}$, have been shown to be required precisely at the neurogenin to neuroD step during primary neurogenesis in the *Xenopus* embryo (Vernon et al., 2003). neuroD (also called Beta2) itself can induce cell cycle withdrawal and *neuroD/Beta2*-null mice have an abnormal number of proliferative cells in the small intestine (Mutoh et al., 1998). Thus, it is possible that the regulation of SRp38 expression by neuroD is secondary to neuroD control of the cell cycle. Conversely, positive regulators of the cell cycle, such as cyclinD1, have been shown to repress the transcriptional activity of neuroD in endocrine cells (Ratineau et al., 2002).

What is the requirement for SRp38 in the early embryo? The use of high-affinity sequence-specific RNA binding had previously been used to deplete SRp38 from mitotic and heat shocked cell extracts (Shin et al., 2004). We used a similar strategy to inhibit the activity of endogenous SRp38 in the early embryo, as well as two different antisense morpholino oligonucleotides (AMOs) to inhibit translation of embryonic mRNA (Fig. 6). Depleting SRp38 activity when Delta activity is inhibited resulted in a synergistic increase in neurogenesis (Fig. 6). There was little effect on the development of whole embryos when they were treated with C3 or the morpholino oligonucleotides, consistent with the idea that SRp38 overlaps in function with other regulators of neurogenesis during development. In the absence of SRp38 function, it may be that the amount of neurogenesis continues to be limited by 'redundant' mechanisms, including appropriate spatial and temporal transcription of the proneural genes, cell cycle regulation of transcription and translation, and coordination of exit from the cell cycle. SRp38 activity would serve to reinforce these other controls and thus only become obvious when the system is sensitized.

An illuminating result of these studies is the identification of the 28S rRNA as a target of SRp38. SRp38 is capable of sequence specific binding and, while an 11 nucleotide consensus binding sequence is known (C3), it provides no insight into the RNA targets of SRp38. Using in vivo immunoprecipitation and subtraction, we have identified a 289 nucleotide RNA containing the peptidyltransferase domain of the 28S ribosomal RNA. This region has been shown to be selectively bound by RNA-binding proteins, in particular by the bacterial DEAD box proteins DbpA and YxiN (Kossen et al., 2002; Nicol and Fuller-Pace, 1995). Binding of SRp38 to this region of the ribosomal RNA suggests a function in ribosome biogenesis or function. It is possible that other regulators of splicing bind to this region of the ribosome or, this function may be unique to the mechanism of SR splicing repressors. Importantly, this result also provides a link between splicing regulation and translation.

Developmental heterogeneity of ribosome composition has been well documented in the slime mold *Dictyostelium discoideum* (Agarwal et al., 1999). Ribosomes in *Dictyostelium* spores are quantitatively and qualitatively different from those in the vegetative state and these differences result from a range of changes, from transcription of ribosome components to protein modifications (reviewed by Ramagopal, 1992). Additional ribosome diversity is likely to result from interaction with cellular proteins. For example, the *fragile X mental retardation protein* (FMRP) has been shown to bind to polyribosomes and regulate translational efficiency (Khandjian et al., 2004; Stefani et al., 2004). It is possible that SRp38 binds to 28s rRNA in a similar mechanism for generating ribosome diversity. In this way, the composition of the ribosome (and presumably, translational efficiency) as well as the repertoire of transcription and signaling factors present would generate specificity in specific cell types. Many unanswered questions remain, such as, what factors dictate the mRNAs that escape from the transcriptional and translational silencing imposed during mitosis? We suggest that *SRp38* is likely to play a role in this process. This hypothesis is based on the fact that SRp38 is required for mitotic splicing inhibition and this function is required for limiting neuronal progenitors. It is also possible that *SRp38* plays a positive role in allowing specific sequences to be processed during mitotic silencing.

It is likely that *SRp38* or genes like *SRp38* play similar roles in other developmental processes. For example, it is known that Notch signaling and cell cycle control are also important for myogenesis. Our data suggest that SRp38 may affect both mesoderm and endoderm development (Fig. 3; Table 1), however, because *SRp38* expression in the *Xenopus* embryo was mostly detected in the neural plate (Fig. 1), we have primarily studied its function in that context. Supporting a general role in the development of animals, the SRp38 protein is found in *Xenopus*, mouse and man and the proteins are functionally interchangeable in our hands (data not shown). This study elucidates a novel mechanism for the control of neurogenesis, while revealing a developmental role for the unusual SR protein SRp38, thus providing an important link between transcriptional and translational regulation of neuronal cell fates.

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