Prdm1a directly activates foxd3 and tfap2a during zebrafish neural crest specification

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SUMMARY
The neural crest comprises multipotent precursor cells that are induced at the neural plate border by a series of complex signaling and genetic interactions. Several transcription factors, termed neural crest specifiers, are necessary for early neural crest development; however, the nature of their interactions and regulation is not well understood. Here, we have established that the PR/SET domain-containing transcription factor Prdm1a is co-expressed with two essential neural crest specifiers, foxd3 and tfap2a, at the neural plate border. Through rescue experiments, chromatin immunoprecipitation and reporter assays, we have determined that Prdm1a directly binds to and transcriptionally activates enhancers for foxd3 and tfap2a and that they are functional, direct targets of Prdm1a at the neural plate border. Additionally, analysis of dominant activator and dominant repressor Prdm1a constructs suggests that Prdm1a is required both as a transcriptional activator and transcriptional repressor for neural crest development in zebrafish embryos.

KEY WORDS: Blimp1, Neural crest, Neural plate border, Prdm1

INTRODUCTION
Neural crest cells (NCCs) are a transient population of stem cell-like progenitors that are born at the dorsal neural tube during vertebrate embryonic development. NCCs are induced at the junction between neural and non-neural ectoderm in a region called the neural plate border (NPB). The induction of NCCs requires interactions of the BMP, Wnt and Fgf signaling pathways. Proper temporal and spatial regulation of these genes is required for induction of NPB specifiers, which include members of the Pax, Msx and Dlx gene families and prdm1a (reviewed by Aybar and Mayor, 2002; Huang and Saint-Jeannet, 2004; Sauka-Spengler and Bronner-Fraser, 2008). After undergoing an epithelial-to-mesenchymal transition (EMT), NCCs migrate away from the dorsal neural tube along specified pathways and differentiate to form one of many derivatives, including tendons, cartilage and bone of the face, Schwann cells and neurons of the peripheral nervous system, and pigment cells. In zebrafish (Danio rerio), the specification of cells at the NPB to commit to the neural crest fate occurs at the end of gastrulation and the beginning of segmentation around the 2-somite stage. During this process, several key genes, which are referred to as neural crest specifiers, become expressed in the NPB following induction of NCCs, including foxd3 (Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006), snail1b or slug (snai2) (Thiess et al., 1995), tfap2a (Barrallo-Gimeno et al., 2004) and sox10 (Dutton et al., 2001; Carney et al., 2006). Although these neural crest specifiers have been well-studied in the context of NCC development, little is known about direct interactions among these genes and how genes that initially pattern the NPB interact with and regulate the genes that are required for subsequent NCC specification.

The Prdm1a transcription factor was identified as an important regulator of neural crest in zebrafish when it was reported that embryos carrying a mutation in prdm1a [the narrowminded (nrd) and the u-boot (ubo) mutants] exhibit a significant reduction in NCCs, as reflected in the downregulation of NCC markers including sox10 and crestin, as well as the partial loss of derivatives such as pigment cells, craniofacial cartilages, and cranial and dorsal root ganglia (Artinger et al., 1999; Roy and Ng, 2004; Hernandez-Lagunas et al., 2005; Birkholz et al., 2009). prdm1a is first expressed broadly in the NPB of zebrafish embryos at 50% epiboly and continues to be expressed in the developing NPB and migrating NCC progenitors, as well as in mesodermal adaxial cells, through the 6-somite stage (Hernandez-Lagunas et al., 2005). It is also expressed later in the developing pharyngeal arches, suggesting an additional role in craniofacial development (Birkholz et al., 2009).

Interestingly, the expression of prdm1a at the developing NPB is conserved in lamprey, the most basal extant vertebrate (Nikitina et al., 2011), suggesting that prdm1 is likely to have a conserved role in early NCC development.

The Prdm1a protein harbors five zinc-fingers for DNA binding as well as a PR/SET domain and Pro/Ser-rich region, which are both thought to be important in protein-protein interactions (Bikoff et al., 2009). Along with the demonstrated role in NCC development, Prdm1a is also necessary for the differentiation of adaxial cells into slow-twitch muscle fiber rather than fast-twitch fiber types in zebrafish (Baxendale et al., 2004; von Hofsten et al., 2008). Consistent with this role, Prdm1a is a key transcriptional repressor of fast muscle-specific genes, possibly through both direct and indirect means (von Hofsten et al., 2008; Wang et al., 2011b).

