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Transcriptional control of Rohon-Beard sensory neuron development at the neural plate border

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Abstract

Rohon-Beard (RB) mechanosensory neurons are among the first sensory neurons to develop, and the process by which they adopt their fate is not completely understood. RBs form at the neural plate border (NPB), the junction between neural and epidermal ectoderm, and require the transcription factor *prdm1a*. Here, we show that prior to RB differentiation, *prdm1a* overlaps extensively with the epidermal marker *dlx3b* but shows little overlap with the neuroectodermal markers *sox3* and *sox19a*. Birthdating analysis reveals that the majority of RBs are born during gastrulation in zebrafish, suggesting that it is during this period that RBs become specified. Expression analysis in *prdm1a* and *neurogenin1* mutant and *dlx3b/dlx4b* morpholino-injected embryos suggests that *prdm1a* is upstream of *dlx3b*, *dlx4b*, and *neurogenin1* at the NPB. mRNA for *neurogenin1* or *dlx3b/dlx4b* can rescue the lack of RBs in *prdm1a*. Based on these data we suggest a preliminary gene regulatory network for RBs development.

Keywords

Rohon-Beard sensory neurons; *prdm1a*; *dlx3b*; *dlx4b*; *neurogenin1*

Introduction

Understanding the process by which cells adopt different fates within the nervous system is a fundamental question of developmental neuroscience. While many neurons share similarities in their development, the series of molecular events that comprise this process is largely different for each type of neuron. Rohon-Beard (RB) sensory neurons are a unique population of neurons that exist in the developing embryos of anamniotes and contribute importantly to the embryonic nervous system. RBs innervate the skin and are the earliest mechanism by which embryos of species such as zebrafish and *Xenopus* detect information about their environment. When their nerve endings are activated by light touch, the embryo contracts muscles on the opposite side of its body to flex the trunk away from the stimulus and swim in the opposite direction (Roberts, 2000). Although the cell bodies of most sensory neurons are located in the peripheral nervous system, the cell bodies of RB sensory neurons lie within the dorsal region of the embryonic spinal cord. Because these neurons are the first mechanosensory neurons to function within the embryo, the molecules involved in their development are among the earliest to play a role in cell fate specification of a neuronal population.

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RBs form at the neural plate border (NPB), the region of the ectoderm that lies between the neural plate and the epidermal ectoderm prior to neurulation. Previous studies demonstrate that interactions between these tissues are sufficient for RB formation. In addition, a small number of genes known to be important for RB development are expressed at the NPB. For example, an intermediate level of BMP signaling that occurs at the NPB is important for development of these neurons (Neave et al., 1997; Nguyen et al., 2000; Rossi et al., 2008). *prdm1a*, a transcription factor that is downstream of BMP signaling and is expressed at the NPB, is also required for development of RBs. Zebrafish embryos with a mutation in *prdm1a*, known as *narrowminded* mutants, fail to develop RBs or display an escape response when touched on the trunk (Artinger et al., 1999; Hernandez-Lagunas et al., 2005). The predicted Prdm1a protein in *narrowminded* mutants contains no DNA binding domain, thus preventing the ability of the protein to act as a transcription factor. While it is clear that *prdm1a* is required for the development of RBs, the molecules that act downstream of *prdm1a* in RB development are still unknown.

prdm1a expression at the NPB precedes markers of differentiated RBs at the NPB, the latter of which are first evident at about 11 hpf. Understanding the role of *prdm1a* and other factors important for cell fate specification at the NPB requires an understanding of the timing with which different NPB fates are adopted. RBs can be “birthdated” due to the fact that neurons cease dividing before they become fully differentiated. Early experiments by Lamborghini (1980) found that in *Xenopus*, the majority of RBs are born by the end of gastrulation. These experiments monitored incorporation of a radioactive nucleotide at progressive stages of gastrulation to identify when different sets of neurons were born in the embryo. Birthdating RBs in zebrafish has yet to be investigated, and determining when RBs undergo their final cell division will be important for identifying the developmental period during which these neurons acquire their fate.

In addition to *prdm1a*, other transcription factors that are expressed at the NPB are also required for the development of RB neurons. For example, the homeodomain-containing transcription factors *dlx3b* and *dlx4b* are expressed in overlapping domains at the NPB in zebrafish embryos (Ellies et al., 1997) and act redundantly in the development of RBs. Preventing translation of a functional protein product for either of these genes using antisense morpholinos has little effect on RB development, but injecting morpholinos for both genes together causes a reduction or loss of RBs at the NPB (Kaji and Artinger, 2004). It is not yet known whether these transcription factors work within the same pathway as *prdm1a* in RB development. Additionally, some transcription factors are known to be required for development of some but not all RBs, such as *tfap2a* and *tfap2c*, which are required for a subset of RBs to develop (Li and Cornell, 2007). Further, *olig4* appears to negatively regulate RBs, and morpholino against this transcription factor results in an increase of RBs (Filippi et al., 2005).

The bHLH transcription factor *neurogenin1* begins to be expressed in a domain suggestive of a potential function in RB development slightly later than *dlx3b* and *dlx4b* and well after the initiation of *prdm1a* expression. At 3 somites, *neurogenin1* is expressed in solid clusters of highly expressing cells in 3 stripes, the lateral most of which corresponds to the NPB (Blader et al., 1997). Loss of function of this gene causes a decrease of RBs (Cornell and Eisen, 2002). When transgenic zebrafish expressing GFP under control of the complete *neurogenin1* regulatory domain are crossed with *prdm1a* mutants, the GFP expression is reduced at the NPB (Blader P, 2003), suggesting that *prdm1a* may regulate *neurogenin1* expression. *neuroD* has been described to be a potent activator of neuronal fate, in that it can convert *Xenopus* ectoderm into neurons (Lee et al, 1995). In zebrafish, *neuroD* is expressed in a subset of primary neurons, including RB neurons, and is a necessary step for neuronal differentiation (Korzh et al, 1998).

