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## Ancestral network module regulating *prdm1* expression in the lamprey neural plate border

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### Abstract

*prdm1* is an important transcriptional regulator that plays diverse roles during development of a wide variety of vertebrate and invertebrate species. *prdm1* is required for neural crest specification in zebrafish, but not in mouse embryos. The role of this gene in neural crest formation in other species has not been examined, and its regulation during embryonic development is poorly understood. Here, we investigate the expression pattern, function and the upstream regulatory inputs into *prdm1* during lamprey neural crest development. *prdm1* is strongly expressed in the lamprey neural plate border, suggesting a conserved ancestral role of this gene in the neural crest formation. We found that lamprey neural plate border expression of *prdm1* is activated by Ap-2 and Msx, but is independent of Pax3/7 and Zic.

### Introduction

*prdm1* (PR domain containing 1, with ZNF domain) or Blimp-1 (B lymphocyte-induced maturation protein 1) is a zinc-finger-containing transcriptional regulator that plays critical developmental roles in a wide range of species including insects, worms, echinoderms, and all vertebrates (Bikoff *et al.*, 2009; John and Garrett-Sinha, 2009). Several studies have demonstrated that *prdm1* acts by recruiting a range of epigenetic histone modifiers (histone methylases and deacetylases) in a sequence-specific manner (Ancelin *et al.*, 2006; Gyory *et al.*, 2004; Su *et al.*, 2009; Yu *et al.*, 2000). *prdm1* plays important roles in cell fate decisions, by repressing large sets of genes and dramatically altering a cell's transcriptional profile. Loss-of-function *prdm1* mutants and morphants exhibit complete loss of the cell type in which the gene is expressed, further underscoring the developmental importance of this gene (Brzezinski *et al.*; Robertson *et al.*, 2007; Roy and Ng, 2004; Vincent *et al.*, 2005; Wilm and Solnica-Krezel, 2005).

The neural crest is a vertebrate-specific multipotent migratory embryonic cell population, which forms most of the peripheral nervous system, cranium and pigment cells. Much progress has been made in the recent years in understanding the genetic mechanisms underlying neural crest formation, and this information has been assembled into a proposed neural crest gene regulatory network (Sauka-Spengler and Bronner-Fraser, 2008; Betancur *et al.*, 2010). Extensive analysis of the neural crest gene regulatory network in the lamprey, the most basal extant vertebrate, demonstrated that there is a great deal of conservation in

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the genetic mechanisms responsible for neural crest formation to the base of vertebrate lineage, particularly at early stages (Sauka-Spengler *et al.*, 2007).

Data from several zebrafish mutants show that *prdm1* is essential for the specification of the common progenitor of the neural crest and Rohon-Beard (RB) sensory neurons. Two *prdm1* fish mutants, *narrowminded* and *U-boot*, exhibit a decrease in the number of neural crest cells, and all neural crest derived structures are much smaller than in the wild-type fish (Artinger *et al.*, 1999; Hernandez-Lagunas *et al.*, 2005; Roy and Ng, 2004). The null *prdm1* mutant, *narrowminded*, also exhibits a complete loss of RB neurons (Hernandez-Lagunas *et al.*, 2005). Interestingly, however, there are no reports on a similar function for *prdm1* or any other member of the *prdm* family in other species. *prdm1* is not expressed in the neural plate border or neural crest of mouse embryos, and no defects in the early steps of neural crest formation are seen in any *prdm1* mouse knockouts (Chang *et al.*, 2002; Robertson *et al.*, 2007; Vincent *et al.*, 2005). It is possible, however, that *prdm1* is expressed and functions in the neural crest component of the branchial arches in the mouse at later stages. Expression of *prdm1* is seen at E10.5 in the mesenchyme of the mouse branchial arches (Chang and Calame, 2002; Robertson *et al.*, 2007). *prdm1*-null mice exhibit a complete loss of all branchial arches posterior to the first arch, which could be due to a cell-autonomous neural crest defect or to a failure of the neural crest migration (Robertson *et al.*, 2007; Vincent *et al.*, 2005). No expression of *prdm1* in neural crest of chick and lamprey embryos has been reported; however it is possible that the embryonic stages examined were too late to detect it (Ha and Riddle, 2003; Hammond *et al.*, 2009). It is not clear at present whether *prdm1*'s role in zebrafish neural crest is specific to the fish lineage, or if this gene has an ancestral role in the neural crest development that had been lost in the mouse. In order to address this question, we isolated a full-length sequence for *prdm1* from an embryonic cDNA library of the sea lamprey *Petromyzon marinus*, and examined *prdm1* expression pattern at a range of embryonic stages. We found strong expression of this gene in the lamprey neural plate border and premigratory neural crest, suggesting an ancestral role in neural crest development. We next investigated *prdm1* loss-of function phenotype and found that it plays a role in the specification of the mesoderm, which precedes and masks any possible function in the neural plate border. Finally, we examined the upstream inputs into *prdm1* in the neural plate border, and found that Msx A and AP-2 function as upstream activators of *prdm1* transcription.