The mouse homolog of Prdm1a, Blimp1 (Prdm1 – Mouse Genome Informatics), is important in the specification of primordial germ cells (Ohihata et al., 2005; Vincent et al., 2005), is required for the functional differentiation of B and T lymphocytes (Turner et al., 1994; Shapiro-Shelaf et al., 2003; Shapiro-Shelaf et al., 2005; Kallies et al., 2006; Martinis et al., 2006), and plays a role in the development of the forelimb, pharyngeal arches, heart and sensory vibrations (Robertson et al., 2007). Although Blimp1 is likely to play a role in NCC differentiation in the pharyngeal arches, it has not been demonstrated to play a role in mouse NCC specification (John

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and Garrett-Sinha, 2009). Several studies on Blimp1 and its human ortholog PRDI-BF1 (PRDM1—Human Genome Nomenclature Committee) have demonstrated that Prdm1 represses multiple target genes through the recruitment of various histone modifying proteins, including histone methyltransferases (Gyory et al., 2004; Ancelin et al., 2006) and histone deacetylases (Yu et al., 2000), or through binding to the Groucho family of co-repressors (Ren et al., 1999). Whereas other members of the PRDM family have intrinsic methyltransferase activity through the PR/SET domain (Hohenauer and Moore, 2012), it appears that Prdm1 lacks this activity (Gyory et al., 2004). This suggests that Prdm1 might depend largely on binding partners to regulate its target genes.

Several of the genes that are downregulated in zebrafish prdm1a-deficient embryos are involved in neural crest specification at the NPB. One of these is the forkhead-box transcription factor foxd3. foxd3 is expressed in the NPB and is required for formation of NCCs and expression of other NCC specifiers, including snail1b and sox10 (Montero-Balaguer et al., 2006; Stewart et al., 2006). Studies in chick and mouse suggest that the role of foxd3 in NCC development is highly conserved (Kos et al., 2001; Teng et al., 2008) and that foxd3 is required for NCCs to maintain their pluripotency (Mundell and Labosky, 2011). Recent work in chick embryos has further identified genomic enhancers that drive Foxd3 expression specifically in the developing neural crest and determined potential transcription factors that bind to and regulate these regions (Simões-Costa et al., 2012); however, the direct regulation of foxd3 in zebrafish NCCs has not previously been studied. Another gene known to be upstream of foxd3 in zebrafish is tfap2a, a well-known neural crest specifier. Tfap2a is a member of the AP-2 family of transcription factors, which play many important roles in embryonic development (Breuer et al., 2004; Eckert et al., 2005). Zebrafish tfap2a mutants display a loss of neural crest derivatives and a reduction in the expression of key NCC specifier genes (Knight et al., 2003; Barrallo-Gimeno et al., 2004; Knight et al., 2004). In double knockdowns of both tfap2a and its redundant family member tfap2c, early markers of NCC specification are lost at the NPB, including foxd3, snail1b, and prdm1a (Li and Cornell, 2007). Furthermore, it has been shown that Tfap2a protein directly activates the expression of sox10 in zebrafish NCCs (Van Otterloo et al., 2012). Additional studies have shown that knockdown of both foxd3 and tfap2a in zebrafish completely ablates NCCs and that these genes together are responsible for turning on key signaling pathways for neural crest induction (Arduini et al., 2009; Wang et al., 2011a). Despite their demonstrated importance in regulating early NCC development, the genes responsible for directly regulating tfap2a and foxd3 in the neural crest are unknown.

Here, we ascertain the Prdm1a gene regulatory network for neural crest specification in zebrafish. Via knockdown and mRNA rescue experiments, we show that two candidate Prdm1a targets, foxd3 and tfap2a, are able to rescue NCC specification in prdm1a knockdown embryos. We demonstrate that Prdm1a binds directly to enhancer regions for foxd3 and tfap2a, positively regulating the activity of these enhancers at the NPB. Thus, Prdm1a is a transcriptional activator of these key neural crest genes, revealing for the first time that Prdm1a can act as a transcriptional activator in vertebrates. In addition, Prdm1a dominant activator and dominant repressor constructs must both be present to rescue migratory NCCs in prdm1a−/− embryos. From these data, we propose that Prdm1a functions as a transcriptional activator and transcriptional repressor of target genes during development, and that both roles are crucial for formation of the neural crest.

MATERIALS AND METHODS

Zebrafish

Zebrafish were maintained as described (Westerfield, 1993). Wild-type (WT) strains include AB, TAB and EKK lines (ZIRC) and mutant lines include prdm1a<sup>mo1005</sup> (nr4) (Artinger et al., 1999; Hernandez-Lagunas et al., 2011) and foxd3<sup>2010</sup> (formerly sym1) (Stewart et al., 2006). Developmental staging followed published standards (Kimmel et al., 1995). All experiments utilizing zebrafish are approved by UC Denver IACUC and conform to NIH regulatory standards of care and treatment.

Morpholino and mRNA injections

Morpholino oligonucleotides (Gene Tools) were injected at the 1- to 2-cell stage together with Rhodamine dextran (Molecular Probes). Morpholinos include prdm1a E121 splice site injected at 4 ng (Hernandez-Lagunas et al., 2005), foxd3 5'UTR and ATG morpholinos at 2 ng and 4 ng each (Montero-Balaguer et al., 2006), tfap2a 5.1 MO at 4 ng (Knight et al., 2003) and tfap2c MO at 5.5 ng (Li and Cornell, 2007). mRNA sequences were prepared from whole-embryo cDNA and cloned into pCS2+ using the following primers: foxd3, 5'-AATAGAGATCCTGCGACCACATGCTGTCGAGG-GCA-3' and 5'-GCCGGTCTAGATCATGAGAAGGCATTTGCTGTCAGTA-ACGCCGT-3'; prdm1a (Hernandez-Lagunas et al., 2005). The tfap2a plasmid was a gift from T. Williams (University of Colorado, Boulder, CO, USA) (Li and Cornell, 2007). mRNA was synthesized using the mMESSAGE mMACHINE Kit (Ambion) and injected at the 1-cell stage in the following doses: foxd3 at 40 pg, tfap2a at 86 pg, prdm1a at 75 pg, and gfp at 67 pg. mRNA and morpholinos were co-injected into embryos at the 1-cell stage for rescue experiments.

