Published in final edited form as:

Dev Dyn. 2009 October ; 238(10): 2575–2587. doi:10.1002/dvdy.22090.

***prdm1a* is necessary for posterior pharyngeal arch development in zebrafish**

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Abstract

Multiple tissue interactions and signaling within the pharyngeal arches are required for development of the craniofacial skeleton. Here, we focus on the role of the transcription factor *prdm1a* in the differentiation of the posterior skeleton. *prdm1a* is expressed in the presumptive pharyngeal arch region and later in an endodermal pouch, the otic vesicle, and pharyngeal teeth. *prdm1a* mutants display a reduction in pharyngeal arch markers, a loss of posterior ceratobranchial cartilages, and a reduction in most neural crest-derived dermal bones. This is likely caused by a decrease in the number of proliferating cells but not an increase in cell death. Finally, a reduction in two key developmental signaling pathways, Fgf and retinoic acid, alters *prdm1a* expression, suggesting that *prdm1a* expression is mediated by these signaling pathways to pattern the posterior craniofacial skeleton. Together, these results indicate an essential role for *prdm1a* in the development of the zebrafish craniofacial skeleton.

Keywords

prdm1a; neural crest; craniofacial development; zebrafish; pharyngeal arches; endodermal pouches; Fgf signaling; retinoic acid signaling NIH R01 DE017699; F32 HD056779; F32 DE018594

INTRODUCTION

Neural crest cells are a multipotent migratory cell population found in all vertebrate embryos that generate a diverse array of tissue types during development (Le Douarin, 1982; LaBonne and Bronner-Fraser, 1998). Specifically, cranial neural crest cells (CNCCs) give rise to a majority of the peripheral nervous system and ectomesenchymal derivatives (cartilage, bone, and connective tissues) of the head (Le Douarin, 1982; Raible et al., 1992; Schilling and Kimmel, 1994; Trainor and Krumlauf, 2001; Graham, 2003). In all vertebrates, including humans, a series of pharyngeal arches develops on the lateral surface of the head, and these are the templates for the adult craniofacial structures. Perturbation of neural crest cell (NCC) differentiation results in many human craniofacial birth defects. A better understanding of cell fate determination, migration, and differentiation of neural crest cells during formation of the craniofacial skeleton is an important step towards developing approaches to prevent and repair neural crest cell-associated birth defects.

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Cranial neural crest cells migrate ventrolaterally along three distinct streams from the posterior midbrain and segments of the hindbrain, called rhombomeres (r), into the pharyngeal arches (Le Douarin, 1982; Schilling and Kimmel, 1994; Schilling, 1997). Neural crest cells from the midbrain, r1, and r2 populate the first pharyngeal arch. Cells mainly from r4 (some from r3 and r5) populate the second pharyngeal arch, and cells from r6–r8 populate the posterior pharyngeal arches, specifically arches 3–7 in zebrafish. Each arch gives rise to specific cartilages of the pharyngeal skeleton. In zebrafish, the first (mandibular) arch derivatives include Meckel's cartilage and the palatoquadrate, the second (hyoid) arch structures include the ceratohyal and hyosymplectic cartilages, and the third through seventh arches form the posterior ceratobranchial cartilages (Schilling and Kimmel, 1997). The cranial skeleton of zebrafish is composed of a dorsal neurocranium and a ventral viscerocranium (Kimmel et al., 1998) with the entire viscerocranium developing from the ventrally migrating CNCCs that populate the arches. Although much is known from previous studies about zebrafish craniofacial development, very little is known about the specific development of the more posterior arches.

Development of the pharyngeal arches is particularly interesting because they develop from all three germ layers and require multiple signaling pathways for proper patterning (Schilling and Kimmel, 1997; Graham and Smith, 2001; Clouthier and Schilling, 2004). Each pharyngeal arch has a mesodermal core surrounded by CNCCs, is covered externally by ectoderm and is separated from other arches by an endodermal pouch (Graham and Smith, 2001; Graham, 2003). Complex signaling pathways such as Fibroblast Growth Factor (Fgf), retinoic acid (RA), Hedgehog (Hh), Bone Morphogenetic Proteins (Bmps), Wnt, and Endothelins regulate downstream genes that play a role in neural crest cell induction, migration, patterning of the pharyngeal endodermal pouches, and the eventual differentiation of ectomesenchymal derivatives (David et al., 2002; Crump et al., 2004; Eberhart et al., 2006; Kopinke et al., 2006; Blentic et al., 2008; Sperber and Dawid, 2008).

A number of studies have focused on the role of the transcription factor *prdm1a* - PR domain containing 1a, with ZNF domain - during both invertebrate and vertebrate embryonic development, suggesting that *prdm1a* is very highly conserved (Baxendale et al., 2004; Roy and Ng, 2004; Hernandez-Lagunas et al., 2005; Vincent et al., 2005; Wilm and Solnica-Krezel, 2005; Lee and Roy, 2006; Mercader et al., 2006; Robertson et al., 2007; Elworthy et al., 2008; von Hofsten et al., 2008). In mice, *Prdm1* (also known as *Blimp-1*) drives differentiation of plasma cells from B-cells (Shaffer et al., 2002; Shapiro-Shelef et al., 2003), represses genes such as *c-myc* and *pax5* that maintain a B-cell state and promote a plasma cell fate (Lin et al., 1997; Lin et al., 2000; Lin et al., 2002), and promotes a progenitor fate in embryonic skin that defines the sebaceous gland (Horsley et al., 2006). Mouse *Prdm1* is expressed in the branchial arches starting at embryonic day (E) 8.5 and in the more posterior arches over the next 24 hrs. At E 9.5, *Prdm1* is restricted to the endoderm of the first arch but has expanded into the endoderm, ectoderm, and mesenchyme of the more posterior second and third arches. *Prdm1* transcripts are barely detectable in pharyngeal tissues beyond E10.5 (Vincent et al., 2005). The targeted disruption of *Blimp1* throughout early mouse development is embryonic lethal and suggests that *Prdm1* functions in multiple cell types during development (Shapiro-Shelef et al., 2003; Robertson et al., 2007). Robertson et al (Robertson et al., 2007) circumvented the early lethality of *Blimp1* null embryos by using a *Sox2-Cre* to knock out *Blimp1* only in the embryo proper and demonstrated the requirement for *Blimp1* in the function of multipotent progenitor cell populations in the posterior forelimb, caudal pharyngeal arches, secondary heart field, and sensory vibrissae. Specifically in the arches, *Sox2-Cre- Blimp1* mutant embryos from E10.5 onwards show that, while the jaws derived from the maxillary and mandibular arches of the first arch form normally, none of the tissue structures formed from the more caudal arches were present.

