Laminins, via heparan sulfate proteoglycans, participate in zebrafish myotome morphogenesis by modulating the pattern of Bmp responsiveness

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There was an error published in *Development* **138**, 97-106.

The sequence of the *lamc1* morpholino was incorrect (a T was omitted). The corrected sentence appears below.

The MO (Gene Tools) used against *lamc1* was 5′-TGTGTCCTTTTGCTATTGCGACCTC-3′ (Parsons et al., 2002).

The authors apologise to readers for this mistake.

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SUMMARY

In zebrafish, Hedgehog-induced Engrailed expression defines a muscle fibre population that includes both slow and fast fibre types and exhibits an organisational role on myotome and surrounding tissues, such as motoneurons and lateral line. This Engrailed-positive population is restricted in the myotome to a central domain. To understand how this population is established, we have analysed the phenotype of the *sly/lamc1* mutation in the Laminin γ1 chain that was shown to specifically affect Engrailed expression in pioneers. We find that the *sly* mutation affects Engrailed expression in the entire central domain and that Hedgehog signalling does not mediate this effect. We show that Bmp-responding cells are excluded from the central domain and that this pattern is modulated by laminins, but not by Hedgehog signalling. Knockdown of Bmp signalling rescues Engrailed expression in the *sly* mutant and ectopically activates Engrailed expression in slow and fast lineages in wild-type embryos. Last, extracellular matrix-associated heparan sulfate proteoglycans are absent in *sly* and their enzymatic removal mimics the *sly* phenotype. Our results therefore show that laminins, via heparan sulfate proteoglycans, are instrumental in patterning Bmp responsiveness and that Bmp signalling restricts Engrailed expression to the central domain. This study underlines the importance of extracellular cues for the precise spatial modulation of cell response to morphogens.

INTRODUCTION

Production of muscle fibre populations with distinct characteristics is essential for the morphogenesis of a functional myotome. In zebrafish, Engrailed expression defines a muscle fibre population that is restricted in the myotome to a central domain. This Engrailed-positive population encompasses both slow and fast fibre types and exhibits an organisational role on myotome and surrounding tissues such as motoneurons and lateral line. The aim of this study is to understand how this Engrailed-positive central domain is established.

A zebrafish primary myotome is composed of two major skeletal muscle fibre types: the superficial slow muscle fibres and deep fast muscle fibres (for a review, see Ochi and Westerfield, 2007). Engrailed transcription factor is expressed in a subset of both fibre types. Engrailed-positive pioneer slow muscle fibres and medial fast fibres (MFFs) are grouped together in a central domain of the myotome (Hatta et al., 1991; Wolff et al., 2003). Engrailed expression thus defines a muscle fibre population that encompasses two muscle fibre types. Engrailed-expressing pioneers exhibit an unusual shape, stretching from the somite lateral surface to the notochord. Low Engrailed-positive medial fast fibres surround the pioneers. Whereas fast and slow muscle fibres constitute the operational musculature of the swimming larva, pioneers are a particular cell population in that their position and morphology prefigure the horizontal myoseptum (Felsenfeld et al., 1991). The pioneer presence is associated with the formation of the horizontal myoseptum that gives zebrafish somites their characteristic chevron shape (van Eeden et al., 1996) and constitutes a myotendinous junction, functionally analogous to the mammalian tendon (Bassett and Currie, 2003).

Hedgehog (Hh) signalling, originating from the notochord, has been shown to commit slow muscle precursors to their slow and pioneer fates and to differentially activate *engrailed* expression in these precursors (Hirsinger et al., 2004; Wolff et al., 2003). Specification of pioneer precursors requires a higher concentration and longer exposure to Hedgehog signalling compared with slow precursors (reviewed in Ochi and Westerfield, 2007). It is unclear how this is achieved considering that both cell types derive from the same pool of precursors, the adaxial cells, which are expected to be all exposed to similar Hedgehog signalling. Indeed, adaxial cells are organised in a transient epithelial monolayer abutting the notochord; it is when they are anchored in the peri-notochordal basement membrane that they commit to their fates (Hirsinger et al., 2004). As the somite matures, they reorganise to stack up on one another while elongating on the antero-posterior axis and then slow muscle fibres migrate across the field of fast muscle precursors to reach their final superficial location (Devoto et al., 1996).

Low-Engrailed-positive medial fast fibres are specified in an Hh-dependent manner. They appear in the vicinity of the pioneers, at a later stage when the slow muscle fibres have migrated laterally (Wolff et al., 2003). The mechanisms that delay *engrailed* activation and limit it to a subset of fast muscle precursors are not understood.

The *sly/lamc1* mutation disrupts the gene for Laminin γ1 chain. Engrailed expression is specifically affected in pioneers at 24 hours post-fertilisation (hpf); basement membranes are disorganised and non-functional and somites are mis-shaped (Parsons et al., 2002; Stemple et al., 1996; Wiellette et al., 2004). Laminins are

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extracellular matrix $\alpha_x\beta_x\gamma_x$ trimeric proteins, the deposition of which (in particular that of Laminin111) is the first and necessary step to assemble basement membrane (for a review, see Li et al., 2003). Laminin γl chain is part of 10 Laminins out of 18, including Laminin111. Laminins are found in close proximity to developing muscle cells, i.e. in the peri-notochordal basement membrane, around forming somites and then, around myotomes including vertical myosepta (Parsons et al., 2002; Snow and Henry, 2009).

