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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), *snai1b* or *slug* (*snai2*) (Thisse 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.

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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 *prdm1* 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 (Ohinata et al., 2005; Vincent et al., 2005), is required for the functional differentiation of B and T lymphocytes (Turner et al., 1994; Shapiro-Shelef et al., 2003; Shapiro-Shelef et al., 2005; Kallies et al., 2006; Martins et al., 2006), and plays a role in the development of the forelimb, pharyngeal arches, heart and sensory vibrissae (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

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 *snai1b* 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 (Brewer 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*, *snai1b* 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*^{m805} (*nrd*) (Artinger et al., 1999; Hernandez-Lagunas et al., 2011) and *foxd3*^{zdf10} (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* E2I2 splice site injected at 4 ng (Hernandez-Lagunas et al., 2005), *foxd3* 5′UTR and ATG morpholinos at 2 ng or 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′-AATAAGGATCCGCCGCCACCATGACCCTGTCTGGAGGCA-3′ and 5′-GCCGGTCTAGATCATTGAGAAGGCCATTTCGATAACGCTG-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 (Thisse and Thisse, 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: *snai1b* (primers 5′-GCTAGGGATCCGCCGCCACCATGCCACGCTCATTTCTTGTCA-3′ and 5′-GAATTCTAGATGTGTGTCCACTAGAGCGCC-3′); *foxd3* (see above); *sox10* (Olesnicky 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 Biotin System (Perkin Elmer, NEL700A001KT) followed by streptavidin Alexa Fluor 488 antibody (Invitrogen, 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 (Genomatix) were amplified by PCR from WT zebrafish genomic DNA using the following primers (5′-3′): *foxd3*E1, GGGGACAAGTTTGTACAAAAAAGCAGGCTACTGAACGCTGTGTGTCCAG and GGGGACCACTTTGTACAAGAAAGCTGGGTAAATTATCCAACTTGGATGAGCG; *tfap2a*E2, GGGGACAAGTTTGTACAAAAAAGCAGGCTTACATATGATCCTTATGCCATTCAG and GGGGACCACTTTGTACAAGAAAGCTGGGTATGGCAACTGCACTAACCTTCA. Each primer contained attB1 (forward primers) or attB2 (reverse primers) sites for Gateway cloning into the pGreenE vector (from D. Meulemans Medeiros, University of Colorado, Boulder, CO, USA) (Garnett et al., 2012). Plasmid containing the enhancer sites or empty plasmid alone expressing GFP under the cFos minimal promoter was injected into single-cell embryos at 80-100 pg. Mutated enhancer sequences were synthesized using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, 200518). GFP pixel intensity was calculated in Adobe Photoshop by converting images to grayscale, normalizing for background autofluorescence, outlining each embryo, and measuring the average pixel intensity value within the outlined region (using the Histogram function).

Chromatin immunoprecipitation

The protocol for chromatin immunoprecipitation (ChIP) on zebrafish embryos was optimized from published work (Wardle et al., 2006; von

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* morphants 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

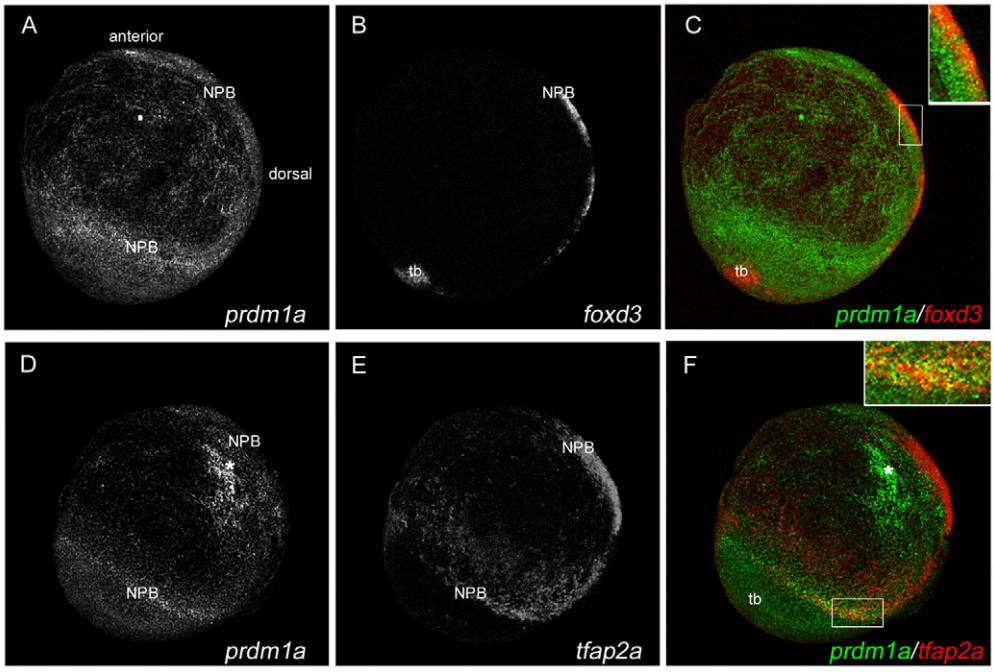

Fig. 1. *prdm1a* is co-expressed with *foxd3* and *tfap2a* at the NPB. Confocal micrograph projections of double fluorescent *in situ* hybridization (ISH) of 2-somite (11 hpf) wild-type (WT) zebrafish embryos for *prdm1a* with *foxd3* (**A-C**) and for *prdm1a* with *tfap2a* (**D-F**). *prdm1a* (green) is co-expressed with both *foxd3* (red, C) and *tfap2a* (red, F) at the NPB, as represented in yellow in the merged images (see insets in C and F). All images are lateral views with anterior to the top, dorsal to the right. Asterisk indicates non-specific staining. NPB, neural plate border; tb, tailbud.