## Results and Discussion

### ***prdm1* expression during sea lamprey embryonic development suggests ancestral roles in the development of neural crest and branchial arches**

In order to determine if *prdm1* is present in the lamprey embryo, we screened the *Petromyzon marinus* embryonic cDNA library (Sauka-Spengler *et al.*, 2007) using a fragment of zebrafish *prdm1* containing a conserved PR ( a type of SET protein-protein interaction) domain. We found a 5kb clone encoding a 1014-amino acid protein that corresponded to the full-length lamprey *prdm1* (GenBank accession number JN242000). Analysis of the conserved domains using Prosite identified an SET domain and five C2Hs zinc fingers. Comparative sequence analysis revealed high levels of sequence conservation with *prdm1* proteins from other vertebrates in the SET domain and the zinc finger domains, but low levels of similarity in the rest of the protein sequence (Figure 1). Interestingly, lamprey *prdm1* does not show any conservation in the region corresponding to the Groucho binding site, which is highly conserved among other vertebrates.

We analyzed the expression pattern of *prdm1* during lamprey embryonic development starting at E4.0 to E20. At E4.0 (early neurula), *prdm1* is expressed throughout the ectoderm with the exception of the neural plate, with particularly high expression at the neural plate

border and anteriorly in the preplacodal domain (Figure 2A, B). *prdm1* transcripts are also present at relatively low levels in the anterior endomesoderm and paraxial mesoderm (Figure 2C). As neurulation progresses, *prdm1* expression in the ectoderm decreases, while that in the neural plate border and the preplacodal domain becomes enhanced (Figure 2D–H). Expression of *prdm1* is particularly prominent in the premigratory neural crest (Figure 2G), but is turned off as soon as the neural crest starts migrating (compare Figure 2G to 2I). By E6.5, *prdm1* expression remains only in the posterior dorsal neural tube. Additionally, it is expressed in the branchial arches (Figure 2I–M). This expression persists until E14 in the posterior arches. Comparison of the expression pattern of *prdm1* to that of *Col2a2* (which marks the neural crest, Figure 2O) suggests that *prdm1* is expressed in the endodermal component and a subset of the neural-crest-derived component of the branchial arches (lateral neural crest). At later stages (E20) *prdm1* expression is seen in the branchial arches, which by that time have become cartilaginous (Figure 2N) (Cerny et al, 2010). Expression in the somites is initiated at E7 and continues until E18 (Figure 2J, L, M), as described previously by Hammond *et al* (2009).

In the lamprey *P. marinus* *prdm1* is expressed in the neural plate border as early as mid-gastrula (E4), and persists anteriorly until the start of neural crest migration (E5.5). It is maintained in the posterior dorsal neural tube until E9. This expression pattern is consistent with what has been reported for zebrafish (Hernandez-Lagunas *et al.*, 2005; Roy and Ng, 2004), and suggests a likely ancestral role for this gene in the neural crest specification.