To address the question of early cell fate decisions at the NPB with regards to RB development, we characterized gene expression at the neural plate border and examined transcriptional pathways required in RB specification. We examined *prdm1a* expression with relation to neural and epidermal markers, and found extensive overlap with the epidermal marker *dlx3b*, less overlap with a marker of the neural ectoderm, *sox3*, and no overlap with neural *sox19a*. Birthdating analysis revealed that the majority of RBs undergo their final cell division in zebrafish during gastrulation stages when the neural plate border is present within the embryo, similar to what is observed in *Xenopus*. In order to place *prdm1a* in a molecular pathway, we examined gene expression in *prdm1a* mutants during these gastrula stages. *dlx3b* was previously shown to be down-regulated in *prdm1a* mutants, and we found that expression of the closely related gene *dlx4b* is also decreased in *prdm1a*-deficient embryos. Importantly, mRNA for *dlx3b* and *dlx4b* can rescue the lack of RBs in *prdm1a* mutant embryos. Similarly, we found that *neurogenin1* expression is decreased or absent at the NPB in *prdm1a* mutants and that *neurogenin1* mRNA can rescue the lack of RBs in *prdm1a* mutant embryos. Thus we propose here a gene regulatory network (GRN) for RB development where both *dlx3b/4b* as well as *neurogenin1* and *neuroD* are downstream of *prdm1a* in RB formation.

Results

Gene expression profiles during early NPB formation

As a first step to investigating the NPB in detail as it forms, we examined gene expression at the NPB and in the two surrounding tissues, neural and epidermal ectoderm, during the stages when the neural plate is forming. *prdm1a* expression commences at the NPB at 70% epiboly (7.7 hpf; Wilm and Solnica-Krezel, 2005), and thus we began our analysis at this stage. Using *in situ* hybridization, we investigated expression of *dlx3b* (the epidermal side of the NPB, Artinger et al., 1999), *prdm1a* (neural plate border, Hernandez-Lagunas et al., 2005); and *gbx1* (neural tissue, Rhinn et al., 2003). While expression patterns of some of these genes at select stages had already been published, a comprehensive analysis of genes at both sides of the NPB and the NPB itself had not yet been compiled and provides a necessary framework from which to consider the transcriptional regulation of RB development at the NPB.

dlx3b transcripts were found throughout the epidermal ectoderm in the ventral embryo spanning 70% epiboly through 90% epiboly (Figure 1B-D). Over time, the domain of *dlx3b* expression narrowed to include only a discrete region that abuts the NPB but appeared to remain within the epidermal ectoderm (Figure 1 E-F). By the 2 somite stage, *dlx3b* expression was primarily evident in the anterior non-neural ectodermal region of the embryo, rather than spanning the anterior-posterior axis as was observed at tail bud stage. During the stages examined here (70% epiboly through 2 somites), there is an overall movement of cells toward the dorsal midline (Kimmel et al., 1995), and the domain of *dlx3b* expression also followed this pattern by shifting dorsally over time.

prdm1a is expressed at the NPB by 70% epiboly in a broad domain that continues through the next two stages, 80% and 90% epiboly (Figure 1 G-I and Wilm and Solnica-Krezel, 2005). Beginning at tail bud, the domain of *prdm1a* expression at the NPB narrows (Figure 1J-K). Again as cells move toward the dorsal midline during these stages, the domain of *prdm1a* expression at the NPB also shifts dorsally over time. *prdm1a* is also highly expressed in the anteriorly-located prechordal plate during these stages.

The neural marker *gbx1* was also examined to mark neural plate tissue during early neural plate and NPB development. Its expression domain was in the dorsal, neural region of the embryo during each stage examined (Figure 1L-P). At early NPB stages, neural *gbx1*

expression was fairly close to the embryonic margin, which lies in the future posterior region of the embryo. Its expression moved toward the anterior region at later developmental stages. *gbx1* plays an early role in positioning the midbrain-hindbrain boundary during gastrula stages (Rhinn et al., 2003), so its expression can be thought of as a marker of the posterior region of the future cranial neural plate.

Taken together, these expression profiles provide an important foundation for studying the normal development of NPB and NPB cell types. It is important to compile these profiles in order to determine which genes potentially overlap, which will be further examined in the next section, so that we can begin to dissect the GRN for RB development.

prdm1a* at the NPB overlaps with *dlx3b* but shows little overlap with *sox3

Examination of the expression pattern of *prdm1a* relative to markers of the epidermal ectoderm suggests that it may partially overlap with markers of the surrounding tissues. Determining whether *prdm1a* expression at the NPB overlaps with that of markers of the surrounding tissues is key to understanding the tissue contributions to the NPB ectoderm. To investigate the relationship of *prdm1a* expression to genes expressed in the surrounding tissues, double fluorescent *in situ* hybridization was performed to reveal expression patterns of two genes within single embryos. Probes were developed to emit fluorescent signals at two different wavelengths so that their expression patterns could be compared with one another using confocal microscopy (Pineda, et al, 2006).

We observed that at 80% and 90% epiboly, almost the entire *prdm1a* expression domain (Figure 2A, yellow region, and not shown) overlapped with *dlx3b* expression. Because *dlx3b* is expressed throughout the epidermal domain at these stages, its expression domain was much broader than that of *prdm1a*. In subsequent stages, some overlap between *prdm1a* and *dlx3b* was observed, but the expression domains were mostly separate from one another, with *prdm1a* expressed more dorsally than *dlx3b* (Figure 2B-C'). These results indicate that during the earliest stages of NPB development, there is a significant overlap in the expression domains of *dlx3b* and *prdm1a*, but that expression of the two transcription factors segregates by later developmental stages such that *dlx3b* is only expressed in non-neural ectoderm and *prdm1a* continues to be expressed at the NPB.