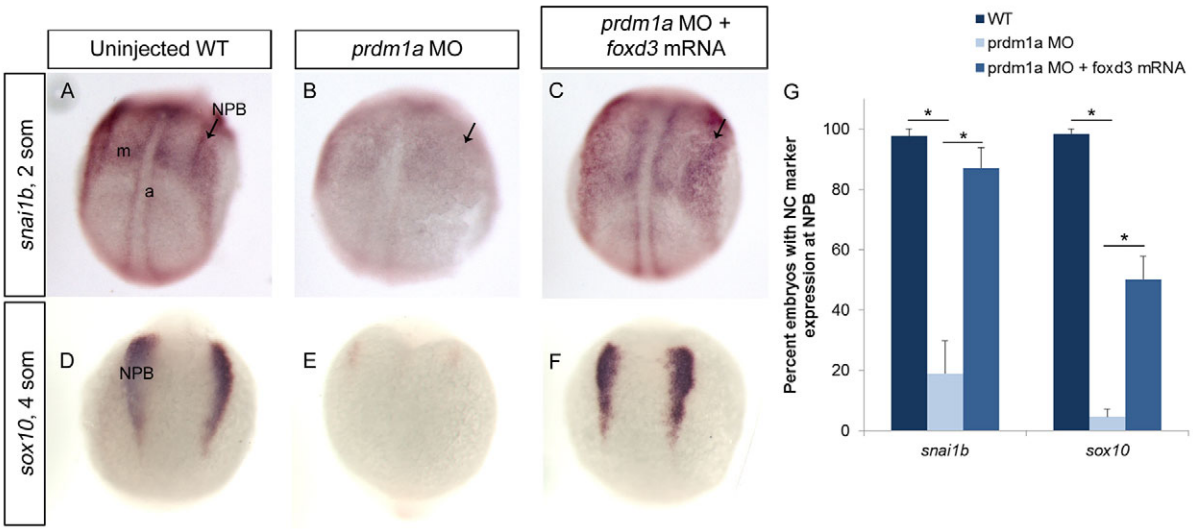

Fig. 2. *foxd3* mRNA rescues NCCs in *prdm1a*-deficient embryos. (**A-F**) ISH for neural crest markers *snai1b* (A-C) and *sox10* (D-F) on 2- to 4-somite (11-12 hpf) uninjected zebrafish embryos (A,D), *prdm1a* morphants (B,E) and with *prdm1a*-MO co-injected with *foxd3* mRNA (C,F). Dorsal view of WT embryos show neural crest expression at the NPB (arrows) for both *snai1b* and *sox10*, with *snai1b* also expressed in the adaxial cells and mesoderm. In *prdm1a* morphants, the expression is reduced at the NPB. However, after co-injection with *foxd3* mRNA, the NPB expression is restored. All images are dorsal views, anterior to the top. a, adaxial cells; m, mesoderm. (**G**) Percentage of embryos expressing each marker. *snai1b*: WT, *n* (number of embryos exhibiting the phenotype in A out of the number of embryos examined)=81/84; *prdm1a*-MO, *n*=13/59; rescue, *n*=41/48. *sox10*: WT, *n*=156/160; *prdm1a*-MO, *n*=4/92; rescue, *n*=39/77. *P<0.05. Error bars indicate s.e.m.

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) embryo 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 *foxd3*E1:GFP construct is expressed at the NPB and to a lesser extent in neighboring domains (Fig. 3D). Double fluorescent ISH 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. 3E,G,H). To confirm that wild-type *prdm1a* mRNA is sufficient to activate the enhancer, we overexpressed *prdm1a* mRNA with *foxd3*E1:GFP and observed a significant increase in GFP pixel intensity over *foxd3*E1: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