Expression pattern of *prdm1* in other embryonic structures is consistent with what was reported in other vertebrates (Chang *et al.*, 2002; Ha and Riddle, 2003).

### Expression of *prdm1* in the neural plate border is downstream of *MsxA* and *AP-2*

Very little is known about regulation of *prdm1* expression during embryonic development, except that its expression in the neural plate border is downstream of Bmp signaling (Hernandez-Lagunas *et al.*, 2005; Roy and Ng, 2004). To address interactions with potentially upstream genes, we investigated the effect of morpholino-mediated loss of function of four genes important for the neural plate border formation on *prdm1* expression. Injection of *MsxA* morpholino in lamprey resulted in a complete loss of *prdm1* transcripts from the ectoderm and the neural plate border on the injected side (73%, n=33) (Figure 3B). When these embryos were allowed to develop to later stages, we observed loss of *prdm1* in the somites and dorsal neural tube on the injected side (Figure 3F, H) (47% at E8.5, n=17), suggesting that *MsxA* also regulates *prdm1* in these tissues. Similarly, loss of *AP-2* resulted in the absence of *prdm1* expression from both the ectoderm and the neural plate on the injected side at E4.5 (74%, n=55) (Figure 3C). Similarly to the phenotype of *MsxA* morphants, loss of *prdm1* expression in the dorsal neural tube was observed in *AP-2*-morpholino-injected embryos at E7 (72%, n=18) (Figure 3I–K). However, the expression of *prdm1* in the somites was not affected (Figure 3I). In contrast to *MsxA* and *AP-2*, neither *Pax3/7* (n=24) nor *Zic* morpholino (n=28) affected *prdm1* expression (Figure 3D and E), suggesting that these two neural plate border specifiers are either downstream of *prdm1* or act in a parallel pathway.

In zebrafish double *tfap2a/c* morphant both *prdm1* expression in the neural plate border and Rohon-Beard neuron formation was reduced, but not completely absent (Li and Cornell, 2007). This data suggests that, consistent with what we observed in the lamprey, *prdm1* expression in at least a subset of the neural plate border-derived cells is downstream of the two zebrafish *AP-2* homologues. Similarly, in triple *MsxB*, *C*, *E* morphants of zebrafish, a loss of a subset, but not all, Rohon-Beard neurons (a cell type dependent on *prdm1* expression) is seen (Phillips *et al.*, 2006). Taken together, these results suggest that there is a conservation of the upstream inputs into *prdm1* between lamprey and zebrafish in a subset

of the neural plate border cells. It is possible that those Rohon-Beard neurons that are still formed in zebrafish in the absence of *tfap2a/c* or *Msx B, C, E* (and the corresponding *tfap2a/c* and *Msx B, C, E*-independent *prdm1* expression domain) is a teleost innovation. *prdm1* regulation in these cells remain to be further explored.

In summary, our experiments demonstrate that *MsxA* and *AP-2* are essential for *prdm1* expression in the neural plate border. Two other neural plate border specifiers, *Pax3/7* and *ZicA*, have no effect on *prdm1* expression, suggesting that they act in a parallel pathway. Figure 5 shows a schematic representation of the current understanding of *prdm1* regulation in the neural plate border.