To determine whether *prdm1a* expression appears in the neuroectoderm during gastrulation, *prdm1a* expression was also compared to those of the neural markers *sox3* and *sox19a* between 80% epiboly and 2 somites. *sox19a* is a fish-specific gene that has widespread expression in the early neuroectoderm, resembling the amniote expression pattern of *sox2*. In zebrafish, *sox2* expression is restricted to the anterior-most region of the embryo rather than being expressed throughout the neural tissue. At mid-gastrulation, *sox3* is expressed in the presumptive forebrain, hindbrain, and spinal cord and is stronger than that of *sox19a* (Okuda et al., 2006). At 90% epiboly, a small amount of overlap was observed between the ventralmost cells in the *sox3* domain and the dorsalmost cells in the *prdm1a* domain (Figure 2D). At tail bud and 2 somites, the domain of *prdm1a* abutted *sox19a* expression but no overlap between these genes was observed (Figure 2E-F'). Examination of the domains at higher magnification confirms the apparent lack of overlap between *prdm1a* and *sox19a* (Figure 2E', F'). This suggests that unlike the overlap with the *dlx3b* in the non-neural ectoderm, *prdm1a* expression is mostly excluded from the neuroectoderm during these stages. Taken together, these expression profiles surprisingly indicate that the NPB expression of *prdm1a* is separate from the neuroectoderm *sox3/19a* expression domain, but not from the *dlx3b* expressing non-neural ectoderm indicating that the NPB primarily consists of non-neural ectoderm. Thus, these data suggest that by the end of gastrulation, the NPB is separate from both non-neural and neural ectoderm and could be viewed as a third division of embryonic ectoderm.

RBs are born during gastrulation in zebrafish

Our investigation of RB development focused on gastrulating embryos due to evidence that these are the stages when the NPB, from which RBs are known to arise, initially forms in zebrafish embryos and the stages during which these neurons are born in *Xenopus*. Further, *prdm1a* is expressed during gastrulation, suggesting that gastrula stages define the developmental window during which RB development initially occurs. In *Xenopus*, the majority of RBs are born during gastrulation (Lamborghini, 1980), and the same has been assumed to be true in zebrafish. To test this assumption, we grew zebrafish embryos that express green fluorescent protein in differentiated RBs (*Tg(neurogenin1:GFP)* embryos; Blader et al, 2003) in embryo media containing BrdU commencing at varying times and extending until 24 hpf, when embryos were fixed and RBs were examined for incorporation of BrdU using confocal microscopy. By labeling during blastula stages when most cells are still dividing, we found that 84% of the RB precursors are incorporating BrdU and co-expressed GFP, suggesting that the majority of the RB precursors were born between blastula and 24 hpf (Figure 3A). Similarly, exposing embryos to BrdU at 50% epiboly (the beginning of gastrulation), resulted in BrdU incorporation in 81% of the RB precursors expressing GFP (Figure 3B). However, with BrdU exposure commencing at the end of gastrulation, tailbud stage, only 34% of RB neurons labeled, while BrdU treatment from the 2 somite stage onward labeled only 19% of RB precursors (Figure 3C,D; Table 1). These results suggest that most of the RBs, 81%, are born by the 2 somite stage and begin to undergo a terminal differentiation step at this stage. These findings demonstrate that the majority of RBs undergo their terminal cell division during gastrula stages in zebrafish.

dlx3b and *dlx4b* act downstream of *prdm1a* in RB development

Having determined that the majority of RBs are born during gastrula stages in zebrafish, we next wanted to determine the molecular mechanism by which these cells develop at the NPB. Because *prdm1a* is required for RB development, we analyzed gene expression in *prdm1a* mutant embryos in an effort to define other genes that act within the same pathway as *prdm1a* in RB development.

Previous analysis of *prdm1a* mutants suggest that *prdm1a* may regulate expression of *dlx3b* (Artinger et al., 1999), and that both *dlx3b* and the closely related transcription factor *dlx4b* are required for proper RB development (Kaji and Artinger, 2004). In about a quarter of embryos obtained from heterozygous *prdm1a* parents, *dlx3b* expression in the posterior NPB was decreased at tail bud stage (n = 10/45, 22%, Figure 4A,B; Table 2). This decrease was also evident when wild type embryos were injected with a splice blocking morpholino to *prdm1a* (n=6/8). The ability of this morpholino to replicate the findings observed in *prdm1a* mutants has been previously demonstrated by our lab (Hernandez-Lagunas et al., 2005), and this was confirmed by an absence of *HuC* mRNA at the NPB (Figure 4E, F). The motor neuron domain is out of focus in these images, but is still present in the morphant embryos.

dlx4b, formerly called *dlx7*, lies on the same chromosome as *dlx3b* and the two genes are thought to be linked in zebrafish as well as mammals (Ellies et al., 1997). These two genes are expressed at the same location across developmental stages in zebrafish (Ellies et al., 1997), so we expected that *dlx4b* expression would be decreased at the NPB in *prdm1a* morpholino-injected embryos. Indeed, *dlx4b* expression was detectable at the NPB in uninjected or control morpholino-injected embryos at tail bud (arrow in Figure 4C), but not in *prdm1a*-morpholino injected embryos (18/19; arrow in Figure 4D). Similar results were found in 2 somite-stage embryos (6/9 embryo displayed a decrease in *dlx4b* at the NPB, not shown).

Often in development, pairs or groups of transcription factors regulate expression of one another, creating a feedback loop in transcriptional pathways. To determine whether this occurs between the *dlx* genes and *prdm1a* at the NPB, we also examined expression of *prdm1a* in embryos in which splicing of Dlx3b and Dlx4b proteins was interrupted. Splice blocking morpholinos for *dlx3b* and *dlx4b* have previously been shown to effectively block formation of their functional protein products (Kaji and Artinger, 2004). Expression of *prdm1a* appeared normal in *dlx3b* and *dlx4b* morpholino injected embryos at both 90% epiboly (Figure 4G, H) and tail bud (Figure 4I, J).