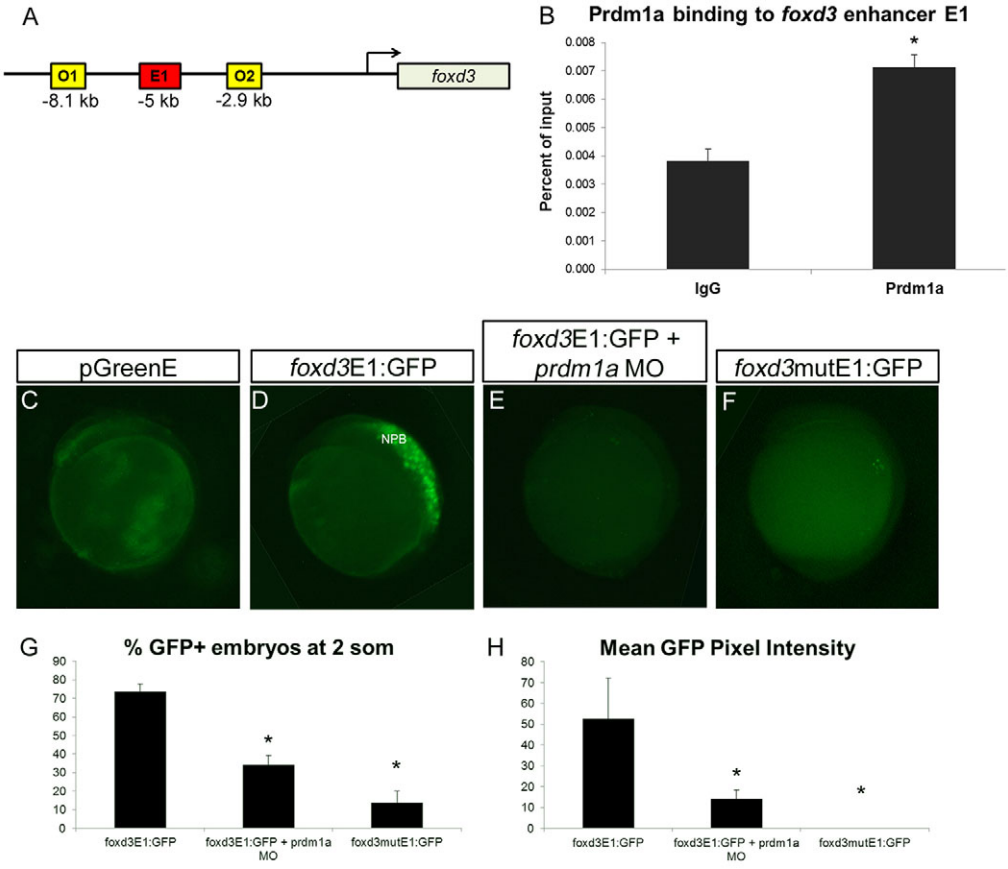

Fig. 3. Prdm1a directly binds and activates a *foxd3* enhancer at the NPB. (**A**) The zebrafish *foxd3* locus showing one putative enhancer (E1) ~5 kb upstream from the transcription start site that contains a binding sequence for Prdm1a, as well as the two off-target sites O1 and O2 used for ChIP. (**B**) Prdm1a ChIP pulls down *foxd3* E1, which is enriched compared with the control IgG pulldown. (**C-F**) Lateral view of embryos injected with empty (no enhancer sequence) pGreenE GFP expression vector (C), *foxd3* enhancer construct *foxd3*E1:GFP (D), *foxd3*E1:GFP with *prdm1a*-MO (E), and the *foxd3* enhancer with a mutated Prdm1a binding site driving GFP as construct *foxd3*mutE1:GFP (F). Specific binding of Prdm1a to the *foxd3* enhancer E1 is illustrated. Lateral views, anterior to the top. (**G**,**H**) The percentage of embryos expressing GFP (G) and the average pixel intensity of GFP (H). (G) *foxd3*E1:GFP, *n*=161/227; *foxd3*E1:GFP + *prdm1a*-MO, *n*=32/102; *foxd3*mutE1:GFP, *n*=29/166. (H) *n*=10 per condition. **P*<0.05. Error bars indicate s.e.m.

2-somites (supplementary material Fig. S5) and is upregulated and expanded in *prdm1a*-overexpressing embryos (supplementary material Fig. S1I,J) 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 *foxd3* or *sox10*, respectively. There is a clear downregulation of both *foxd3* (Fig. 4A,B,G; WT, 88% of embryos express *foxd3* at NPB; *prdm1a*-MO, 8.5% express *foxd3*) 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 *foxd3* and *sox10* are partially rescued at the NPB (Fig. 4C,F,G; *foxd3*, 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 *foxd3*, *tfap2a* expression is probably also modulated by other enhancers that are not regulated by Prdm1a. We co-injected the *tfap2a*E2: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 *tfap2a*E2:GFP construct and injected it into single-cell WT embryos (*tfap2a*MutE2:GFP; sequence in supplementary material Fig. S4B). At 2-somites, GFP expression was reduced to levels similar to those of the wild-type *tfap2a*E2: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 *tfap2a*E2:GFP and observed an increase in GFP pixel intensity compared with *tfap2a*E2: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

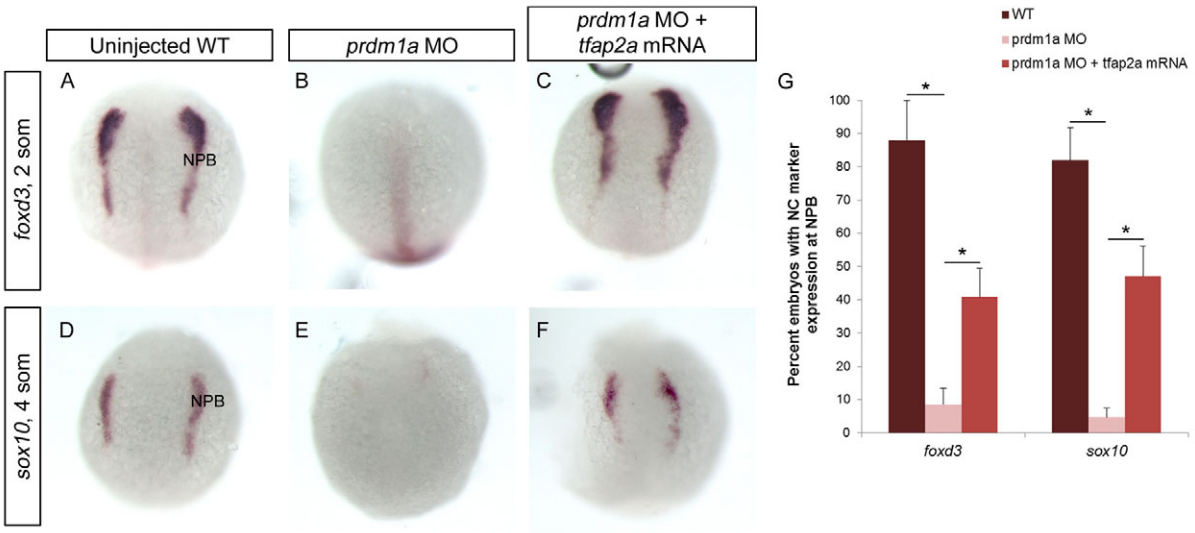

Fig. 4. *tfap2a* mRNA rescues *prdm1a*-deficient neural crest. (**A-F**) Uninjected WT zebrafish embryos (A,D), *prdm1a* morphants (B,E) and *prdm1a* morphants co-injected with *tfap2a* mRNA (C,F) were subject to ISH for the neural crest markers *foxd3* at 2-somites (A-C) and *sox10* at 4-somites (D-F). WT embryos show defined expression of *foxd3* and *sox10* at the NPB, which is significantly decreased in *prdm1a* morphants. *tfap2a* mRNA injection rescues the neural crest in *prdm1a*-deficient embryos. Dorsal views, anterior to the top. (**G**) The percentage of embryos expressing each marker. *foxd3*: WT, *n*=49/58; *prdm1a*-MO, *n*=13/120; rescue, *n*=27/72. *sox10*: WT, *n*=45/56; *prdm1a*-MO, *n*=3/62; rescue, *n*=37/76. **P*<0.05. Error bars indicate s.e.m.

then co-injected *foxd3*E1:GFP or *tfap2a*E2:GFP with *prdm1a*DBD-EnR or *prdm1a*DBD-VP16 and imaged embryos at 2-somites. We observed a loss of GFP expression with both constructs when co-injected with *prdm1a*DBD-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 *prdm1a*DBD-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 *foxd3* 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 *foxd3* and *tfap2a* and assayed for *prdm1a* expression at the NPB by ISH and qRT-PCR. Interestingly, morpholino knockdown of *foxd3* caused an increase in *prdm1a* expression in the NPB at the 2-somite stage (supplementary material Fig. S6A-C), which was confirmed in *foxd3*$^{zdf10/zdf10}$ (*sym1*) mutants (data not shown). As assessed by ISH, the expression domain of *prdm1a* is expanded, especially in the anteriormost region of the NPB where *foxd3* is most highly expressed. This suggests that *foxd3*, 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 *foxd3* 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 *foxd3* or *prdm1a* (data not shown). These data suggest a positive-feedback loop between *prdm1a* and *tfap2a*/*c* during NPB and NCC specification. Interestingly, *foxd3* 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 *foxd3* is downstream of both *prdm1a* and *tfap2a*.