In situ hybridization

Whole-mount RNA in situ hybridization (ISH) was performed as previously described (Thiess and Thiess, 1998). Single-embryo genotyping of prdm1a−/− following ISH was performed as described (Rossi et al., 2009). DIG-conjugated antisense probes were synthesized from full-length transcript sequences in the pCS2+ plasmid to the following genes: snail1b (primers 5'-GCTAGGGATTCCGCGCCAGGTCGTTATTGTTAA-3' and 5'-GAATTCTAGATGTTGGGGTCTAGTGTCGTTAAC-3'; foxd3 (see above); sox10 (Olesenky et al., 2010); crestin (Rubinstein et al., 2000); and tfap2a (from T. Williams). Fluorescent ISH was performed as described (Pineda et al., 2006) and used the TSA Biostain System (Perkin Elmer, NEL700/A001KT) followed by streptavidin Alexa Fluor 488 antibody (Invirogen, S11223) to develop FITC-labeled antisense probes and the Fast Red Kit (Sigma, F4648) to develop DIG-labeled probes.

Enhancer reporter constructs

Evolutionarily conserved regions (ECRs) identified on the ECR Browser (http://ecrbrowser.dcode.org/) containing Prdm1a binding sites identified by MatInspector (Genomatrix) were amplified by PCR from WT zebrafish genomic DNA using the following primers (5'-3'): foxd3E1, GGGGAAC-AAGTTTGTGATACAAAAAGCGAGGCTACTGAACGTGTTGGTGCCAG and GGGAACGCCACTTGTGTAACAAAAGACGCTTACATATGCTGAACGATTGCACTACAG and GGGAGACCTTGTGTCAGTCAAGGCGAACTGACGAGTGGACCTAACCTGTTGACAGTGGGATAGAGCCACCATG. The protocol for chromatin immunoprecipitation (ChIP) on zebrafish genomic DNA using the following primers (5'-3'): foxd3E1, GGGAACG-
Hofsten et al., 2008). The Prdm1a antibody was a generous gift from Philip Ingham. ChIP was performed on 2-somite TAB WT embryos. Approximately 800-1000 embryos were fixed with 1.85% paraformaldehyde for 15 minutes followed by snap freezing. Cells and nuclei were lysed before DNA sonication by Bioruptor (Diagenode) for 45 minutes to create fragments of ~300 bp. Beads incubated with Prdm1a antibody, IgG antibody (Jackson ImmunoResearch) or without primary antibody were added to genomic DNA fragments at 4°C overnight. DNA was eluted from beads at 65°C for 6 hours to overnight and DNA was purified by phenol:chloroform extraction. Quantitative RT-PCR (qPCR) was performed on pulldowns and input DNA using TaqMan primer/probe sets (Applied Biosystems).

**Activator and repressor constructs**

Prdm1aDBD-VP16 and DBD-EnR fusion constructs were generated similarly to previous methods (von Hofsten et al., 2008) and cloned into pCS2+. 100-250 pg of total mRNA from each construct was injected into 1-cell stage embryos.

**Statistics**

All experiments were performed with three or more biological replicates. In addition, qPCR experiments contained at least three technical replicates per biological sample. For statistical analysis, ANOVA followed by Fisher’s LSD test was used unless otherwise noted. Error bars denote s.e.m..

**RESULTS**

**prdm1a is co-expressed with foxd3 and tfap2a at the developing neural plate border**

To determine potential targets for Prdm1a transcriptional regulation, we first identified genes that were co-expressed with prdm1a at the NPB during early neural crest specification. We used double fluorescent RNA in situ hybridization (ISH) to establish the expression of prdm1a with the known NCC specification genes foxd3 and tfap2a. ISH at the 2-somite stage showed that both foxd3 and tfap2a are co-expressed with prdm1a within an overlapping domain at the NPB (Fig. 1). prdm1a and tfap2a are expressed throughout the NPB, whereas foxd3 is primarily expressed in the anterior NPB at this stage. This suggests that foxd3 and tfap2a are good candidates for Prdm1a transcriptional regulation during the initial stages of NCC specification.