### Loss of *prdm1* results in abnormal axis formation

Strong and specific expression of *prdm1* in the neural plate border and premigratory neural crest suggests that the function of this gene is important for the formation of the neural crest in lamprey. In order to examine the role this gene plays in the neural crest development, we designed a translation-blocking morpholino against *prdm1* and injected it into lamprey embryos at the 4-cell stage. To confirm the specificity of *prdm1* morpholino, we used both a standard control morpholino (Sauka-Spengler et al, 2007; Nikitina et al, 2008) and a *prdm1* morpholino with 5 base pair mismatches. Neither of these morpholinos affected the phenotype of the injected embryos. Surprisingly, we found that none of about 300 embryos that received *prdm1* morpholino incorporated it into the ectoderm, compared to ectodermal distribution in 25% of the control morpholino-injected embryos, as expected (compare Figure 4B and 4A). Since *prdm1* is also expressed in the prospective anterior endomesoderm, we speculated that its loss might interfere with mesoderm formation. In an attempt to target the morpholino more specifically to the neural plate border, we injected it into one out of 16 cells at later cleavage stages. This resulted in progressively smaller regions of mesoderm and endoderm containing the morpholino, and never any ectodermal incorporation. A possible explanation for this result is that *prdm1* plays a crucial role in the specification of embryonic layers during and before gastrulation. Continuous *prdm1* expression may maintain ectodermal fate, while transient expression of low levels of *prdm1* may instruct the cells to become paraxial mesoderm. Consistent with this possibility, embryos that received *prdm1* morpholino also exhibited abnormal gastrulation and incorrect positioning of the anterior-posterior axis. Normally, the posterior end of the neural plate is formed just on top of the blastopore, and the head on the other end. In these embryos, we observed that the axis was perpendicular to its normal pattern: i.e. the head and the tail were formed on each side of the blastopore, which in some cases failed to close (asterisk in Figure 4C and 4C').

Thus, we observed *prdm1* morpholino incorporation exclusively in endoderm and mesoderm, and never in the ectoderm, despite the large numbers of the embryos injected. Data from *prdm1* overexpression experiments in *Xenopus* lends some support to the possibility that *prdm1* expression might maintain ectodermal fate (de Souza et al., 1999), whereas transient low levels of *prdm1* expression may promote mesodermal fate. Injection of *prdm1* RNA into *Xenopus* eggs results in inhibition of mesoderm formation and repression of mesodermal markers. Also *prdm1* overexpression in the animal cap explants induces endodermal markers (de Souza et al., 1999). In zebrafish, *prdm1* overexpression results in the loss of anterior-dorsal structures, while MO-mediated loss of function causes an increase in dorsal extension movements and mild dorsalization (Wilm and Solnica-Krezel, 2005).

## Experimental Procedures

### Library screen

A directional high quality full-length cDNA library prepared from embryonic day 2–12 lampreys using Superscript Plasmid System from Invitrogen (Carlsbad, CA) (Sauka-Spengler *et al.*, 2007) was screened at medium stringency using heterospecific probes per standard methods. The probes were synthesized using *Danio rerio prdm1* (Gene Accession Number NM\_199515.2) as a template in GC Rich PCR from Roche (Indianapolis, IN). Primers were synthesized by Integrated DNA Technologies (San Diego, CA) with sequences as follows:

- A2** Forward: 5'-CTCAACTACCCTGCCTCTGG-3'  
Reverse: 5'-GAGTAGTGCGGGTTTGGGTA-3'
- B2** Forward: 5'-ATCGTGGCCTGAACCACTAC-3'  
Reverse: 5'-CCTGTGGAACCTCGTTTCAT-3'
- C2** Forward: 5'-GCAGAAAATGTCCCCAAAGA -3'  
Reverse: 5'-GACTTCTCTCAGGGCTGCTG-3'

The radioactive probe was synthesized from the PCR product with P-32-labeled dATP using Amersham Rediprime II (GE Healthcare). The radioactive probe was hybridized at 52°C and washed at 42°C until radioactivity of the screen no longer decreased. Phosphor cassettes were exposed with the radioactive screen for 24 hours, then scanned and read using the BioArray program (The Biocomputation Group, Philadelphia, PA). Positive clones were selected, amplified, digested, and sequenced using SP6 and T7 promoter primers. Returned sequences were used in BLAST searches (more dissimilar sequences, discontinuous megablast) against the NCBI nucleotide collection. Sequences that returned with matches to *prdm1* homologs in other species were sequenced to full length.