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

Our data on the *foxd3* 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 *prdm1a*DBD-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 *prdm1a*DBD-EnR mRNA partially rescued the slow-twitch muscle phenotype of *prdm1a*$^{-/-}$ as assayed by *prox1* expression (supplementary material Fig. S7A-C). Correspondingly, injection of *prdm1a*DBD-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*

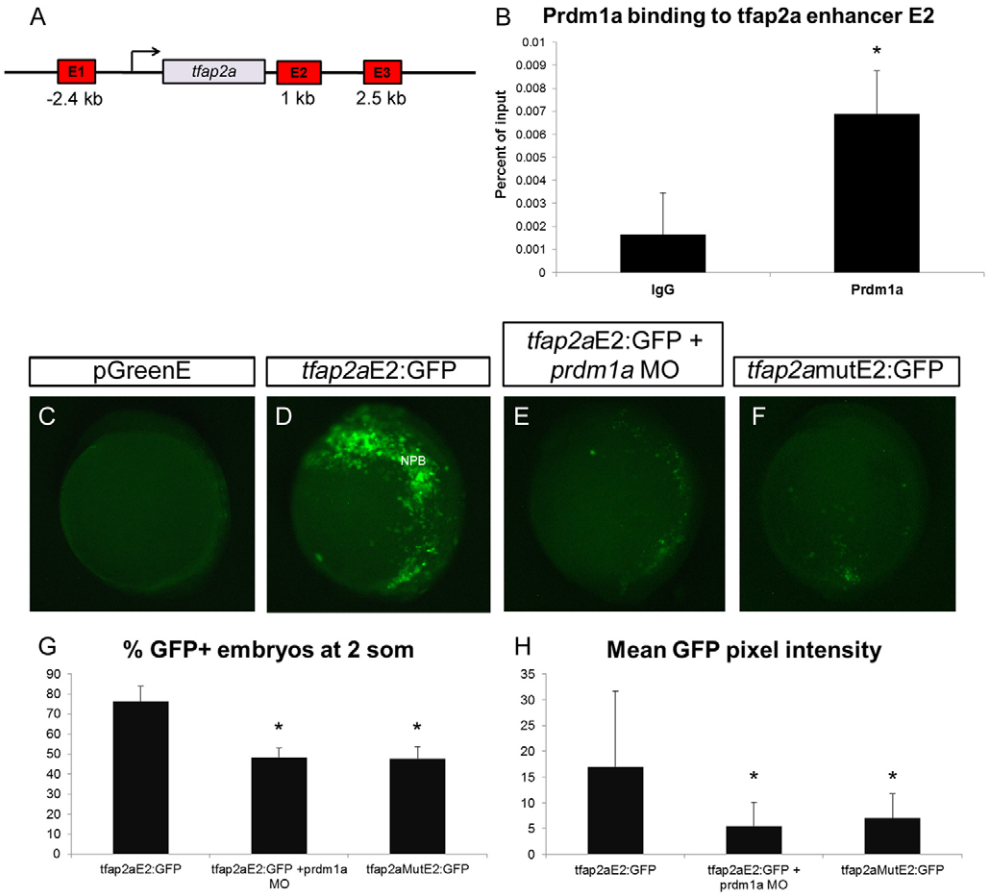

Fig. 5. Prdm1a directly binds and activates a *tfap2a* enhancer at the NPB. (**A**) The zebrafish *tfap2a* locus showing the three putative enhancers E1, E2 and E3 (distance from the *tfap2a* transcription start site is indicated) that contain Prdm1a binding sequences and were analyzed by ChIP. (**B**) Prdm1a ChIP pulls down *tfap2a* E2, which is enriched compared with IgG. (**C-F**) Two-somite embryos injected with pGreenE GFP expression plasmid (C), *tfap2a*E2 driving GFP as construct *tfap2a*E2:GFP (D), *tfap2a*E2:GFP with *prdm1a*-MO (E), and *tfap2a*E2 with a mutated Prdm1a binding site driving GFP as construct *tfap2a*mutE2:GFP (F). Specific binding of Prdm1a to an enhancer for *tfap2a* is illustrated. (**G**,**H**) The percentage of embryos expressing GFP (G) and the average pixel intensity of GFP (H). (G) *tfap2a*E2:GFP, *n*=55/68; *tfap2a*E2:GFP + *prdm1a*-MO, *n*=43/88; *tfap2a*E2mut:GFP, *n*=47/100. (H) *tfap2a*E2:GFP, *n*=23; *tfap2a*E2:GFP + *prdm1a*-MO, *n*=14; *tfap2a*E2mut:GFP, *n*=10. **P*<0.05. Error bars indicate s.e.m.

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 *prdm1a*DBD 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 a transcriptional repressor.

To determine whether expression of *prdm1a*DBD-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 *prdm1a*DBD-VP16 injection in *prdm1a*$^{-/-}$, whereas *prdm1a*DBD-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 *prdm1a*DBD-VP16 and/or *prdm1a*DBD-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