**foxd3 rescues the prdm1a loss-of-function neural crest phenotype**

In prdm1a mutant and morphant zebrafish, it is known that the expression of the early neural crest specifier foxd3 is downregulated (Hernandez-Lagunas et al., 2005), and in prdm1a-overexpressing embryos foxd3 is upregulated within the NPB compared with wild type (WT) (supplementary material Fig. S1G,H) and gfp mRNA-injected controls (data not shown). To determine whether foxd3 is a candidate for direct regulation by Prdm1a, we performed rescue experiments of the prdm1a knockdown neural crest phenotype with overexpression of foxd3 mRNA. WT embryos were injected at the single-cell stage with prdm1a-MO alone or were co-injected with prdm1a-MO and foxd3 mRNA. Embryos were fixed at 2- or 4-somites and ISH was performed for the neural crest markers snai1b and sox10. At 2-somites, snai1b expression is highly downregulated at the NPB in prdm1a morphant embryos compared with WT (Fig. 2A-C,G; WT, 98% of embryos positive for snai1b expression at NPB; prdm1a-MO, 19% express snai1b in NPB), similar to what is observed in prdm1a mutants (Artinger et al., 1999). Interestingly, snai1b is also downregulated in the mesoderm in both prdm1a mutants and mutants, suggesting that prdm1a might also modulate some unknown indirect interactions between the NPB and mesodermal tissue or that prdm1a expression in the adaxial cells also regulates snai1b in the mesoderm directly. Co-injection of foxd3 mRNA with the prdm1a-MO rescued the expression of snai1b at the NPB (87% of embryos), whereas co-injection with gfp mRNA as a negative control did not rescue NPB expression (n=18, data not shown). Expression of sox10, another neural crest specifier, is almost completely absent in prdm1a morphant embryos (5% of embryos express sox10) and co-injection of foxd3 is able to rescue the expression of sox10 at the NPB at the 4-somite stage (Fig. 2D-G; prdm1a-MO + foxd3 mRNA, 50% of embryos express sox10). The rescue of NCC specification with foxd3 mRNA was confirmed in prdm1a-/- embryos (data not shown).

**Prdm1a directly binds to and activates an enhancer for foxd3**

To determine whether foxd3 is a direct target of the Prdm1a transcription factor, we searched for the Prdm1a consensus binding
sequence, AG(T/C)GAAAG(T/C)(G/T) (italics indicate conserved core of the binding sequence), in putative enhancers around the foxd3 locus. The ECR Browser was used to identify evolutionarily conserved regions (ECRs) between zebrafish, mouse and human that could serve as enhancers for foxd3. The ECR sequences were then analyzed using MatInspector to search for conserved transcription factor binding sites. One putative enhancer containing the Prdm1a consensus binding sequence was identified ~5 kb upstream of the foxd3 start site (foxd3 E1, Fig. 3A). Chromatin immunoprecipitation (ChiP) was performed on 2-somite WT embryos using a published rabbit polyclonal antibody to Prdm1a (von Hofsten et al., 2008) and qPCR was performed on the pulldown genomic DNA using primers and a probe designed to span the putative binding site within foxd3 E1. The E1 putative enhancer was enriched in the Prdm1a-bound lysate compared with the IgG (Fig. 3B) and no-primary antibody (data not shown) controls. To confirm the specificity of the Prdm1a antibody, we also performed ChiP on 24-hpf (hours post-fertilization) embryonic lysates and performed qPCR using primers and probes against two known target enhancers for myosin heavy chain (MyHC) and myosin light chain (MyLC) as positive controls (von Hofsten et al., 2008). Both of these enhancers were bound by Prdm1a antibody and detected by qPCR (supplementary material Fig. S2A). qPCR was also performed on four off-target genomic regions (Upstream 1 and 2 and Downstream 1 and 2) flanking the highly bound MyHC enhancer; Prdm1a did not bind these regions, demonstrating the specificity of the antibody (supplementary material Fig. S2B,C). We also designed primers and probes to off-target-flanking regions of foxd3 E1, referred to as foxd3 off-target 1 (O1) and foxd3 off-target 2 (O2). Again, the Prdm1a antibody did not pull down the off-target regions, demonstrating that the binding of Prdm1a to foxd3 E1 is specific (supplementary material Fig. S2D).

To test whether E1 is a functional enhancer for foxd3 at the NPB, we transiently expressed GFP under the control of the entire 558 bp enhancer in embryos. As expected for transient transgenes, both GFP protein and mRNA expression were mosaic (Fig. 3; supplementary material Fig. S3). At 2-somites, however, the foxd3E1::GFP construct is expressed at the NPB and at a lesser extent in neighboring domains (Fig. 3D). Double fluorescentISH of gfp and foxd3 mRNA demonstrated that the majority of gfp colocalizes with endogenous foxd3 mRNA (supplementary material Fig. S3A-C). Therefore, the foxd3 E1 enhancer is sufficient to drive expression in foxd3-expressing NPB cells but is not sufficient to limit expression to these cells. Most likely, elements within the several other conserved foxd3 genomic regions outside of E1 repress foxd3 expression outside the NPB. The broad expression of prdm1a beyond the endogenous foxd3 domain further supports this model (Fig. 1). If Prdm1a binding to E1 activates transcription in these cells, the predicted Prdm1a consensus binding element should be necessary. Indeed, mutation of this site in E1 caused dramatic loss of GFP expression (Fig. 3F-H). Moreover, depletion of prdm1a by MO injection also caused a severe reduction in GFP expression from the wild-type enhancer at the 2-somite stage. This was evident in both the percentage of embryos expressing detectable GFP and the intensity of GFP expression, as measured by pixel intensity (Fig. 3G-H). To confirm that wild-type prdm1a mRNA is sufficient to activate the enhancer, we overexpressed prdm1a mRNA with foxd3E1::GFP and observed a significant increase in GFP pixel intensity over foxd3E1::GFP-expressing embryos (supplementary material Fig. S1A,B,E).