These findings suggest that transcriptional regulation of *dlx3b* and *dlx4b* by *prdm1a* at the NPB is unidirectional. This, in combination with previous findings indicating a requirement for *dlx3b* and *dlx4b* in RB development (Kaji and Artinger, 2004), suggests that *prdm1a* is required upstream of *dlx3b* and *dlx4b* in the development of RBs at the NPB. However, additional experiments were required to test this functional relationship.

Because *dlx3b* and *dlx4b* appear to act downstream of *prdm1a* in the formation of RBs, we predicted that mRNA for these *dlx* genes would be able to rescue the lack of RBs in *prdm1a* mutants. In order to test this, 75-100 pg *dlx3b* and *dlx4b* mRNA was injected into embryos from *prdm1a* heterozygous parents. Embryos were stained at the 2-3 somite stage for *HuC* mRNA, and the number of *HuC* positive neurons at the NPB (presumptive RBs) was blindly scored. To determine which of the embryos were homozygous mutants, each embryo was subsequently genotyped. In homozygous *prdm1a* mutants that were not injected or were injected with mRNA for GFP as a control, typically no *HuC* positive cells were observed at the NPB (Figure 5A, B; 9/10 and 5/5 respectively). On the other hand, in six out of seventeen homozygous mutants injected with mRNA for *dlx3b* and *dlx4b*, *HuC* positive cells were observed at the NPB (Figure 5D; arrows). The number of these *HuC* positive neurons ranged from two to thirteen on one side of the embryo, compared to an average of 9.7 RBs per side in heterozygous and wild type embryos. These data provide further evidence that *dlx3b* and *dlx4b* act downstream of *prdm1a* in the development of RBs at the NPB.

***neurogenin1* acts downstream of *prdm1a* in RB development**

neurogenin1 is known to be required for RB development, and is expressed in the RB-forming NPB (Blader et al., 1997). Previous findings suggest that *neurogenin1* may be regulated by *prdm1a*; when transgenic zebrafish expressing GFP under control of the complete *neurogenin1* regulatory domain were crossed with *prdm1a* mutants, the embryos displayed a reduction in GFP expression at the NPB (Blader P, 2003). We tested this by examining expression of *neurogenin1* in *prdm1a* mutant embryos. When *prdm1a* heterozygous parents were crossed, a reduction of *neurogenin1* was observed in approximately a quarter of the embryos. At both tail bud (Figure 6A,B) and 2 somites (Figure 6C,D; Table 2), the lateral stripe that corresponds to the NPB was either reduced or absent (12/52 and 11/43, respectively; arrows in Figure 6A-D). PCR genotyping of individual embryos lacking the lateral stripe of *neurogenin1* expression confirmed that the embryos displaying this reduction were *prdm1a* $-/-$.

Conversely, when *prdm1a* expression was analyzed in *neurogenin1* mutant embryos, no changes in its expression were observed. No differences in expression were observed at 80% epiboly (n=24), 90% epiboly (n=24), tail bud (Figure 6E; n=21), or 2 somites (Figure 6F, n=19). This suggests that *neurogenin1* functions downstream of *prdm1a* at the NPB. To test whether *neurogenin1* mRNA can rescue the lack of RBs in *prdm1a* mutants, we injected 100 pg *neurogenin1* mRNA into progeny of *prdm1a* heterozygous parents. After staining these embryos for *HuC* and counting the presumptive RBs in these embryos, they were individually genotyped as described above. In seven out of fourteen homozygous mutant

embryos, a partial or full rescue of RBs was observed at the NPB, with the number of RBs on one side of the embryo ranging from 3 to 28 (Figure 7, white arrows in C). In addition, we noticed an increase in the number of *HuC* positive presumptive RBs at the NPB in some wild type embryos injected with *neurogenin1* mRNA compared to uninjected controls (data not shown), although this result was not consistent across experiments.

Because both *dlx3b/4b* and *neurogenin1* appear to act downstream of *prdm1a* in the specification of RB neurons, we tested whether these transcription factors regulate transcription within the same pathway. No change in *dlx3b* expression was observed in *neurogenin1* mutant embryos, suggesting that *dlx3b* does not act downstream of *neurogenin1* (Figure 8C,D). Similarly, embryos injected with *dlx3b* and *dlx4b* morpholino displayed no change in *neurogenin1* expression in the trunk region at tail bud (Figure 8A,B) or 3 somites (Kaji and Artinger, 2004). This suggests that transcriptional regulation of *neurogenin1* and the *dlx* genes by *prdm1a* is initially in separate pathways. To further explore this, we injected a small number of *neurogenin1* mutant embryos with 75pg *dlx3b* and *dlx4b* mRNA and asked whether RB neurons were rescued in these embryos. We observed no rescue of RBs (n=4, and data not shown), in contrast to the results obtained when mRNA for these *dlx* genes was injected in to *prdm1a* mutant embryos. These results support our hypothesis that *neurogenin1* and *dlx3b/dlx4b* act in separate pathways in RB development. We were unable to perform the converse experiment, to ask whether *neurogenin1* mRNA rescues RBs in *dlx3b/dlx4b* morpholino-injected embryos, because the volume of morpholino and mRNA required for this experiment would be lethal to the embryos.

We also examined expression of *neuroD*, a transcription factor downstream of *neurogenin1* at the NPB (Cornell and Eisen, 2002), in *prdm1a* mutants and in wild type embryos injected with *dlx3b* and *dlx4b* morpholino. We found that in *prdm1a* mutants, *neuroD* expression was decreased at the NPB at 3 somites (7/22; Figure 8G, H). Further, in *dlx3b/dlx4b* morphants, *neuroD* was reduced or absent at the NPB at 3 somites (Figure 8E,F). These data in combination demonstrate that *neuroD* expression is downstream of *prdm1a*, *dlx3b/4b*, and *neurogenin1* and suggest a possible mechanism by which *dlx3b/dlx4b* and *neurogenin1* may converge in RB development.

