Expression of *patched*, *prdm1* and *engrailed* in the lamprey somite reveals conserved responses to Hedgehog signalling

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Abstract

In the zebrafish embryo, expression of the *prdm1* and *patched1* genes in adaxial cells is indicative of their specification to give rise to slow twitch muscle fibres in response to Hh signalling. Subsets of these slow twitch muscle progenitors activate *engrailed* (*eng*) strongly in response to high level Hh signalling, and differentiate into muscle pioneer cells, which are important for subsequent development of the horizontal myoseptum. In addition, *eng* is expressed more weakly in medial fast fibres in response to lower Hh levels. Somite morphology in the lamprey, an agnathan (jawless) vertebrate, differs significantly from that of teleosts. In particular, the lamprey does not have clear epaxial/hypaxial domains, lacks a horizontal myoseptum and does not appear to possess distinct populations of fast and slow fibres in the embryonic somite. Nevertheless, Hh is expressed in the midline of the lamprey embryo, and we report here that, as in zebrafish, homologues of *patched* and *prdm1* are expressed in adaxial regions of the lamprey somite, and an *eng* homologue is also expressed in the somite. However, the lamprey adaxial region does not exhibit the same distinct adaxial cell morphology as in the zebrafish. In addition, the expression of *follistatin* is not excluded from the adaxial region, and *eng* is not detected in discrete muscle pioneer-like cells. These data suggest the presence of conserved responses to Hh signalling in lamprey somites, although the full range of effects elicited by Hh in the zebrafish somite is not recapitulated.

Introduction

Cells derived from vertebrate somites give rise to a wide variety of structures including the vertebrae and ribs, the dermis of the skin and the skeletal muscles of the back, body wall and limbs. They also provide cues that guide the migration of neural crest cells and spinal neurons. Evolutionary changes in somite patterning can therefore have a considerable effect on the adult form of an organism. Somite development in gnathostome (jawed) vertebrates is relatively well understood both morphologically and molecularly, but comparatively little is known of the molecular mechanisms underlying somite development in agnathan, or jawless, vertebrates. These are the most basal group of extant vertebrates, positioned phylogenetically between the non-vertebrate chordates and the gnathostome vertebrates, and are therefore ideally placed for investigating the evolution of somite development. Lampreys are one of only two agnathan groups alive today.

The somitic muscles of all gnathostome vertebrates contain both slow and fast twitch muscle fibres. In zebrafish, the first slow muscle fibres form from a distinct adaxial cell population, which is obvious both morphologically and by its expression of *myoD* and *myf5* even before the somite becomes epithelialised (Chen et al., 2001; Devoto et al., 1996; Weinberg et al., 1996). This adaxial population gives rise to slow muscle cells, including a migratory population, which travels through the somite to form a superficial slow muscle layer, and muscle pioneer cells, which remain medial and are believed to be important for the subsequent formation of the horizontal myoseptum (Devoto et al., 1996). A similar adaxial cell population that gives rise to migratory slow muscle is also present in *Xenopus* and sturgeon and is therefore likely to have been the ancestral condition for the sarcopterygians (lungfish, coelacanths, tetrapods) and actinopterygians (ray-finned fish) (Grimaldi et al., 2004; Steinbacher et al., 2006). Similarly, a horizontal myoseptum in the embryonic somite is present in all gnathostomes, except for the sarcopterygians, in which it is thought to have been secondarily lost (Gemballa et al., 2003). Where present, the horizontal myoseptum physically divides the gnathostome somite into distinct epaxial and hypaxial domains. Defined epaxial and hypaxial domains are, however, present in all gnathostomes, whether or not the horizontal myoseptum is present.

Lamprey somite morphology differs in several important respects from that of zebrafish and other gnathostomes (Kusakabe and Kuratani, 2005). In particular, lamprey somites have no horizontal myoseptum, nor any obvious morphological distinction between hypaxial and epaxial muscle (Peters and Mackay, 1961). As in gnathostomes, however, two varieties of muscle fibre are recognizable in the adult somitic muscle. Here, muscle fibres are stacked in horizontal blocks, each containing approximately four layers of central or fast fibres, covered dorsally and ventrally by parietal or slow fibres (Flood et al., 1977; Peters and Mackay, 1961; Teräväinen, 1971). Historically, however, no distinction between fast and slow fibres has been identified in embryonic or larval lamprey younger than 10 cm ammocoetes, and at early stages the larval somitic muscle is arranged very differently: triangular muscle lamellae are stacked horizontally and covered by a single layer of lateral fibres (Flood et al., 1977; Nakao, 1976; Peters and Mackay, 1961).

In zebrafish, early somite patterning is dependent on Hedgehog (Hh) signalling from the midline, which specifies different slow and fast muscle cell types according to both concentration and time of exposure to the signal (Ingham and Kim, 2005; Lewis et al., 1999b; Wolff et al., 2003). A comparatively low dose of Hh is required at the shield stage for the specification of slow muscle from the adaxial cells, while at slightly later stages, higher levels are required to specify the Engrailed (Eng)-positive muscle pioneer cells that give rise to the horizontal myoseptum (Lewis et al., 1999b; Wolff et al., 2003). Later still, migration of slow muscle cells displaces fast muscle cells, bringing the latter into the vicinity of the notochord, where they respond to medium levels of Hh signalling, resulting in the specification of Eng-expressing medial fast fibres. Mutations in genes encoding components of the Hh pathway eliminate all or a subset of these cell types. In the zebrafish *smu* mutant, in which the Hh transducing protein Smo is completely inactivated, both the superficial slow fibres and the muscle pioneers fail to be specified, and no horizontal myoseptum forms (Barresi et al., 2000). This early Hh signalling is dependent on the activity of Gli proteins, and is disrupted by the *yot*/*gli2* mutation, which acts as a dominant repressor of Hh target genes (Karlstrom et al., 2003). Adaxial cells are present in both *smu*/*smo* and *yot*/*gli2* mutant embryos, but differentiate by default into fast fibres (Hirsinger et al., 2004; Lewis et al., 1999b). In contrast, in the *syu*/*shha* mutant, in which Hh

pathway activation is partially attenuated, cell types that require higher level Hh signalling (muscle pioneers and medial fast fibres) are lost, but specification of a reduced number of superficial slow fibres, for which lower levels of Hh signalling are sufficient, does occur (Ingham and Kim, 2005; Lewis et al., 1999b; Schauerte et al., 1998; Wolff et al., 2003).

Based on the morphological differences between lamprey and gnathostome somites, and the known roles for Hh signalling in patterning the somite in selected gnathostomes, two somewhat contradictory theories concerning the role of Hh signalling in lamprey somite development can be proposed. Firstly, it has been noted that lamprey somites—on the basis of their pattern of innervation and lack of a horizontal myoseptum—resemble those of zebrafish Hh pathway mutants such as *yot/gli2* (Kusakabe and Kuratani, 2005). A prediction arising from this comparison is that Hh signalling plays little or no role in lamprey somite patterning, suggested as a possible hypothesis by Kusakabe and Kuratani (Kusakabe and Kuratani, 2007). A second study, however, suggests that Hh-dependent generation of migratory slow muscle from adaxial cells is likely to have occurred in the last common ancestor of lampreys and primitive jawed fishes, predicting an important role for Hh signalling in lamprey somite patterning (Grimaldi et al., 2004). As discussed above, slow myogenesis from an adaxial cell population occurs in *Xenopus*, sturgeon and zebrafish. This process is known to be Hh-dependent in *Xenopus* as well as in zebrafish, suggesting that Hh-dependent adaxial slow myogenesis occurred in the last common ancestor of the sarcopterygians and the actinopterygians (Grimaldi et al., 2004). Additional evidence that superficial slow fibres are present not only in these groups but also in more primitive fish—including shark and arguably also adult lamprey (Flood et al., 1977)—led Grimaldi and colleagues (2004) to postulate that Hh-dependent adaxial myogenesis might be the ancestral condition for lampreys and all gnathostome vertebrates.

In the lamprey, as in zebrafish, Hh is expressed in midline tissues adjacent to the somites (Osorio et al., 2005). With the aim of understanding the role that Hh signalling plays in lamprey somite patterning, we have examined expression of a lamprey homologue of the gene encoding the Hh receptor, *Ptc*, itself a target of Hh

activity, to ascertain whether lamprey somites actively transduce Hh signalling. In *Drosophila*, zebrafish and amniotes, *ptc* transcription is strongly up-regulated in response to Hh, and so high level *ptc* expression indicates cells in which the Hh signal is being actively transduced (Concordet et al., 1996; Goodrich et al., 1996; Hidalgo and Ingham, 1990; Lewis et al., 1999a). In addition, we have examined the expression of lamprey homologues of three further genes that are regulated by Hh in the gnathostome somite. Firstly, we cloned and analysed expression of a lamprey homologue of *prdm1*: *prdm1a* is a vital downstream component of the zebrafish slow muscle specification cascade (Baxendale et al., 2004). In zebrafish, expression of *prdm1a* is activated in adaxial cells in response to Hh signalling and is itself both necessary and sufficient to drive slow myogenesis. Secondly, we examined expression of a lamprey homologue of *follistatin (fst)*. Expression of *fst* in the gnathostome somite is inhibited by Hh: it is specifically excluded from adaxial cells in zebrafish and salmon (Bauer et al., 1998; Macqueen and Johnston, 2008), an effect that we show to be Hh-dependent in the zebrafish (Supplementary Fig. 1), and is down-regulated by notochord signals in the chick, an effect that can be mimicked by Shh protein (Amthor et al., 1996). Lastly, we cloned and analysed expression of a lamprey homologue of the *engrailed* (*eng*) genes that are strongly expressed in zebrafish muscle pioneer cells in response to high level Hh signalling from the midline and at lower levels in medial fast fibres in response to sub-maximal levels of Hh (Ekker et al., 1992; Hatta et al., 1991; Roy et al., 2001; Wolff et al., 2003).

We find that all four genes—*ptc, prdm1, fst* and *eng*—are expressed in the lamprey somite. Although we cannot define a morphologically distinct adaxial cell population in the early somite, the expression of *ptc* and *prdm1* is higher in adaxial regions, suggesting that somite cells are responsive to Hh signalling from the midline, and that such signalling results in the activation of *prdm1* expression. Expression of *eng* in the lamprey somite is weak, and present in a broad domain, rather than in discrete muscle pioneer-like cells, while the expression of *fst* is not excluded from adaxial regions as in other species. These results suggest that Hh signalling does play a role in patterning the lamprey somite, but that the full set of responses seen in gnathostomes is not found. Staining with antibodies that mark different myosin types also reveals

differences that suggest myosin types have diverged between agnathans and gnathostomes.