Together, these data support direct binding of Prdm1a to the E1 enhancer to activate foxd3 transcription at the NPB, and suggest that this interaction promotes the specification of neural crest by foxd3.

tfap2a is downstream of prdm1a in neural crest specification

To identify additional candidates for Prdm1a transcriptional regulation, we analyzed the expression of other neural crest specifiers in prdm1a morphant embryos by ISH. We found that the AP-2 family member tfap2a is downregulated in the NPB of prdm1a morphants at...
2-somites (supplementary material Fig. S5) and is upregulated and expanded in prdm1a-overexpressing embryos (supplementary material Fig. S11J) compared with controls. To examine whether tfap2a is a candidate for direct regulation by Prdm1a, we performed rescue experiments with tfap2a mRNA in prdm1a mutants (data not shown) and morphants, and performed ISH at 2- or 4-somites for tfap2a or sox10, respectively. There is a clear downregulation of both tfap2a (Fig. 4A,B,G; WT, 88% of embryos express tfap2a at NPB; prdm1a-MO, 8.5% express tfap2a) and sox10 (Fig. 4D,E,G; WT, 81.9% express sox10; prdm1a-MO, 4.7% express sox10) in the prdm1a morphants compared with WT, and when tfap2a mRNA is co-expressed both tfap2a and sox10 are partially rescued at the NPB (Fig. 4C,F,G; tfap2a, 40.9% rescued; sox10, 47% rescued). These data suggest that tfap2a is directly downstream of prdm1a in the NCC specification pathway and is a candidate for Prdm1a transcriptional regulation.

Prdm1a directly targets and regulates a tfap2a enhancer

Three putative enhancers, each containing a consensus binding site for Prdm1a, adjacent to the tfap2a gene locus (Fig. 5A; E1, 2.4 kb upstream of tfap2a start site; E2, 1 kb downstream of the tfap2a gene; and E3, 2.5 kb downstream) were identified as described above. ChIP was performed on 2-somite WT embryos using the Prdm1a antibody, followed by qPCR for each of the putative tfap2a enhancers. Only one putative enhancer region, tfap2a E2, was pulled down by the Prdm1a antibody (compared with IgG control antibody, Fig. 5B; E1 and E3 in supplementary material Fig. S2E).

To show that the 741 bp E2 region is a functional enhancer for tfap2a at the NPB, we drove GFP transiently under the control of tfap2a E2 and performed imaging at 2-somites. GFP expression was seen mostly in the NPB in a broad domain similar to endogenous tfap2a expression (Fig. 5D), and double fluorescent ISH showed mosaic colocalization of gfp and tfap2a mRNA at the NPB (supplementary material Fig. S3D-F); although similar to that of tfap2a, tfap2a expression is probably also modulated by other enhancers that are not regulated by Prdm1a. We co-injected the tfap2aE2-GFP construct with prdm1a-MO and observed strong reduction of GFP expression (Fig. 5E,G,H). Additionally, we mutated the Prdm1a consensus binding site in the tfap2aE2-GFP construct and injected it into single-cell WT embryos (tfap2aMutE2-GFP, sequence in supplementary material Fig. S4B). At 2-somites, GFP expression was reduced to levels similar to those of the wild-type tfap2aE2-GFP construct in prdm1a knockdown embryos, but was not completely ablated (Fig. 5F-H), suggesting that although Prdm1a is a strong regulator of this enhancer, it likely is not the only direct activator of tfap2a E2 at the NPB. To confirm regulation by prdm1a, we overexpressed prdm1a mRNA with tfap2aE2-GFP and observed an increase in GFP pixel intensity compared with tfap2aE2-GFP alone (supplementary material Fig. S1C,D,F). Altogether, these data indicate that Prdm1a binds to and activates an enhancer for tfap2a at the NPB and is sufficient to drive tfap2a specification of NCCs.

Prdm1a EnR repressor and VP16 activator constructs directly regulate enhancers for foxd3 and tfap2a

To further investigate Prdm1a regulation of the identified enhancers for foxd3 and tfap2a, we created dominant activator and dominant repressor constructs for Prdm1a by fusing the Prdm1a zinc-finger DNA-binding domain (DBD) with either the Engrailed (EnR) repressor or VP16 activator transcriptional regulation domains. We
then co-injected fox3E1::GFP or tfap2aE2::GFP with prdm1aDBD-EnR or prdm1aDBD-VP16 and imaged embryos at 2-somites. We observed a loss of GFP expression with both constructs when co-injected with prdm1aDBD-EnR, suggesting that the enhancers were directly repressed (Fig. 6A,B,F,G), and an expansion or increase of GFP expression when co-expressed with prdm1aDBD-VP16 (Fig. 6C,H, quantified in 6D,E,I,J), suggesting that they were directly activated in the embryo. These results further demonstrate that Prdm1a directly binds and regulates these enhancers at the NPB.