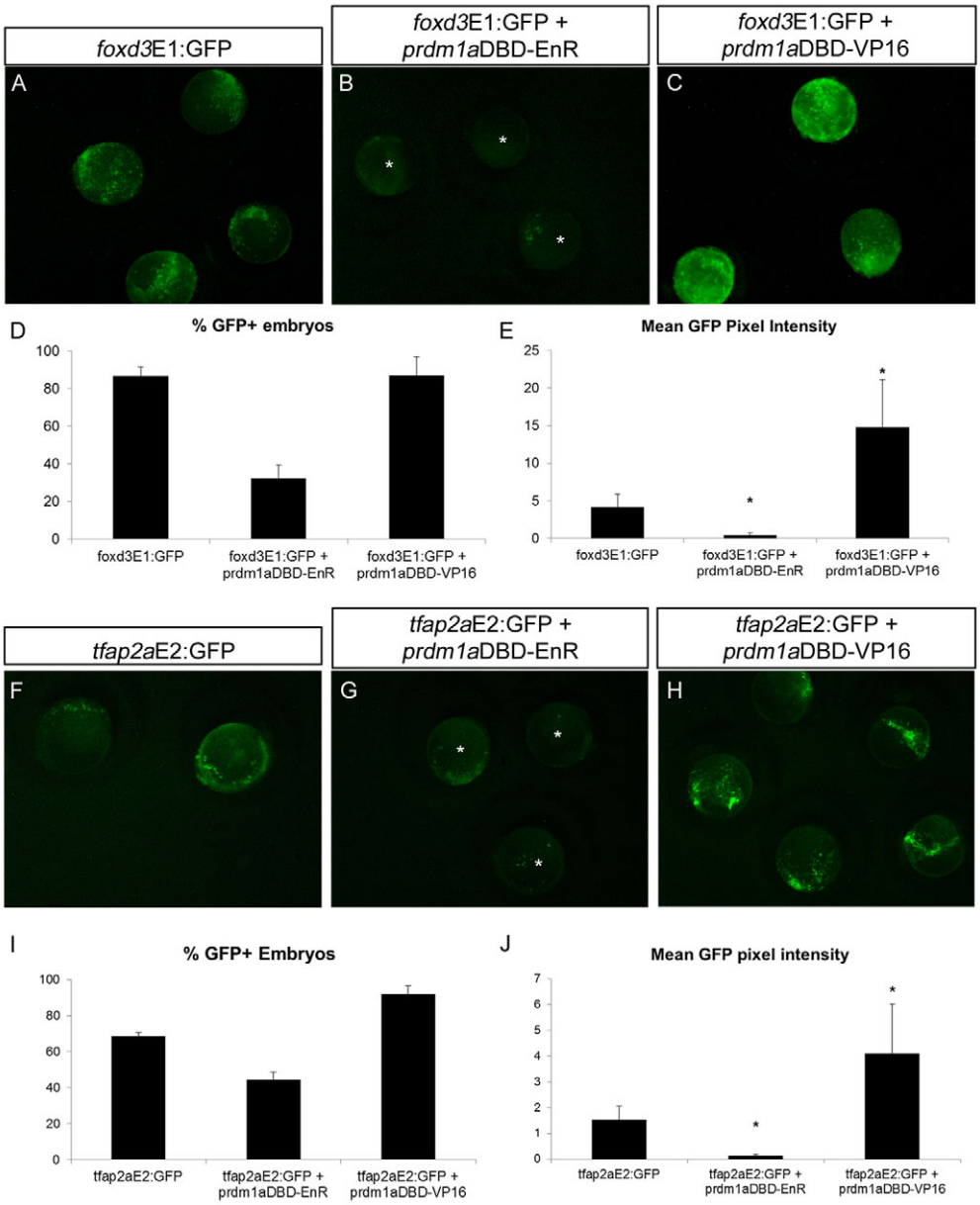

Fig. 6. Prdm1a dominant activator and dominant repressor constructs directly regulate *foxd3* and *tfap2a* enhancers. (**A-C**) Zebrafish embryos at the 2-somite stage expressing *foxd3*E1:GFP alone (A) or with *prdm1a*DBD-EnR dominant repressor (B) or *prdm1a*DBD-VP16 dominant activator (C). GFP expression is downregulated when *foxd3*E1:GFP is co-injected with *prdm1a*DBD-EnR (asterisks) and upregulated when the enhancer construct is co-expressed with *prdm1a*DBD-VP16. (**D**,**E**) The percentage of embryos expressing GFP (D) and GFP pixel intensity (E) in A-C. (D) *foxd3*E1:GFP, *n*=19/22; EnR, *n*=6/19; VP16, *n*=28/32. (E) *foxd3*E1:GFP, *n*=17; EnR, *n*=10; VP16, *n*=10. (**F-H**) Two-somite embryos expressing *tfap2a*E2:GFP with *prdm1a*DBD-EnR also exhibit downregulated GFP (G, asterisks), whereas when co-expressed with *prdm1a*DBD-VP16 they display increased GFP expression (H). (**I**,**J**) Percentage of GFP-positive embryos (I) and GFP pixel intensity (J) in F-H. (I) *tfap2a*E2:GFP, *n*=11/16; EnR, *n*=16/36; VP16, *n*=36/39. (J) *tfap2a*E2:GFP, *n*=11; EnR, *n*=14; VP16, *n*=12. **P*<0.05. Error bars indicate s.e.m.