Materials and Methods

Isolation and sequencing of lamprey cDNA

A 179bp fragment and a 175bp fragment corresponding respectively to the SET domain and the zinc finger domain of *prdm1* were amplified from *Lampetra fluviatilis* stage 22/23 oligo (dT)-primed cDNA by degenerate PCR using primers: SETF: CATCCCCAAGGGCACCMGNTTYGGNCC, SETR: GGGCGGGGTTCACGTANCGCATCCART and ZINCF: GAACGGCAAGATCAAGTACGARTGYAAYRT, ZINCR: GCCGGTGTGCACCAGGWWRTGYTTYTG. Primers were designed with CODEHOP (Rose et al., 1998) using a CLUSTALW alignment of mouse, human, zebrafish, fugu, *Xenopus* and *Drosophila prdm1* sequences and the *Petromyzon marinus* codon usage table (http://bioinformatics.weizmann.ac.il/blocks/help/CODEHOP/codon.html). RNA was extracted from stage 22/23 *L. fluviatilis* embryos using Trizol (Sigma), cDNA was transcribed using a Superscript III first strand synthesis kit (Invitrogen) and PCR was carried out using Diamond Taq polymerase for GC-rich DNA (Bioline). PCR fragments were cloned into the pCR2.1 TOPO vector (Invitrogen). Sequencing was performed by the Genetics Core Facility, University of Sheffield, using an ABI 3730 capillary sequencer. *prdm1* sequences have been deposited with Genbank (accession numbers: FJ376435, FJ376436). Comparison of the *L. fluviatilis* cDNA sequence fragments with *P. marinus* genomic sequence suggests that there may be more than one *prdm1* gene in each species, but we have been unable to isolate full-length sequences.

The *engrailed* (*eng*) fragment was PCR-amplified from cDNA obtained from an embryonic *P. marinus* cDNA library (kindly provided by J. Langeland) using the following primers: PmEnF: GCAGGCCCGCGGATCCGCAA , PmEnR: TAGAGGCCCTGCGCGAGCAAC. Primers were designed from genomic DNA sequence freely accessible from the *P. marinus* genome sequencing project (Washington University; http://genome.wustl.edu). A 278 bp sequence was amplified and cloned into the pGEM T-easy vector (Promega). Sequencing was performed by the Zoology Core Molecular Laboratory (University of Oklahoma) using an ABI 3730

capillary sequencer. The *eng* sequence has been deposited with Genbank (accession number: FJ358499).

Isolation and sequencing of *P. marinus ptc* (DQ370170) and *fst* (DQ370171) cDNA sequences are described in our previous paper (Hammond and Whitfield, 2006).

Phylogenetic analysis

Protein sequence alignments were created using CLUSTALW, accessed through the workbench interface (http://workbench.sdsc.edu). Alignments for Ptc and Follistatin were previously published (Hammond and Whitfield, 2006); alignments for Prdm1 and Engrailed are shown in Supplementary Fig. 2. Phylogenetic relationships were analysed by neighbour joining using the PHYLIP v3.67 package (J. Felsenstein (2007), http://evolution.genetics.washington.edu/phylip.html). Bootstrapping, using 100 replicates, was carried out to estimate the degree of support for internal branches. Trees are shown in Supplementary Fig. 3.

In situ hybridisation and photography

All in situ hybridisation was carried out using *P. marinus* embryos. Acquisition and staging of embryos, synthesis of digoxygenin-labelled *patched* (*ptc*), *follistatin* (*fst*), *prdm1* and *eng* riboprobes, in situ hybridisation, clearing of embryos and photography were all carried out as previously described (Hammond and Whitfield, 2006). In situ hybridisations using the sense strand for all probes were carried out and none showed specific expression (data not shown). Whole mount specimens were cleared using Murray's clear (2:1 benzyl benzoate:benzyl alcohol), which we find more effective than glycerol for the yolky lamprey embryo. For Fig. 5d', images were collected on a Zeiss Axioimager Z1 equipped with the Apotome module for optical sectioning. Fluorescence of the NBT/BCIP precipitate within tissue was imaged using a Cy7 filter set and optically sectioned as described (Trinh et al., 2007).

Antibody staining

P. marinus and *L. fluviatilis* embryos were acquired, staged and fixed as described (Hammond and Whitfield, 2006) except for *L. fluviatilis* embryos used for mAb S58 (DHSB) staining, which were fixed in Carnoy's fix as described (Barresi et al., 2000).

Staining protocols for mAb MF20 (DHSB) and mAb F59 (DHSB) (Bader et al., 1982) were as described (Du et al., 1997), with the following modifications to increase penetration of the antibody. After rehydration, the lamprey embryos were washed in H_2O for 5 min, acetone (-20°C) for 7 min, H_2O for 5 min and PBS, 0.1% Tween-20 for 5 min. The embryos were then incubated at 37°C for 2 hours in 10µg/ml proteinase K (Sigma), fixed in 4% paraformaldehyde for 20 min at room temperature and washed 5 times for 5 min in PBS, 0.1% Tween-20 before blocking. Primary antibodies were used at the following concentrations: mAb S58, 1 in 20; mAb F59, 1 in 20; MF20, 1 in 500. Biotinylated anti-mouse IgA (1 in 200) and streptavidin Alexa 568 (1 in 500) were used for fluorescent detection of S58; all other stainings were detected using biotinylated anti-mouse IgA (for S58 detection, 1 in 200) or biotinylated anti-mouse IgG (1 in 200) and developed using the Vectastain ABC kit (Vector Laboratories) and DAB as previously described (Du et al., 1997).

Histology

Thick sections (approximately 25-50μm) were cut by hand using a hypodermic needle, and mounted for photography in DPX mounting medium (Sigma). Vibratome sections were cut at 30μm. For resin sections, embryos were first orientated in 1% low melting point agarose, and then embedded in JB4 resin (Polysciences). Sections were cut at 6 or 7μm and where appropriate were lightly counterstained with either eosin or toluidine blue before mounting in DPX.

Results

Expression of *patched* in the lamprey embryo and larva

To investigate whether Hh may be actively transduced in the lamprey somite, we examined expression of a previously reported 747bp fragment of a *P. marinus* homologue of *patched* (*ptc*) (Hammond and Whitfield, 2006). Phylogenetic analysis of the predicted protein sequence reveals that lamprey Ptc clusters with, but is basal to, vertebrate Ptc2 proteins (Supplementary Fig. 3). This cluster includes zebrafish Ptc1 in our analysis (Supplementary Fig. 3). Although we have not isolated a second lamprey *ptc*, it is possible that another *ptc* gene, more similar to *Ptc1*, may also exist.

Lamprey *ptc* is expressed in a pattern highly reminiscent of zebrafish *ptc1* (Concordet et al., 1996), with extensive expression in ventral regions of the CNS (Fig. 1). At stage 22/23, *ptc* expression in the neural tube is detected ventrally on either side of the floorplate (Fig. 2c-c″) and later, by stage 24-25, this begins to restrict to a more central domain in anterior regions of the embryo (Fig. 2a-a″). Other domains of expression include the pharyngeal arches (Fig. 1a-d) and the otic vesicle (Hammond and Whitfield, 2006).

As in the zebrafish, we also see strong *ptc* expression in the lamprey somite. At stage 21, as the somites are beginning to form, we detect high levels of *P. marinus ptc* medially, adjacent to the notochord and neural tube in both the presomitic mesoderm (PSM) and in the newly formed somites (Fig. 1e). Expression is seen throughout the anteroposterior (AP) and dorsoventral (DV) extent of the somite, becoming weaker with increasing distance from the midline. This pattern is seen in the PSM and in the youngest somites until stage 26 (Fig. 2c-c″). Strong *ptc* expression later restricts along the AP axis, becoming confined to a smaller central and medial region of the somite but remaining detectable throughout the DV extent of the tissue (Fig. 2b′, b″). This pattern is observable in the more mature somites by early stage 23 (Fig. 1f, f′). In the oldest somites, from late stage 23/early stage 24 onwards, *ptc* expression disappears entirely (Fig. 2a-a″). Note that all three stages of *ptc* expression can be observed within a single *P. marinus* embryo, since somites are added sequentially and are youngest at the posterior of the animal (e.g. Fig. 2d, stage 24/25).

Cloning and expression of *prdm1* in the lamprey embryo and larva

The *positive regulatory domain containing 1a* gene (*prdm1a*; formerly *blimp1*) is transcriptionally regulated by Hh signalling in the zebrafish somite and is of particular interest, since it is both activated in zebrafish adaxial cells in response to Hh signalling and is itself necessary and sufficient for slow fibre specification of these cells (Baxendale et al., 2004). We have cloned 179bp and 175bp fragments from *L. fluviatilis* that are highly homologous to the PR (SET) domain and zinc finger domain respectively of *Prdm1* genes in a variety of species (Supplementary Fig. 2a), and have used these as probes for *prdm1* expression in *P. marinus* embryos. In situ hybridisation with these fragments detects transcripts in the developing somites (described below), in a pattern similar to that seen for zebrafish *prdm1a*. The SET domain probe alone shows a similar pattern (data not shown), whereas the zinc finger domain probe alone hybridises only very weakly; sequence analysis suggests that the zinc finger fragment may correspond to an alternative *prdm1* gene (data not shown). Phylogenetic analysis of the SET domain protein sequence suggests that lamprey Prdm1 clusters with, but is basal to, other vertebrate Prdm1 proteins, apart from zebrafish Prdm1c, which has a very divergent SET domain (Sun et al., 2008) (Supplementary Fig. 3). The zinc finger fragment was too highly conserved to generate a meaningful phylogenetic tree.