tfap2a and fox3 form a reciprocal feedback loop with prdm1a at the NPB

We next explored the genetic hierarchy of these conserved transcription factors during neural crest specification. To determine whether there are reciprocal interactions between prdm1a and its targets, we used morpholinos to knockdown fox3 and tfap2a and assayed for prdm1a expression at the NPB by ISH and qRT-PCR. Interestingly, morpholino knockdown of fox3 caused an increase in prdm1a expression in the NPB at the 2-somite stage (supplementary material Fig. S6A-C), which was confirmed in fox3zdf10/zdf10 (syml) mutants (data not shown). As assessed by ISH, the expression domain of prdm1a is expanded, especially in the anteriormost region of the NPB where fox3 is most highly expressed. This suggests that fox3, once activated by Prdm1a, restricts the expression of prdm1a in the presumptive neural crest; however, whether this is a direct interaction is not yet known.

We next assessed potential regulation of prdm1a by tfap2a. In tfap2a morphants, prdm1a expression was unchanged (data not shown). It is known, however, that in zebrafish NCC development tfap2a shares redundant activity with its family member tfap2c. Upon knockdown of both tfap2a and tfap2c we observed decreased expression of prdm1a as well as of fox3 by qRT-PCR and ISH (supplementary material Fig. S6D-H), confirming previously published data (Li and Cornell, 2007). tfap2c-MO alone did not decrease the expression of fox3 or prdm1a (data not shown). These data suggest a positive-feedback loop between prdm1a and tfap2a/c during NPB and NCC specification. Interestingly, fox3 overexpression in prdm1a morphants is unable to rescue tfap2a expression at the NPB, indicating a genetic hierarchy of NPB and neural crest specifiers in which fox3 is downstream of both prdm1a and tfap2a.

Prdm1a functions as both a transcriptional activator and repressor during neural crest development

Our data on the fox3 and tfap2a enhancers suggest that Prdm1a is a transcriptional activator during NCC specification; however, previous work on Prdm1a in other cell types and model systems has demonstrated that Prdm1a functions primarily as a transcriptional repressor of target genes. Our previously published microarray (Olesnicky et al., 2010) as well as mRNA-seq data from embryos at this early stage (unpublished data) demonstrate both upregulation and downregulation of various genes in prdm1a knockdown embryos as compared with WT embryos, suggesting that, if these targets are direct, Prdm1a might have both transcriptional activator and repressor functions during embryogenesis. To demonstrate the mode of Prdm1a transcriptional regulation during NCC development, we expressed the prdm1aDBD-VP16 dominant activator and EnR dominant repressor constructs in prdm1a mutants. In order to ensure the efficacy of our constructs during embryonic development, we analyzed their ability to rescue slow-twitch muscle development in prdm1a−/−, as previously reported (von Hofsten et al., 2008). Injection of prdm1aDBD-EnR mRNA partially rescued the slow-twitch muscle phenotype of prdm1a−/− as assayed by prox1 expression (supplementary material Fig. S7A-C). Correspondingly, injection of prdm1aDBD-VP16 produced precocious F310 immunoreactivity in fast-twitch muscle (supplementary material Fig. S7D-F), demonstrating that each of these constructs is effective at regulating established target genes.

To determine the role of Prdm1a regulation of target genes in neural crest development, we injected these constructs into prdm1a
mutants and assessed the NCC marker crestin at 24 hpf. In prdm1a−/− embryos, NCCs were highly reduced compared with WT, and in these mutants we rarely observed NCCs in the anterior trunk or in more than seven somite lengths (Fig. 7A,B,F; NCCs in 6% of prdm1a−/− embryos as confirmed by single-embryo genotyping). Thus, our criteria for embryos with NCC rescue were: (1) the presence of NCCs in the anterior trunk and (2) instances of NCCs migrating in seven or more somites. In DBD-VP16- or DBD-EnR-injected mutants, NCCs were absent in most embryos (Fig. 7C,D,F), suggesting that neither the dominant activator nor the dominant repressor form of Prdm1a is sufficient for NCC development. Injection of prdm1aDBD alone also did not rescue NCCs, suggesting that transcriptional activation and repression are required for Prdm1a function (supplementary material Fig. S7G-I). However, when both the VP16 and EnR fusion mRNAs were co-injected into prdm1a mutants, NCCs were rescued in 41% of the mutant embryos (Fig. 7E,F) to levels similar to those seen in prdm1a mRNA rescue of prdm1a−/− (supplementary material Fig. S7J-L) (Hernandez-Lagunas et al., 2005). This suggests that, for migratory NCC development, Prdm1a is required both as a transcriptional activator and as a transcriptional repressor.