### Lamprey husbandry and embryo culture

Adult lampreys (*Petromyzon marinus*) were obtained from Hammond Bay Biological Station, Millersburg, MI, USA. The animals were housed in a Caltech lamprey facility as previously described (Nikitina *et al.*, 2009a). They were matured by expanding their daylight cycle and raising the temperature by 1°C per day until 18°C was reached. Eggs were harvested from gravid females, and fertilized in 100–200ml of 18°C spring water (Sparkletts). The eggs were washed after 15 minutes to prevent hyperspermy. After 6 hours when the embryos had undergone the first division, they were transferred to 0.1X MMR (Marc's Modified Ringer's solution) media, which was then replaced every day to prevent fungal or other infections. Embryo fixation was done with MEMFA (4% formaldehyde, 0.1M MOPS (pH 7.4), 1 mM MgSO<sub>4</sub>, 2 mM EGTA), dehydrated, and stored in MeOH at –20°C.

### Morpholino injection

FITC-labeled translation blocking morpholinos against lamprey Pax3/7, ZicA, MsxA, and AP-2 were obtained from Gene Tools (Philomath, OR, USA). Their sequences were as follows (Sauka-Spengler *et al.*, 2007; Nikitina *et al.*, 2008):

- Pax3/7 MO: 5'-TGTCCTGGTGCCGGGCGCATCATCC-3'; Msx MO: 5'-GACCGCGAAGCGAAATGCGTTCATG-3'; ZicA Mo: 5'-CGCCTCCAGAAGCATCGCGTGCGGT-3'; AP2 Mo: 5'-

CCTGTAATTTCAAAGCATGACTCC-3'; control Mo: 5'-

CCTCTTACCTCAGTTACAATTTATA-3'

FITC-labeled translation blocking morpholino targeting lamprey Blimp-1 (5'-ATCACCATGCGCCCTCACCGCTGTT-3') was injected into a single blastomere at 4-, 8-, 16- and 32-cell stage, and the embryos were cultured and collected as previously described (Nikitina *et al.*, 2009; McCauley and Bronner-Fraser, 2006). A 5-nucleotide mismatch morpholino (5'-ATCtCCAcGCGCCCTCAtCGCaGTT-3') was used as a negative control.

### ***In situ* hybridization**

*In situ* hybridization was performed as described (Nikitina *et al.*, 2009c). Following color development, the embryos were transferred to 7.5% gelatin/15% sucrose/PBS, then mounted in 20% gelatin/PBS and frozen in liquid nitrogen. Cryosections (10–12µm) were collected on Super Frost Plus slides (Fischer Scientific, Pittsburg, PA).

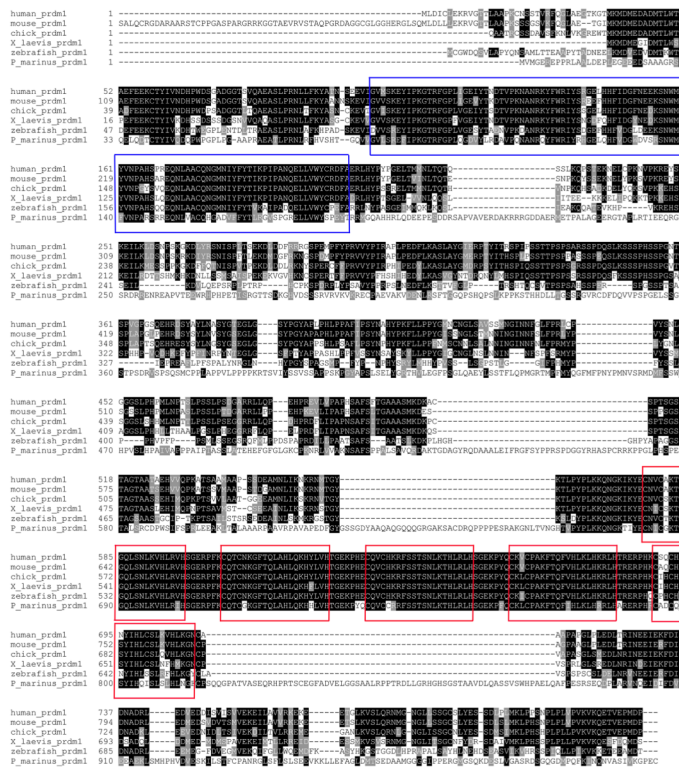
### **Conclusion**

The expression pattern of *prdm1* in the lamprey *P. marinus* suggests that it may play an evolutionarily conserved role in early events of neural crest specification. We show that *prdm1* expression in the neural plate border is turned on by AP-2 and Msx1, but is independent of Pax3/7 and Zic. In addition, loss-of-function experiments suggest an early role in anterior endomesoderm specification.