In posterior (younger) lamprey somites at stage 23 the *prdm1* probe detects transcripts in cells located medially, close to the notochord, and in a separate, more lateral domain (Fig. 2e, h-l). Expression is maintained in the somites until at least stage 26. This is similar, but not identical, to the pattern of *prdm1a* expression during zebrafish somite development, which is restricted to the adaxial cells until the onset of lateral migration of the slow muscle cells in each somite, when expression decreases (Baxendale et al., 2004). Interestingly, in lampreys, we see a lateral shift in the expression domain of *prdm1* as the somite develops, which may be indicative of a similar cell migration (see arrows in Fig. 2h-l). We also detect lamprey *prdm1* expression in the otic vesicle, similar to the zebrafish *prdm1* genes (Fig. 2e). In addition, there is a low level of signal throughout the lamprey embryo, which could in part be due to background arising from the small size of the riboprobes used. We do not, however, detect specific *prdm1* staining in the branchial arches, neural crest and

apical ectodermal ridge of the fins, where *prdm1* genes are expressed in other species (Chang et al., 2002; de Souza et al., 1999; Ha and Riddle, 2003; Lee and Roy, 2006; Roy and Ng, 2004; Vincent et al., 2005; Wilm and Solnica-Krezel, 2005). It is interesting to note that while the adaxial expression of *prdm1a* in the zebrafish somite is downstream of Hh signalling, these other domains of *prdm1a* expression are not (Baxendale et al., 2004). Similarly, the lateral domains of *prdm1* expression in the lamprey somite seem unlikely to be Hh-dependent.

Expression of *follistatin* in the lamprey embryo and larva

Follistatin (fst) encodes a Bmp/TGFβ antagonist and is expressed strongly during gnathostome somite development. We were particularly interested to document *fst* expression during lamprey somite development, since it is repressed by Hh during chick somite patterning (Amthor et al., 1996); similarly, in zebrafish somites, expression of *fst1* and *fst2* is specifically excluded from adaxial cells (Bauer et al., 1998) in a Hh-dependent manner (Supplementary Fig. 1). Zebrafish *fst2*, which is expressed later than *fst1*, is also excluded from the region prefiguring the horizontal myoseptum, formation of which is dependent on adaxial cell derivatives, the muscle pioneer cells (Bauer et al., 1998; Dal-Pra et al., 2006).

We therefore examined expression of a previously cloned *P. marinus* homologue of *fst* corresponding to the entire open reading frame (Hammond and Whitfield, 2006). As expected, phylogenetic analysis of the predicted protein sequence shows that this clusters with, but is basal to, the gnathostome Fsts (Supplementary Fig. 3). Like zebrafish *fst1*, lamprey *fst* is expressed in the otic vesicle, as previously reported (Hammond and Whitfield, 2006). Similar to zebrafish *fst1* and *fst2* expression, we also detect strong *P. marinus fst* expression in the mesoderm of the pharyngeal arches, beginning at stage 24 and persisting until at least stage 29, although staining becomes less strong by this time (Fig. 3a-d, g). *P. marinus fst* expression also appears in the mandibular arch at stage 21-22. This becomes strong by stage 23 (Fig. 3a, e, f) and persists, splitting into two separate domains by stage 25 (Fig. 3b-d). At stage 29 an additional expression domain appears in the dorsal tail fin (Fig. 3h-j). Unlike in zebrafish, however, we do not detect extensive *fst* expression within the lamprey brain.

As in zebrafish we also see extensive *fst* expression within the lamprey somite. *fst* is initially expressed throughout the bulk of the somite, first appearing between stages 22 and 23, after the somites become epithelialised, and some time after the initiation of *ptc* expression in the somites (Fig. 3f, 4c-c″). In contrast to the situation in gnathostomes, lamprey *fst* expression is, if anything, initially stronger adaxially. Later, *fst* expression is detectable in a domain directly adjacent to the notochord as well as in a larger more dorsal region (Fig. 4b-b″). Similar *fst1* expression is seen in zebrafish posterior somites at the 25-somite stage, where the ventral domain is thought to represent sclerotomal expression and the dorsal domain myotomal expression (Bauer et al., 1998). Later still, lamprey *fst* expression restricts to a thin lateral and posterior domain, remaining stronger dorsally (Fig. 4a-a″). By analogy with amniotes, where dermomyotomal *fst* expression is detected (Albano et al., 1994; Connolly et al., 1995), this lateral domain of *fst* expression may mark the lamprey equivalent of the dermomyotome, a structure previously identified in *Lethenteron japonicum* by its expression of *pax3/7* (Kusakabe and Kuratani, 2005). Macqueen and Johnston (Macqueen and Johnston, 2008) report that salmon *fst* is expressed in a similar posterior and lateral region of the somite, which will give rise to the dermomyotome. Note that as for *ptc*, all *fst* somite expression patterns can be observed at different axial levels of a single lamprey embryo; Fig. 4a-d shows somite expression in an early stage 26 embryo in which the youngest somites (c) are at the posterior and the more mature somites (a) at the anterior.

To summarise, in contrast to the situation in gnathostomes, *fst* is not excluded from the adaxial region of lamprey somites, despite the overall similarity of lamprey and gnathostome *fst* expression. Note that, as in lamprey, there appears to be a single *fst* gene in all gnathostomes examined except for the Ostariophysi, which includes the zebrafish, where there are two *fst* genes. This is thought to be the result of duplication during the teleost whole genome duplication, with subsequent loss of *fst2* in clades outside the Ostariophysi (Macqueen and Johnston, 2008).

Expression of *engrailed* in the lamprey somite

The *engrailed1a, b* and *2* (*eng*) genes are expressed strongly in zebrafish muscle pioneer cells (Ekker et al., 1992; Hatta et al., 1991) in response to high level Hh signalling from the midline (Roy et al., 2001; Wolff et al., 2003). Lamprey *eng* expression was of special interest to us, since in the zebrafish, muscle pioneer cells are required for the formation of the horizontal myoseptum, a structure that the lamprey lacks. Based on staining with αEnhb1, a polyclonal antibody raised against the mouse En protein, lamprey *eng* expression was previously reported in the midbrain hindbrain boundary region, the mandibular arch and the tail bud (Holland et al., 1993). However, no mention was made of expression in the somites. We therefore cloned and analysed expression of a 278 bp fragment of a *P. marinus* homologue of *eng*. Phylogenetic analysis of the predicted protein sequence reveals that this Eng fragment clusters with the single Eng proteins of amphioxus and *Ciona* rather than with the separate Eng1 and Eng2 clusters of the gnathostomes (Supplementary Fig. 3).

Consistent with the expression of Eng in other vertebrate species, and in line with the pattern revealed by immunostaining in the lamprey, the *P. marinus eng* probe detects transcript in the midbrain-hindbrain boundary (mhb) region from stage 23 (Fig. 4e); in addition, we detected weak expression in the somites (Figs. 4, 5). Note that, as for *prdm1*, there is also a low level of signal throughout the embryo, which could in part be due to background arising from the small size of the riboprobe. At stage 23, expression is restricted to the medial part of the somite, in a pattern that has distinct similarities with lamprey *ptc* expression (Fig. 4). In the youngest somites, at the posterior of the embryo, *eng* expression is detected throughout the AP extent of the somite, extending laterally in rays from the adaxial region (Fig. 4e, h'). In older, more anterior somites, *eng* expression becomes restricted with respect to the AP axis in each somite, so that it is present in a central stripe that extends throughout the DV extent of the somite, slightly displaced from the notochord, and similar to both *ptc* and *prdm1* expression (Fig. 4e, f, f'). By stage 27, *eng* expression is detected in the bulk of the somite at most axial levels (Fig. 5). As the staining in the somites was weak, we confirmed the expression pattern by imaging the near-infra red fluorescence of the NBT/BCIP precipitate; this shows that the signal is cytoplasmic, and confined to the somite, and thus appears to be specific (Fig. 5d, d'). However, high levels of

eng expression typical of zebrafish muscle pioneer cells are not present in lamprey somites.

The adaxial region of the lamprey somite is not morphologically distinct from the rest of the somite

In zebrafish, the adaxial region is morphologically distinct and visible as a defined layer of cuboidal cells adjacent to the notochord, from a stage well before overt epithelialisation of the somite until the epithelialised somite is a few hours old (Devoto et al., 1996). To identify any such region within the lamprey somite we have analysed lamprey somite morphology histologically, from stages prior to somite epithelialisation until the somite is well formed and consists of horizontally stacked muscle lamellae covered by a lateral cell layer (Fig. 6). Early lamprey somites consist of a ball of cells (Fig. 6b, c) with one side of this ball in direct contact with the notochord. Unlike in zebrafish, we find no strong evidence—based on morphology alone—for a distinct adaxial region during this period, although we do see occasional cells that might represent adaxial cell equivalents (arrows, Fig. 6).

Expression of later markers of slow muscle

In zebrafish, *prdm1a* is required for commitment to the slow muscle fate and is expressed at high levels in developing slow muscle fibres (Baxendale et al., 2004). Thus adaxial expression of *prdm1* in the lamprey somite might also suggest the presence of slow fibre fate in the early larval lamprey. To investigate this further we have incubated stage 25 to stage 28 lamprey larvae with the S58 antibody, which in zebrafish and chick embryos specifically stains the slow twitch muscle fibres, and the F59 antibody, which shows strong staining in slow twitch muscle fibres and weak staining in fast twitch fibres in zebrafish (Barresi et al., 2000; Devoto et al., 1996). In lamprey, both S58 and F59 antibodies strongly stain the head muscles and the heart, but expression in the trunk muscles is restricted to a few fibres in the anterior somites (Fig. 7a-c and data not shown). The lack of staining in posterior somites does not necessarily indicate that slow muscle is absent here; it is possible that the epitopes that these antibodies recognise have diverged between lamprey and zebrafish.

To determine whether the antibodies can penetrate throughout the lamprey myotome, we also stained embryos with the pan muscle marker MF20, which labels all differentiated muscle in every species analysed. While the staining of this antibody is strong throughout the trunk of the embryos, no staining was found in either the heart or the head muscles, again suggesting that the myosin isoforms in these muscles have diverged between jawed fishes and agnathans (Fig. 7d). This builds on previous work by Kusakabe et al. (2004), who noted that while the muscle actin gene *LjMA2* is expressed in both myotomal and head musculature, the myosin heavy chain (MyHC) genes *LjMyHC1* and *LjMyHC2* are detected only in cells originating in the myotome. Lamprey head and myotomal muscles therefore differ in their expression of genes encoding contractile protein isoforms. Likewise, in the zebrafish, there is significant heterogeneity of slow MyHC gene expression in different regions of the body (Elworthy et al., 2008).

Discussion

Lamprey somites are likely to be responsive to Hh signalling

As noted by Kusakabe and Kuratani (2005), lamprey somites bear at least a superficial resemblance to those of mutant zebrafish in which Hh signalling is defective. During zebrafish somite development, Hh signalling is crucial for the acquisition of slow muscle fibre identity by adaxial cells (Blagden et al., 1997; Du et al., 1997; Lewis et al., 1999b; Wolff et al., 2003). Consequently, in zebrafish carrying strong mutations in Hh pathway genes, slow muscle and its derivatives, including the muscle pioneers and the horizontal myoseptum, are absent or severely reduced (Barresi et al., 2000; Lewis et al., 1999b). Thus, as in lamprey, there is no longer a clear physical distinction between epaxial and hypaxial domains of the somite in these zebrafish mutants.