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

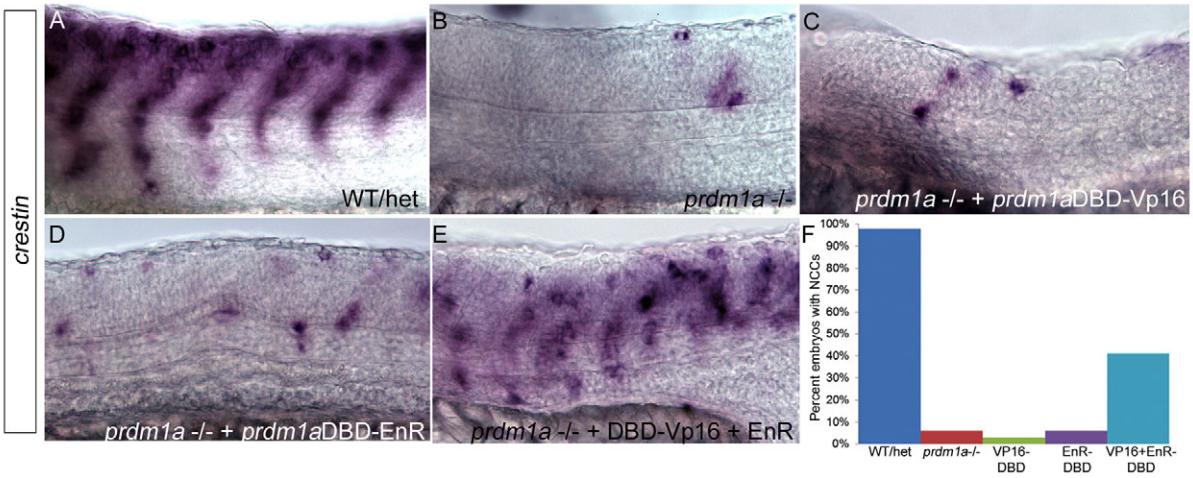

Fig. 7. *prdm1a*DBD-VP16 and *prdm1a*DBD-EnR together rescue NCCs in *prdm1a* mutant embryos. (**A-E**) Lateral views of WT and *prdm1a*$^{-/-}$ zebrafish embryos at 24 hpf, dorsal to the top. ISH for *crestin* expression in WT and/or heterozygotes reveals migrating NCCs in the trunk (A). *prdm1a* mutant embryos have little NCC migration and most do not exhibit migration in more than seven somites (B). Injection of *prdm1a*DBD-VP16 (C) or *prdm1a*DBD-EnR (D) alone cannot rescue NCCs in *prdm1a* mutants. However, injection of *prdm1a*DBD-VP16 and *prdm1a*DBD-EnR together rescues *crestin* expression in *prdm1a*$^{-/-}$ embryos and NCCs of rescued animals are able to migrate similarly to WT (compare E with A), suggesting that Prdm1a must function as both a transcriptional activator and repressor for migratory NCCs to develop. *prdm1a*$^{-/-}$ embryos were identified by their curved tail, U-shaped somites and fin mesenchyme defects, and confirmed by genotyping. (**F**) The percentage of embryos with NCCs present in seven or more somites. Sample size: Prdm1a mutants alone (with NCCs in seven or more somites), *n*=7/106; Prdm1a mutants injected with Vp16, *n*=2/55; Prdm1a mutants injected with EnR, *n*=2/29; Prdm1a mutants injected with both prdm1a-DBD-Vp16 and EnR, n=52/125.

by interacting with the Groucho family of transcriptional co-repressors 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 fates (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 *Krox1*) 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 nonskeletogenic 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,

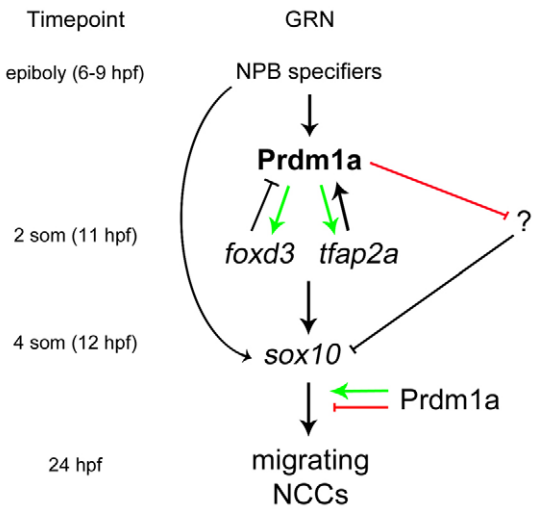

Fig. 8. Model of the Prdm1a gene regulatory network for NCC specification. Prdm1a is activated at the NPB by NPB specifiers during gastrulation and epiboly. Prdm1a directly activates *foxd3* and *tfap2a* at 2-somites (green arrows) and these genes in turn feedback on *prdm1a* through either direct or 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.

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 *prdm1* in early NCC development in mammals (our unpublished data). Interestingly, *prdm3* is also important for cranial NCC maintenance in zebrafish (Ding et al., 2013) and is expressed in cranial NCCs in *Xenopus* (Mead et al., 2005).

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 experimement 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
Supplementary material available online at
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References

Ancelin, K., Lange, U. C., Hajkova, P., Schneider, R., Bannister, A. J., Kouzarides, T. and Surani, M. A. (2006). Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. *Nat. Cell Biol.* **8**, 623-630.
Arduini, B. L., Bosse, K. M. and Henion, P. D. (2009). Genetic ablation of neural crest cell diversification. *Development* **136**, 1987-1994.
Artinger, K. B., Chitnis, A. B., Mercola, M. and Driever, W. (1999). Zebrafish narrowminded suggests a genetic link between formation of neural crest and primary sensory neurons. *Development* **126**, 3969-3979.
Aybar, M. J. and Mayor, R. (2002). Early induction of neural crest cells: lessons learned from frog, fish and chick. *Curr. Opin. Genet. Dev.* **12**, 452-458.
Barrallo-Gimeno, A., Holzschuh, J., Driever, W. and Knapik, E. W. (2004). Neural crest survival and differentiation in zebrafish depends on mont blanc/tfap2a gene function. *Development* **131**, 1463-1477.
Baxendale, S., Davison, C., Muxworthy, C., Wolff, C., Ingham, P. W. and Roy, S. (2004). The B-cell maturation factor Blimp-1 specifies vertebrate slow-twitch muscle fiber identity in response to Hedgehog signaling. *Nat. Genet.* **36**, 88-93.
Bikoff, E. K., Morgan, M. A. and Robertson, E. J. (2009). An expanding job description for Blimp-1/PRDM1. *Curr. Opin. Genet. Dev.* **19**, 379-385.
Birkholz, D. A., Olesnicky Killian, E. C., George, K. M. and Artinger, K. B. (2009). Prdm1a is necessary for posterior pharyngeal arch development in zebrafish. *Dev. Dyn.* **238**, 2575-2587.
Brewer, S., Feng, W., Huang, J., Sullivan, S. and Williams, T. (2004). Wnt1-Cre-mediated deletion of AP-2alpha causes multiple neural crest-related defects. *Dev. Biol.* **267**, 135-152.
Carney, T. J., Dutton, K. A., Greenhill, E., Delfino-Machín, M., Dufourcq, P., Blader, P. and Kelsh, R. N. (2006). A direct role for Sox10 in specification of neural crest-derived sensory neurons. *Development* **133**, 4619-4630.
Davidson, E. H., Rast, J. P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C. H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C. et al. (2002a). A genomic regulatory network for development. *Science* **295**, 1669-1678.
Davidson, E. H., Rast, J. P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C. H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C. et al. (2002b). A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. *Dev. Biol.* **246**, 162-190.
de Souza, F. S., Gawantka, V., Gómez, A. P., Delius, H., Ang, S. L. and Niehrs, C. (1999). The zinc finger gene Xblimp1 controls anterior endomesodermal cell fate in Spemann's organizer. *EMBO J.* **18**, 6062-6072.
Ding, H. L., Clouthier, D. E. and Artinger, K. B. (2013). Redundant roles of PRDM family members in zebrafish craniofacial development. *Dev. Dyn.* **242**, 67-79.
Dutton, K. A., Pauliny, A., Lopes, S. S., Elworthy, S., Carney, T. J., Rauch, J., Geisler, R., Haffter, P. and Kelsh, R. N. (2001). Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development* **128**, 4113-4125.
Eckert, D., Buhl, S., Weber, S., Jäger, R. and Schorle, H. (2005). The AP-2 family of transcription factors. *Genome Biol.* **6**, 246.
Garnett, A. T., Square, T. A. and Medeiros, D. M. (2012). BMP, Wnt and GF signals are integrated throughevolutionarily conserved enhancers to achieve robust expression of Pax3 and Zic genes at the zebrafish neural plate border. *Development* **139**, 4220-4231.

