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Xaml1/Runx1 is required for the specification of Rohon-Beard sensory neurons in *Xenopus*

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Abstract

Lower vertebrates develop a unique set of primary sensory neurons located in the dorsal spinal cord. These cells, known as Rohon-Beard (RB) sensory neurons, innervate the skin and mediate the response to touch during larval stages. Here we report the expression and function of the transcription factor *Xaml1/Runx1* during RB sensory neurons formation. In *Xenopus* embryos *Runx1* is specifically expressed in RB progenitors at the end of gastrulation. *Runx1* expression is positively regulated by Fgf and canonical Wnt signaling and negatively regulated by Notch signaling, the same set of factors that control the development of other neural plate border cell types, i.e. the neural crest and cranial placodes. Embryos lacking *Runx1* function fail to differentiate RB sensory neurons and lose the mechanosensory response to touch. At early stages *Runx1* knockdown results in a RB progenitor-specific loss of expression of *Pak3*, a p21-activated kinase that promotes cell cycle withdrawal, and of *N-tub*, a neuronal-specific tubulin. Interestingly, the pro-neural gene *Ngnr1*, an upstream regulator of *Pak3* and *N-tub*, is either unaffected or expanded in these embryos, suggesting the existence of two distinct regulatory pathways controlling sensory neuron formation in *Xenopus*. Consistent with this possibility *Ngnr1* is not sufficient to activate *Runx1* expression in the ectoderm. We propose that *Runx1* function is critically required for the generation of RB sensory neurons, an activity reminiscent of that of *Runx1* in the development of the mammalian dorsal root ganglion nociceptive sensory neurons.

Keywords

Rohon-Beard; Sensory neurons; Runx1; Ngnr1; N-Tub; Pak3; *Xenopus*

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Introduction

The ectoderm of the vertebrate embryos can be divided into three regions at the end of gastrulation: the neural plate, which is the precursor of the central nervous system, the non-neural ectoderm forming the epidermis, and the neural plate border (NPB) that arises at the boundary between the neural plate and the non-neural ectoderm. The NPB is the source of two important cell populations: the neural crest (NC) and the pre-placodal ectoderm (PE). The NC is located lateral to the neural plate but is excluded from its most anterior region. NC cells will migrate in the periphery and give rise to a broad array of derivatives including craniofacial structures, the pigment cell lineage and peripheral nervous system (LeDouarin et al., 2004). The PE is restricted to the anterior-most region of the neural plate and lateral to the NC. The PE will eventually segregate into individual cranial placodes to give rise to the sensory organs in the head (Schlosser, 2010; Park and Saint-Jeannet, 2010a).

In anamniotes such as the frog *Xenopus laevis* the NPB gives rise to two additional cell populations: the hatching gland (HG) cells and a group of primary neurons known as Rohon-Beard (RB) sensory neurons. The HG is located in the outer layer of the ectoderm of the anterior neural folds, medial to the prospective NC. The HG produces proteolytic enzymes, which digest the vitelline envelope and jelly coat to release the tadpole into the environment (Drysdale and Elinson, 1991). The RB sensory neurons arise from the posterior-most region of the NPB. At the end of neurulation, these neurons are located in the dorsal spinal cord and innervate the skin to mediate the escape response to touch at the larval stages (Roberts and Smyth, 1974). Later in development RB neurons will undergo apoptosis (Lamborghini, 1987) and their function will be assumed by the NC-derived dorsal root ganglia neurons (reviewed in Roberts, 2000). Genes typically expressed in RB sensory neuron progenitors are also detected in two additional primary neuron subpopulations confined to a more medial region of the neural plate, the primary interneurons and motoneurons. This is the case, for example, of the basic helix loop helix (b-HLH) gene neurogenin-related-1 (*Ngnr1*; Ma et al., 1996), and the neural-specific tubulin gene, *N-Tubulin* (*N-Tub*; Chitnis et al., 1995). The absence of molecular markers restricted to RB sensory neuron progenitors has made it difficult to analyze the more unique requirements of this population of primary neurons in terms of specification and differentiation.

The gene *Runx1* encodes a runt domain transcription factor with a critical role in hematopoietic stem cell formation and definitive hematopoiesis in mammals (reviewed in Swiers et al., 2010). *Runx1* is also expressed in a subpopulation of dorsal root ganglia (DRG) sensory neurons involved in pain transduction and regulates aspects of the differentiation of this group of nociceptive neurons (reviewed in Stifani and Ma, 2009). In *Xenopus* in addition to its expression and function in blood progenitors, *Xaml1/Runx1* is expressed in RB neuron precursors at the end of gastrulation (Tracey et al., 1998), and therefore represents a unique tool to analyze the development of this population of primary sensory neurons. Moreover little is known of the role *Xaml1/Runx1* in the formation of these mechanosensory neurons.

Here we describe the detailed expression pattern of *Runx1* in RB progenitors as compared to other primary neuron-specific genes. We characterize the regulatory inputs controlling *Runx1* expression at the NPB and analyze the consequences of *Runx1* knockdown on the sensory function of *Xenopus* tadpoles. We also analyze the position of *Runx1* in the regulatory cascade leading to RB sensory neurons specification. Our findings indicate that *Runx1* function is critically required in RB progenitors to promote cell cycle exit and neuronal differentiation, and that *Runx1* is acting in parallel with *Ngnr1* to regulate sensory neuron formation.

Materials and Methods

Plasmid constructs

Vertebrate *Runx1* genes are expressed from two alternative promoters, a distal (P1) and a proximal (P2), that encode isoforms with distinct amino-terminal sequences (Supplementary Fig S1), here referred to as *Runx1* (accession # BC057739.1) and *Xaml1* (accession # AF035446), respectively. *Xenopus laevis Runx1* (pCMV-Sport6) was obtained from Open Biosystems. *Xaml1* was amplified by PCR from stage 30 cDNA using primers based on the published sequence (Tracey et al., 1998), and subcloned into pGEM-T Easy (Promega). Both ORFs including 9bp (*Runx1*) and 14bp (*Xaml1*) upstream of the ATG were amplified by PCR and subcloned into pCS2+ expression plasmid digested with ClaI and XbaI. These two constructs were used to test the specificity of the translation blocking morpholino antisense oligonucleotides (Supplementary Fig S1). We generated a hormone-inducible version of *Ngnr1* (Ma et al., 1996) by sub-cloning the coding region of *Ngnr1* into pCS2+GR (*Ngnr1*-GR). All constructs were sequenced and the corresponding proteins monitored using an in vitro transcription/translation assay.

In vitro transcription/translation

The in vitro transcription/translation coupled rabbit reticulocyte lysate system (SP6-TNT, Promega) was performed following the manufacturer recommendations (Promega), in the presence of ³⁵S-methionine. The reaction was resolved on a NuPAGE BIS-Tris gel (Invitrogen). The gel was dried using GelAir Drying System (Bio-Rad) and the product of the TNT reaction was detected by exposure onto a BioMax film (Kodak).

Morpholino antisense oligonucleotides

β -Catenin (β catMO; 25 ng; Heasman et al., 2000), Wnt8 (Wnt8MO; 30 ng; Park and Saint-Jeannet, 2008), Fgf8a (Fgf8aMO; 50 ng; Fletcher et al., 2006), Pax3 (Pax3MO; 50 ng; Monsoro-Burq et al., 2005; Hong and Saint-Jeannet, 2007), Zic1 (Zic1MO; 45 ng; Sato et al., 2005; Hong and Saint-Jeannet, 2007) and control (CoMO; 60 ng) morpholino antisense oligonucleotides were purchased from Gene-Tools LLC (Philomath, OR). To interfere with *Runx1* function we used two translation blocking and a splice blocking morpholinos. *Runx1*MO (CACTATGTGAGGCCATTGCGTTTC) and *Aml1*MO (GGGATACGCATCACAACAAGCCTGG) specifically block translation of *Runx1* (P1 promoter) and *Xaml1* (P2 promoter) mRNA, respectively. The specificity of *Runx1*MO and *Aml1*MO was tested in an in vitro transcription/translation coupled rabbit reticulocyte lysate assay (Supplementary Fig S1). Based on *Xenopus tropicalis* genome information (Ensembl Gene ID: ENSXETG00000014140), several intronic regions within *Runx1* were selected as candidate sites for a splice-inhibitory morpholino. Primers flanking these introns in the *Xenopus laevis Xaml1/Runx1* mRNA sequence were used to amplify *Xenopus laevis* genomic DNA fragments, which were cloned into pGEM-T (Promega) and sequenced. We designed a splice-inhibitory morpholino (*Runx1*SMO: AAACAGAGCCAGGGTCTTACCTTGA) targeting the Exon1-Intron1 junction (Supplementary Fig S1).