The results of our gene expression analysis, however, provide evidence that Hh may play a role in specifying cell identity in the lamprey somite. Homologues of *patched (ptc),* which is both a target of and receptor for Hh signalling, and of *prdm1* (*prdm1a* is a target of Hh signalling in zebrafish somites), are expressed adaxially in lamprey and zebrafish somites. In contrast, expression of both *ptc1* and *prdm1a* is lacking in zebrafish *smu* mutants in which Hh signalling is absent (Barresi et al., 2000; Baxendale et al., 2004). Lamprey somites cannot, therefore, be considered to be the equivalent of somites of zebrafish embryos in which Hh signalling is completely absent. They might, however, be more similar to the situation in the *syu* zebrafish mutant, in which *shha* is mutated but *shhb* (formerly *twhh*; also expressed in the midline) is unaffected. The consequent attenuation of Hh pathway activation in *syu* homozygotes results in the loss of muscle pioneers, medial fast fibres and the horizontal myoseptum, but superficial slow fibres, which require lower levels of Hh, differentiate, though in reduced numbers (Lewis et al., 1999b; Schauerte et al., 1998; Wolff et al., 2003). Likewise, both *ptc* and *prdm1a* are expressed in *syu*$^{+}$ animals, although at reduced levels (Lewis et al., 1999b) and data not shown (KH).

The *eng* expression pattern, although weak, is similar to that of *ptc*, suggesting that *eng* may also be under the control of Hh signalling in the lamprey somite. Somitic expression of *eng* genes has been described in both amphioxus (a non-vertebrate

chordate) (Holland et al., 1997) and dogfish (a chondrichthyan vertebrate) (Tanaka et al., 2002), organisms that lie on either side of the agnathan vertebrates on the evolutionary tree. These studies support the idea that a common ancestor of all chordates expressed *engrailed* in segmented mesoderm. In amphioxus, *eng* (*AmphiEn*) is expressed in a posterior stripe in the first eight somites, which form by enterocoely, but not in the more posterior somites, which pinch off from the tailbud (Holland et al., 1997). Interestingly, Hh expression in amphioxus midline tissues is present during formation of the first anterior *eng*-expressing somites, but lost at later stages (Shimeld, 1999). Expression of a *Gli* gene in amphioxus somites provides further evidence that the somitic mesoderm is Hh-responsive in this species (Shimeld et al., 2007).

While our paper was under revision, an expression analysis of four *Eng* genes in *L. japonicum* was published (Matsuura et al., 2008); our *eng* fragment is the *P. marinus* homologue of *LjEngrailedB*. The expression of *LjEnB* is similar but not identical to the pattern we see for *P. marinus eng*: weak expression of *LjEnB* in the trunk somites was reported to be non-specific. Our sense controls and fluorescence data indicate that the *P. marinus* staining is weak but specific; species differences, probe length, length of time in the staining reaction or difference in clearing agents may account for the differences seen. Interestingly, Matsuura et al. report expression of *LjEnA* in a subset of rostral somites (Matsuura et al., 2008).

Not all downstream effects of Hh within the somite are conserved between lamprey and zebrafish

Despite the similarities described above, the full range of responses to Hh signalling found in zebrafish does not appear to be present in lamprey somites. Firstly, we find that lamprey *fst* is initially expressed throughout the somite, in contrast to both zebrafish *fst* genes, which are specifically excluded from the adaxial region (Bauer et al., 1998) in response to Hh-mediated signalling from the midline (Supplementary Fig. 1). The expression of lamprey *fst*, therefore, does resemble the situation in *smu* mutant zebrafish, in which *fst1* and *fst2* are no longer excluded from the adaxial region (Supplementary Fig. 1).

Secondly, *eng* expression in the lamprey somite does not resemble *eng* expression in gnathostome somites. In zebrafish, *eng1a, eng1b* and *eng2a* are expressed in muscle pioneer cells in the centre of the somite directly adjacent to the notochord, and more weakly in medial fast fibres, but expression does not extend into dorsal and ventral regions of the somite (Ekker et al., 1992) (http://zfin.org, expression pattern database). This suggests that the *eng*-expressing cells in the lamprey are unlikely to represent muscle pioneer-like cells at the stages examined (stages 23-27). Muscle pioneers are, however, likely to be specific to the teleost lineage, having so far only been described in the zebrafish; no such adaxial *eng*-expressing muscle pioneer cells have been identified in the somites of either sturgeon or amniotes (Steinbacher et al., 2006). It is also worth noting that the notochord of the lamprey is larger, in proportion to somite size, than that of zebrafish, which may account for the broader domain of *eng* expression in the lamprey.

In the avian somite, despite the lack of muscle pioneer cells and a horizontal myoseptum, expression of *Engrailed1* (*En1*) defines the epaxial region of the dermomyotome and myotome, where it is established and maintained by signals (most likely including Hh) from the notochord, floorplate, dorsal neural tube and surface ectoderm (Ahmed et al., 2006; Cheng et al., 2004). Since *eng* is expressed in the lamprey myotome, and in a subset of amphioxus somites, it is unlikely that de novo expression of an *Engrailed* gene marked the transition from a uniform agnathan somite to one with distinct epaxial and hypaxial domains in gnathostomes. Rather, changes to the pattern of existing *Engrailed* expression, involving restriction either to a central region prefiguring the horizontal myoseptum (as in teleosts), or to an *En*-positive epaxial and *En*-negative hypaxial domain (as in amniotes), may have been a key event in the emergence of an epaxial-hypaxial distinction at the agnathan/gnathostome transition.

ptc and *prdm1* expression domains define an adaxial region in the lamprey myotome

Only a few studies to date have described molecular regionalisation within the lamprey somite. Two of these concern development of the sclerotomal compartment, which expresses *Sox9* and two isoforms of *Col2α1* (Zhang et al., 2006), together with

Parascleraxis (Freitas et al., 2006). Lateral regions of the somite (the presumed dermomyotome) express *Col2α1a* (Zhang et al., 2006) and *Pax3/7* (Kusakabe and Kuratani, 2005). *PitxA* is also expressed in ventrolateral regions of the somite (Boorman and Shimeld, 2002), while *Tbx15/18* is expressed in the anterior region (Freitas et al., 2006). Similarly there is evidence of a degree of regionalisation in the amphioxus somite: *dach* and *msx* mark a dorsal region, *zic* marks a dorsolateral region that does not contribute to the myotome and *foxD* is expressed in the medial myogenic region (Candiani et al., 2003; Gostling and Shimeld, 2003; Sharman et al., 1999; Yu et al., 2002).

Of particular interest in the context of lamprey myotomal patterning, it has been shown that at stage 25 a region of the lamprey myotome directly adjacent to the notochord is molecularly distinct: while *LjMyHC2* is expressed throughout the myotome, *LjMyHC1* and *LjMA2* are specifically excluded from the cells nearest to the notochord (Kusakabe and Kuratani, 2005; Kusakabe et al., 2004). Since in zebrafish both *ptc* and *prdm1a* are strongly expressed in adaxial cells and are required for specification of slow muscle fibres from these cells (Baxendale et al., 2004; Lewis et al., 1999b; Wolff et al., 2003), our finding that both *ptc* and *prdm1* are also expressed adaxially in lamprey suggests that the lamprey adaxial region is not only molecularly distinct, but may have functional similarities with the zebrafish adaxial region. This is consistent with the idea, proposed by Grimaldi et al. (2004), that Hh-dependent adaxial slow myogenesis occurred in the last common ancestor of lamprey and the jawed fishes.

Conclusion

The muscle cells of the somite are generally characterised on the basis of the twitch isoforms of myosin that they express. Previously thought to contain only fast twitch fibres, the lamprey embryonic and early larval myotome was thought to be more uniform than in gnathostome species. However, this study has revealed that—despite the lack of *eng*-expressing muscle pioneer-like cells—the lamprey somite shows considerable complexity of pattern, including several characteristics of slow twitch muscle differentiation. Far from being a homogeneous population of cells lacking the diversity of cell types found in gnathostome somites, the lamprey somite contains

several different cell types defined by different domains of expression of homologues of four targets of the Hh signalling pathway.

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Figure Legends

Figure 1: Expression of *patched* in *P. marinus* embryos

(a-c, e-g) Lateral views: anterior to left, dorsal to top. (d) Hand cut section through the pharyngeal arch region of a stage 23 embryo, taken along line d (shown in panel a). b, brain; nt, neural tube; ot, otic vesicle; ph, pharyngeal arch; s, somites; zli, *ptc* expression surrounding the zona limitans intrathalamica. Panels f and g are composites of two images. Scale bars, 250μm, except (d), 100μm.

Figure 2: Expression of *patched* and *prdm1* in the somites of *P. marinus* embryos

Panels a-d: *patched* expression in the somites of stage 24-25 *P. marinus* embryos. Sections and views were taken at the levels shown in panel (d). Sections were hand cut at approximately 50μm; other views are of whole-mount preparations. Anterior to the left in dorsal and lateral views; dorsal to the top in lateral and transverse views. n, notochord. Panel d is a composite of 2 images. Scale bars, (a, b, c) transverse: 50μm, dorsal and lateral: 100μm, (d) 500μm.

Panels e-l: *prdm1* expression in *P. marinus* embryos. (e, f) Lateral views. (g-i) Dorsal views; anterior to left. (h) More anterior (older) somites at stage 23; (i) more posterior (younger) somites at stage 23. (h') shows an enlarged view of a stage 23 somite at the level of k in panel (h). (j-l) Hand cut transverse sections, approximately 50μm thick, taken at stage 23 at the levels shown in panels (e, h, i). (l') Enlarged view of right side of the section shown in (l). Arrow * denotes medial *prdm1* expressing cells pre-migration; arrow l shows possible migratory cells; arrows j and k denote possible post-migratory cells. lat, lateral; med, medial; o, otic vesicle. Scale bars, (e, f) 250μm, (g-i) 100μm, (j-l) 50μm. In situ hybridisations for the embryos shown here were carried out using a mixture of the SET domain and zinc finger domain probes, but the expression pattern is indistinguishable from hybridisations using the SET domain alone (data not shown).