Gyory, I., Wu, J., Fejér, G., Seto, E. and Wright, K. L. (2004). PRDI-BF1 recruits the histone H3 methyltransferase G9a in transcriptional silencing. *Nat. Immunol.* **5**, 299-308.
Hernandez-Lagunas, L., Choi, I. F., Kaji, T., Simpson, P., Hershey, C., Zhou, Y., Zon, L., Mercola, M. and Artinger, K. B. (2005). Zebrafish narrowminded disrupts the transcription factor prdm1 and is required for neural crest and sensory neuron specification. *Dev. Biol.* **278**, 347-357.
Hernandez-Lagunas, L., Powell, D. R., Law, J., Grant, K. A. and Artinger, K. B. (2011). prdm1a and olig4 act downstream of Notch signaling to regulate cell fate at the neural plate border. *Dev. Biol.* **356**, 496-505.
Hinman, V. F. and Davidson, E. H. (2003). Expression of AmKrox, a starfish ortholog of a sea urchin transcription factor essential for endomesodermal specification. *Gene Expr. Patterns* **3**, 423-426.
Hohenauer, T. and Moore, A. W. (2012). The Prdm family: expanding roles in stem cells and development. *Development* **139**, 2267-2282.
Huang, X. and Saint-Jeannet, J. P. (2004). Induction of the neural crest and the opportunities of life on the edge. *Dev. Biol.* **275**, 1-11.
John, S. A. and Garrett-Sinha, L. A. (2009). Blimp1: a conserved transcriptional repressor critical for differentiation of many tissues. *Exp. Cell Res.* **315**, 1077-1084.
Kallies, A., Hawkins, E. D., Belz, G. T., Metcalf, D., Hommel, M., Corcoran, L. M., Hodgkin, P. D. and Nutt, S. L. (2006). Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat. Immunol.* **7**, 466-474.
Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
Knight, R. D., Nair, S., Nelson, S. S., Afshar, A., Javidan, Y., Geisler, R., Rauch, G. J. and Schilling, T. F. (2003). lockjaw encodes a zebrafish tfap2a required for early neural crest development. *Development* **130**, 5755-5768.
Knight, R. D., Javidan, Y., Nelson, S., Zhang, T. and Schilling, T. (2004). Skeletal and pigment cell defects in the lockjaw mutant reveal multiple roles for zebrafish tfap2a in neural crest development. *Dev. Dyn.* **229**, 87-98.
Kos, R., Reedy, M. V., Johnson, R. L. and Erickson, C. A. (2001). The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. *Development* **128**, 1467-1479.
Li, W. and Cornell, R. A. (2007). Redundant activities of Tfap2a and Tfap2c are required for neural crest induction and development of other non-neural ectoderm derivatives in zebrafish embryos. *Dev. Biol.* **304**, 338-354.
Lin, K. I., Angelin-Duclos, C., Kuo, T. C. and Calame, K. (2002). Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *Mol. Cell. Biol.* **22**, 4771-4780.
Lister, J. A., Cooper, C., Nguyen, K., Modrell, M., Grant, K. and Raible, D. W. (2006). Zebrafish Foxd3 is required for development of a subset of neural crest derivatives. *Dev. Biol.* **290**, 92-104.
Livi, C. B. and Davidson, E. H. (2006). Expression and function of blimp1/krox, an alternatively transcribed regulatory gene of the sea urchin endomesoderm network. *Dev. Biol.* **293**, 513-525.
Martins, G. A., Cimmino, L., Shapiro-Shelef, M., Szabolcs, M., Herron, A., Magnusdottir, E. and Calame, K. (2006). Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat. Immunol.* **7**, 457-465.
Mead, P. E., Parganas, E., Ohtsuka, S., Morishita, K., Gamer, L., Kuliyev, E., Wright, C. V. and Ihle, J. N. (2005). Evi-1 expression in Xenopus. *Gene Expr. Patterns* **5**, 601-608.
Minokawa, T., Wikramanayake, A. H. and Davidson, E. H. (2005). cis-Regulatory inputs of the wnt8 gene in the sea urchin endomesoderm network. *Dev. Biol.* **288**, 545-558.
Montero-Balaguer, M., Lang, M. R., Sachdev, S. W., Knappmeyer, C., Stewart, R. A., De La Guardia, A., Hatzopoulos, A. K. and Knapik, E. W. (2006). The mother superior mutation ablates foxd3 activity in neural crest progenitor cells and depletes neural crest derivatives in zebrafish. *Dev. Dyn.* **235**, 3199-3212.
Mundell, N. A. and Labosky, P. A. (2011). Neural crest stem cell multipotency requires Foxd3 to maintain neural potential and repress mesenchymal fates. *Development* **138**, 641-652.
Nikitina, N., Tong, L. and Bronner, M. E. (2011). Ancestral network module regulating prdm1 expression in the lamprey neural plate border. *Dev. Dyn.* **240**, 2265-2271.
Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S. C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A. et al. (2005). Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* **436**, 207-213.
Olesnicky, E., Hernandez-Lagunas, L. and Artinger, K. B. (2010). prdm1a Regulates sox10 and islet1 in the development of neural crest and Rohon-Beard sensory neurons. *Genesis* **48**, 656-666.
Pineda, R. H., Svoboda, K. R., Wright, M. A., Taylor, A. D., Novak, A. E., Gamse, J. T., Eisen, J. S. and Ribera, A. B. (2006). Knockdown of Nav1.6a Na^+ channels affects zebrafish motoneuron development. *Development* **133**, 3827-3836.