In zebrafish, there are two well characterized *prdm1a* mutant alleles, *narrowminded*^{tm805} and *U-boot* (*ubo*^{tp39}), where a null and missense hypomorphic mutation are observed, respectively. These studies show that *prdm1a* is required for neural crest and Rohon-Beard (RB) sensory neuron specification, downstream of Bmp signaling (Roy and Ng, 2004; Hernandez-Lagunas et al., 2005; Rossi et al., 2009). More recent results also suggest that *prdm1a* regulates *dlx3b/4b* expression in the non-neural ectoderm and *neurogenin1* expression in RB sensory neurons (Rossi et al., 2009). In addition to this role, *prdm1a* is also required in the specification of slow twitch muscle fibers (Elworthy et al., 2008; von Hofsten et al., 2008), has an early function during gastrulation in the formation of head structures, and acts downstream of retinoic acid, Wnt, and Fgf signaling during fin development (Mercader et al., 2006). Taken together, these data demonstrate that *prdm1a* is required for many aspects of embryogenesis. Expression of *prdm1a* in pharyngeal arch regions of *Xenopus* and zebrafish (de Souza et al., 1999; Wilm and Solnica-Krezel, 2005) is suggestive of a role in head development, but how *prdm1a* functions in the specification and differentiation of the zebrafish craniofacial skeleton is not yet known.

Here we focus on the role of *prdm1a* in the differentiation of neural crest cells into the craniofacial skeleton. We show that *prdm1a* is expressed throughout the pharyngeal arch region and a loss of *prdm1a* results in malformed endodermal pouches and a loss of ectomesenchymal derivatives. Our analysis also indicates that *prdm1a* expression in the arch region requires Fgf and retinoic acid signaling. This work provides insight into a novel role for *prdm1a* during zebrafish craniofacial development.

RESULTS

prdm1a is expressed in the developing zebrafish embryo

Previous data has shown that *prdm1a* is required in many cell types in the zebrafish but how *prdm1a* functions in the specification and differentiation of the zebrafish craniofacial skeleton is not yet known. We performed a series of *in situ* hybridizations in zebrafish embryos beginning at 12 hours post fertilization (hpf) and determined that *prdm1a* is expressed in a correct temporal and spatial pattern suggestive of a role in the differentiation of craniofacial structures. At 12 hpf (~5 somites), *prdm1a* is expressed in the polster, the anterior part of the prechordal plate which gives rise to the hatching gland (Kimmel et al., 1995), at the neural plate border where neural crest cells have begun to migrate in the cranial region, and the two stripes of adaxial (slow muscle precursor) cells (Fig. 1A). There is also faint expression in the otic placode (Fig. 1A, asterisk). By 16 hpf, neural crest migration is well underway and *prdm1a* is now expressed in the condensing presumptive pharyngeal arch region (Fig 1B; par). At 20 hpf, the NCCs have finished their migration and the endodermal pouches are beginning to form. *prdm1a* expression in the posterior arch region remains strong and expression in the fin bud can be detected (Fig. 1C). By 32 hpf, 5 endodermal pouches have formed (Crump et al., 2004; Kopinke et al., 2006) but *prdm1a* expression is restricted to the arch region posterior to the otic vesicle (Fig. 1D–F). Between 36 and 40 hpf, *prdm1a* continues to be broadly expressed in the posterior pharyngeal arches and is also seen in the apical ectodermal ridge (AER) of the developing fin bud (Fig. G–H). From 44 hpf to 56 hpf, *prdm1a* expression resolves and becomes restricted to the pharyngeal teeth, which are located deep in the posterior, ventral portion of the pharynx and are attached to the 5th ceratobranchial cartilage (Fig. 1I–L). Additionally, by 44–52 hpf, *prdm1a* is expressed in what appears to be an endodermal pouch (Fig. 1K–L, arrow). Wilm and Solnica-Kretzel (Wilm and Solnica-Kretzel, 2005) have previously shown that *prdm1a* is expressed in the arches at 72 hpf and in the arches and the developing neuromasts of the posterior lateral line at 120 hpf. This data shows that *prdm1a* is expressed in the pharyngeal arch region during the stages where multiple tissue interactions are necessary for the proper development of the craniofacial skeleton.

***prdm1a* is expressed in multiple cell types within the zebrafish head**

We next utilized plastic sectioning (at 4µm) to obtain better resolution and to determine whether *prdm1a* was expressed in multiple tissue layers within the pharyngeal arches. Thin sectioning revealed that, at approximately 52 hpf, *prdm1a* is expressed in the neural retina (Fig. 2B), the otic vesicle and an endodermal pouch (Fig. 2C), and the pharyngeal teeth (Fig. 2D), which are located deep in the posterior, ventral portion of the pharynx, attached to the 5th ceratobranchial cartilage. A pre-initiation stage of tooth development begins at 44 hpf with an initiation stage from 48–52 hpf. Final morphogenesis of the tooth can be observed at 56 hpf (Yelick and Schilling, 2002; Jackman et al., 2004). *prdm1a* is expressed in the forming tooth bud (Fig. 2D, pt) as well as in the mesenchyme of the 5th ceratobranchial cartilage (arch 7) on which the pharyngeal teeth form (Fig. 2D, cb5).