Embryo injections and in situ hybridization

Embryos were staged according to Nieuwkoop and Faber (1967). Fgf8a (2 μ g; Christen and Slack, 1997), Notch-ICD (0.5 ng; Chitnis and Kintner, 1996) and *Ngnr1*-GR (0.5 ng; Perron et al., 1999) mRNAs were synthesized *in vitro* using the Message Machine kit (Ambion, Austin, TX). Synthetic mRNA and morpholino antisense oligonucleotides were injected in the animal pole of 2-cell stage embryos. All embryos were co-injected with the lineage tracer β -gal mRNA (β -gal; 0.5 ng) to identify the injected side. *Ngnr1*-GR (0.5 ng) injected

embryos were treated with 10 μ M dexamethasone (Sigma) in NAM 0.1X at stage 10.5 or stage 12.5, as described (Gammill and Sive, 1997). Untreated sibling embryos were used as a control (not shown). For in situ hybridization embryos were fixed in MEMFA and were successively processed for Red-Gal (Research Organics) staining to detect β -gal activity, and in situ hybridization. Antisense DIG-labeled probes (Genius kit, Roche) were synthesized using template cDNA encoding *Xaml1/Runx1* (Tracey et al., 1998), *Xhe* (Katagiri et al., 1997), *N-Tub* (Chitnis et al., 1995), *Pak3* (Souopgui et al., 2002), *Ngnr1* (Ma et al., 1996), *Islet1* (Brade et al., 2007), *Krox20* (Bradley et al., 1993), *Snail2* (Mayor et al., 1995), *XK81* (Jonas et al., 1989), *Kv1.1* (Burger and Ribera, 1996) and *Ccndx* (Chen et al., 2007). Whole-mount in situ hybridization was performed as previously described (Harland, 1991). For *in situ* hybridization on sections, embryos were fixed in MEMFA for 1 hour, embedded in Paraplast+, 12 μ m sections cut on a rotary microtome and hybridized with the appropriate probes as described (Henry et al. 1996). Sections were then briefly counter stained with Eosin.

Proliferation assay

For phosphohistone H3 detection (Saka and Smith, 2001), Sox9MO-injected albinos embryos were fixed in MEMFA. Embryos were incubated successively in α -phosphohistone H3 antibody (Upstate Biotechnology; 1 μ g/ml) and anti-rabbit IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch; 1:1000). Alkaline phosphatase activity was revealed using NBT/BCIP (Roche). Fluorescein lysine dextran (FLDX; MW 10,000, Molecular Probes) was used as a lineage tracer to identify the injected side.

Touch response assay

During embryogenesis *Xenopus* embryos develop a dual touch sensory system largely mediated by RB sensory neurons (Roberts and Smyth, 1974). The touch response assay and quantification was performed as previously described (Fein et al., 2008). Briefly, the dorsal trunk of stage 32 embryos was gently touched or stroked with a metal probe. After a period of approximately 3 seconds the probe was reapplied for a total of 10 trials. Responses were scored as follows: 0, no response; 0.5, non-swimming response (restricted trunk bend); 1.0, normal swimming response. The scores of each of the 10 trials were summed to yield a final touch response score between 0 and 10.

Results

Xaml1/Runx1 is expressed in Rohon-Beard sensory neurons

The cloning and expression of *Xaml1/Runx1* has been previously reported (Tracey et al., 1998), however this initial study was primarily focused on *Runx1* expression in the hematopoietic lineage. To further evaluate the developmental expression of *Runx1* in the ectoderm, we performed whole-mount in situ hybridization on embryos at various stages. At the neurula stage (stage 15) the *Runx1* expression domain in the ectoderm is located posterior to the prospective NC and HG, as seen by *Snail2* and *Xhe* expression, respectively (Fig 1A–C). *Runx1* expression is restricted to a region of the NPB that is also posterior to the hindbrain marker *Krox20* (Fig 1D). Transverse sections indicate that *Runx1* is expressed in the deep layer of the ectoderm, lateral to the neural plate (Fig 1E–F), which anatomically corresponds to the position of the prospective RB sensory neurons (Roberts, 2000). By stage 23, the neural plate has folded into a tube, resulting in the repositioning of *Runx1*-expressing cells to the dorsolateral region of the spinal cord (Fig 1G–I). At this stage *Runx1* is also detected in blood progenitors in the ventral mesoderm (Fig 1H), as previously reported (Tracey et al., 1998). Around stage 28, additional domains of expression of *Runx1* include the olfactory epithelium and the developing statoacoustic ganglia associated with the otic

vesicle (Fig 1J; Park and Saint-Jeannet, 2010b). *Runx1* expression persists in RB sensory neurons at least up to stage 45 (Fig 1K).

***Runx1* is co-expressed with other primary neuron-specific genes**

We next performed a comparative analysis of *Runx1* expression to that of classic markers for primary neurons such as *N-Tub* and *Ngnr1*. *Runx1* is first detected at stage 13 in RB progenitors, where it colocalizes with *Ngnr1*, which is also expressed in progenitors of primary interneurons and motoneurons within the neural plate (Chitnis et al., 1995; Ma et al., 1996; Fig 2A). The onset of *Runx1* expression is slightly later than the stage at which RB progenitors have been birthdated in *Xenopus*, around stage 11.5 to 12 (Lamborghini, 1980; Jacobson and Moody, 1984). *N-Tub* expression in RB progenitors is initiated around stage 15 as well as in the other two primary neuron subpopulations (Fig 2A). At this stage, *N-Tub*, *Ngnr1*, and *Runx1* are all co-expressed in RB progenitors. This co-localization was confirmed by in situ hybridization on adjacent sections of the same embryo (Fig 2B). By stage 19, as the neural plate folds to form a tube, *Runx1* and *N-Tub* are still co-expressed in RB progenitors, however *Runx1* expression is more sparse than that of *N-Tub* suggesting that *Runx1* is only expressed in a subset of RB cells (Fig 2A). At this stage *Ngnr1* expression appears to be more medial than that of *N-Tub* and *Runx1* as *Ngnr1* is now downregulated in RB progenitors (Fig 2A). In situ hybridization on adjacent sections of stage 19 embryos using all three probes confirmed the loss of *Ngnr1* expression in RB progenitors (Fig 2C), while the *Runx1* expression domain largely overlaps with that of *N-Tub* in the dorsolateral region of the spinal cord containing RB progenitors (Fig 2C). In other regions of the spinal cord *Ngnr1* overlaps with *N-Tub* in the prospective primary interneurons and motoneurons. These results show that *Runx1*, *Ngnr1* and *N-Tub* are initially co-expressed in RB progenitors, however by neural tube closure *Ngnr1* expression is lost in this population of primary neurons.

Fgf, Wnt and Notch signaling regulate *Runx1* expression at the NPB

The formation of NPB cells requires attenuation of Bmp signaling in the ectoderm through the activity of Bmp antagonists produced by the axial mesoderm. However, changes in Bmp signaling in the ectoderm are not sufficient to specify the NPB and other signaling pathways have been implicated in this process including Fgf, canonical Wnt and Notch signaling (reviewed in Knecht and Bronner-Fraser, 2002; Huang and Saint-Jeannet, 2004). A recent study indicates that like other NPB cell types RB sensory neuron formation requires Bmp activity (Rossi et al., 2008). We decided to determine whether other signaling pathways were also implicated in the generation of RB neurons. We specifically analyzed the role of Fgf8a and Wnt8, two ligands implicated in NPB induction in *Xenopus* (Hong and Saint-Jeannet, 2007; Hong et al., 2008). Overexpression of Fgf8a by injection of Fgf8a mRNA resulted in a dramatic ventrolateral expansion of the *Runx1* expression domain in all embryos examined (Table 1; Fig 3). *N-Tub* and *Ngnr1* expression domains were also expanded in a majority of embryos. The expansion of these genes was associated with a loss of epidermal keratin (Supplementary Fig S2). Conversely, knockdown of Fgf8a using a splice-blocking morpholino antisense oligonucleotide (Fgf8aMO) caused a severe reduction of all three genes in most embryos examined (Table 1; Fig 3). Interference with canonical Wnt signaling by injection of Wnt8 or β -catenin morpholino antisense oligonucleotides (Wnt8MO and β -catMO) had a similar outcome: a reduction of *Runx1* expression domain at a high frequency, as well as a reduction of *N-Tub* and *Ngnr1* in all three populations of primary neurons (Table 1; Fig 3). Because the formation of primary neurons is negatively regulated by Notch signaling (Chitnis et al., 1995), we next asked whether Notch signaling had an inhibitory effect on *Runx1* expression. We found that expression of mRNAs encoding an activated form of Notch (Notch-ICD; Chitnis and Kintner, 1996) also represses *Runx1* expression (Table 1; Fig 3), consistent with a previous study (Perron et al., 1999).

Taken together, these results indicate that *Runx1* expression in RB progenitors is negatively regulated by Notch signaling, and under the positive influence of Fgf and canonical Wnt signaling.