Discussion

The data presented here reveal the expression of *prdm1a* with relation to neural and epidermal markers, and help elucidate the function of *prdm1a* in RB specification in gastrulating zebrafish embryos. We show that *prdm1a* overlaps to a larger extent with non-neural ectoderm expression of *dlx3b* than with neural markers at the developing NPB, and that the majority of RBs are born during these gastrula stages. *prdm1a* was shown to act upstream of *dlx3b* and *dlx4b*, two transcription factors previously found to be required for RB development. Similarly, *prdm1a* appears to regulate transcription of *neurogenin1* at the NPB. mRNA for *dlx3b* and *dlx4b* or *neurogenin1* can partially rescue the presumptive RBs at the NPB.

RBs are born during gastrulation in zebrafish

Our birthdating results closely resemble previous findings using ³H-thymidine in *Xenopus* where the majority of RBs are born during gastrulation (Lamborghini, 1980). This suggests that in both species, most RBs undergo their terminal cell division around the time of neural tube closure and hours before they send out processes and become functional. This protracted period between the time when RBs are born and the period in which they become functional suggests that a series of signaling molecules and transcription factors are likely

required for RBs to become fully functional. Here, we focused on the early stages of RB development by investigating the molecules involved in RB specification.

Epidermal ectoderm contributes significantly to RB neurons

Transplantation analysis in which ectopic interactions between neural and epidermal ectoderm were created in *Xenopus* revealed that both neural and epidermal ectoderm can contribute to RBs (Rossi et al., 2008). Here we show that markers of both neural and epidermal ectoderm overlap with expression of the NPB marker *prdm1a*, but that the overlap with *dlx3b* is greater than with the neuroectodermal markers *sox3* or *sox19a*. This suggests that contrary to what we showed in *Xenopus* under experimental conditions, the primary tissue contribution to the NPB in situ is likely predominantly non-neural ectoderm rather than neural. The lack of apparent overlap of *prdm1a* with markers of either neural or epidermal tissue later in gastrulation suggests that the older NPB is distinct from either tissue.

RBs and neural crest cells require a similar set of genes at the developing NPB

Another population of cells, neural crest cells, also arises from the NPB. Neural crest cells are a stem cell-like population that forms in developing vertebrate embryos and subsequently differentiates into a diverse array of cell types including most of the peripheral nervous system. The molecules important for neural crest cell development have been widely studied, and have been placed in a neural crest gene regulatory network (NC GRN). This network is based primarily on expression of these factors in multiple vertebrates as well and non-vertebrate chordates. The network includes inducers, genes that are required to induce neural crest cell fate (BMPs, FGFs, and Wnts), NPB specifiers, genes expressed in the NPB domain (*zic1*, *msx1*, *msx2*, *dlx3*, *dlx5*, *pax3*, and *pax7*), neural crest specifiers, genes that are expressed in the neural crest domain (*snail1*, *snail2*, *sox8*, *sox9*, *sox10*, *foxd3*, *ap-2*, *twist*, *c-myc* and *id* family members), and several effector genes, which are expressed in specific subsets of neural crest cell derivatives (Sauka-Spengler and Bronner-Fraser, 2008).

Because both RBs and neural crest cells arise from the NPB, some of the molecules that make up the NC GRN, particularly the inducers and NPB specifiers, are likely to be important in development of both cell types. Indeed, BMP signaling is required for proper development of both RBs and neural crest cells of the trunk (Marchant et al., 1998; Neave et al., 1997; Nguyen et al., 1998; Rossi et al., 2008; Tribulo et al., 2003). *prdm1a*, in addition to being required for RB development in zebrafish, is also essential for development of many trunk neural crest cells. *prdm1a* mutants display a reduction in pre-migratory trunk neural crest cells and many trunk neural crest derivatives (Artinger et al., 1999). Due to its function in the development of both RBs and trunk neural crest cells, *prdm1a* likely acts at the level of NPB specifiers.

While *dlx3b* and *dlx4b* loss of function embryos display a reduction in RBs, the development of trunk NCCs is not perturbed in these morphants (Kaji and Artinger, 2004). This suggests that *dlx3b* and *dlx4b* are among the earliest transcription factors differentially required for RBs but not trunk NCCs at the NPB. *dlx* genes are known to have a role in positioning the NPB in *Xenopus* (Woda JM, 2003), and *dlx3b* is considered an NPB specifier according to the NC GRN (Sauka-Spengler and Bronner-Fraser, 2008). The fact that in zebrafish the *dlx* genes appear to be more specifically required for RBs and not NPB cell fates in general may point to species differences in the NC GRN.

dlx3b, *dlx4b*, and *neurogenin1* are required downstream of *prdm1a* in RB development

Our rescue experiments reveal that *dlx3b* and *dlx4b* function downstream of *prdm1a* in RB development. Expression analysis shows that while *neurogenin1* also acts downstream of

prdm1a, the *dlx* genes and *neurogenin1* do not appear to regulate one another. This dual requirement for *dlx* and *neurogenin1* may add to the regulation required to specify RBs only within the NPB domain (see below).