Figure 3: Expression of *follistatin* in *P. marinus* embryos

(a-f; i, j) Lateral views; anterior to left, dorsal to top. (g) 10μm horizontal section through the pharyngeal arch region of a stage 26 embryo counterstained with eosin. (h) Hand cut transverse section taken through the dorsal tail fin at stage 29. f, dorsal

tail fin; m, mandibular arch; ot, otic vesicle; ph, pharyngeal mesoderm; s, somites. Scale bars, 250μm except (g, h) 50μm.

Figure 4: Expression of *follistatin* and *engrailed* in the somites of *P. marinus* embryos

Panels a-d: Expression of *follistatin* in the somites of stage 26 *P. marinus* embryos. Sections and views were taken at the levels shown in panel (d). Sections (a, b, c) were hand cut at approximately 50μm; other views are of whole-mount preparations. Anterior is to the left in dorsal and lateral views; dorsal to the top in lateral and transverse views. n, notochord. Scale bars, (a, b, c) transverse: 50μm, lateral and dorsal: 100μm, (d) 250μm.

Panels e-h: Expression of *engrailed* in the somites of stage 23 *P. marinus* embryos. (e, e') Lateral views. (e') is an enlarged lateral view taken at the level of arrow f in panel (e); anterior to the top, dorsal to the right. (f-h) Sections and dorsal views were taken at the levels shown by arrows in panel (e). (f) Shows a dorsal view of expression in somites on one side of the embryo; the notochord is at the top of the picture. (g) and (h) show dorsal views of somites on both sides of the notochord. Transverse sections were hand cut at approximately 50μm. Dorsal is to the top in sections and dorsal views; anterior is to the left in dorsal views. mhb, midbrain-hindbrain boundary; n, notochord. Scale bars, (e) 250μm; (f-h) 100μm (f'-h'); 50μm.

Figure 5: Expression of *engrailed* in the somites of a stage 27 *P. marinus* embryo

Sections and views were taken at the levels shown in the bottom panel (e). Sections (a, b, c, d, d') were cut at 30μm using a vibratome; other views are of whole mount preparations. (d') shows near-infra red fluorescence of the NBT/BCIP precipitate. The bright puncta are non-specific staining; the signal is weak but localizes to the cytoplasm of somitic cells, and is absent from the surface ectoderm, notochord and neural tube. Anterior is to the left in dorsal and lateral views; dorsal to the top in lateral and transverse views. mhb, midbrain-hindbrain boundary; n, notochord; nt, neural tube. Scale bars, 50μm.

Figure 6: Morphology of the developing lamprey somite

Transverse 6 μm resin sections stained lightly with toluidine blue. (a) Section through presomitic mesoderm at stage 24. (b) Section through central somites at stage 22. (c) Section through central somites at stage 22-23. (d) Section through more mature somites at stage 24. Note, however, that all these stages can be observed at stage 24 with the youngest somites seen at the posterior and the more mature somites at the anterior. lam, muscle lamellae; lat, lateral cells; n, notochord; nt, neural tube; som, somite. Arrows indicate cells abutting the notochord that may have distinct adaxial morphology. Scale bars, 50μm.

Figure 7: S58 and MF20 immunohistochemistry

Lateral views; anterior to left, dorsal to top. (a, d) Immunohistochemistry detected using DAB staining. The S58 antibody marks the head and heart muscles (a) and the MF20 antibody marks all skeletal muscles (d). (b, c) Immunohistochemistry detected using Alexa 568; higher power views of the regions boxed in panel (a). The S58 antibody marks the head muscles (b) and the heart (c). h, heart; hd, head muscle. Scale bars, 500μm.

Supplementary data

Analysis of *fst1* and *fst2* expression in *smu* zebrafish embryos

Methods: In situ hybridisation was carried out and hand cut sections produced as described (Hammond et al., 2003) using DIG-labelled *fst1* and *fst2* riboprobes (Bauer et al., 1998; Dal-Pra et al., 2006). Embryos were cultured as previously described (Westerfield, 1995). At 20S *smu* homozygotes were separated from siblings on the basis of morphology. At 10S this was not possible; at this stage eight embryos were chosen at random for sectioning, of which six showed the wild-type *fst1* expression pattern (as previously described (Bauer et al., 1998)) and two showed the mutant pattern (Supplementary Fig. 1).

Results: At the 10S stage *fst1* is expressed strongly throughout the first five somites, excluding the adaxial region. In *smu* homozygotes, in which Hh signalling is absent, expression encroaches into the adaxial region (Supplementary Fig. 1). Later, and in more posterior somites, wild-type *fst1* expression becomes more complex, splitting into two separate domains (Bauer et al., 1998). This is similar to *fst* expression in more mature lamprey somites (Fig. 4b). *fst2*, which is expressed later than *fst1*, is specifically excluded from the adaxial cells and the region that will give rise to the horizontal myoseptum, formation of which is dependent on adaxial cell derivatives, the muscle pioneer cells (Bauer et al., 1998; Dal-Pra et al., 2006). In *smu* homozygotes *fst2* expression encroaches into both of these regions.

Supplementary Figure 1: Expression of zebrafish *fst1* and *fst2* in somites of wild-type and *smu* embryos

Transverse hand cut sections approximately 50μm thick. (a, b) 10S *smu* homozygous mutant and sibling embryos. Sections taken through the anterior five somites. (c, d) 20S *smu* homozygous mutant and sibling embryos. Sections taken through the central somites. Note that in wild-type embryos *fst* expression is excluded from adaxial cells (*fst1* and *fst2*) and from the region of the somite that will give rise to the horizontal myoseptum (*fst2*), while in *smu* homozygotes *fst* expression expands into these regions. ad, adaxial cells; hms, horizontal myoseptum region; n, notochord (in d: position of notochord, which was lost from this section). Scale bars, 50μm.

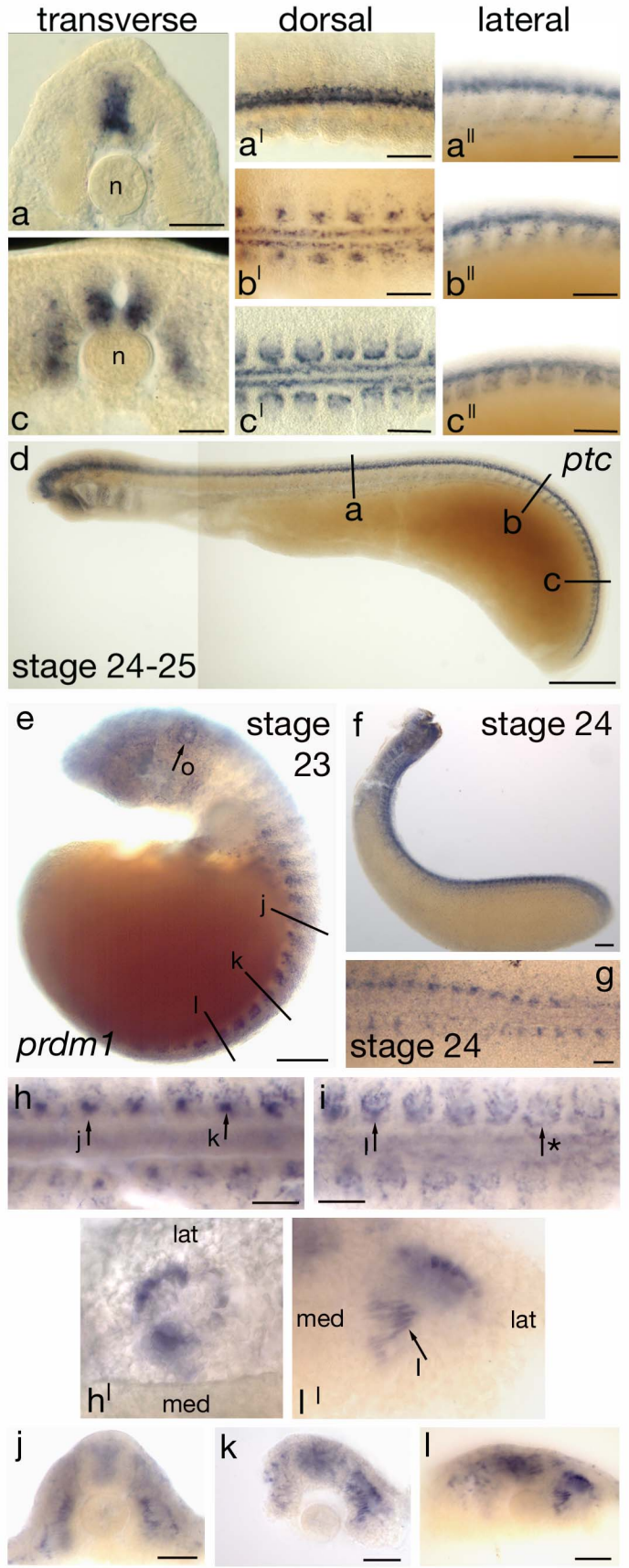
Supplementary Figure 2: ClustalW alignments of *L. fluviatilis* Prdm1 and *P. marinus* Engrailed protein sequences with those of other species

Identical residues are boxed in black, similar residues are grey and non-conserved residues are white. (A) The *L. fluviatilis* Prdm1 SET domain fragment has 69.5% 67.8%, 67.8%, 67.8%, 66.1%, 45% and 39% identity with zebrafish Prdm1b, zebrafish Prdm1a, chicken Prdm1, mouse Prdm1, *Xenopus* Prdm1, *Drosophila* Prdm1 and zebrafish Prdm1c sequences, respectively. The *L. fluviatilis* Prdm1 zinc finger fragment has 93.1%, 91.4%, 91.4%, 91.4%, 81% and 72.4% identity with *Xenopus* Prdm1, chicken Prdm1, mouse Prdm1, zebrafish Prdm1a, *Drosophila* Prdm1 and zebrafish Prdm1c, respectively. Note that the published sequence for zebrafish Prdm1b does not include this domain. (B) The *P. marinus* Eng fragment has 66.3% identity with chick Eng1, mouse Eng2 and zebrafish Eng2b, 67.4% identity with mouse Eng1 and zebrafish Eng1b, and 58.2%, 60.9%, 65.2%, 66.7%, 68.5% and 69.6% identity with *Ciona* Eng, *Drosophila* Eng, chick Eng2, zebrafish Eng1a, zebrafish Eng2a and amphioxus Eng, respectively.