The NPB specifiers *Pax3* and *Zic1* regulate *Runx1* expression

Downstream of these signaling events *Pax3* and *Zic1* are two genes activated early at the NPB and required for the development of three distinct lineages: the NC, PE and HG (Monsoro-Burq et al., 2005; Sato et al., 2005; Hong and Saint-Jeannet, 2007). At early neurula stages *Pax3* and *Zic1* expression overlaps with the *Runx1* expression domain in RB progenitors (Fig 4A) suggesting that these factors may also regulate *Runx1* expression and RB sensory neuron formation. To test this possibility, we used Pax3- and Zic1-specific morpholino antisense oligonucleotides (Pax3MO and Zic1MO), which block Pax3 and Zic1 translation, respectively. Unilateral injection of either Pax3MO or Zic1MO at the 2-cell stage inhibited *Runx1* expression in a vast majority of the embryos (Table 2; Fig 4B). Pax3 or Zic1 knockdown also prevented *Ngnr1* and *N-Tub* expression in RB progenitors as well as in the other two primary neuron populations (Table 2; Fig 4B), consistent with the expression of *Pax3* and *Zic1* in the lateral neural plate (Hong and Saint-Jeannet, 2007). These results demonstrate that the NPB specifiers *Pax3* and *Zic1* are functioning upstream of *Runx1*, *Ngnr1* and *N-Tub*, and suggest that Pax3 and Zic1 are required for the formation of all four NPB cell types: the NC, PE, HG and RB progenitors.

Embryos lacking *Runx1* function fail to differentiate RB sensory neurons and lose response to touch

To determine whether *Runx1* function is required for RB neuron development we performed *Runx1* knockdown in developing embryos using morpholino antisense oligonucleotides. A conserved feature of the vertebrate *Runx* genes is their expression from two adjacent promoters (distal, P1 and proximal, P2) encoding two isoforms with distinct amino-terminal sequences (reviewed in Blyth et al., 2005). We designed two morpholinos to specifically interfere with the translation of each isoform, *Runx1MO* (P1 promoter) and *Aml1MO* (P2 promoter) (Supplementary Fig S1). We also designed a third morpholino (*Runx1SMO*) that blocks *Runx1* splicing at the Exon1-Intron-1 junction (Supplementary Fig S1). To evaluate the formation of RB sensory neurons in morphant embryos we analyzed the expression of the potassium-gated channel *Kv1.1*, which is normally expressed in differentiated RB neurons (Burger and Ribera, 1996). Bilateral injection of either morpholino in the animal region of 2-cell stage embryos resulted in a severe reduction or loss of *Kv1.1* expression in the dorsal spinal cord at stage 32 (Fig. 5A–B). In these embryos the development of other neuronal subpopulations in the spinal cord was largely unaffected (Fig. 5B), as exemplified by unperturbed expression of *Ccndx*, a gene expressed in motoneurons (Chen et al., 2007).

Because RB neurons serve as primary mechanosensory neurons and are involved in the response to touch in the developing embryo, we next analyzed the behavior of morphant embryos to determine the functional consequences of the loss of *Runx1*. Bilateral injection of any one of the three morpholinos led to a significant reduction in touch sensitivity in a dose dependent manner (Fig 5C–D; Supplemental movies). The fact that two translation blocking morpholinos and a splice-inhibitory morpholino give identical results establishes the strong specificity of the phenotype. These results indicate that *Runx1* function is critically required for the formation of RB sensory neurons and is essential for the development of a functional mechanosensory system at larval stages.

Runx1 regulates *Pak3*, *Islet1* and *N-Tub* expression in RB progenitors

In order to gain insights into the mechanisms by which *Runx1* regulates RB sensory neuron formation, we analyzed the influence of *Runx1* function on the expression of several

components of the proneural gene network regulating the emergence of primary neurons at the neurula stage. These genes include: *Ngnr1*, a b-HLH proneural factor, and an upstream regulator of the pathway (Ma et al., 1996); *Islet 1*, a LIM homeodomain transcription factor involved in neuronal specification (Brade et al., 2007); *Pak3*, a p21-activated kinase 3 essential for cell cycle withdraw (Souopgui et al., 2002); and *N-Tub*, a marker of neuronal differentiation (Chitnis et al., 1995). *Runx1* knockdown by injection of *Runx1*MO, *Aml1*MO or *Runx1*SMO resulted in a similar phenotype characterized by a reduction of *Islet1*, *Pak3* and *N-Tub* expression in RB progenitors at the neurula stage (Table 3; Fig 6A–B). In contrast *Ngnr1* was either unaffected or expanded in morphant embryos (Table 3; Fig 6A–B). Phosphohistone H3 staining did not show any significant change in the rate of cell proliferation at the neural plate border of morphant embryos (Supplementary Fig S2). Altogether these experiments indicate that *Runx1* is functioning upstream of *Pak3*, *Islet1* and *N-Tub* in the regulatory cascade leading to RB progenitors formation, and suggest that *Runx1* may regulate the differentiation of these cells by promoting cell cycle withdrawal.

***Ngnr1* is not sufficient to activate *Runx1* expression**

To further evaluate the role of *Ngnr1* in the regulation of *Runx1* expression in RB sensory neurons we expressed an hormone inducible version of *Ngnr1* in which *Ngnr1* was fused to the hormone-binding domain of human glucocorticoid receptor (*Ngnr1*-GR). The activity of this fusion protein can be regulated by addition of dexamethasone to the embryo culture medium (Kolm and Sive, 1995). Embryos injected with 0.5 ng of *Ngnr1*-GR mRNA were treated with dexamethasone at the early (stage 10.5) or late (stage 12.5) gastrula stages and analyzed for gene expression at the neurula (stage 15) or tailbud (stage 27) stages. Activation of *Ngnr1*-GR at the gastrula stages resulted in a reduction of the NC-specific gene *Snail2*, and a dramatic expansion of *N-Tub* and *Pak3* expression domain at the neurula stage, converting the entire ectoderm into primary neuron progenitors (Table 4; Fig 7A), as previously reported (Ma et al., 1996; Olson et al., 1998; Perron et al., 1999). For both genes the lateral expansion was more pronounced for a treatment with dexamethasone at stage 10.5 (Fig 7A). In contrast, *Runx1* expression appeared more diffuse or partially inhibited after dexamethasone treatment at stage 10.5, while for a later treatment (stage 12.5) a majority of the embryos were unaffected (Table 4; Fig 7A), suggesting that *Ngnr1* expression is not sufficient to induce *Runx1*, consistent with a previous study (Perron et al., 1999). Interestingly, at later stage these embryos showed massive ectopic *Islet1* and *Kv1.1* expression in the ectoderm without any upregulation of *Runx1* expression (Table 4; Fig 7B). These results indicate that *Ngnr1* has the ability to promote the induction of neuronal sensory characteristics in the ectoderm, and this is occurring in a *Runx1* independent manner, suggesting the existence of two distinct and parallel regulatory pathways controlling sensory neurons formation in *Xenopus*.

Discussion

In this study we provide novel information on the regulatory mechanisms controlling the specification and differentiation of RB sensory neurons in *Xenopus*, focusing on the activity of the transcription factor *Xaml1/Runx1*. *Runx1* is detected in RB progenitors at the end of gastrulation (stage 13) and its expression is regulated by the same factors that control the development of other NPB cell types. *Runx1* is specifically required for the expression of the *p21-activated serine/threonine kinase 3 (Pak3)*, allowing RB progenitors to exit the cell cycle and initiate differentiation. In the absence of *Runx1*, RB sensory neurons failed to form resulting in embryos with impaired mechanosensory function. These results indicate that *Runx1* is critically required for RB sensory neurons formation in *Xenopus*, reminiscent of *Runx1* function in the development of the mammalian DRG nociceptive sensory neurons (Chen et al., 2006; Marmigere et al., 2006; Kramer et al., 2006).

Recent transplantation experiments have shown that RB neurons form as the result of an inductive interaction between the neural and non-neural ectoderm in *Xenopus* (Rossi et al., 2008). In these experiments *Bmp4* protein was reported to induce RB neurons ectopically, as assayed by *XHox11L2* expression, and was shown to be required for RB induction at sites of newly formed neural plate-epidermal boundaries (Rossi et al., 2008). Other studies using *N-Tub* as a marker have proposed a role for Fgf, Wnt and Notch signaling in RB neuron induction (Bang et al., 1999; Pera et al., 2003; Fletcher et al., 2006; Garcia-Morales et al., 2009). However, because *N-Tub* is expressed in all three subpopulations of primary neurons, it is difficult in some of these studies to determine whether the effects observed reflect changes in RB progenitor fate or a broader effect on the development of other populations of primary neurons. Our work using *Runx1* as an early marker for RB progenitors demonstrates unambiguously that in addition to *Bmp*, canonical Wnt, Fgf and Notch signaling are all implicated at some level in the induction of RB progenitors. It is interesting that the same set of signals regulating the formation of the NC, PE and HG at the NPB are also involved in the induction of RB progenitors (McGrew et al., 1999; Brugmann et al., 2004; Glavic et al., 2004a; Glavic et al., 2004b; Ahrens and Schlosser, 2005; Hong and Saint-Jeannet, 2007; Hong et al., 2008). How these signals are integrated at the NPB to generate distinct fates is an important and still unresolved question.