Although these data in combination suggest an epistatic relationship, we cannot confirm a linear pathway or direct interactions of these transcription factors simply based on expression patterns and rescue experiments. However, we predict that there are complex interactions between *prdm1a*, *dlx3b*, and *neurogenin1* that act cell autonomously in the same cell but also non-cell autonomously between neighboring adjacent cells. Our expression data for *prdm1a* and *dlx3b* show some overlap in expression during early gastrulation, suggesting that they may act cell autonomously to promote RB fate. Consistent with this, previous experiments suggest that *prdm1a* is required cell autonomously for RB development (Artinger et al., 1999). However, we have also shown that *dlx3b/dlx4b* are required non-cell autonomously for RB formation (Kaji and Artinger, 2004). Thus, *dlx3b* may function in the neighboring domain to *prdm1a* to refine the region where RB neurons can form. For a non-cell autonomous signal to impart information to neighboring cells, a secreted factor would be required. Based on our work in *Xenopus* and preliminary zebrafish experiments, the signal is likely to involve BMP signaling and remains to be directly tested.

neurogenin1 has been shown to act cell autonomously to rescue *foxd3*-expressing neural crest cells in the absence of functional *mind bomb*, a ubiquitin ligase required for Notch signaling (Cornell and Eisen, 2002). Most likely *neurogenin1* also acts cell autonomously for RB neuron development, however it is possible that it also has a non-cell autonomous function. We know from our results that *dlx3b/4b* cannot rescue *neurogenin1* mutants, suggesting that there is a split in the pathway at this level. Because both *neurogenin1* and *dlx3b* can both rescue RB neuron development, we hypothesize that *dlx3b/dlx4b* and *neurogenin1* can independently rescue RBs since either is independently capable of affecting *neuroD* expression. Further experiments will be required to increase our understanding of this relationship.

RBs neurons are restricted to forming only at the NPB

We have shown that *prdm1a* is both necessary and sufficient for RB development. Embryos lacking *prdm1a* do not form RBs and have reduced neural crest cells. Overexpression of *prdm1a* promotes the number of RBs within the spinal cord (Hernandez-Lagunas et al. 2005) and there is a trend toward increased RBs earlier in the NPB domain in younger embryos). At both developmental stages, increased RBs were restricted to the region of the embryo in which they are typically found. Thus, *prdm1a* may be the primary mechanism by which RB neurons are restricted to the NPB domain. Similarly, *dlx3b/4b* overexpression and rescued embryos only produce RB neurons within the NPB domain. However, *neurogenin1* overexpression caused ectopic RBs both within the neural plate border as well as the non-neural ectoderm, suggesting that *neurogenin1* can convert non-neural ectoderm into neuronal fate. Because *dlx* and *neurogenin1* act downstream of *prdm1a* in seemingly independent pathways, this could be an additional mechanism by which RBs are specified only at the NPB domain.

Model for RB Gene Regulatory Network

Based on these and previous data, we propose a simplified temporal epistatic model for development of RBs at the NPB (Figure 9A). First, the induction of NPB fates requires BMP signaling. Zebrafish embryos with mutations in the BMP signaling pathway show a lack of RB neuron and neural crest cell fates suggesting they are required for induction of these fates. In addition, the expression of *prdm1a* is lost at the NPB but not in other regions of the embryo, indicating that BMP signaling acts upstream of *prdm1a* at the NPB (Hernandez-

Lagunas et al., 2005). This finding, combined with data in *Xenopus* revealing that BMP4 can induce RBs suggests that BMP signaling contributes to the induction of an NPB that is competent to form NPB cell fates and express *prdm1a* (Neave et al., 1997; Nguyen et al., 2000; Rossi et al., 2008). Analysis of embryos with a mutation in *prdm1a* reveals its requirement for RBs (Artinger et al., 1999; Hernandez-Lagunas et al., 2005), and previous experiments also reveal a requirement for *dlx3b* and *dlx4b* in RB development. The data presented here show that *dlx3b* and *dlx4b* act downstream of *prdm1a* in the specification of RBs. Importantly, expression of *bmp2b* depends on functional *dlx3b* and *dlx4b* (Kaji and Artinger, 2004), suggesting that BMP signaling may function at multiple points along the pathway. Similar to the *dlx* genes, *neurogenin1* also acts downstream of *prdm1a* in RB specification but in a pathway that appears to be separate to that of *dlx3b* and *dlx4b*. Expression analysis of *neuroD* in *prdm1a* mutants and *dlx3b/dlx4b* morphants, combined with previous reports of its expression in *neurogenin1* loss of function embryos, suggests that *neuroD* expression may be the step at which these separate pathways converge in RB development.

In order to place these data in the broader context of RB development, we constructed a classification scheme that begins to categorize the important players in RB development into putative components of an RB GRN. In addition, we included other genes previously shown to be required for RB development (Figure 9B). Neural plate border inducers include Wnts as well as BMPs, and we categorize *prdm1a* as an NPB specifier because it is expressed at the NPB and, when mutated, shows an effect on both neural crest and RB neuron specification. *tfap2a/c* (Li and Cornell, 2006), *dlx3b/4b* (this work and Kaji and Artinger, 2004), *neuroD* and *neurogenin1* (Cornell and Eisen, 2002) appear to act as RB specifiers since they are expressed in RBs and loss of their function results in defects in RB development with or without affecting neural crest cells as well. Finally, and *msxB*, *msxC*, and *msxE* are potential RB effectors that are required downstream of initial specification for differentiation of an RB neuronal fate (Phillips et al, 2006). *olig4* has been shown to be an important player in development of RBs and neural crest cells, but it remains to be determined where it fits into the model (Filippi et al, 2005). As this is a first attempt at such an RB GRN, this classification scheme will likely be modified as future experiments further the inter-relationships between molecules essential for NPB and RB development. Further studies will help to better define the genetic cascade at work and reveal potential for feedback loops during the early stages of RB development.

The data presented here contribute importantly to our understanding about the process of cell fate specification of RB sensory neurons, which are among the earliest mechanosensory neurons to function within the embryo. Further, the simplified model for an RB GRN that we present provides a hypothesized framework to consider in future studies on the development of neural plate border cell fates.

Experimental Procedures

Zebrafish maintenance

Zebrafish were maintained according to Westerfield (Westerfield, 1993) and embryos were staged according to Kimmel et al. 1995. Lines included wild type AB and TAB (ZIRC), *neurogenin1(neurog)^{hi1059Tg/+}* from ZIRC), *prdm1a* (*nrd^{m805}* from our lab), and *Tg(neurogenin1:GFP)/Tg(-8.4ngn1:GFP)* from P. Blader. Note that previously published data regarding *neurogenin1* loss of function describes work with a dominant negative constructed by Cornell and Eisen (2002), but a mutant has subsequently been made available (Golling et al., 2002) that is used here.