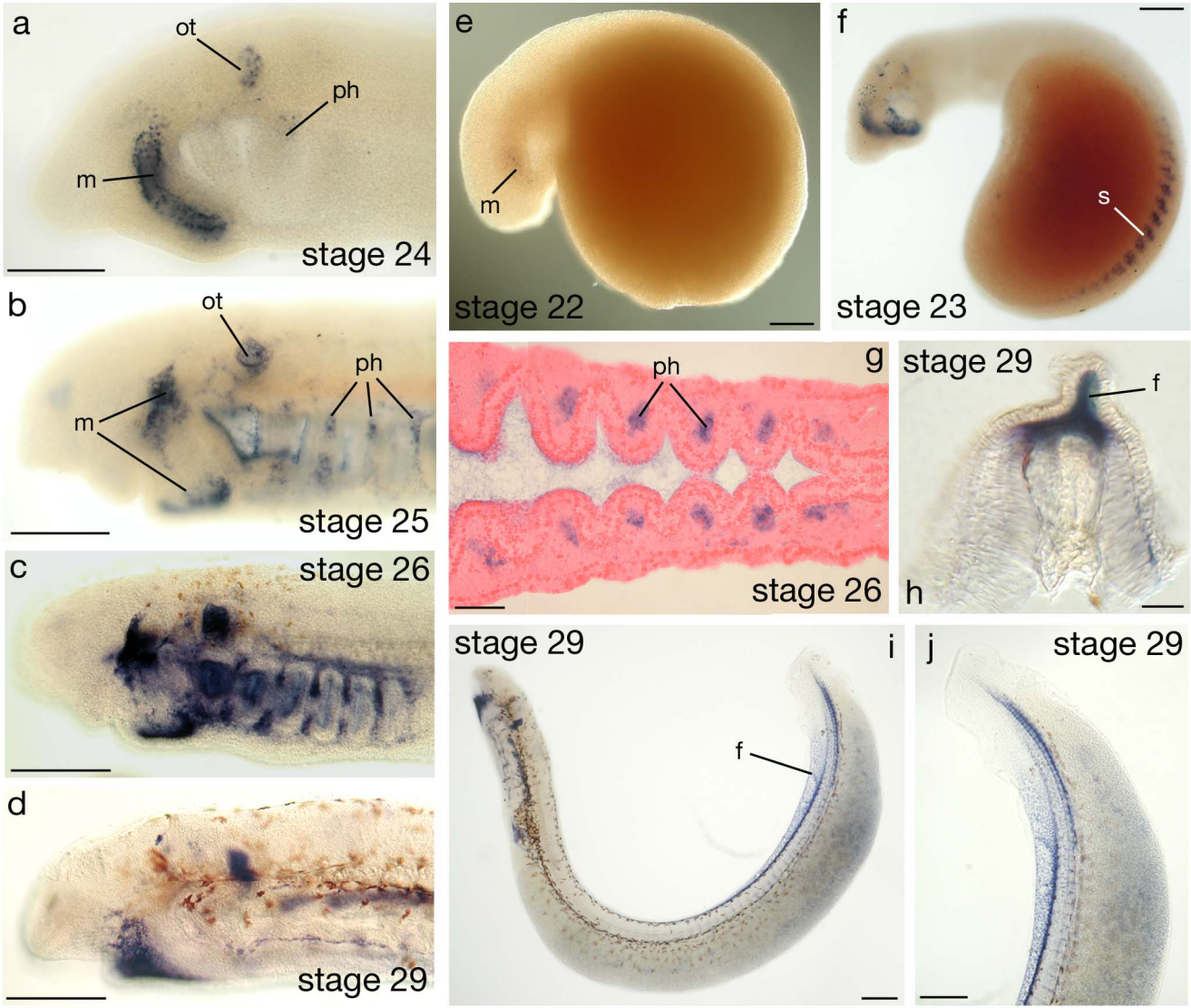
Supplementary Figure 3: Neighbour-joining phylogenetic trees for Patched (Ptc), Prdm1, Follistatin (Fst) and Engrailed (Eng) protein sequences

Neighbour joining trees were constructed using the regions of each protein that are homologous to our lamprey fragments. Scale bars indicate relative evolutionary distance. Percentage bootstrap values (based on 100 replicates) are indicated by figures adjacent to nodes. (A) Lamprey (*P. marinus*) Ptc clusters with, but basal to the gnathostome Ptc2 clade. Although we have not identified a second lamprey Ptc, we cannot exclude the possibility that a second lamprey Ptc clustering with the Ptc1 clade may exist. Note that zebrafish *ptc* genes were named in the order in which they were isolated rather than their similarity to other *ptcs*. (B) The lamprey Prdm1 SET domain clusters basal to the gnathostome Prdm1 SET domains apart from zebrafish Prdm1c, which is very different to all the other vertebrate Prdm1 proteins analysed. We did not construct a tree for the zinc finger domain of Prdm1, since this domain is so highly conserved between species. Note that zebrafish Prdm1a and Prdm1b may have resulted from a species-specific duplication (Sun et al., 2008). (C) Lamprey (*P. marinus*) Fst falls basal to the gnathostome Fsts. (D) Lamprey (*P. marinus*) Eng

clusters with amphioxus Eng and *Ciona* Eng, outside the gnathostome Eng1 and Eng2 clusters.



Hammond et al., Figure 2



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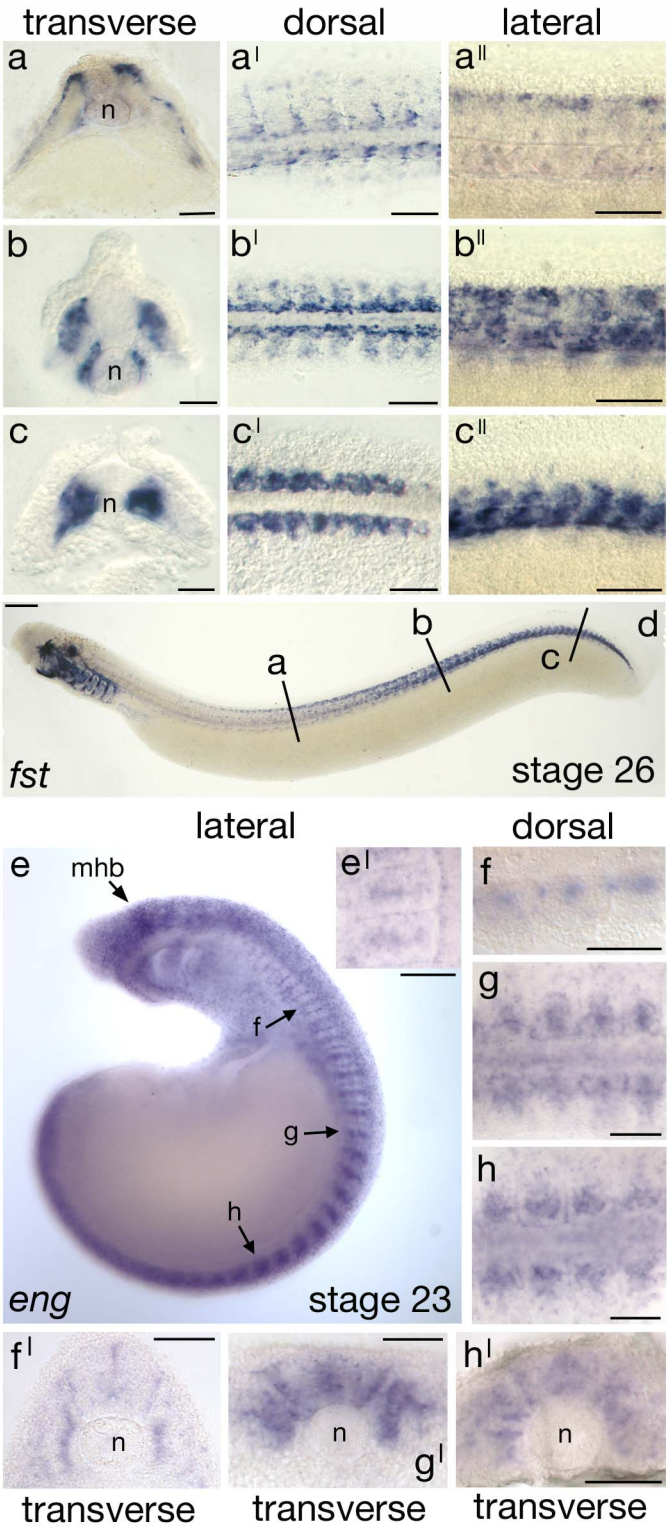