Downstream of these signaling molecules a number of genes are broadly activated at the NPB. These genes are referred as NPB specifiers and include members of the *Zic*, *Pax*, *Dlx* and *Msx* families of transcriptional regulators. In turn these genes are responsible for the activation of a subset of genes with more restricted expression domains, known as NC specifiers or PE specifiers (Meulemans and Bronner-Fraser, 2004; Litsiou et al., 2005). *Pax3* and *Zic1* are two NPB specifiers that have the ability, alone or in combination, to regulate the formation of three distinct fates at the NPB. *Pax3* and *Zic1* are critical for HG and PE fate, respectively, while in combination they synergize to specify the NC (Monsoro-Burq et al., 2005; Sato et al., 2005; Hong and Saint-Jeannet, 2007). Our observations indicate that *Runx1* is also under the control of *Pax3* and *Zic1*, suggesting that the regulatory network underlying the emergence of the NC and PE (Meulemans and Bronner-Fraser, 2004; Litsiou et al., 2005) can be extended to RB sensory neurons, and we propose that *Runx1* represents a bona fide RB specifier downstream of the NPB specifiers *Pax3* and *Zic1* (Fig 8). Recent work in zebrafish indicates that the transcription factor *prdm1a* is an important upstream regulator of NPB cell fates, through the selective activation of two target genes, *sox10* in the NC, and *islet1* in RB neurons (Olesnicki et al., 2010), and the repression of the basic helix-loop-helix gene *olig4* (Hernandez-Lagunas et al., 2011). In light of these results it would be of particular importance to also evaluate the role of *Prdm1* in the regulation of cell fate at the NPB in *Xenopus*.

Loss of *Runx1* function using 3 distinct morpholino antisense oligonucleotides interfering either with *Runx1* translation or splicing show an extremely consistent phenotype at the tadpole stage, characterized by the failure to form RB neurons in the dorsal spinal cord. Later, morphant tadpoles have a defective escape response to touch, consistent with the loss of RB neurons. These observations demonstrate that *Runx1* is critically required for the development of RB sensory neurons and the establishment of the larval mechanosensory system. Vertebrate *Runx* genes are expressed from two alternative promoters, the distal P1 and proximal P2 encoding isoforms with distinct amino-terminal sequences (reviewed in Blyth et al., 2005). This is also the case for *Xenopus Runx1*, which has two isoforms that differ by a few amino acids. Interestingly in *Xenopus* elimination of one of the isoforms using *Runx1MO* or *Aml1MO* appears to be sufficient to abrogate all mechanosensory functions. Moreover, the splice blocking MO (*Runx1SMO*) that is believed to interfere with both isoforms does not produce a phenotype that is any stronger than each translation blocking morpholino individually. This suggests that the formation of RB progenitors is

extremely sensitive to Runx1 dosage. This dosage sensitivity has also been described in the context of Runx1 function during hematopoiesis. For example loss of *P1-Runx1* in the mouse embryo is reminiscent of the *Runx1* heterozygote phenotype with sufficient definitive hematopoietic cells to permit embryonic survival. With loss of *P2-Runx1*, in contrast, definitive hematopoiesis is dramatically affected, resulting in a phenotype resembling the Runx1 null (Bee et al., 2009; 2010).

RB progenitors constitute one of the three groups of primary neurons specified at the end of gastrulation in anamniotes. The other two, the primary motoneurons and interneurons, are confined to the medial neural plate. The differentiation of the primary neurons is driven by proneural transcription factors, which promote the activation of a number of factors required for cell fate determination, cell cycle exit and terminal differentiation. In *Xenopus* most of these factors have fairly similar expression patterns in all three primary neuron populations, suggesting that the formation of primary neurons is regulated by the same mechanisms (reviewed in Henningfeld et al., 2007). The b-HLH gene *Ngnr1* is at the top of this regulatory cascade (Ma et al., 1996). *Ngnr1* indirectly activates Pak3, a p21-activated serine/threonine kinase 3, which promotes cell cycle withdrawal, thereby allowing neuronal differentiation to proceed (Souopgui et al., 2002). Our results using morpholinos interfering with Runx1 function demonstrate that Runx1 is also required for *Pak3* and *N-Tub* expression in RB progenitors. In these embryos *Ngnr1* expression was unperturbed or expanded. A similar phenotype was observed using a dominant negative form of *Runx1* (Tracey et al., 1998; not shown). These results indicate that *Runx1* can regulate the generation of RB cells independently of *Ngnr1*. Interestingly, while *Runx1* and *Ngnr1* are initially coexpressed in RB progenitors (stage 13), a few hours later (stage 19) *Ngnr1* is no longer detected in RB cells (Fig 2). Transient *Ngnr1* expression in RB progenitors distinguishes this cell type from the other two populations of primary neurons, and suggests that the formation of this primary neuron subtype is regulated by distinct mechanisms. One interpretation of our results is that Runx1 is required in RB progenitors to downregulate *Ngnr1*. In the absence of Runx1 function, *Ngnr1* is maintained in RB progenitors preventing their differentiation.

Misexpression of *Ngnr1* is known to repress NC fate by converting the entire ectoderm into primary neuron progenitors (Ma et al., 1996; Olson et al., 1998; Perron et al., 1999). Interestingly, unlike *N-Tub* and *Pak3*, we found that *Runx1* was not ectopically induced upon *Ngnr1* misexpression, while *Islet-1* and *Kv1.1* were dramatically upregulated throughout the ectoderm at the tailbud stage. One interpretation is that *Ngnr1* can bypass the need for *Runx1* to induce sensory neuron characteristics in the ectoderm, suggesting the existence of two distinct pathways regulating the emergence of sensory neurons. Alternatively, it is also possible that *Runx1* and *Ngnr1* are involved in the differentiation of distinct classes of sensory neurons. Additional studies will be needed to fully evaluate these possibilities, define the interplay between these factors, and identify the downstream targets they regulate to establish the identity of this cell population. Interestingly, a recent study is also pointing to the existence of *Ngnr1*-dependent and *Ngnr1*-independent pathways in the specification of cranial sensory neurons in *Xenopus* (Schlosser et al., 2008).

In the mouse DRG, Runx1 is expressed in a subpopulation of sensory neurons involved in pain transduction, the nociceptive neurons. Runx1 is required for the generation of nociceptive neurons during embryonic and early postnatal phases of DRG development (Chen et al., 2006; Marmigere et al., 2006; Kramer et al., 2006). There is an interesting parallel between Runx1 function in these two anatomically distinct groups of sensory neurons across species. It has been proposed that the NC may have evolved from a subset of RB progenitors that had delaminated from the dorsal spinal cord and migrated in the periphery, to eventually give rise to the modern DRG sensory neurons (Fritzscht and Northcutt, 1993; reviewed in Donohue et al., 2008). Consistent with the idea of a common

origin to both RB and NC cells, in zebrafish the segregation of the two fates is tightly linked and depends on Notch/Delta signaling (Cornell and Eisen, 2000; 2002). Moreover, the transcription factor *prdm1a* is required for specification of both RB and NC cells (Artinger et al., 1999; Roy and Ng, 2004; Rossi et al., 2009; Olesnicki et al., 2010). The conserved function of Runx1 in anamniote RB sensory neurons and in the NC-derived DRG sensory neurons of higher vertebrates may represent additional evidence in support of the evolutionary derivation of these cells.

Highlights

- *Runx1* is expressed in Rohon-Beard (RB) progenitors at the end of gastrulation.
- In the absence of Runx1 function RB sensory neurons failed to form.
- The resulting embryos lose the mechanosensory response to touch.
- Runx1 is critically required for RB sensory neurons formation in *Xenopus*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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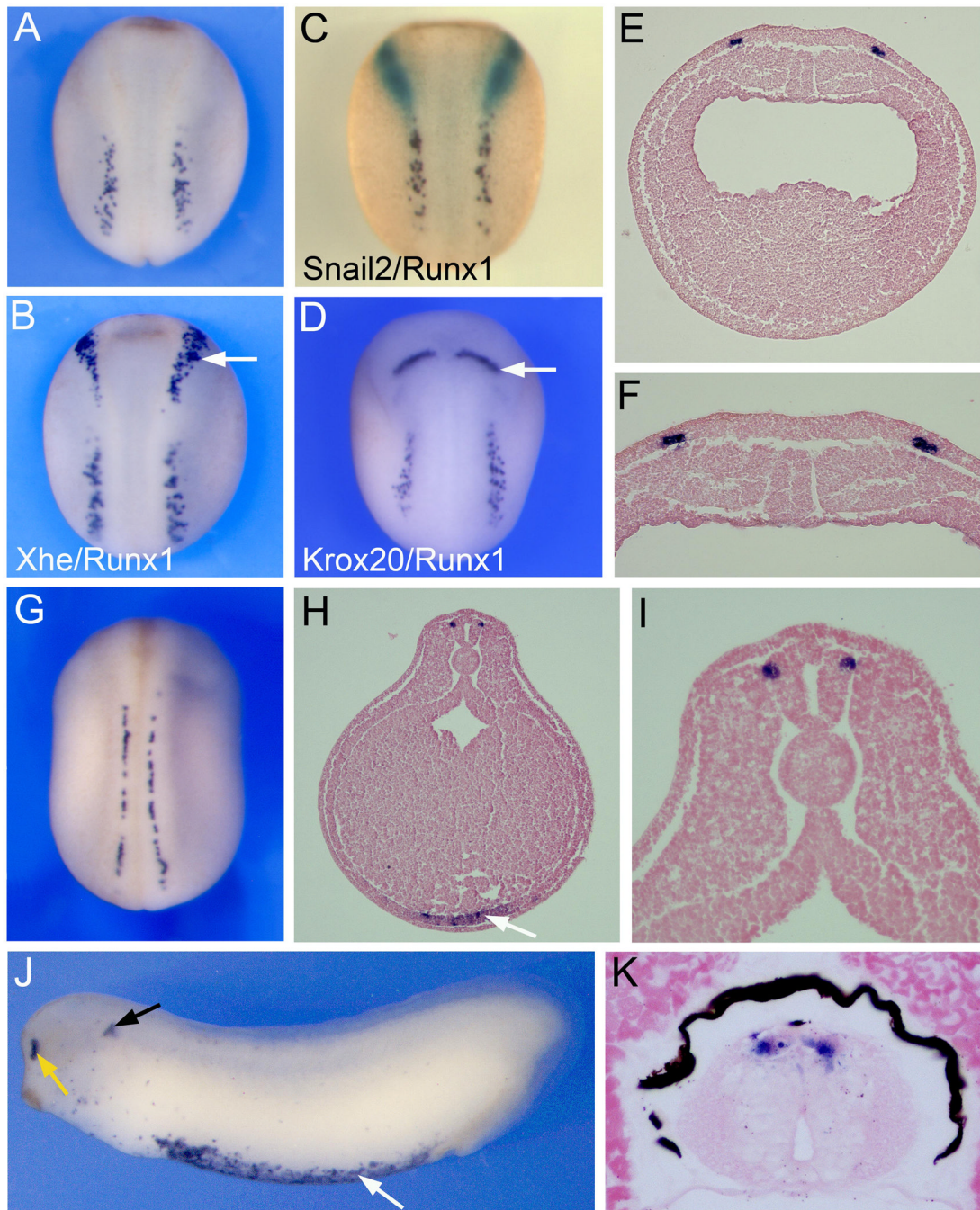