***in situ* hybridization (ISH)**

Conventional whole-mount ISH was performed as described in Thisse (Thisse, 1998), using the following DIG-labeled antisense RNA probes: *dlx3b*, *gbx1* (from M. Brand), *prdm1a*, *dlx4b*, and *neurogenin1*. BM Purple (Roche) was used to develop the alkaline phosphatase reaction.

Fluorescent ISH was performed using the protocol described in Pineda et al., 2006, in which a DIG-conjugated probe was developed using a fast red kit (Sigma F4648) and a fluorescein-conjugated probe was developed using a TSA kit (Perkin Elmer NEL741). In addition to the probes listed above, *sox3* (ZIRC, P40 RR12546) and *sox19a* (ZIRC, P40 RR12546) were also used.

BrdU Incorporation

Embryos were placed in 10mM BrdU (Sigma B5002) and 1% DMSO in embryo media at various stages and grown in the media until 24 hpf. Embryos were processed for BrdU immunohistochemistry (anti-BrdU from Chemicon International) as described in Grant et al., 2005, with a 1 hour wash in methanol at -20° , and a 20 minute wash in Proteinase K (10ug/ml) at RT prior to 2 N HCl washes. The secondary antibody used was goat anti-mouse Alexa 594.

mRNA and morpholino injections

mRNAs were synthesized using the Ambion Message Machine kit from PCS2+ constructs and 100 pg each mRNA was injected into the embryonic cell at the one-cell stage. The *neurogenin1* in PCS2+ construct was a kind gift from P. Blader. *dlx3b* and *dlx4b* were cloned into the PCS2+ expression vector using the following primers with 24 hpf cDNA: CCGTAGGATCCCAACAGAGGGAGTGTGAGAAAGCA (F) and CGCGCGAATTCAAATGGTTCGAGCACCACCGT (R) for *dlx3b* and CGCGCAGGCCTCGGAGCTCGAGTCAGTCAGAAACA (F) and CGCGATCTAGAAGCTACTGAAGTGTAATATGGGAGTTTCGGG (R) for *dlx4b*.

Morpholinos to *prdm1a* (12 ng splice blocking morpholino to intron 2 splice donor, Baxendale et al., 2004), *dlx3b* and *dlx4b* (20 ng each splice blocking morpholinos, Kaji and Artinger, 2004) were injected into the embryonic cell(s) at the 1-2 cell stage.

Genotyping

To genotype single embryos, DNA was extracted by placing embryos in lysis buffer at 98° for 10 minutes, incubating with 5 ul Proteinase K (10mg/ml) at 55° for one hour, and incubating again at 98° for 10 minutes. The primer sequences TTCAGTCAAGACCTAAGCCCGC (F) and CAAAAACATCTTAAGGAAGAGGGCAG (R) were used, followed by a 2 hour digest with the restriction enzyme *fokI*.

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Figure 1. Detailed analysis of gene expression at early NPB stages

(A-P) Lateral views of wild type embryos with dorsal toward the right and anterior toward the top. (A) Schematic of the approximate location of the NPB, between dorsal neural ectoderm and ventral epidermal ectoderm, in gastrula stage zebrafish embryos. (B-F) *dlx3b* expression from 70% epiboly through 2 somites. *dlx3b* transcripts are found throughout the epidermal ectoderm at 70% epiboly (B), 80% epiboly (C), and 90% epiboly (D). At tail bud, the expression domain narrows to a more discrete domain near the NPB (E), and at 2 somites the posterior portion of this domain is mostly gone (F). (G-K) *prdm1a* expression. *prdm1a* transcripts are found at the NPB at each of these stages, but the domain narrows and moves toward the dorsal midline over time. Strong expression is also evident in the prechordal plate at anterior tip of embryo. (L-P) *gbx1* is expressed in dorsal neural tissue, and its domain shifts toward the future anterior as development proceeds. (A) was modified, with permission, from Kimmel et al., 1995.

Figure 2. Overlap of the *prdm1a* expression domain with *dlx3b* is greater than with neural markers *sox3* and *sox19a*

Confocal micrographs of wild type embryos mounted to best observe potential overlap of expression at the NPB; (A-B',D) mounted laterally with dorsal toward the right, (C-C',FF') mounted dorsally with anterior toward the top, and (E-E') mounted dorsolaterally with anterior toward the top. (A-C') *prdm1a* in green, *dlx3b* in red, overlap in yellow. (D) *prdm1a* in green, *sox3* in red, overlap in yellow. (E-F') *prdm1a* in red, *sox19a* in green. The *prdm1a* domain overlaps extensively with *dlx3b* at 90% epiboly (yellow in A), with less overlap at tail bud (B,B'), and very little overlap by 2 somites (C,C'). (D) A small amount of overlap of *prdm1a* and *sox3* is observed at 90% epiboly (yellow). Transcripts for *sox19a* (green) and *prdm1a* (red) abut one another, but do not overlap with each other, at tail bud (E-E') and 2 somites (F-F'). This is especially evident at 20X (E',F'). *prdm1a* is also expressed in the adaxial cells in the mesoderm near dorsal midline at tail bud and 2 somites. ac, adaxial cells, npb, neural plate border. A-F, 10x; B'-F', 20x.

Figure 3. RB sensory neurons are born during gastrulation in zebrafish

(A-D) Confocal micrographs of dorsal views of the spinal cord in 24 hpf

Tg(neurogenin1:GFP) embryos, in which RB neurons express GFP. Embryos were treated with BrdU from high stage (A), 50% epiboly (B), tail bud (C), or 2 somites (D) through 24 hpf. In embryos treated from high stage or 50% epiboly (A,B), most of the RBs show BrdU incorporation, while in embryos in which BrdU exposure began at tail bud (C) or 2 somites (D), few RBs show BrdU incorporation.