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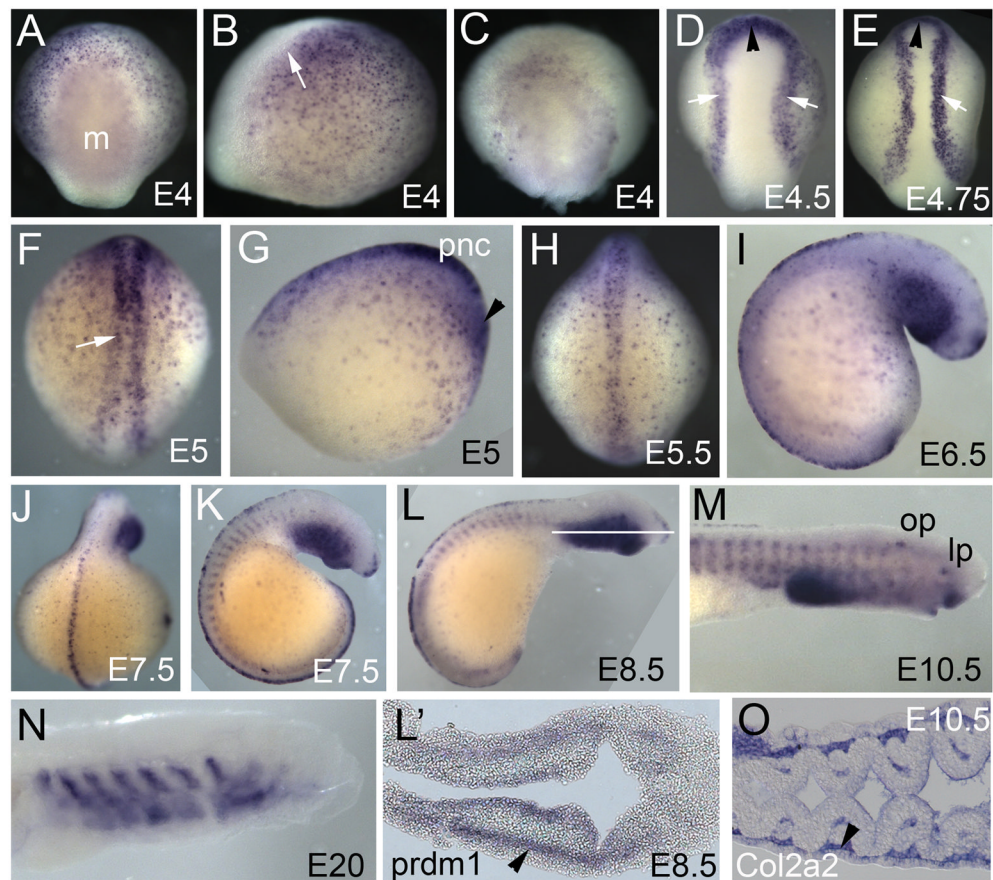
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**Figure 1. Sequence alignment of vertebrate Prdm1 protein sequences to deduced *P. marinus* protein sequence**  
 Identical amino acids are highlighted in black, amino acids with similar properties in grey. The SET domain is boxed in blue, and the five zinc fingers in red. Sequences used for alignment: human (CAQ52608.1), mouse (AAI29802.1, chicken (ENSGALP00000024778), *Xenopus laevis* (AAH60348.1), zebrafish (AAR87139.1)