To determine whether expression of prdm1aDBD-VP16 and DBD-EnR can rescue prdm1a−/− NCCs at earlier stages, we injected DBD-VP16 and DBD-EnR into prdm1a−/− embryos and performed ISH for foxd3 and tfap2a at 2-somites. As expected of directly activated targets, foxd3 and tfap2a mRNA expression was rescued by prdm1aDBD-VP16 injection in prdm1a−/−, whereas prdm1aDBD-EnR was unable to rescue (supplementary material Fig. S8A-H). To determine the effect on NCC specification at the intermediate stage of 4-somites, we assayed for rescue of sox10 expression in prdm1a mutants injected with prdm1aDBD-VP16 and/or prdm1aDBD-EnR. Interestingly, sox10 expression at the NPB was partially rescued with DBD-VP16 or DBD-EnR alone, as well as with both injected together (supplementary material Fig. S8I-M). This suggests that Prdm1a regulates sox10 both by activating genes that positively regulate it, such as tfap2a and foxd3, and by repressing genes that encode repressors of sox10, allowing for its activation by other genes that are parallel to the Prdm1a pathway (Fig. 8). Furthermore, Prdm1a must have roles as an activator and repressor of additional targets required for migratory NCCs, as both the activator and repressor forms are needed to rescue crestin-positive NCCs at later stages.

**DISCUSSION**

These studies demonstrate the role of Prdm1a as a master regulator of neural crest specification in zebrafish embryogenesis by directly binding to and activating the transcription of several hallmark NCC specifier gene enhancers. We have identified foxd3 and tfap2a, both crucial NCC specifiers, as candidate genes downstream of Prdm1a during NPB and NCC specification. We have demonstrated that foxd3 and tfap2a colocalize with prdm1a at the NPB in zebrafish embryos and that prdm1a is both required and sufficient to drive their expression at the NPB. Further, both foxd3 and tfap2a rescue the reduced NCC phenotype of prdm1a-deficient embryos. We identified Prdm1a consensus binding sites in putative enhancers for both of these genes and via ChiP confirmed Prdm1a binding at these sites during NCC specification. GFP reporter constructs using these putative enhancers confirmed that they drive expression at the NPB and are directly activated by Prdm1a. Although these reporter assays demonstrate Prdm1a regulation of these enhancers, it is important to
note that there are other potential regulators of foxd3 and tfap2a that are also likely to contribute to their regulation at the NPB. These studies demonstrate a novel role for Prdm1a as a transcriptional activator of genes required for NCC specification and development in zebrafish embryos.

These data allow us to assemble a new hierarchy of genes that contributes to our understanding of the gene regulatory network driving NCC specification (Fig. 8). Previous work has shown that tfap2a, along with its redundant family member tfap2c, are upstream of foxd3 (Li and Cornell, 2007), and we have confirmed that tfap2a/c also regulate prdm1a through a positive-feedback loop within the NPB. The subsequent gene cascade for NCC specification is well characterized, with foxd3 and tfap2a required for the expression of additional early neural crest genes including snai1b and sox10. From our work, we now know that Prdm1a, possibly along with other transcriptional regulators and/or co-factors, is directly upstream of foxd3 and tfap2a and is potentially a master regulator of the initiating steps in the specification of NCCs from the NPB in addition to potentially regulating specification of the NPB itself (Rossi et al., 2009). Prdm1a might also have a role in repressing genes that are inhibitors of NCC specification and appears to regulate NCCs after their initial specification; its action as both a transcriptional activator and repressor of genes is required for migratory NCCs.

Our findings also reveal a novel mechanism of Prdm1a transcriptional regulation of vertebrate target genes. Previous work on the Prdm1a protein in zebrafish as well as its homologs Blimp1 in mouse and PRDI-BF1 in human has demonstrated that Prdm1 is a transcriptional repressor of its direct targets. In zebrafish muscle cells, Prdm1a binds to and represses sox6, a repressor of slow-twitch muscle genes, and may also target fast-twitch fiber genes in order to promote slow muscle differentiation (von Hofsten et al., 2008). In mammalian B cells, Blimp1 is considered a master regulator of plasma cell differentiation as it directly represses several genes associated with the mature B cell program and cell cycle. Specifically, Blimp1 recruits histone deacetylases to repress transcription from the c-Myc (Yu et al., 2000) and Pax5 (Lin et al., 2002) promoters. Additionally, Blimp1 represses expression of the interferon-β gene.
by interacting with the Groucho family of transcriptional corepressors as well as with G9a (Ehmt2) methyltransferase (Ren et al., 1999; Gyory et al., 2004). Interestingly, the Pro/Ser-rich region, as well as the zinc-finger domain, of Blimp1 seems to be responsible for interaction with each of these transcriptional co-factors. This, combined with the fact that Blimp1/Prdm1 does not appear to have any intrinsic histone methyltransferase activity through its PR/SET domain, suggest that Prdm1 regulates targets primarily through the recruitment of binding partners.