Figure 4. *prdm1a* affects *dlx3b* and *dlx4b* expression unidirectionally at the NPB

(A-B) *dlx3b* expression is decreased at the NPB in *prdm1a* mutant embryos (B) compared to WT embryos (A) at tail bud. (C-F) Embryos injected with antisense oligonucleotide morpholino to *prdm1a* show a decrease in *dlx4b* at the NPB at tail bud (D) and an absence of *HuC*-positive RBs (F) at 2 somites compared to un-injected controls (C, E). Conversely, *prdm1a* expression at the NPB shows no change in *dlx3b/4b* morpholino injected embryos (compare un-injected controls at 90% epiboly (G) and tail bud (I) to morphants (H,J)). Lateral (A-B, E-J) or dorsal (C-D) views with anterior toward the top.

Figure 5. *dlx3b* and *dlx4b* function downstream of *prdm1a* to specify RB sensory neurons
Dorsal views with anterior toward the top. (A) Wild type embryo and (B-D) *prdm1a* homozygous mutant embryos stained for *HuC*, 2-3 somites, dorsal views with anterior toward the top. (A) *HuC* stains RBs, motoneurons (mn), and interneurons (in). (B) Un-injected *prdm1a* mutants lack RBs at the NPB. (C-D) mRNA for *dlx3b* and *dlx4b* partially rescues the RB phenotype to a varying degree in *prdm1a* mutants (arrows).

Figure 6. *neurogenin1* expression is decreased at the NPB in *prdm1a* mutants

Lateral (A,B, E, F) or dorsal (C, D) views with anterior toward the top. *neurogenin1* is expressed in three pairs of stripes at tail bud (A-B) and 2 somites (C-D). The most lateral stripes on either side of dorsal midline correspond to the NPB (arrows in A, C). This stripe of expression is reduced or missing in homozygous mutant *prdm1a* embryos (arrows in B, D). Conversely, *prdm1a* expression at tailbud and 2 somites stage is normal in *neurogenin1(neurog1)* mutant embryos (E,F).

Figure 7. *neurogenin1* functions downstream of *prdm1a* to specify RB sensory neurons
Dorsal views with anterior toward the top. (A) Wild type embryo and (B, C) *prdm1a* homozygous mutant embryos stained for *HuC*, 2-3 somites (A) *HuC* stains RBs, motoneurons (mn), and interneurons (in). (B) Un-injected *prdm1a* mutants lack RBs at the NPB. (C) mRNA for *neurogenin1* partially rescues the RB phenotype to a varying degree in *prdm1a* mutants (arrows).

Figure 9. Model for transcriptional control of cell fate specification at the neural plate border
 (A) Summary of our data, showing the epistatic relationships observed. *prdm1a*, which is downstream of BMP signaling, is required for the specification of RBs, and acts upstream of *neurogenin1* and *dlx3b/4b* in apparently separate pathways. These pathways may converge in regulating expression of *neuroD* in the RB-forming region of the embryo. (B) A preliminary classification scheme for an RB GRN. Further studies will be required to better define direct interactions and reveal potential for feedback loops during the early stages of RB development.

Table 1

Birthdating of RB sensory neurons

Average # of RBs expressing BrdU	high	50%epiboly	shield	70%epiboly	90%epiboly	tailbud
	82.3% +/-6.6%	81.5% +/-5.8%	77.3% +/-12.1%	66.9% +/-11.2%	49.4% +/-5.3%	33.7% +/-4.5%

* At least three embryos were averaged per stage

Table 2

Summary of observed gene expression in mutant and morphant embryos

Mutant [*] (M) or Morphant (MO)	Stage	Expression Analyzed	Observed Expression	n =
<i>prdm1a</i> (M)	70% epiboly	<i>dlx3b</i>	No change	20
<i>prdm1a</i> (M)	80% epiboly	<i>dlx3b</i>	No change	23
<i>prdm1a</i> (M)	90% epiboly	<i>dlx3b</i>	No change	31
<i>prdm1a</i> (M)	Tail bud	<i>dlx3b</i>	Decreased at NPB	10/45 (22%)
<i>prdm1a</i> (MO)	Tail bud	<i>dlx3b</i>	Decreased at NPB	6/8
<i>prdm1a</i> (MO)	Tail bud	<i>dlx4b</i>	Decreased at NPB	18/19
<i>prdm1a</i> (MO)	2 somites	<i>dlx4b</i>	Decreased at NPB	6/9
<i>prdm1a</i> (M)	Tail bud	<i>neurogenin1</i>	Lateral stripe reduced or absent	12/52 (23%)
<i>prdm1a</i> (M)	2 somites	<i>neurogenin1</i>	Lateral stripe reduced or absent	11/43 (26%)
<i>prdm1a</i> (M)	3 somites	<i>neuroD</i>	Lateral stripe reduced or absent	7/22 (32%)
<i>dlx3b/dlx4b</i> (MO)	90% epiboly	<i>prdm1a</i>	No change	9
<i>dlx3b/dlx4b</i> (MO)	Tail bud	<i>prdm1a</i>	No change	11
<i>neurogenin1</i> (M)	80% epiboly	<i>prdm1a</i>	No change	24
<i>neurogenin1</i> (M)	90% epiboly	<i>prdm1a</i>	No change	24
<i>neurogenin1</i> (M)	Tail bud	<i>prdm1a</i>	No change	21
<i>neurogenin1</i> (M)	2 somites	<i>prdm1a</i>	No change	19
<i>neurogenin1</i> (M)	80% epiboly	<i>dlx3b</i>	No change	23
<i>neurogenin1</i> (M)	90% epiboly	<i>dlx3b</i>	No change	21
<i>neurogenin1</i> (M)	tail bud	<i>dlx3b</i>	No change	26

* For mutant embryos, 25% of each clutch is expected to be homozygous mutant. Cases in which morpholino was injected into wild type embryos are shaded in gray.