Figure 1. Expression of *Runx1/Xam1* in Rohon-Beard sensory neurons by whole-mount in situ hybridization

(A–F) *Runx1* expression in stage 15 embryos. (A) *Runx1* is detected at the posterior portion of the NPB. (B) Double in situ hybridization for *Runx1* and the HG marker *Xhe*. *Runx1* positive cells are located posterior to HG cells (arrow). (C) Double in situ hybridization for *Runx1* and the neural crest marker *Snail2*. *Runx1* expression (purple) is distinct from and posterior to the neural crest forming region (*Snail2*, green staining). (D) Double in situ hybridization for *Runx1* and the hindbrain marker *Krox20* (arrow). Panels (A–D), dorsal view, anterior to top. (E) Transverse section, dorsal to top, showing that *Runx1* is restricted to two discrete domains at the neural plate border. (F) Higher magnification of the neural

plate region of the embryo shown in (E). (G) At stage 22 as the neural tube closes, *Runx1* expression is restricted to the dorsal aspect of the spinal cord. Dorsal view, anterior to top. (H–I) Transverse section through a stage 23 embryo, dorsal to top. *Runx1* is confined to the dorso-lateral region of the spinal cord. *Runx1* is also detected ventrally in the lateral plate mesoderm, precursor of the hematopoietic lineage (arrow). (I) Higher magnification of the neural tube region of the embryo shown in (H). (J) *Runx1* expression in a stage 28 embryo. *Runx1* is detected in the olfactory epithelium (yellow arrow), periotic mesenchyme (black arrow), and blood precursors (white arrow). Lateral view, anterior to left. (K) Transverse section through the spinal cord of a stage 45 embryo shows *Runx1* expression in RB sensory neurons in the dorsal spinal cord. Dorsal to top.

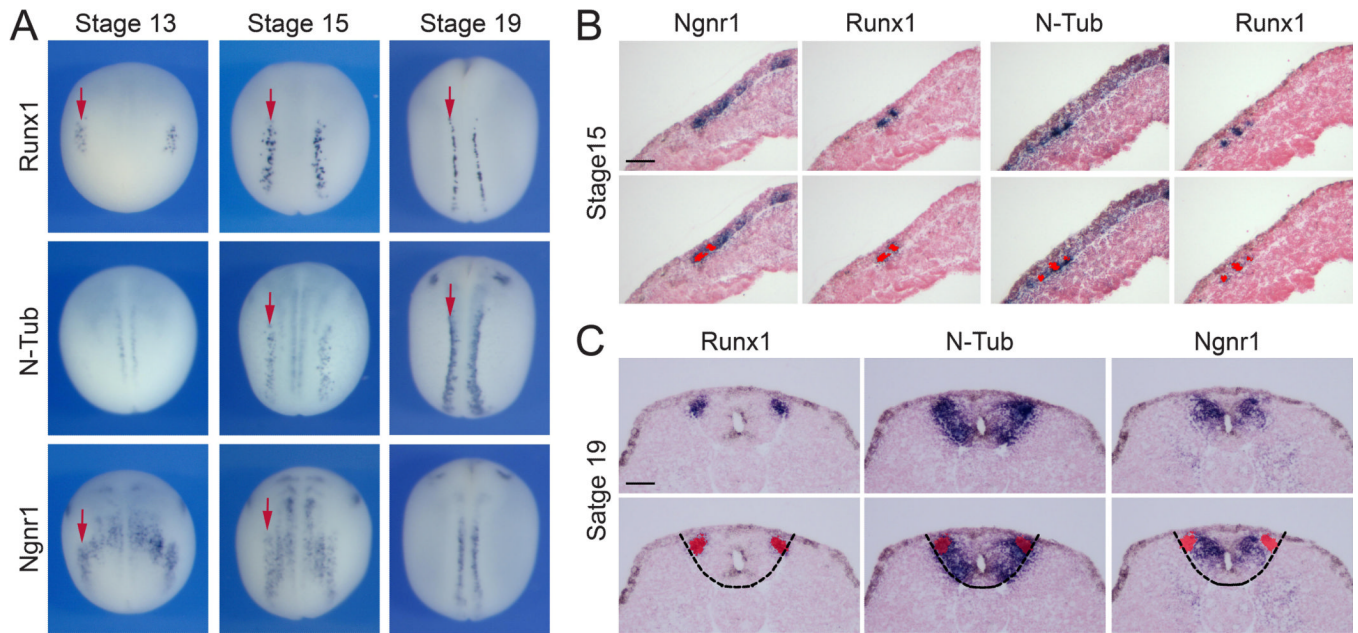


Figure 2. Comparative expression of *Runx1*, *N-Tub* and *Ngnr1* in primary neurons

(A) Developmental expression of *Runx1*, *N-Tub*, and *Ngnr1* at the neural plate border in stage-matched embryos. The arrows indicate the position of the row of RB sensory neurons. Dorsal views, anterior to top. (B–C) In situ hybridization on adjacent transverse sections of stage 15 and stage 19 embryos, dorsal to top. (B) At stage 15 *Runx1* is co-expressed with *N-Tub* and *Ngnr1*, as indicated by the red overlay in the lower panels. (C) At stage 19 while *Runx1* and *N-Tub* are still co-expressed, *Ngnr1* expression is lost in the RB neurons population, as indicated by the red overlay in the lower panels. The dotted lines demarcate the position of the spinal cord.