Pharyngeal arch markers are reduced in *prdm1a* mutants

We were next interested in whether *prdm1a* was required in multiple embryonic cell types that pattern the pharyngeal arches. To determine this, we looked at molecular markers for CNCCs, chondrogenesis, cranial ganglia, and endoderm in *prdm1a* mutants. *dlx2a* is expressed in the migrating CNCC that contribute to the pharyngeal arches and later compose the neural crest-derived structures within the arch. At 24 hpf, we saw a severe reduction in *dlx2a* expression in the posterior arches in *prdm1a* mutants, while the anterior expression is slightly reduced (Fig. 2E–F). A reduction in *dlx2a* expression is suggestive of a post-migratory effect of *prdm1a* on ectomesenchymal CNCCs that are filling the pharyngeal arches. Additionally, we looked at *barx1* expression, a gene that is necessary for proper patterning of osteochondrogenic progenitors (Sperber and Dawid, 2008) and is expressed in an overlapping domain to *dlx2a* in the more ventral and central arch region at 28 hpf (Olesnick Killian et al., 2009). In *prdm1a* mutants, *barx1* expression is normal in arch 1 and slightly reduced in the arch 2 ventral domain but is significantly reduced in the posterior arches (Fig. 2G–H). Since we observed a reduction in neural crest and chondrogenesis markers, we wanted to verify whether *prdm1a* was required for NCC migration. To determine the role of *prdm1a* in NCC migration, we completed time lapse confocal microscopy from 10–25 hpf and determined that neural crest cells migrate normally in three streams to populate the pharyngeal arches in *prdm1a* mutants (Supplementary movie 1 and 2), again suggesting that *prdm1a* plays a post-migratory role. Neural crest cells that populate the more dorsal region of the arches differentiate into the neurons and glia of the cranial ganglia. In *prdm1a* mutants, *neuroD* is expressed in the neurons of the developing cranial ganglia but is reduced in the trigeminal ganglia (Artinger et al., 1999; Roy and Ng, 2004) and posterior lateral line ganglia (Fig. 2I–J; arrows). Interestingly, the ganglia forming around the otic vesicle appear normal. Finally, to determine the affect of *prdm1a* loss on the endoderm, we examined the expression of *zn8* (see below) and *nkx2.3*, a marker of pharyngeal pouch endoderm specification. As compared to wildtype, *nkx2.3* was reduced in the more posterior pharyngeal pouches, suggesting that the endoderm is specified, but reduced in *prdm1a* mutants (Fig. 2K–L). Together, these data suggest that *prdm1a* is expressed at multiple developmental time points and strongly suggests that a loss of *prdm1a* has effects in multiple tissues required for proper zebrafish pharyngeal arch morphogenesis.

Loss of *prdm1a* results in defects in posterior endodermal pouch morphogenesis

As discussed above, we have determined that *prdm1a* is not required for NCCs to migrate in three streams to populate the pharyngeal arches (see Fig. S1). However, the loss of *prdm1a* in the arches results in defects in endodermal pouch patterning and a subsequent loss of pharyngeal cartilage and bone. In zebrafish, the pharyngeal endoderm invaginates to form six endodermal pouches that separate the arches and are required for the development of the craniofacial skeleton (Graham and Smith, 2001; Crump et al., 2004). To look further at endodermal pouch patterning, we stained embryos at 48 hpf with anti-*zn8* antibody, which is

expressed in endodermal pouches and motor neurons, within the *tg{prdm1a::GFP}* transgenic background (Fig. 3A–B). In control embryos, endodermal pouch development proceeds from anterior to posterior and by 38 hpf, all pouches have formed (Crump et al., 2004). In *prdm1a* mutant embryos, morphogenesis of the endodermal pouches is disrupted (Fig. 3B). Pouches 1 and 2 are often present and may only be mildly affected, still providing enough structure for arches 1 and 2 to form normally. The more posterior pouches (3–6) are not well organized or simply do not form (Fig. 3A and 3B) suggesting that the earlier defects in pouch formation may result in a loss of cartilage and other ectomesenchymal derivatives at later time points in *prdm1a* mutants.

Loss of *prdm1a* results in craniofacial defects

Previous studies have shown that the endodermal pouches play an important role in the differentiation of NCCs into craniofacial skeleton (Crump, et al 2004; Graham, et al 2005; Piotrowski, et al 2000). Because *prdm1a* mutants have defects in posterior pharyngeal pouch development, we stained 6 dpf embryos with alcian blue to determine if there are defects in visceroskeletal development (Fig. 3C–F; for review of craniofacial skeletal elements see (Schilling, 1997; Knight and Schilling, 2006). Loss of *prdm1a* does not appear to affect the first arch cartilages, as Meckel's cartilage and the palatoquadrate form normally (Fig. 3D). The second arch structures have mild defects, with the hyosymplectic forming normally and the ceratohyal often either compressed (Fig. 3D, ch) or inverted (outlined in Fig. 3F). These second arch defects are likely due to a loss of support from missing ceratobranchials. Arches 3–7 are the most severely affected in *prdm1a* mutants. Ceratobranchial 1 (cb1) is sometimes present but ceratobranchials 2–5 and the pharyngeal teeth (on cb5) are completely absent (Fig. 3D, arrowhead to cb1) suggesting that *prdm1a* is required for posterior cartilage formation.

Because neural crest cells also give rise to dermal bone, we examined the formation of these derivatives in *prdm1a* mutants. Dermal bone is a type of bone that ossifies directly from membrane without a cartilaginous precursor and occurs only in the skull region as compared to endochondral, or cartilage replacement, bone (Cubbage, 1996; Yelick and Schilling, 2002). There are 74 bones comprising the head skeleton of the mature zebrafish and rudiments of 9 of them develop in the pharyngeal arches by 6 dpf (Cubbage, 1996). We focused mainly on 2 dermal bones, the branchiostegal rays (br) and opercle (op), and an endochondral bone, cb5. At 6 dpf, control embryos labeled with alizarin red to detect bone showed 2 branchiostegal rays (br) and the normal fanning shape of the opercle (op) as well as bone formation and the pharyngeal teeth on cb5 (Fig. 3E; the opercle is present on both sides). However, in *prdm1a* mutants, many of the anterior dermal bones are reduced or missing (Fig. 3F). It appears only neural crest derived dermal bone is affected as the cleithrum, a mesodermally-derived component of the pectoral girdle, is still present (Cubbage, 1996; McGonnell, 2001). Multiple craniofacial structures are missing in *prdm1a* mutants indicating that *prdm1a* is critical for the proper formation of the posterior cartilage and both dermal and endochondral bone of the zebrafish head skeleton.

Misexpression of *prdm1a* results in ectopic cartilage

We next asked whether supplying ectopic *prdm1a* would result in craniofacial patterning defects. Previous experiments suggest that *prdm1a* is sufficient to produce ectopic neural crest cells within the migratory pathway (Hernandez-Lagunas et al., 2005) and thus, we wanted to determine the effects on cartilage formation. Embryos were injected with 150 pg of *prdm1a*-RNA at the one-cell stage and allowed to develop to 6 dpf. The skeletons were dysmorphic (n=13/26), typically exhibiting ectopic cartilage and interestingly, neurocranium defects. Ectopic cartilage often formed at the midline where both sides of Meckel's cartilage meet (Fig. 3G). Upon further dissection of the cartilages, we observed there was a fusion of

the pterygoid process of the palatoquadrate with the trabeculae (Fig. 3H, asterisk). This typically only occurred on one side of the embryo and resulted in a failure of fusion of the trabeculae and subsequent development of the ethmoid plate. These results indicate that *prdm1a* is sufficient to cause ectopic cartilage formation.