Ren, B., Chee, K. J., Kim, T. H. and Maniatis, T. (1999). PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes Dev.* **13**, 125-137.
Robertson, E. J., Charatsi, I., Joyner, C. J., Koonce, C. H., Morgan, M., Islam, A., Paterson, C., Lejsek, E., Arnold, S. J., Kallies, A. et al. (2007). Blimp1 regulates development of the posterior forelimb, caudal pharyngeal arches, heart and sensory vibrissae in mice. *Development* **134**, 4335-4345.
Rossi, C. C., Hernandez-Lagunas, L., Zhang, C., Choi, I. F., Kwok, L., Klymkowsky, M. and Artinger, K. B. (2008). Rohon-Beard sensory neurons are induced by BMP4 expressing non-neural ectoderm in Xenopus laevis. *Dev. Biol.* **314**, 351-361.
Rossi, C. C., Kaji, T. and Artinger, K. B. (2009). Transcriptional control of Rohon-Beard sensory neuron development at the neural plate border. *Dev. Dyn.* **238**, 931-943.
Roy, S. and Ng, T. (2004). Blimp-1 specifies neural crest and sensory neuron progenitors in the zebrafish embryo. *Curr. Biol.* **14**, 1772-1777.
Rubinstein, A. L., Lee, D., Luo, R., Henion, P. D. and Halpern, M. E. (2000). Genes dependent on zebrafish cyclops function identified by AFLP differential gene expression screen. *Genesis* **26**, 86-97.
Sauka-Spengler, T. and Bronner-Fraser, M. (2008). A gene regulatory network orchestrates neural crest formation. *Nat. Rev. Mol. Cell Biol.* **9**, 557-568.
Shapiro-Shelef, M., Lin, K. I., McHeyzer-Williams, L. J., Liao, J., McHeyzer-Williams, M. G. and Calame, K. (2003). Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* **19**, 607-620.
Shapiro-Shelef, M., Lin, K. I., Savitsky, D., Liao, J. and Calame, K. (2005). Blimp-1 is required for maintenance of long-lived plasma cells in the bone marrow. *J. Exp. Med.* **202**, 1471-1476.
Simões-Costa, M. S., McKeown, S. J., Tan-Cabugao, J., Sauka-Spengler, T. and Bronner, M. E. (2012). Dynamic and differential regulation of stem cell factor FoxD3 in the neural crest is Encrypted in the genome. *PLoS Genet.* **8**, e1003142.
Smith, J. and Davidson, E. H. (2008). Gene regulatory network subcircuit controlling a dynamic spatial pattern of signaling in the sea urchin embryo. *Proc. Natl. Acad. Sci. USA* **105**, 20089-20094.
Stewart, R. A., Arduini, B. L., Berghmans, S., George, R. E., Kanki, J. P., Henion, P. D. and Look, A. T. (2006). Zebrafish foxd3 is selectively required for neural crest specification, migration and survival. *Dev. Biol.* **292**, 174-188.
Teng, L., Mundell, N. A., Frist, A. Y., Wang, Q. and Labosky, P. A. (2008). Requirement for Foxd3 in the maintenance of neural crest progenitors. *Development* **135**, 1615-1624.
Thisse, C and Thisse, B. (1998). High resolution whole-mount *in situ* hybridization. *The Zebrafish Science Monitor* **5**.
Thisse, C., Thisse, B. and Postlethwait, J. H. (1995). Expression of snail2, a second member of the zebrafish snail family, in cephalic mesendoderm and presumptive neural crest of wild-type and spadetail mutant embryos. *Dev. Biol.* **172**, 86-99.
Turner, C. A., Jr, Mack, D. H. and Davis, M. M. (1994). Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* **77**, 297-306.
Van Otterloo, E., Li, W., Garnett, A., Cattell, M., Medeiros, D. M. and Cornell, R. A. (2012). Novel Tfap2-mediated control of soxE expression facilitated the evolutionary emergence of the neural crest. *Development* **139**, 720-730.
Vincent, S. D., Dunn, N. R., Sciammas, R., Shapiro-Shalef, M., Davis, M. M., Calame, K., Bikoff, E. K. and Robertson, E. J. (2005). The zinc finger transcriptional repressor Blimp1/Prdm1 is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. *Development* **132**, 1315-1325.
von Hofsten, J., Elworthy, S., Gilchrist, M. J., Smith, J. C., Wardle, F. C. and Ingham, P. W. (2008). Prdm1- and Sox6-mediated transcriptional repression specifies muscle fibre type in the zebrafish embryo. *EMBO Rep.* **9**, 683-689.
Wang, W. D., Melville, D. B., Montero-Balaguer, M., Hatzopoulos, A. K. and Knapik, E. W. (2011a). Tfap2a and Foxd3 regulate early steps in the development of the neural crest progenitor population. *Dev. Biol.* **360**, 173-185.
Wang, X., Ono, Y., Tan, S. C., Chai, R. J., Parkin, C. and Ingham, P. W. (2011b). Prdm1a and miR-499 act sequentially to restrict Sox6 activity to the fast-twitch muscle lineage in the zebrafish embryo. *Development* **138**, 4399-4404.
Wardle, F. C., Odom, D. T., Bell, G. W., Yuan, B., Danford, T. W., Wiellette, E. L., Herbolsheimer, E., Sive, H. L., Young, R. A. and Smith, J. C. (2006). Zebrafish promoter microarrays identify actively transcribed embryonic genes. *Genome Biol.* **7**, R71.
Westerfield, M. (1993). *The Zebrafish Book: A Guide For The Laboratory Use Of Zebrafish (Brachydanio rerio)*. Eugene, OR: University of Oregon Press.
Yu, J., Angelin-Duclos, C., Greenwood, J., Liao, J. and Calame, K. (2000). Transcriptional repression by blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. *Mol. Cell. Biol.* **20**, 2592-2603.