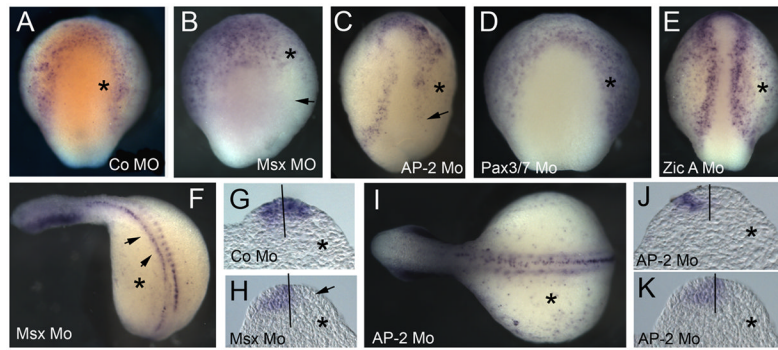




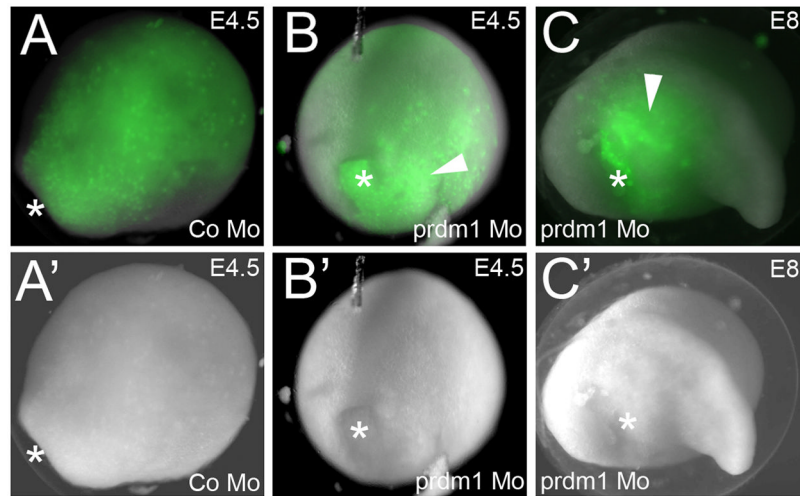
**Figure 2. Expression pattern of *prdm1* in sea lamprey embryos**

(A–N) whole mount *in situ* hybridization with the full-length lamprey *prdm1* probe. Orientation: anterior is at the top in dorsal views, or in the top right-hand corner in side views. (L', O) 12 micron sections through the stained embryos. (A) Dorsal view of an E4.0 embryo, showing *prdm1* transcripts expressed uniformly throughout the ectoderm and the neural plate border, but absent from the neural plate. (B) Side view of the same embryo. (C) Dorsal view of the embryo in (A) with its ectoderm removed, demonstrating that *prdm1* is also expressed in the paraxial mesoderm at this stage. (D and E) Starting at E4.5 (D), high levels of *prdm1* are expressed in the neural plate border (white arrows) and in the preplacodal domain (black arrowhead), and lower levels in the ectoderm. (E) A similar pattern of *prdm1* expression is seen 6 hours later at E4.75. (F) Dorsal and (G) side view of the same E5.0 embryo, showing strong expression in the premigratory neural crest (white arrow). (H) Embryo at E5.5. *prdm1* is turned off in the neural crest as it starts to migrate. (I) Lateral view of an E6.5 embryo. *prdm1* transcripts are no longer seen in the anterior dorsal neural tube. (J) Dorsal and (K) side view of an E7.5 embryo. *prdm1* is expressed in the trunk dorsal neural tube, in the branchial basket and in somites. (L) E8.5 embryo showing *prdm1* expression in the branchial arches. (M) Side view of the head of an E10.5 embryo, showing *prdm1* expression in the lips and lens (lp) and otic placodes (op). (N) Side view of E20 embryo, showing *prdm1* expression in the cartilage of the branchial arches. (L') Transverse section through the head of the E8.5 embryo in (L) showing *prdm1* expression in the endoderm and the lateral region of the neural crest (black arrowhead, compare to panel O). (O) Section through an E10.5 embryo stained for Col2a2, which at that stage is expressed in the neural crest components of the branchial arches. Black arrow marks the lateral component of the neural crest that corresponds to the *prdm1* expressing cells in (L').