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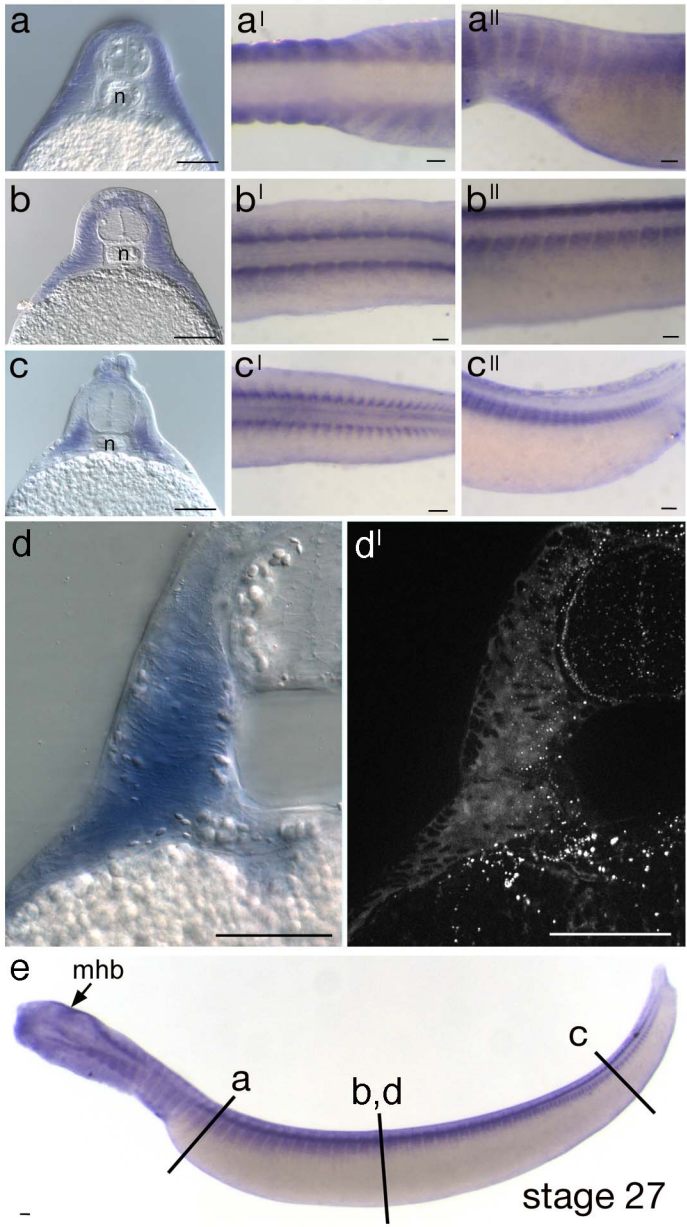
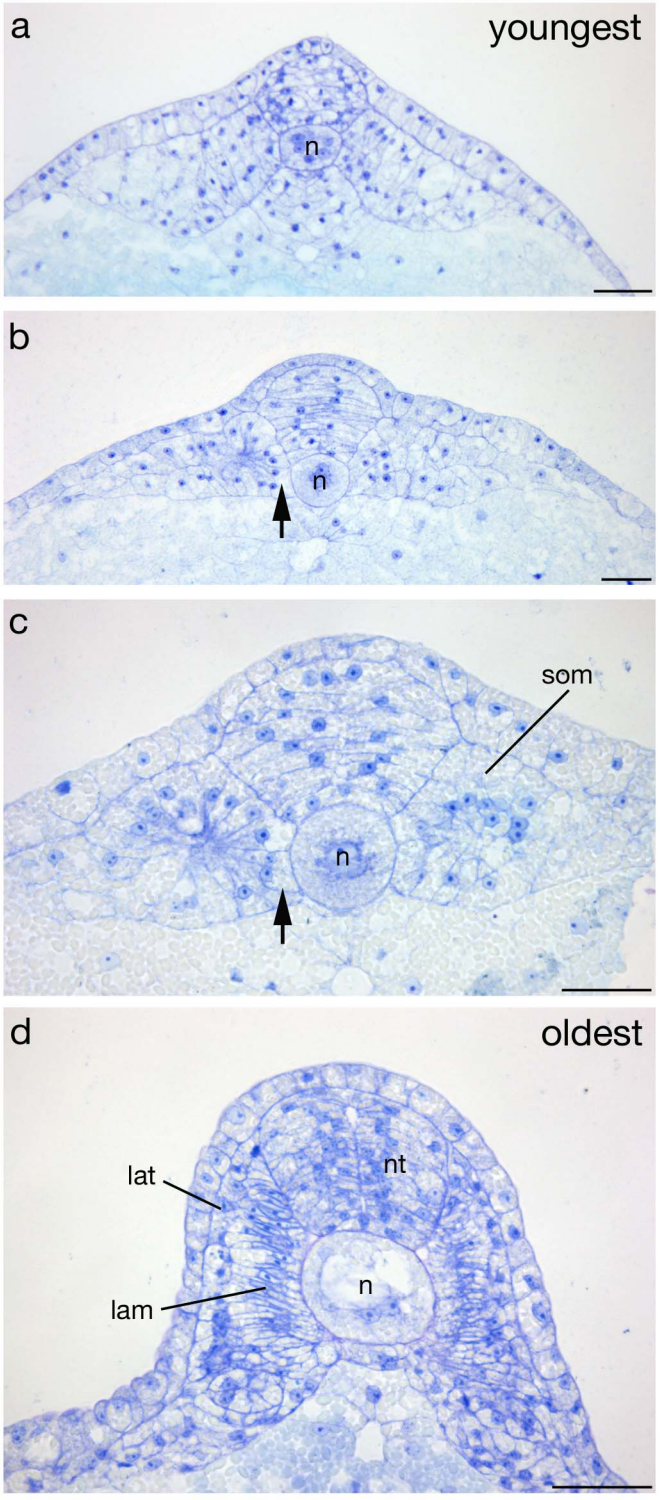
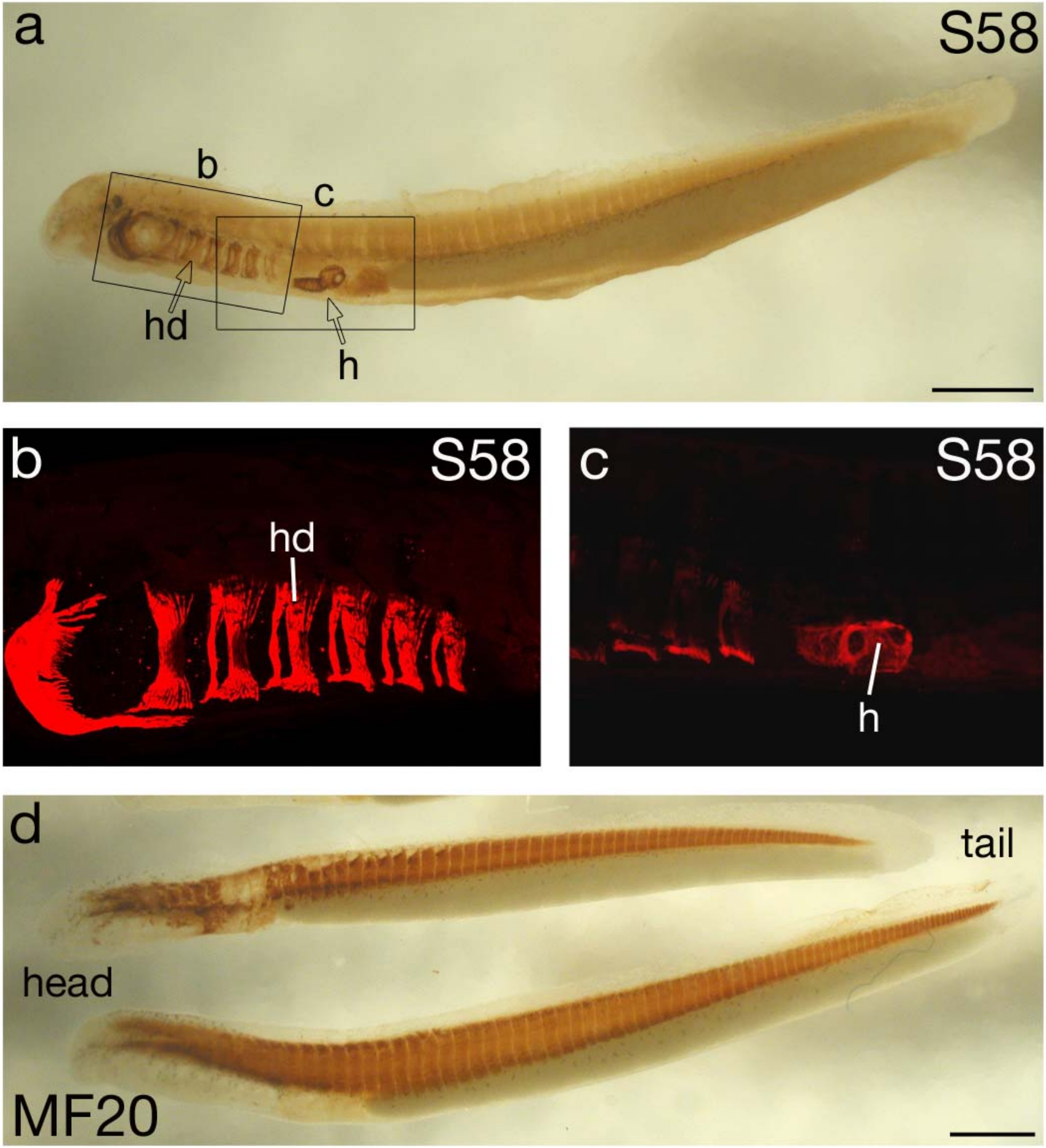