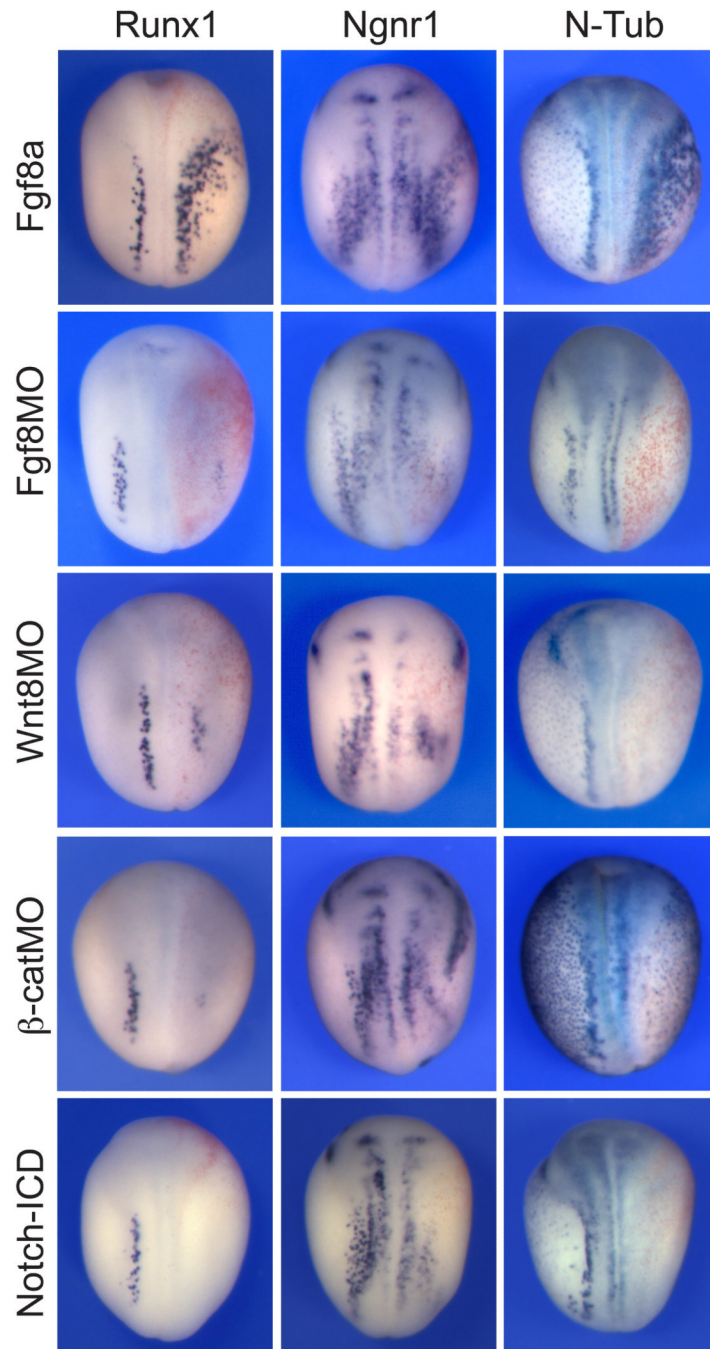


Figure 3. Wnt, Fgf and Notch signaling pathways regulate *Runx1*

Overexpression of Fgf8a by injection of Fgf8a mRNA in one blastomere at the 2-cell stage results in an expansion of the *Runx1* expression domain. The expression domain of *N-Tub* and *Ngnr1* is also expanded in these embryos. Knockdown of Fgf8a (Fgf8aMO) causes a severe reduction of all three genes. Similarly, interference with canonical Wnt signaling, by injection of Wnt8MO or β -catMO, reduces *Runx1*, *N-Tub* and *Ngnr1* expression in all three populations of primary neurons. Expression of an activated form of Notch (Notch-ICD) also represses *Runx1* expression. In all panels embryos are viewed from the dorsal side, anterior to top. The injected side is on the right.

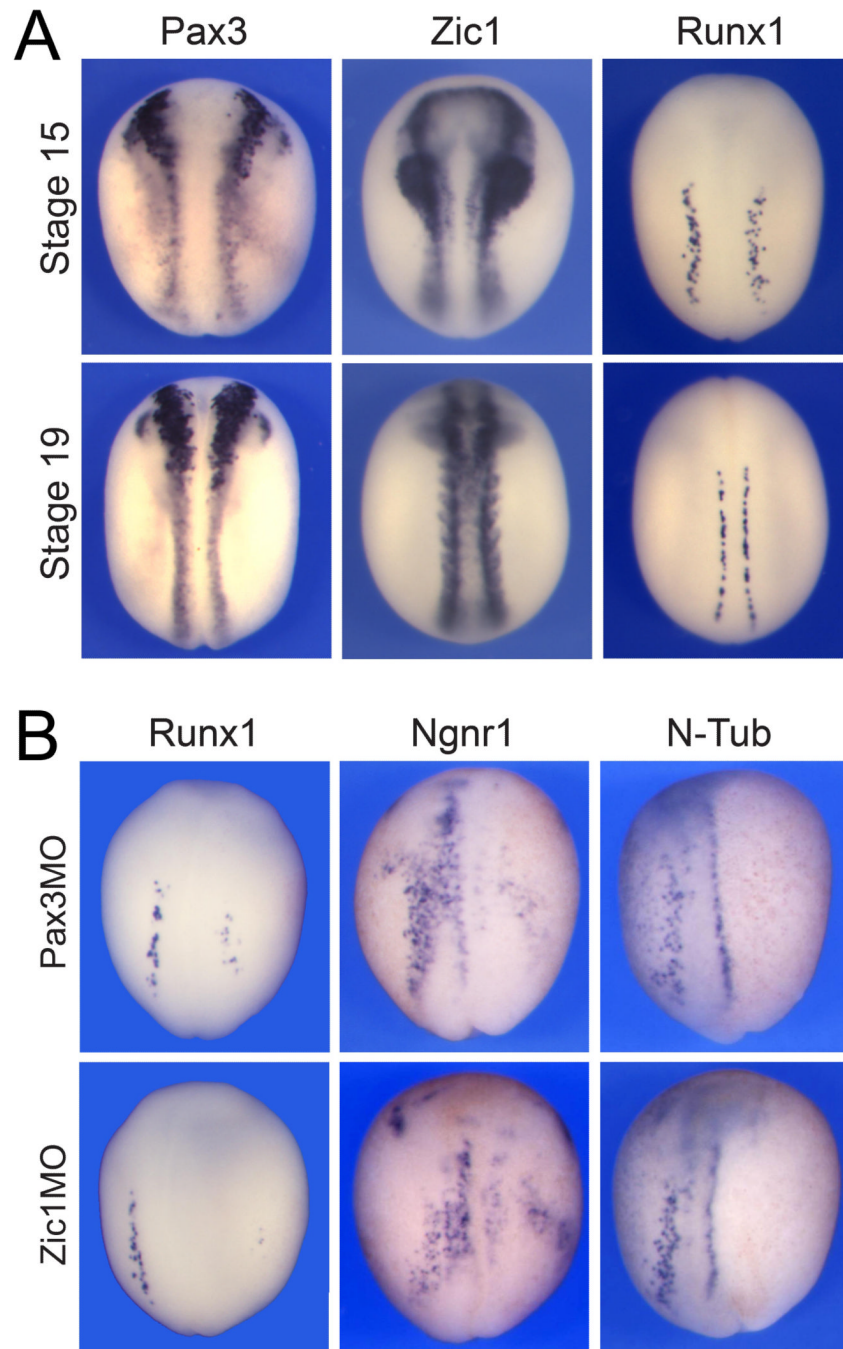


Figure 4. *Runx1* expression in RB sensory neurons depends on Pax3 and Zic1
 (A) Comparative expression of *Pax3*, *Zic1* and *Runx1* in stage-matched embryos indicate that the *Runx1* expression domain overlaps with that of these two NPB specifiers. (B) Embryos injected with Pax3MO (50 ng) or Zic1MO (45 ng) in one blastomere at the 2-cell stage exhibit a strong reduction of *Runx1* as well as *N-Tub* and *Ngnr1* expression in RB progenitors. The injected side is on the right. In all panels embryos are viewed from the dorsal side, anterior to top.

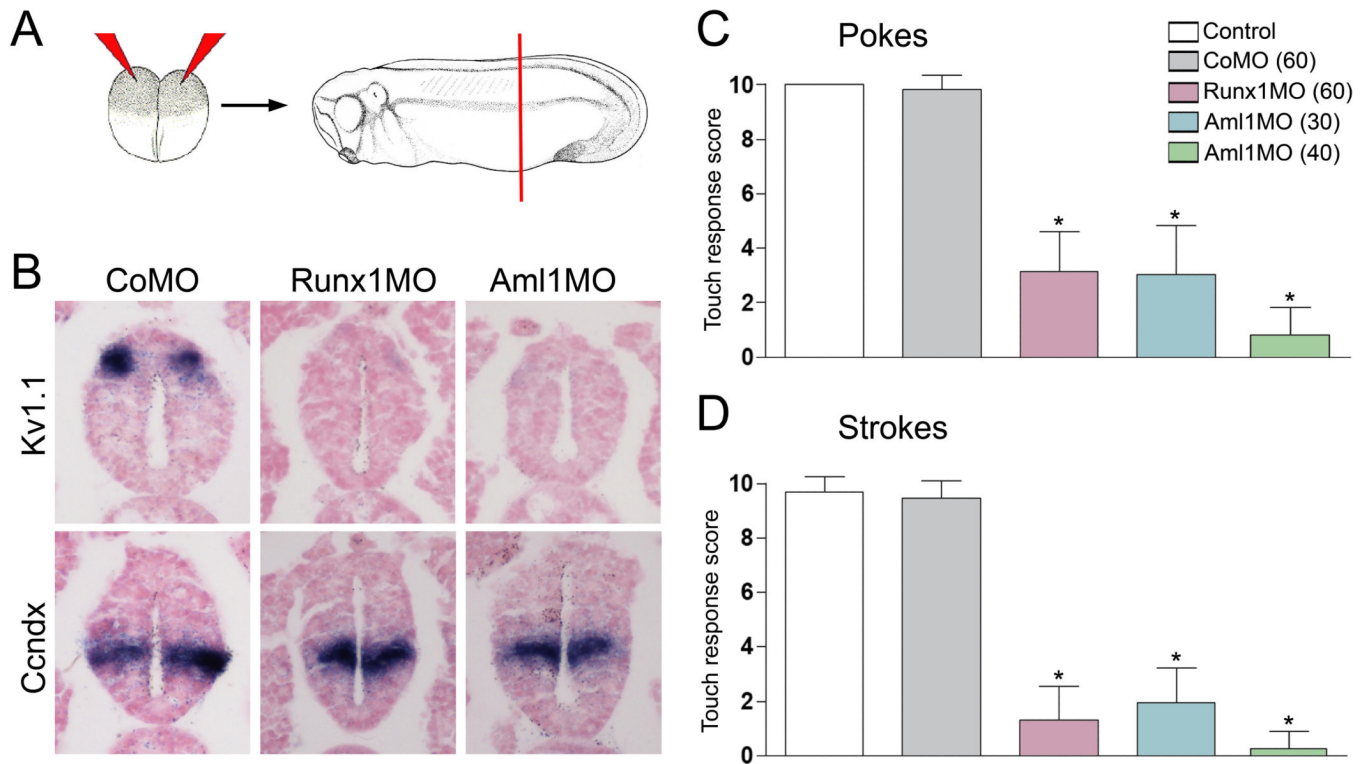


Figure 5. Runx1-deficient tadpoles lack Rohon-Beard sensory neurons and lose response to touch