Abbreviations: m-paraxial mesoderm, pnc – premigratory neural crest, op-otic placode, lp-lens placode. White arrows indicate neural plate border, black arrowheads – preplacodal domain.

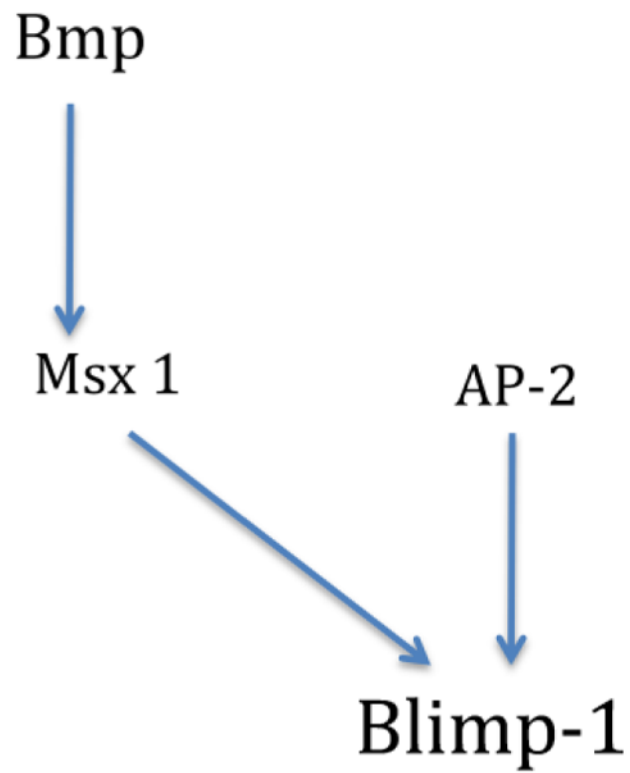


**Figure 3. Expression of *prdm1* in the neural plate border is regulated by AP-2 and Msx A**  
*prdm1* is detected by *in situ* hybridization in morpholino-injected embryos. Top row: E4.5 embryos injected with control (A) Msx A (B), AP-2 (C), Pax3/7 (D) and Zic A (E) morpholinos. All embryos are shown in dorsal view with anterior facing up. In all embryos, morpholino was incorporated into the right side (marked with an asterisk). Arrows in (B) and (C) indicate loss of *prdm1* expression on the injected side. (F) MsxA-morpholino-injected embryo at E8.5, showing loss of *prdm1* expression in somites and reduction of expression in half of the neural tube (arrowheads). (H) Section through the embryo in (F) showing loss of *prdm1* expression in one half of the neural tube (arrow). (G) Section through an E8.5 embryo injected with the control morpholino. (I) E7.5 embryo injected with AP-2 morpholino, showing that *prdm1* expression in the somites is not affected (asterisk marks the injected side). (J, K) Sections through the embryo in panel (I), showing loss of *prdm1* expression on the injected side (asterisk).



**Figure 4. *prdm1* Mo never incorporates in ectoderm**

(A–C) Fluorescent images showing incorporation of FITC-labeled morpholino (green). (A'–C') Bright field images of the same embryos. (A, A') E4.5 embryo injected with a control morpholino, showing incorporation of the morpholino throughout the dorsal ectoderm. The embryo is shown in dorsal view, with the anterior in the top right-hand corner. Asterisk denotes the position of the blastopore. (B, B') E4.5 embryo injected with *prdm1* morpholino. Morpholino is incorporated in the mesoderm and endoderm (white arrowhead). The embryo is shown with the dorsal side facing up, and the posterior (blastopore, marked with an asterisk) facing towards the viewer. (C, C') *prdm1* morpholino-injected embryo at E8. Orientation: the head is facing right, and the dorsal side is facing up. Asterisk denotes the abnormal position of the blastopore.



**Figure 5.**  
Schematic representation of *prdm1* upstream regulatory inputs in the neural plate border.