In contrast to published reports, our new findings indicate that, in zebrafish NCC specification, Prdm1a also has transcriptional activator function; it binds and activates the transcription of foxd3, tfap2a, and potentially other genes as well. Additionally, our results from the super-activator and super-repressor experiments suggest that Prdm1a is required to act as both a transcriptional activator and repressor to drive the development of NCCs. How this dual function works in the embryo remains unknown; it is unclear whether Prdm1a performs these roles differently in different cells types, at different developmental stages, or on specific targets. Prdm1a contains several potential protein-protein interaction domains, suggesting that different transcriptional co-factor binding partners might help facilitate the switch in the mode of Prdm1a regulation. As Prdm1a has not been shown to have any intrinsic transcriptional regulation abilities, it stands to reason that these additional co-factors are required to mediate Prdm1a regulation of target genes. We hypothesize that Prdm1a acts as a transcriptional repressor during NPB stages to repress neural plate and non-neural ectoderm from expanding into the NPB, thus specifying the neural crest domain. One candidate for Prdm1a transcriptional repression is olig4, a transcription factor that is expressed within the interneuron domain of the neural plate, is upregulated in prdm1a morphants and is known to repress NPB and NCC fate (Hernandez-Lagunas et al., 2011). Once the NPB is specified, Prdm1a then activates the neural crest specifiers foxd3, tfap2a and others to promote the NCC fate. At the same time, foxd3 is likely to repress prdm1a from the NPB, potentially to maintain and specify the fate of the NCCs from the NPB. Prdm1a also appears to activate and repress genes required for NCC migration, such as adhesion and EMT genes, possibly regulating their expression during initial neural crest specification (Fig. 8).

Prdm1 is highly conserved in vertebrates and some echinoderms (Davidson et al., 2002a; Hinman and Davidson, 2003; John and Garrett-Sinha, 2009), especially within the key PR/SET and zinc-finger domains (Nikitina et al., 2011). In the basal vertebrate lamprey, prdm1 is expressed in the developing NPB and is regulated by several NPB regulators including Msx-1 and AP-2. In zebrafish, prdm1a expression is also dependent on two AP-2 family members, tfap2a and tfap2c (Li and Cornell, 2007), and here we now demonstrate a positive reciprocal interaction whereby Prdm1a, in turn, directly activates tfap2a at the NPB. Interestingly, in the echinoderm sea urchin, the prdm1 homolog blimp1 (also known as Kro1) is important in specifying the endomesoderm through the Wnt8 pathway, and directly activates both the Wnt8 and Otx genes rather than acting as a transcriptional repressor (Davidson et al., 2002a; Davidson et al., 2002b; Hinman and Davidson, 2003; Minokawa et al., 2005). Additional targets within the endoderm include eve and hox11/13b, which also appear to be transcriptionally activated by blimp1 (Livi and Davidson, 2006). In addition to its demonstrated role as an activator of genes in the endoderm, blimp1 represses its own expression in the mesoderm, probably through direct binding (Livi and Davidson, 2006), and directly represses the delta repressor HesC within the non-skeletogenic mesoderm (Smith and Davidson, 2008). This combination of roles for blimp1 within different tissue types in the sea urchin embryo suggests an evolutionarily conserved ability for prdm1 to function as both a transcriptional activator and repressor during embryonic development, and further supports the role of prdm1 as a master regulator of developmental pathways.

In the vertebrate Xenopus, prdm1 is expressed in the NPB of embryos and knockdown of prdm1 causes malformation of the head,
potentially as a result of NCC defects (de Souza et al., 1999). In addition, prdm1 is induced at an ectopic NPB following neural plate grafts into the non-neural ectoderm (Rossi et al., 2008); however, the exact role of prdm1 in Xenopus NCC development remains unclear. In mouse, Blimp1 does not appear to have a role in neural crest specification directly, but does play a role in craniofacial development (Vincent et al., 2005), suggesting that the role of prdm1 in early NCC development might not be conserved in mammals. However, we have recently determined that the prdm1 family member Prdm3 (Mecom – Mouse Genome Informatics) is expressed in migratory NCCs in the mouse embryo and thus might have assumed the role of Blimp1 in specifying slow-twitch muscle fiber identity in response to Hedgehog signaling. Nat. Genet. 36, 88-93.

In conclusion, these studies demonstrate a novel role for Prdm1a as a transcriptional activator of the gene regulatory network required for neural crest specification in zebrafish, and suggest that Prdm1a functions as both a transcriptional activator and repressor of multiple targets in different tissues and at different time points during neural crest development.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
Experiments were designed by D.R.P. and K.B.A. All experiment were performed by D.R.P. with technical help from L.H.-L. with genotyping and the dominant activator/repressor experiments, and from K.L. with the CHIP D.R.P. and K.B.A. analysed and interpreted data, and wrote the manuscript. All authors commented on the manuscript.

Supplementary material
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Fig. 8. Model of the Prdm1a gene regulatory network for NCC specification. Prdm1a is activated at the NPB by NPB specifiers during gastrulation and epiboly. Prdm1a directly activates foxd3 and tfap2a at 2-somites (green arrows) and these genes in turn feedback on prdm1a through either direct or indirect mechanisms (black arrows). Prdm1a also directly represses genes that are repressors for neural crest specification as marked by sox10 at 4-somites (red capped arrow). Additionally, sox10 expression and NCC specification are likely to be regulated by other genes that are parallel to the Prdm1a pathway of regulation (curved black arrow). Prdm1a also transcriptionally activates and represses genes required for the further development of NCCs during migratory stages. Green arrows denote direct activation, red arrows represent direct repression, and black arrows/capped arrows show activation or repression through indirect or unknown mechanisms. GRN, gene regulatory network; NCC, neural crest cell; NPB, neural plate border; som, somite.

References