(A) Two-cell stage embryos received a bilateral injection of control (CoMO), Runx1 (Runx1MO) or Aml1 (Aml1MO) morpholino antisense oligonucleotides. At stage 28 the corresponding embryos were sectioned in the trunk region (red line) and analyzed by in situ hybridization. (B) Expression of Rohon-Beard (Kv1.1) and motor neuron (Ccndx) marker genes in the spinal cord of morphant embryos. Runx1MO and Aml1MO show a loss of Kv1.1 expression, while the ventral motor neurons are largely unaffected. (C–D) At stage 32 Runx1MO and Aml1MO injected embryos have a severely reduced response to touch (pokes and strokes) as compared to control uninjected or control morpholino (CoMO) injected embryos. (C) Pokes: Control (uninjected), 10.00 ± 0.00 (n=30); CoMO (60 ng), 9.83 ± 0.53 (n=30); Runx1MO (60 ng), 3.14 ± 1.45 (n=28); Aml1MO (30 ng), 3.03 ± 1.82 (n=20); Aml1MO (40 ng), 1.00 ± 1.19 (n=15). (D) Strokes: Control (uninjected), 9.68 ± 0.56 (n=30); CoMO (60 ng), 9.47 ± 0.63 (n=30); Runx1 MO (60 ng), 1.30 ± 1.26 (n=28); Aml1MO (30 ng), 1.95 ± 1.28 (n=20); Aml1MO (40 ng), 0.33 ± 0.75 (n=15). Statistical significance was determined using one-way ANOVA. The values are presented as mean SEM; * = $P < 0.0001$, versus Control and CoMO).

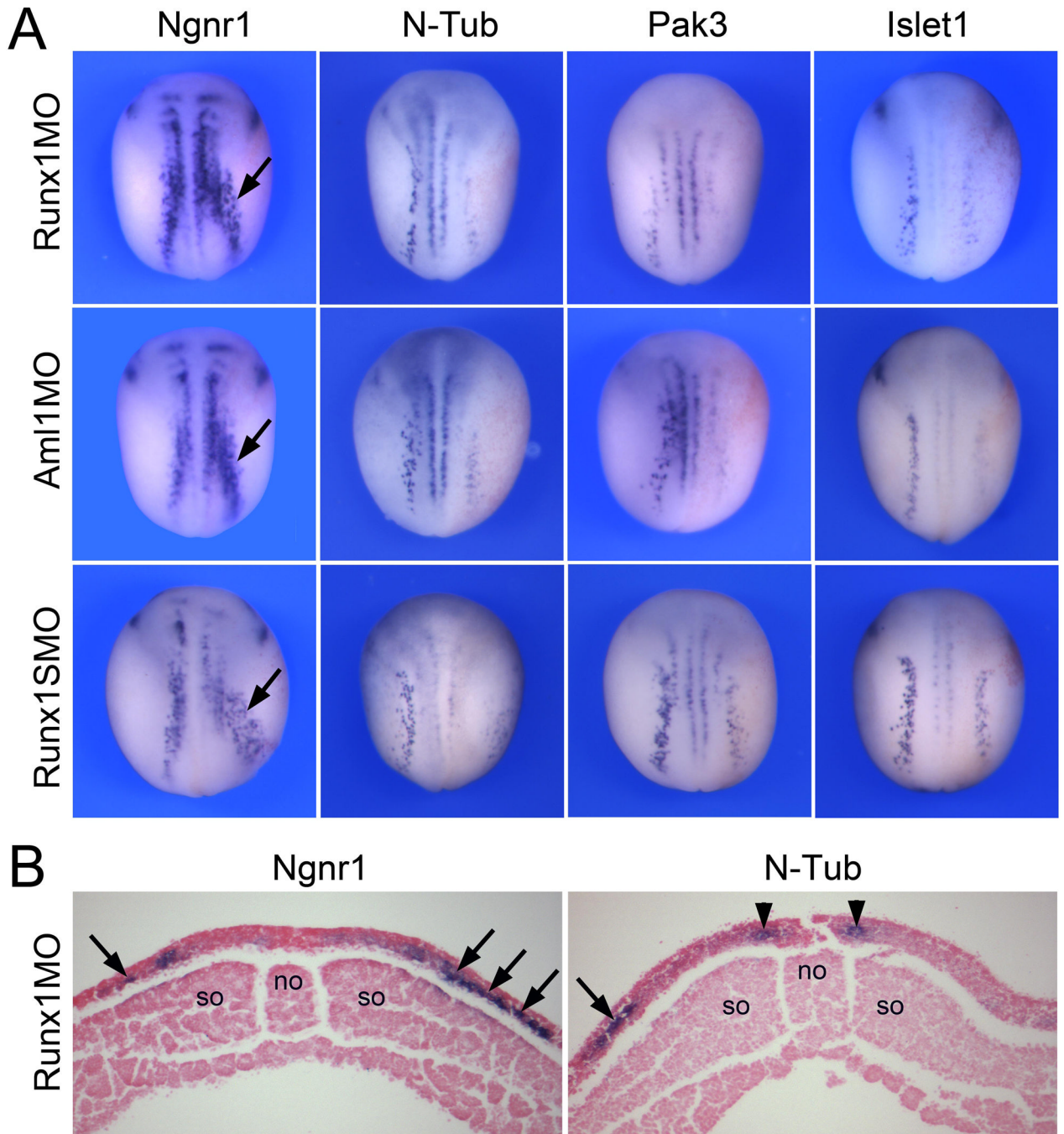


Figure 6. Runx1-deficient embryos downregulate *N-Tub*, *Pak3* and *Islet1*

(A) Embryos were injected in one blastomere at the 2-cell stage with Runx1MO, Aml1MO or Runx1SMO and analyzed at stage 15 for the expression of *Ngnr1*, *N-Tub*, *Pax3* and *Islet1*. The RB expression domain of *N-Tub*, *Pak3* and *Islet1* is reduced while *Ngnr1* expression is expanded (arrows). In all panels the injected side is on the right. Dorsal view anterior to top. (B) Transverse sections of representative Runx1MO-injected embryos. The expression of *N-Tub* is lost in RB progenitors while *N-Tub* expression in primary motor neurons precursors is unaffected (arrow heads). *Ngnr1* expression is expanded (arrows). The injected side is on the right. Dorsal to top. no, notochord; so, somites.