***prdm1a* is necessary for cell proliferation within the pharyngeal arches**

Because we observe a loss in posterior cartilage and bone, we examined the effect of the loss of *prdm1a* on cell death and proliferation within the pharyngeal arches. *prdm1a* plays a role in regulating proliferation in the mouse immune system and might therefore be a mechanism by which *prdm1a* regulates differentiation in the pharyngeal arch. First, we examined cell death in *prdm1a* mutants between 24 and 48 hpf. *prdm1a* mutant embryos have no increase in apoptosis as compared to controls (Fig. S1), suggesting that the loss of cartilage in *prdm1a* mutants is most likely not due to an increase in cell death. We next examined the levels of cellular proliferation, using a phosphohistone H-3 antibody (PH3, Upstate Biotechnology), between 24 and 48 hpf to determine if *prdm1a* is required for proliferation of cartilage progenitors in the arches (Fig. 4). At 24 hpf and 36 hpf, there was not a significant difference in the number of proliferating cells in control embryos as compared to *prdm1a* mutant embryos (Fig. 4C). However, there was a significant decrease in the number of dividing cells in 48 hpf embryos ($n=13$; Fig. 4B, C). *prdm1a* mutants (average PH3⁺ cells per embryo in outlined domain = 37) exhibited a 1.7-fold decrease ($p<0.0001$) in dividing cells as compared to control embryos (average PH3⁺ cells per embryo = 64) (Fig. 4A, C), suggesting that a loss of *prdm1a* results in a reduction of proliferating cells within the pharyngeal arch region. This suggests that *prdm1a* is necessary for proliferation of cells within the pharyngeal arches at an early stage of chondrogenesis, but not at earlier stages in neural crest development.

Chondrogenic markers are downregulated in *prdm1a* mutants

We next sought to quantify changes in chondrogenic gene expression to determine whether *prdm1a* was necessary for the expression of *dlx2b*, *barx1*, *sox9a*, *col2a1*, and *chm*. Although we have *in situ* hybridization data showing that *barx1* (Fig. 2H) and *dlx2b* (data not shown) are reduced in *prdm1a* mutants, other *in situ* experiments were inconclusive. We therefore chose to use quantitative real-time polymerase chain reaction (qPCR) to quantify the reduction in gene expression in *prdm1a* mutants (Figure S2). Analysis was done in whole embryos at 48 and 72 hpf. We chose to look at both 48 hpf, a period of arch outgrowth before appearance of any cartilages, and 72 hpf, after cartilage differentiation has begun. At 48 hpf, none of the genes were significantly changed (data not shown). However, at 72 hpf, *dlx2b*, *barx1*, and *col2a1* were significantly decreased in *prdm1a* mutants. *dlx2b* is a molecular marker expressed in zebrafish tooth germs over many developmental stages (Jackman et al., 2004) and as shown in Figure 1, *prdm1a* is expressed in the developing pharyngeal teeth, suggesting that *prdm1a* could regulate tooth development. In *prdm1a* mutants, *dlx2b* was reduced 4-fold (fold change = -4.11 , p -value > 0.0004) indicating that *prdm1a* is required for *dlx2b* expression. Confirming our *in situ* hybridization results, *barx1* was reduced over 2-fold (fold change = -2.36 , p -value > 0.002) in *prdm1a* mutants, as was *col2a1* (fold change = -2.5 , p value > 0.03). This data indicates that *prdm1a* is required for tooth morphogenesis and during early stages of chondrogenesis. The other chondrogenic genes, *sox9a* and *chm*, were not differentially expressed in *prdm1a* mutants suggesting that these genes are functioning or being regulated independently of *prdm1a*.

***prdm1a* functions downstream of Fgf and RA signaling**

In addition to multiple tissue requirements in the arches, many signaling pathways are also required for proper morphogenesis of the pharyngeal skeleton. To examine the genetic control of *prdm1a*, we focused on major signaling pathways previously shown to function in

the pharyngeal arches: Fibroblast growth factor (Fgf), retinoic acid (RA) and Hedgehog (Hh) signaling. We were specifically interested in the role of *prdm1a* during arch condensation and pouch morphogenesis so we chose to treat the embryos with various pharmacological agents beginning at 29 hpf and analyze *prdm1a* expression with *in situ* hybridization at 35 hpf.

Fgfs are a family of extracellular signaling molecules that have been shown to be important regulators of pharyngeal arch development (Piotrowski and Nusslein-Volhard, 2000; Crump et al., 2004). Fgfs are required for differentiation and survival of post migratory NCC (Blentic et al., 2008) and for pouch morphogenesis in the pharyngeal pouches (Crump et al., 2004). To determine if *prdm1a* expression is regulated by Fgf signaling, we treated embryos with SU5402, an inhibitor that binds to Fgf receptors and blocks Fgf signaling (Mohammadi et al., 1997; David et al., 2002). A loss of Fgf signaling resulted in a reduction of *prdm1a* expression in the eye and complete loss of expression in the arches, fin bud, and otic vesicle (Fig. 5C-D, as compared to vehicle controls, Fig. 5A-B). With all treatments, *prdm1a* expression remained in the hatching gland and cloaca (data not shown), providing an internal control for our *in situ* hybridizations. This data suggests that *prdm1a* is regulated by Fgf signaling. Previous work has shown that treatment with SU5402 from the one-somite stage to 24 hpf does not have an effect on *prdm1a* expression in the arch region (Mercader et al., 2006) and work in our lab has shown a decrease in but not a complete loss of *prdm1a* expression when treating embryos from 24–28 hpf (data not shown). This indicates that Fgf signaling is critical for the regulation of *prdm1a* expression from 29–35 hpf.

RA signaling regulates multiple aspects of embryonic development, including craniofacial patterning (Mark et al., 2004). More specifically, RA is known to be required for endodermal pouch morphogenesis (Kopinke et al., 2006). In order to determine whether *prdm1a* expression is regulated by RA signaling, we treated embryos with either DEAB (50 μ M), a competitive, reversible inhibitor of retinaldehyde dehydrogenases (Perz-Edwards et al., 2001; Kopinke et al., 2006) or ectopic RA (0.1 μ M). With a loss of RA signaling from 29–35 hpf, *prdm1a* expression was reduced in the pharyngeal arch region and eye, but only slightly reduced in the fin bud (Fig. 5E-F). Expression in the otic vesicle, hatching gland, and cloaca (not shown) remained similar to control. Embryos treated with RA showed a dramatic increase in *prdm1a* expression. Not only was *prdm1a* expression increased in the posterior arch region, it also expanded anteriorly and ventrally (Fig. 5G-H). However, expanded expression appeared limited to pharyngeal arch tissues - *prdm1a* was not expanded into the dorsal midline (Fig. 5H). This data indicates that *prdm1a* is being regulated by RA signaling in the pharyngeal arch tissues.