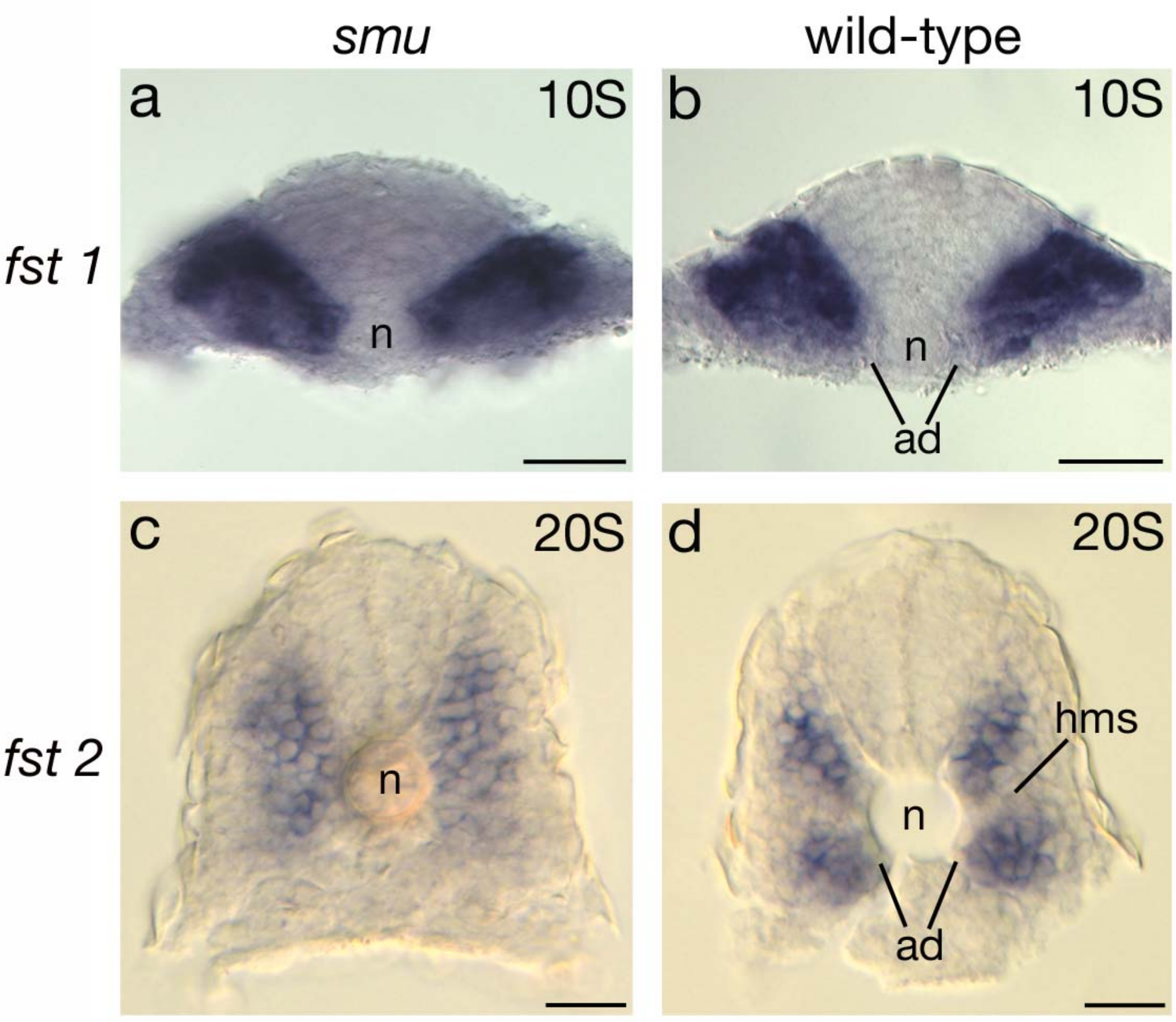
Hammond et al. Figure 5



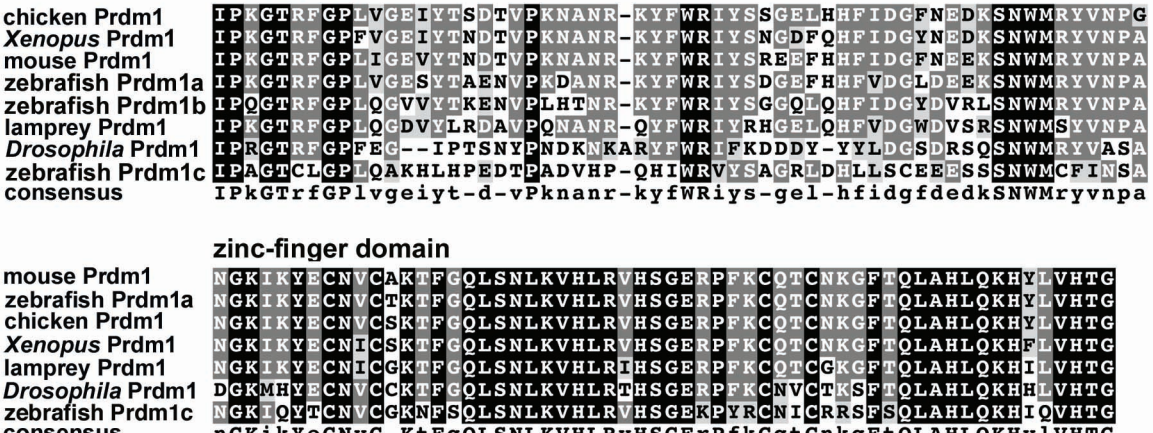
Hammond et al., Figure 6



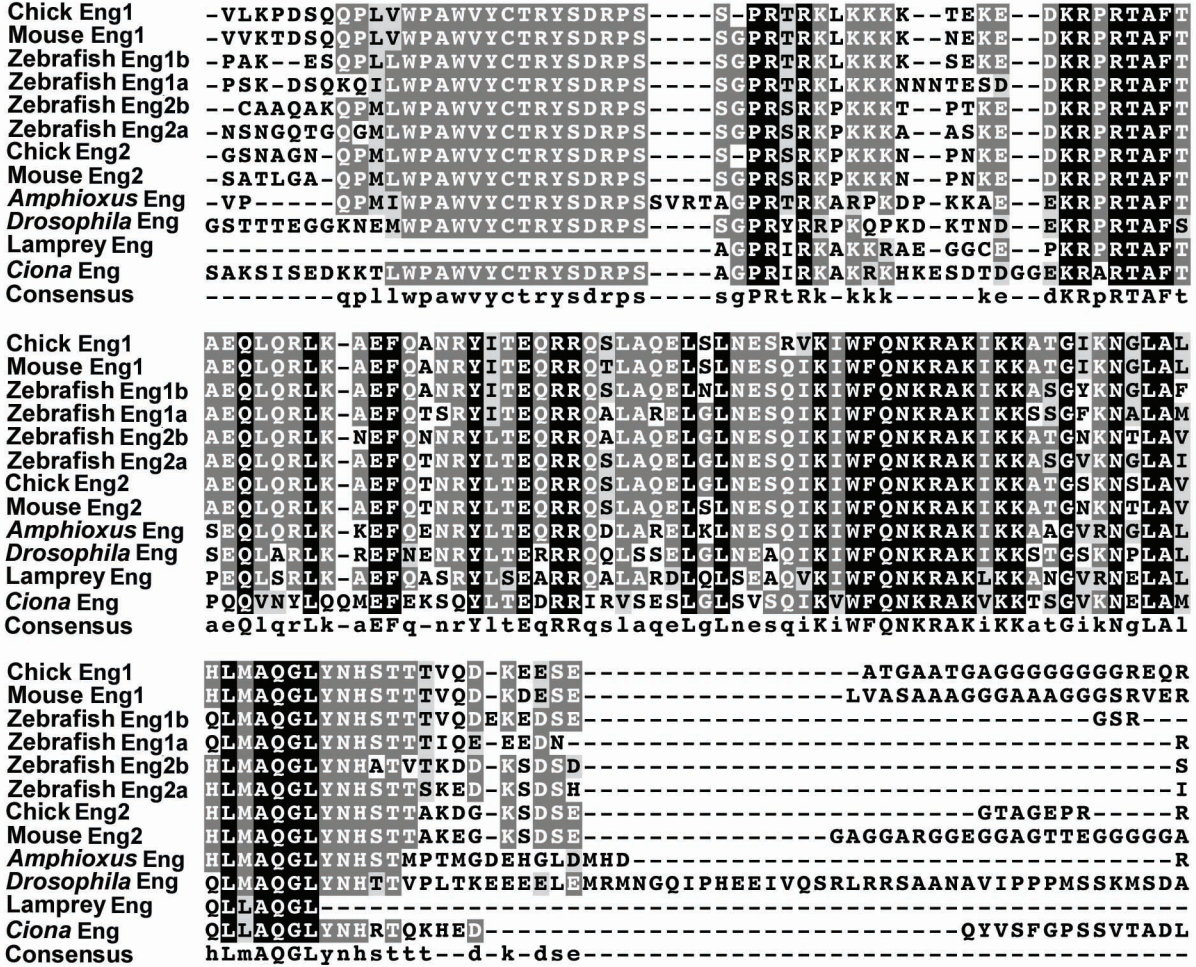
Hammond et al. Figure 7



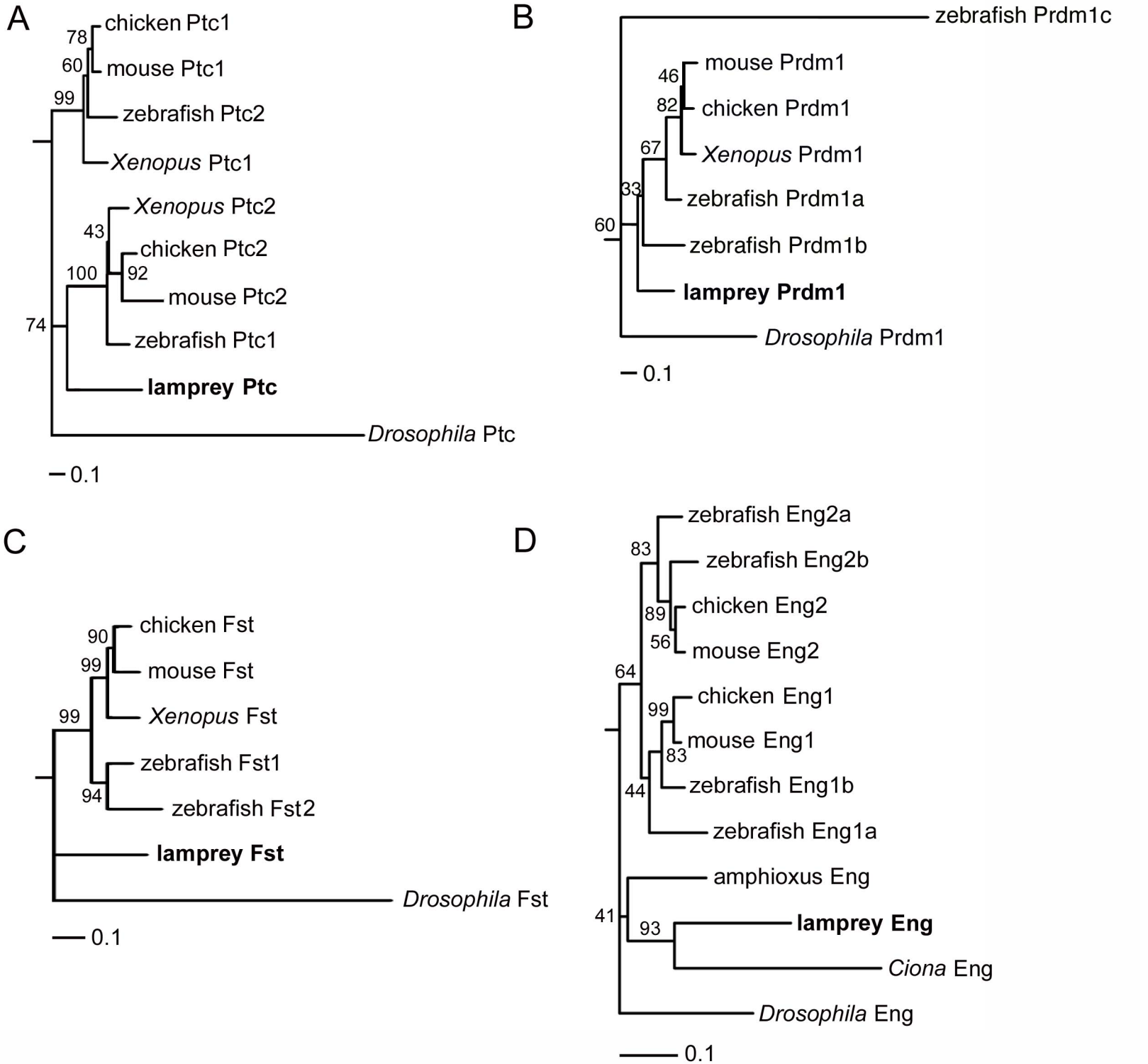
Hammond et al., Supplementary FIgure 1



Hammond et al., Supplementary Figure 2a



Hammond et al., Supplementary Figure 2b



Hammond et al., Supplementary Figure 3