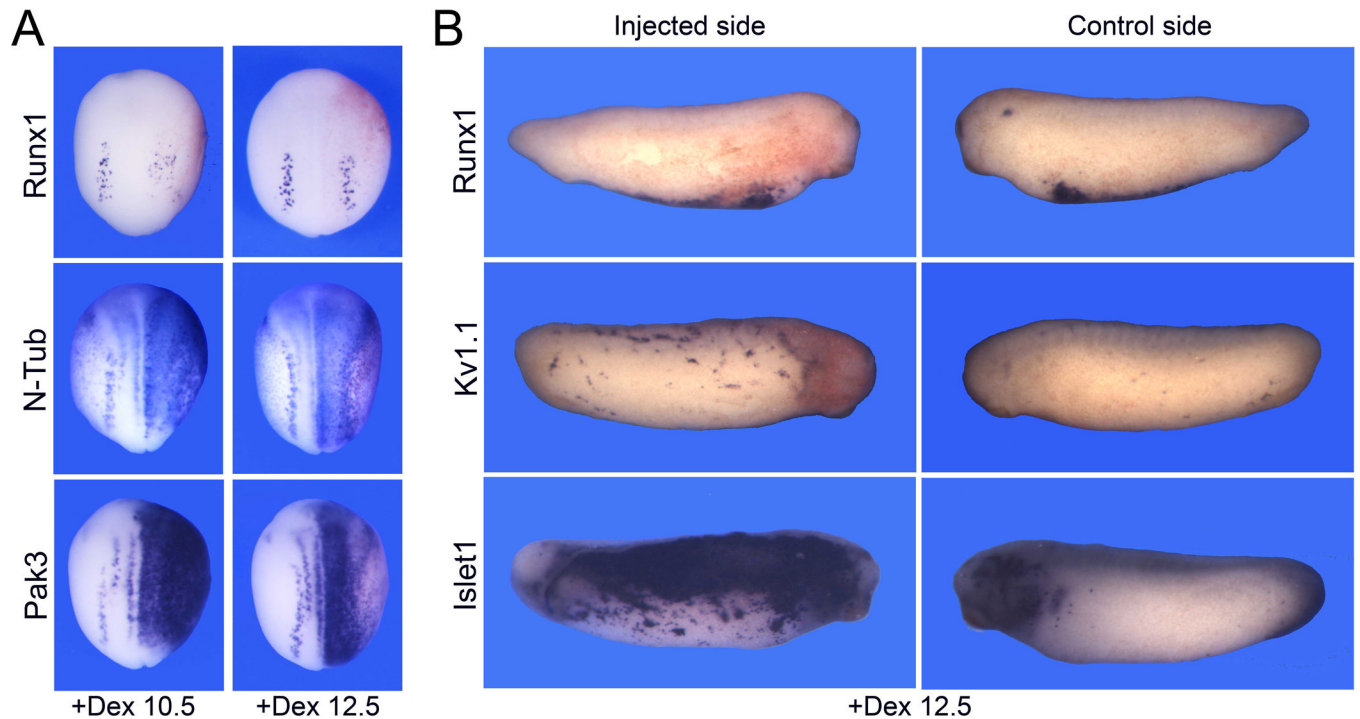


Figure 7. *Ngn1* expression is not sufficient to activate *Runx1* expression

(A) Embryos at the 2-cell stage were injected in one blastomere with 0.5 ng of *Ngn1*-GR mRNA. Embryos were subsequently incubated with dexamethasone at early (+Dex 10.5) or late (+Dex 12.5) gastrula stages, and fixed at stage 15 for detection of *Runx1*, *N-Tub* or *Pak3* by whole mount in situ hybridization. *N-Tub* and *Pak3* are dramatically upregulated while the *Runx1* expression domain is only marginally affected. The injected side is on the right. Dorsal view anterior to top. (B) At the tailbud stage (stage 27) these embryos show ectopic *Kv1.1* and *Islet1* expression in the ectoderm, independently of any upregulation of *Runx1*. Lateral views dorsal to top. Control and injected sides of the same embryo are shown for comparison.

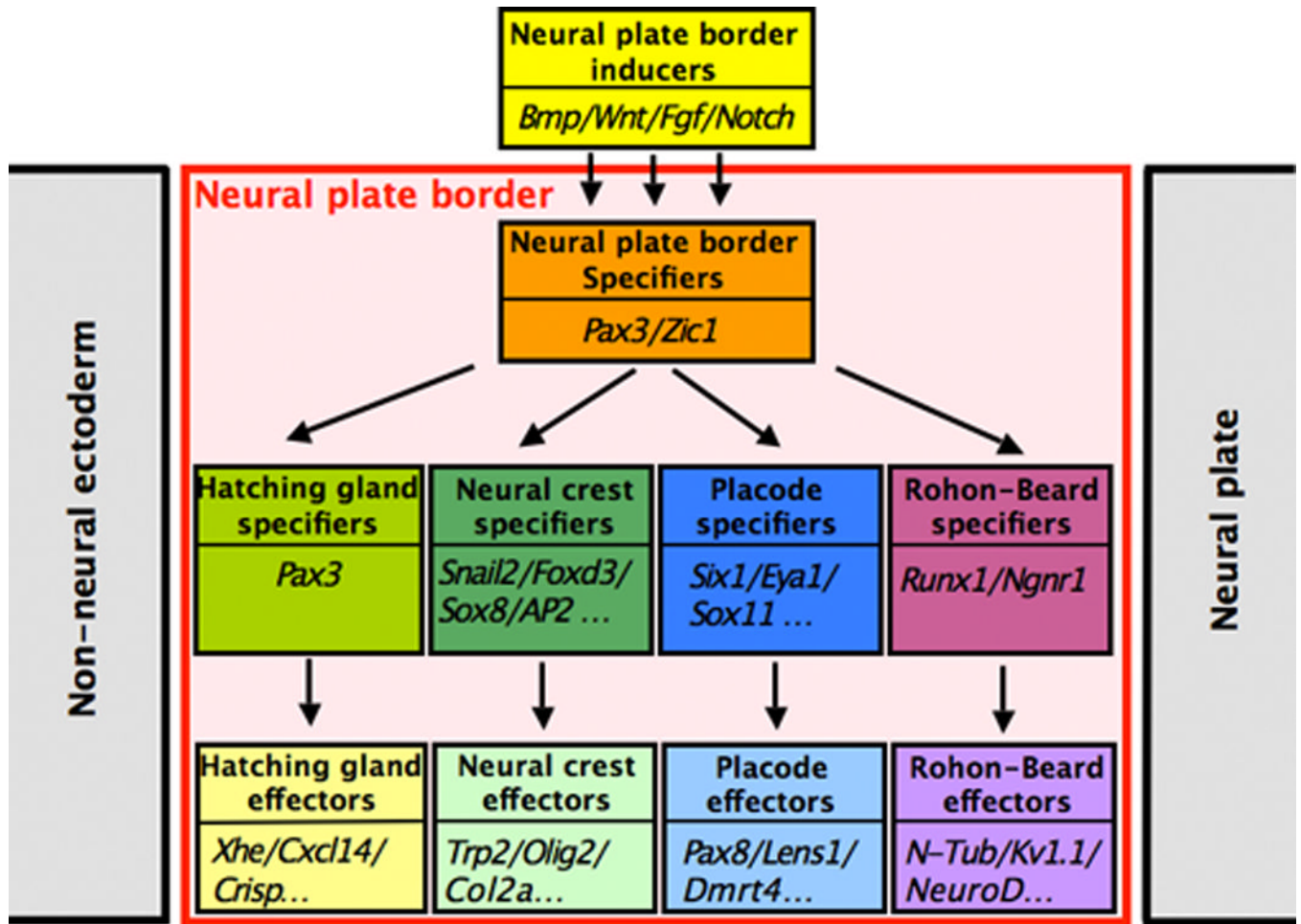


Figure 8. Model of the gene regulatory network regulating cell fate at the neural plate border
 This model is an extension of the model proposed by Meulemans and Bronner-Fraser (2004) and Litsiou et al., (2005) for NC and PE specification. Based on our current observations and other studies (Hong and Saint-Jeannet, 2007) this regulatory cascade has been expanded to include two additional NPB cell types, RB neurons and HG cells.

Table 1

Regulation of Runx1 expression by Fgf, Wnt and Notch signaling

Injection	Probe	N	Normal	Reduced/Lost	Expanded/Ectopic
Fgf8a	Runx1	52	0%	0%	100%
	Ngnr1	57	2%	2%	96%
	N-Tub	45	9%	7%	84%
Fgf8MO	Runx1	76	15%	84%	1%
	Ngnr1	47	34%	66%	0%
	N-Tub	43	10%	88%	2%
Wnt8MO	Runx1	51	4%	96%	0%
	Ngnr1	53	11%	89%	0%
	N-Tub	52	2%	98%	0%
β-CatMO	Runx1	32	0%	100%	0%
	Ngnr1	26	0%	100%	0%
	N-Tub	29	0%	100%	0%
Notch ICD	Runx1	37	0%	100%	0%
	Ngnr1	33	6%	73%	21%
	N-Tub	42	0%	100%	0%

Table 2Pax3 and Zic1 regulates *Runx1* expression at the NPB

Injection	Probe	N	Normal	Reduced/Lost	Expanded/Ectopic
Pax3MO	Runx1	48	6%	94%	0%
	Ngnr1	47	21%	79%	0%
	N-Tub	44	7%	93%	0%
Zic1MO	Runx1	56	4%	96%	0%
	Ngnr1	52	16%	67%	17%
	N-Tub	39	5%	95%	0%

Table 3

Summary of Runx1 knockdown phenotype at the neurula stage

Injection	Probe	N	Normal	Reduced/Lost	Expanded/Ectopic
Aml1MO	N-Tub	78	13%	87%	0%
	Pak3	80	29%	70%	1%
	Islet1	37	22%	78%	0%
	Ngnr1	81	42%	14%	44%
Runx1MO	N-Tub	70	20%	79%	1%
	Pak3	40	33%	65%	2%
	Islet1	35	31%	69%	0%
	Ngnr1	73	42%	0%	57%
Runx1SMO	N-Tub	41	17%	83%	0%
	Pak3	46	24%	76%	0%
	Islet1	45	20%	80%	0%
	Ngnr1	45	58%	9%	33%

Table 4

Summary of the phenotype of Ngnr1 misexpression

Injection	Probe	N	Normal	Reduced/Lost	Expanded/Ectopic
Ngnr1-GR +Dex 10.5 (Stage 15)	Snail2	32	6%	94%	0%
	Runx1	69	7%	86%	7%
	N-Tub Pak3	88 65	0% 0%	0% 0%	100% 100%
Ngnr1-GR +Dex 12.5 (Stage 15)	Snail2	27	0%	100%	0%
	Runx1	55	66%	7%	27%
	N-Tub Pak3	62 62	0% 0%	3% 0%	97% 100%
Ngnr1-GR +Dex 12.5 (Stage 27)	Runx1	38	100%	0%	0%
	Kv1.1	44	9%	0%	91%
	Islet1	36	0%	0%	100%