Because *prdm1a* has previously been shown to be regulated by Shh signaling in the development of smooth muscle (Roy et al., 2001; Baxendale et al., 2004), and Hh is required for condensation and differentiation of CNCCs at the midline (Eberhart, et al 2006; Wada, et al 2005) we were interested in determining if Hh regulates *prdm1a* expression in the arches. Previous studies to determine the precise temporal requirements for Hh showed that removal of Hh signaling using cyclopamine (CyA) between 30–35 hpf eliminated the ethmoid plate while still forming the trabecular cartilages (Wada et al., 2005). However, treatment from 29–35 hpf with CyA did not effect *prdm1a* expression (data not shown), suggesting that Hh does not regulate *prdm1a* expression during this specific time point of arch development.

DISCUSSION

The analysis presented here indicates a requirement for *prdm1a* in zebrafish craniofacial development. We have shown that *prdm1a* is expressed at various developmental time points in multiple tissues and a loss of *prdm1a* leads to cartilage defects in the more

posterior pharyngeal arches. Several other studies have shown that *prdm1a* functions in zebrafish during smooth muscle development (Baxendale et al., 2004; Roy and Ng, 2004; Hernandez-Lagunas et al., 2005; Vincent et al., 2005; Wilm and Solnica-Krezel, 2005; Lee and Roy, 2006; Mercader et al., 2006; Robertson et al., 2007; Elworthy et al., 2008; von Hofsten et al., 2008), limb induction (Mercader et al., 2006) and specification of neural crest and sensory neuron progenitors (Roy and Ng, 2004; Hernandez-Lagunas et al., 2005; Rossi et al., 2009). However, this is the first in depth study focusing on a role in craniofacial morphogenesis.

A role for *prdm1a* in neural crest and endoderm patterning in the pharyngeal arch of zebrafish

During craniofacial development, multiple tissue interactions are required for proper patterning of craniofacial structures, including cross talk between CNCCs, ectoderm, mesoderm and endoderm. It is the CNCCs that generate both of the ectomesenchymal (bone, cartilage, etc) and non-ectomesenchymal (neurons, glia, melanocytes) derivatives. Mis-patterning or a disruption in signaling between any of these tissues can cause defects in craniofacial development. Interestingly, *prdm1a* mutants have defects in both CNCC and endoderm tissue derivatives. In this and previous studies, we have shown that a loss of *prdm1a* results in defects in NCC derived tissues including cartilage and dermal bones, cranial ganglia, and Rohon-Beard neurons and melanocytes (Hernandez-Lagunas et al., 2005). In addition, in this study we have shown that a loss of *prdm1a* results in defective endodermal pouch development and morphogenesis, with a reduction in the endodermal expression of *nkx2.3*, suggesting *prdm1a* is required for endoderm development as well. Importantly, previous studies suggest that the proper patterning of neural-crest derived cartilage and bone in the pharyngeal arches requires the interaction specifically between migrating neural crest cells and the pharyngeal endoderm (Piotrowski and Nusslein-Volhard, 2000; David et al., 2002; Crump et al., 2004). Because *prdm1a* is expressed in both critical tissues, it may be a mediator relaying molecular signals from the endoderm to neural crest cells and other tissues or vice versa.

Because of the diversity of phenotypes that we observe, it is likely that *prdm1a* is involved in pharyngeal arch patterning at multiple timepoints. While migration is essentially normal, it is possible that a loss of *prdm1a* during early development may reduce the number of neural crest cells specified or migrating in the streams (Hernandez-Lagunas et al., 2005). However, we feel that this is unlikely, since *prdm1a* is not expressed in migrating NCCs and the total number of NCCs recover in the cranial region by the 10 somite stage from the earlier specification deficit that is observed (see below). At the time that CNCC reach the arches in *prdm1a* mutants, our time lapse observations within the *tg[sox10::egfp]* line show that essentially normal numbers of neural crest cells are migrating. We thus believe that the CNCC population is not significantly different from that of wildtype levels. In addition, while expression of specific markers cannot reliably predict cell number, the expression domains of both *snail2* and *dlx2a* at 5 and 10 somites stages, respectively, are similar in size to that of wildtype embryos (Artinger, et al 1999). Thus, we predict that *prdm1a* likely plays a later but separable role in patterning of the arches. As development progresses, reduced cellular proliferation in the arch region and the reduction of chondrogenic markers in *prdm1a* mutants suggest that *prdm1a* functions during cartilage development. Whether *prdm1a* functions in the rapid cartilage morphogenesis, the extended period of cartilage outgrowth (Kimmel et al., 1998), or other step of cartilage development has yet to be determined.

prdm1a mutants are interesting in the fact that they do not exhibit any type of anterior craniofacial defect, consistent with the specific expression within the posterior arch region. Many craniofacial mutants that display posterior arch defects also have defects in the more

anterior arches or are missing all posterior structures except for the pharyngeal teeth (Neuhauss et al., 1996; Piotrowski et al., 1996; Schilling et al., 1996). However, in *prdm1a* mutants, Meckel's cartilage and the palatoquadrate are present and form normally. The second arch derivatives also develop normally with the hyosymplectic and developing jaw joint fully intact. Often the ceratohyal is inverted or compressed but is always present. The primary defect of *prdm1a* mutants is a loss of the posterior ceratobranchial cartilages, most often affecting arches 4–7. These results suggest that *prdm1a* is primarily involved in the development of the posterior arch region, likely in the endoderm and NCCs themselves. This defect in craniofacial cartilage described here was not initially identified in the *prdm1a*^{m805} allele on the EKK background (Artinger, et al 1999). It was only after crossing it into a WIK or AB background that we observed the defect, suggesting that some strain differences exist in zebrafish. In addition, a similar craniofacial defect was described using the *prdm1a* Morpholino (Wilm and Solnica-Krezel, 2005). Thus these data confirm the role of *prdm1a* in zebrafish craniofacial development, similar to those defects observed in the knockout mice (Robertson et al, 2007).

***prdm1a* is a potential effector of Fgf and RA signaling in the posterior pharyngeal arches**

Both Fgf and RA signaling pathways are required for various aspects of zebrafish craniofacial development. Interestingly, *prdm1a* mutants have similar craniofacial phenotypes as Fgf- and RA- deficient zebrafish (Begemann et al., 2001; David et al., 2002; Walshe and Mason, 2003; Crump et al., 2004; Kopinke et al., 2006). Loss of *fgf3* function in *ace* mutants results in a loss of cartilage of the third-sixth pharyngeal arches while having little effect on the first, second, or seventh arches (Walshe and Mason, 2003; Crump et al., 2004), while the knockdown of *fgf8* results in variable defects in pouch structure. While the mutant phenotype in the posterior arches is similar, a key difference in *prdm1a* mutants is that they are always missing the seventh arch derivative and the pharyngeal teeth. This is interesting, since previous studies in zebrafish have shown a requirement for redundant Fgf signaling in tooth development (Jackman et al., 2004). Moreover, Fgf3 and Fgf8 influence the segmentation of the pharyngeal arches by promoting cranial neural crest survival and cellular proliferation (Walshe and Mason, 2003; Crump et al., 2004), suggesting that *prdm1a* is being targeted by more than one Fgf signal. Our data shows that a loss of *prdm1a* results in a reduction in proliferating cells in the arch region supporting the idea that in the arches, similar to the fin bud (Mercader et al., 2006), *prdm1a* is downstream of Fgf signaling at the time the posterior pouches are formed around 38 hpf. Similarly, in the mouse immune system, *Prdm1* is required to repress genes that are required for proliferation and thus promotes differentiation. Taken together, it is likely that *prdm1a* mediates an Fgf signal to regulate cell proliferation and promote differentiation of NCCs to a cartilage cell fate.

In addition to its more classical roles in development, retinoic acid is also required for morphogenesis and segmentation of the pharyngeal arches. Specifically, loss of RA signaling during gastrulation results in a loss of postotic neural crest cells potentially caused by misspecification of the posterior rhombomeres (Kopinke et al., 2006). However, the hindbrain specific expression of *krox20* in rhombomeres 3 and 5 is normal in *prdm1a* mutants (data not shown), suggesting that *prdm1a* is not mediating RA signaling during gastrulation. Additionally, a loss of RA signaling post-gastrulation has no effect on neural crest migration and no observed increase in cell death in either neural crest cells or the endoderm (Kopinke et al., 2006), similar to the phenotype we observe in *prdm1a* mutants. Interestingly, at 6 dpf, *prdm1a* mutants and embryos treated with DEAB from 29–35 hpf have normal anterior cartilages, but are both missing posterior cartilages (data not shown). Kopinke et al (2006) showed that treatment with DEAB from 24–30 hpf resulted in the formation of only 4 endodermal pouches and formation of arch cartilages 1–4. We observed the same phenotype when treating embryos from 29–35 hours. *neckless* (*nls*) mutants, in

which *raldh2* is inactive and retinoic acid biosynthesis is decreased, are missing ceratobranchial cartilages 2–5 and have an inverted ceratohyal cartilage (Begemann et al., 2001). However, unlike *prdm1a* mutants, *nls* (*raldh2*) mutants have reduced first and second arch cartilages. These data suggest that *prdm1a* is regulated post-gastrulation by RA signaling.

Previous work has focused on the regulation of *prdm1a* by sonic hedgehog (Shh) to specify the slow twitch muscle cell fate (Roy et al., 2001; Baxendale et al., 2004). At the neural plate border, expression of *prdm1a* is unaffected in a Shh signaling mutation *slow muscle omitted* (*smoothened*) mutants, suggesting that *Shh* does not have a role in neural plate border specification (Wilm and Solnica-Krezel, 2005). In later craniofacial development, treatment of embryos with cyclopamine does not affect *prdm1a* expression (this study). Ectopic expression of *prdm1a* results in formation of extra chondrocytes extending from the midline of the Meckel's cartilage and a fusion of the pterygoid process of the palatoquadrate with a disfigured trabeculae, similar to the phenotype that has previously been reported with treatment of exogenous *Shh* (Wada et al., 2005). However, further experiments are required to determine the interaction of *prdm1a* and *Shh* during craniofacial development. In *prdm1a* mutants, there is no loss or malformation of the pterygoid process or trabecular organization, suggesting that *prdm1a* is not necessary for the formation of these structures. However, overexpression of *prdm1a* may result from early aberrant NCC migration at the midline or later defects in condensation of the trabeculae.

Thus, the phenotype we observe in *prdm1a* mutants suggests that *prdm1a* is required for proper patterning of the endodermal pouches and subsequent development of ectomesenchymal derivatives of the neural crest. Our data also suggests that *prdm1a* is being influenced by a combination of Fgf and RA signaling. This conclusion is supported by the strikingly similar phenotypes of Fgf and RA mutants as compared to *prdm1a* mutants.

The function of *prdm1a* in pharyngeal arch development

Prdm1 gene homologs in humans and mice were first studied for their involvement in immune responses (Turner et al., 1994; Shaffer et al., 2002; Shapiro-Shelef et al., 2003). Subsequent studies now indicate that *Prdm1a/Blimp-1* is an evolutionarily conserved protein that regulates the development of many tissues and functions in a variety of different organisms. While *Prdm1a/Blimp-1* appears to function mainly as a repressor (Lin et al., 1997; Lin et al., 2000; Lin et al., 2002); (Klein et al., 2003; Tamura et al., 2003; Gyory et al., 2004; von Hofsten et al., 2008), more evidence is emerging to suggest that it can function in activating certain target genes as well (Mercader et al., 2006; Rossi et al., 2009).

In mice, *Blimp1* mutant embryos exhibit defects in formation of the caudal branchial arches (Vincent et al., 2005; Robertson et al., 2007). Similar to what we see in zebrafish, only the first arch forms and the more posterior arches are completely lost in *Blimp1* null embryos (Vincent et al., 2005). This is thought to be caused by a failure to expand and maintain signaling capabilities, specifically *Fgf8* and *Tbx1*, within the pharyngeal arch epithelium (Robertson et al., 2007). Interestingly, while the craniofacial defects are conserved, the early neural plate border defects were not observed in the mouse knockouts of *Blimp1*. We suspect there has been a divergence in gene function within the PRDM gene family. In mouse, there are 16 members, while in *Fugu rubripes* and likely in zebrafish, there are 15, many with overlapping patterns of expression with *prdm1a* in one or all tissues (Fumasoni, et al 2007, Sun, et al 2008). The number of genes in the family suggests that the PRDM genes may have some redundant as well as specific roles in development. Thus, elucidating the role of all the PRDM family members in both zebrafish and mice will add to our understanding of both NCC specification and differentiation into craniofacial structures.

It is likely that additional signaling pathways, such as Bmp, Wnt, and Shh, which regulate *prdm1a* during other aspects of development (Roy et al., 2001; Baxendale et al., 2004; Hernandez-Lagunas et al., 2005; Mercader et al., 2006), are also contributing to the function of *prdm1a* during pharyngeal arch development. Future studies of *prdm1a* in the pharyngeal arches will aim to elucidate specifically where *prdm1a* is functioning in the pharyngeal tissues and whether specific pathways are working directly or indirectly to regulate *prdm1a* expression and pattern the posterior craniofacial skeleton.

EXPERIMENTAL PROCEDURES

Zebrafish maintenance and genetics

Zebrafish were maintained according to Westerfield (Westerfield, 1995). Developmental time points refer to hours (hpf) or days post fertilization (dpf) and were staged according to (Kimmel et al., 1995). The *prdm1a* mutant strain (*nrd^{m805}*) was isolated from a small scale *in situ* hybridization screen described in a previous publication (Artinger et al., 1999), and is currently on a mixed background. Control embryos are wildtype or heterozygous *prdm1a*^{+/-} unless otherwise noted.

Whole mount *in situ* hybridization and immunohistochemistry

Whole mount *in situ* hybridization was performed according to Thisse (Thisse et al., 1993; Thisse and Thisse, 1998) using digoxigenin labeled (Roche) RNA probes and visualized using BM Purple substrate (Roche). The following anti-sense probes were used: *prdm1a* (Hernandez-Lagunas et al., 2005), *dlx2a* (Jackman et al., 2004), *neuroD* (Korzh et al, 1998), *nkx2.3* (gift from T. Piotrowski), and *barx1* (Sperber and Dawid, 2008). For plastic sections, embryos were embedded using JB-4 plastic (Polysciences Inc) and sectioned at 4 μ m. Immunohistochemistry was performed as described (Ungos et al., 2003) and the following antibodies were used: zn8 (ZIRC, NIH-NCRR #RR12546) @ 1:25, anti-phosphohistone-H3 (Upstate) @ 1:500, and Alexa568 goat anti-mouse @ 1:750. Apoptosis was determined by TUNEL labeling using fluorescein-dUTP (TMR-Red, Roche).

Whole mount skeletal staining

For cartilage detection, 5–7 dpf larvae were incubated in 0.1% Alcian Blue solution, cleared with acidic ethanol, and stored in glycerol (Schilling et al., 1996). For cartilage and bone visualization, larvae were incubated in 0.02% Alcian Blue and 0.01% Alizarin Red in acid free conditions, cleared using ethanol and glycerol/KOH washes, and stored in 50% glycerol/0.1% KOH at 4°C (Walker et al., 2006).

RNA microinjection

1 μ l of *prdm1a*-RNA was co-injected with rhodamine-dextran (Molecular Probes) for a final concentration of 150 pg.

Quantitative Real-Time PCR

Total RNA was extracted from 48 and 72 hpf control and *prdm1a* mutant embryos with TRIzol/chloroform, DNaseI treated, and column purified (Omega Bio-Tek, Inc.). PCR primers were designed using the Roche Universal Probe Library (UPL) for Zebrafish Assay Design Center (Supplementary Table). Primers were synthesized by Integrated DNA Technologies (IDT). All primer sets spanned an exon-exon junction to avoid errors due to contaminating genomic DNA. Probes were selected from the Roche UPL (Supplementary Table). Amplification and detection of the fluorescence were measured using a Stratagene Mx3005p. All signals were normalized against β -actin and fold change ratios were calculated for *prdm1a* mutant samples as compared to controls. The fold change values in

Figure S2 were determined by comparing mRNA expression in *prdm1a* mutants to control embryos.

Drug treatments

Embryos were raised in embryo media to 24 hpf, manually dechorionated, and transferred to 6-well tissue culture plates. Control embryos were soaked in either 0.2% DMSO or ethanol in embryo media. Fgf inhibition was performed by incubating embryos in 20 μ M SU5402 (a gift from Linda Barlow). To examine retinoic acid signaling, embryos were soaked in either 50 μ M DEAB (4-(Diethylamino)benzaldehyde; Sigma-Aldrich) or 0.1 μ M RA (All Trans Retinoic acid, Sigma-Aldrich). To examine Hedgehog signaling, embryos were soaked in 100 μ M CyA (cyclopamine; Sigma).

Microscopy

Embryos processed for *in situ* hybridization were mounted in 80% glycerol or 3% methylcellulose and imaged using a Zeiss Stemi2000 or Olympus SZX16 dissecting microscope. Fluorescent images were captured using an Olympus SZX16 fluorescent microscope or an FV1000 confocal microscope. For live imaging, 10 hpf embryos were mounted in 0.7% low melt agarose in fish water and oriented in a slightly dorso-lateral angle. Neural crest cell migration was followed using a Zeiss LSM 510 META confocal microscope equipped with a heated stage set to 28°C. Z stack images were taken once every 5 minutes until 25 hpf.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Tom Schilling, David Stock, Tatjana Piotrowski, Steve Sperber, Bill Jackman, and ZIRC (P40 RR012546-NIH-NCRR) for reagents; Phil Ingham and Stone Elworthy for sharing the *prdm1a*-gfp line; David Clouthier and members of the Artinger lab for helpful comments on the manuscript; Morgan Singleton for extraordinary fish care; David Stock and Sarah Wise for help with sectioning; Lee Niswander and Christina Pyrgaki (UC-Denver) and David Bonislawski (UM) for use and assistance with the confocal microscopes; and Corbin Schwanke in the UM Molecular Biology Core Facility (P20 RR017670 and P20 RR015583-NCRR-COBRE). We gratefully acknowledge the support of NIH P30 NS048154 Neuroscience Zebrafish Core, NIH HD050698 and DE017699 to K.B.A, NIH F32 HD056779 to E.O.K., NIH UO1-ES016102 to K.M.G. and F32 DE018594 to D.A.B.

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Fig. 1. *prdm1a* expression in the developing zebrafish embryo

A: At the beginning of CNCC migration (12 hpf), *prdm1a* is expressed at the neural plate border (npb), in the adaxial cells (ac), and in the prechordal plate (pcp). It may also be expressed in the otic placode (asterisk) **B:** By 16 hpf, it is expressed in the pharyngeal arch region (par) and hatching gland (hg). **C-E:** At 20 hpf, 24 hpf, and 28 hpf, *prdm1a* expression continues to progress posteriorly in the arch region (par) and can be detected in the developing pectoral fin bud (fb). **F-J:** From 32–44 hpf, expression becomes restricted to the more posterior arches and by 48 hpf, can be detected only in the most posterior arch in the pharyngeal teeth (pt) as well as the AER of the fin bud. **K-L:** At both 52 hpf and 56 hpf, in addition to the arch and fin bud expression, *prdm1a* may be expressed in an anterior endodermal pouch (black arrow). (A) dorsal views, anterior to the top; (B-C) dorsal view, anterior to the left; (D-L) lateral view, anterior to the left. White arrows in D, G mark the location of the otic vesicle; ac, adaxial cells; AER, apical ectodermal ridge; fb, fin bud; hg, hatching gland; npb, neural plate border; par, pharyngeal arch region; pt, pharyngeal teeth; pcp, prechordal plate.

Fig. 2. *prdm1a* is expressed in multiple pharyngeal arch tissues and *prdm1a*^{-/-} shows a reduction in expression in pharyngeal arch markers

A-D: *In situ* hybridization showing *prdm1a* expression in a 52 hpf embryo. **A:** Whole mount embryo in which the RNA probe was overdeveloped with BM purple and white lines indicate the plane of sectioning in the following panels. **B:** *prdm1a* is expressed in the developing neural retina of the eye (black arrows, e) **C:** the otic vesicle (ov) and an anterior endodermal pouch (ep), and **D:** the developing pharyngeal tooth bud (pt) and mesenchyme of the 5th ceratobranchial (cb5). Molecular markers in the pharyngeal arch region in *prdm1a* mutants **E, F:** *dlx2a* expression in the post-migratory neural crest in wildtype (E) and *prdm1a* mutants (F) shows an overall reduction in expression of *dlx2a* throughout the arch region in *prdm1a* mutants, but more strongly reduced posteriorly. **G, H:** *barx1* expression in chondrogenic progenitors is slightly reduced in arch 2 and significantly reduced in arches 3–7 in *prdm1a* mutants (H) as compared to control (G). Bracketed area indicates pharyngeal arch region in wt/het and arrows indicate reduced expression domains in *prdm1a*^{-/-}. **I, J:** *neuroD* expression in neurons of the cranial ganglia shows a reduction of expression in the trigeminal (anterior arrow) and posterior ganglia, including the lateral line ganglia (posterior arrow) in *prdm1a* mutants (J) as compared to control (I), and **K, L:** *nkx2.3* expression in the pharyngeal endoderm of wildtype (K) and *prdm1a* mutants (L). *nkx2.3* is reduced in the posterior arch region in *prdm1a* mutants. Bracketed area indicates pharyngeal arch region. Anterior is to the left (A, E-L). The otic vesicle (ov) is labeled (E-L) as a reference point. cb5, ceratobranchial 5; ep, endodermal pouch; nc, notochord; ov, otic vesicle; pt, pharyngeal tooth; Wt/het, Wildtype/heterozygotes embryo.

Fig. 3. Loss and gain of function of *prdm1a* results in craniofacial defects

A: 48 hpf *prdm1a*-GFP embryo stained with zn8 antibody showing the normal structure of the pharyngeal endodermal pouches **B:** a *prdm1a* mutant (in a *tg{prdm1a::GFP}* background) stained with anti-zn8 shows defects in endodermal pouch patterning. **C:** a 6 dpf wild type larvae stained with alcian blue to detect cartilage **D:** As compared to control, the *prdm1a* mutant larva has a slightly inverted ceratohyal (ch) and is missing the cartilages of pharyngeal arches 4–7. **E:** Control 7 dpf zebrafish stained with alcian blue (cartilage) and alizarin red (endochondral and dermal bone). **F:** As compared to control, the *prdm1a* mutant larva is missing a majority of the dermal bones, as well as the 5th ceratobranchial and pharyngeal teeth, but the cleithrum is present. The otoliths in the ear are also slightly smaller. The inverted ceratohyal is outlined in white. Staining was performed at multiple timepoints and did not reflect any developmental delay in *prdm1a* mutants. Overexpression of *prdm1a*-RNA results in malformed cartilages including **G:** Ectopic cartilage extending from the Meckel's cartilage (arrow) and **H:** a fusion of the pterygoid process of the palatoquadrate with the disarrayed neurocranium (asterisk, fusion point of pq and tr). **A,B:** lateral views, anterior to the left; **C-H:** Ventral views, anterior is to the left. br, branchiostegal ray; cb 1–5, ceratobranchial 1–5; ch, ceratohyal; cl, cleithrum; ep, ethmoid plate; M, Meckel's cartilage; nc, notochord; op, opercle; ot, otoliths; ov, otic vesicle; pq, palatoquadrate; pt, pharyngeal teeth; tr, trabeculae. 1–6 indicate endodermal pouches; Wt/het, Wildtype/heterozygotes embryo.

Fig. 4. *prdm1a* is necessary for proliferation within the arches

A: 48 hpf control embryo stained with anti-phosphohistone H3. **B:** As compared to control, a 48 hpf *prdm1a* mutant embryo has a reduction in proliferation in the arch region **C:** Average cell proliferation at 24, 36, and 48 hpf in control and *prdm1a* mutant embryos. Boxed area indicates approximate region in which cell counts were measured and analyzed using Image J and found to not be different in size (not shown). Number of embryos examined for 24 hpf, n=17; 36 hpf, n=27; and 48 hpf, n=13.

Fig. 5. Fgf and RA signaling regulate *prdm1a* expression in the pharyngeal arches
A,B: Embryos treated with DMSO, as a vehicle control, showing *prdm1a* expression at 35hpf. **C,D:** Treatment with 20 μ M SU5402 from 29–35 hpf shows reduced expression of *prdm1a* in the arches, otic vesicle (arrow), and fin bud. Expression remained in the hatching gland and cloaca (not shown). **E,F:** Treatment with 50 μ M DEAB from 29–35 hpf shows reduced *prdm1a* expression in the posterior arches (par) and mildly in the fin bud (fb). **G,H:** Treatment with 0.1 μ M RA from 29–35 hpf dramatically increased *prdm1a* expression in the arches and slightly in the fin bud. *prdm1a* expansion appears to be endoderm/arch specific as staining does not cross into the dorsal midline/medial endoderm. All embryos were fixed after treatment and *in situ* hybridization performed at 35 hpf. (A,C,E,G) lateral views; dorsal to the top, anterior to the left (B,D,F,H) dorsal views, anterior to the left. e, eye; fb, fin bud; ov, otic vesicle; par, pharyngeal arch region. Bracket in G indicates extent of *prdm1a* expansion.