To understand how the Engrailed-positive central domain is established, we have studied the *sly/lamc1* mutation. Our study shows that it is Bmp signalling that restricts Engrailed expression to the central domain and that this role requires laminins, via heparan sulfate proteoglycans, to adequately pattern the Bmp responsiveness in the myotome.

MATERIALS AND METHODS

Fish strains

Wild-type AB and *sleepy/lamc1^wi390^* (Wiellette et al., 2004) embryos were obtained from zebrafish (*Danio rerio*) lines maintained according to standard procedures (Westerfield, 2000). Embryos were staged by hours post-fertilisation (hpf) and by standard staging criteria (Kimmel et al., 1995). *sly* embryos are morphologically identifiable from the 18 s (somite) stage. For earlier stages and for morpholino-injected embryos, the ratio of embryos with a given phenotype was compared with the expected mutant ratio of 1/4.

lamc1 morpholino injections

To target the maternal/residual contribution of *lamc1*, a translation-blocking morpholino (MO) (Nasevicius and Ekker, 2000) was injected into embryos from an incross between *sly* heterozygote parents. One in four of the embryos are therefore depleted of *lamc1*. The MO (Gene Tools) used against *lamc1* was 5′-TGTGCCTTTTGCTATTGCGACCTC-3′ (Parsons et al., 2002). A volume of 1.5 nl of a 300 μM MO solution was injected into the cell of one-cell stage embryos.

In situ hybridisation and probes

Whole-mount in situ hybridisation reactions were carried out as previously described (Thisse et al., 1993). Riboprobes used detected *eng1a*, *eng2a* (Ekker et al., 1992), *eng1b* (Amores et al., 1998) and *ptc1* (Concordet et al., 1996). Images were acquired using a Leica MZFLIII combined to a Zeiss AxioCam digital camera.

Cyclopamine treatment

Cyclopamine treatment followed a standard method of immersion of chorionated embryos in a 100 μM cyclopamine solution (Toronto Research Chemicals, catalog number C988400, dissolved in Embryo Medium and 0.5% ethanol) from the sphere stage (Hirsinger et al., 2004) until fixation at the 18-20 s stage.

Dorsomorphin treatment

To avoid biological side effects of DMSO, dorsomorphin (SIGMA, catalog number P5499) (Yu et al., 2008) was protonated with an excess of HCl, lyophilised and then re-dissolved in fish water. Dechorionated embryos were immersed in a 30 μM dorsomorphin solution in fish water from the 6 s stage until fixation at 26 hpf. As expected, a subset of dorsomorphin-treated embryos exhibited ectopic tails, which result from Bmp downregulation (wild type, *n*=26/38; *sly*, *n*=16/25) (Pyati et al., 2005; Yu et al., 2008).

Heparinase III and Chondroitinase ABC treatments

Heparinase III (Sigma, catalog number H8891) was dissolved at 0.4-0.5 IU/ml in 20 mM Tris-HCl (pH 7.85), 0.5 mg/ml bovine serum albumin and 4 mM $CaCl_2$ (activator). Chondroitinase ABC (Sigma, catalog number C3667) was dissolved at 5 IU/ml in 50 mM Tris-HCl (pH 7.85), 0.25 mg/ml bovine serum albumin and 60 mM sodium acetate (activator). To monitor the injection, the solutions contained 0.5% of Phenol Red.

Embryos at the 6 or 10 s stage were dechorionated and mounted on their lateral side in a drop of low-melting agarose (1% in Embryo Medium) covered by Embryo Medium on a depression slide. Under a Leica S8Apo stereomicroscope, an approximate total volume of 30-50 nl was injected at three locations (in the anterior and mid-presomitic mesoderm and in the tail bud) using a Marzhauser MM-33 micromanipulator and a MPPI II (Applied Scientific Instrumentation) pressure injector. The embryos were unembedded, incubated in Embryo Medium and analysed after varying intervals (5-12 hours post-injection).

Antibodies and immunostaining

Antibodies used were: monoclonal anti-Myosin F59 (1:5 on sections; DSHB), polyclonal anti-Laminin (1:400 on sections and 1:200 in toto; Sigma, catalog number L-9393), polyclonal anti-phospho-Smad1/5/8 (1:100 on sections and 1:50 in toto; Cell Signaling Technology, catalog number 9511), monoclonal anti-Engrailed 4D9 (1:50 on sections and 1:25 in toto; Santa Cruz Biotechnology, clone sc-53019), polyclonal anti-Prox1 (1:100 in toto; RELIATech GmbH, catalog number 102-PA32AG), monoclonal anti-heparan sulfate (1:200 on sections; US Biological, 10E4 epitope, catalog number H1890), monoclonal anti-chondroitin sulfate (1:200 on sections; Sigma, clone CS-56, catalog number C8035). The monoclonal antibody F59 developed by F. E. Stockdale was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. The Laminin antibody recognises at least Laminin111 in zebrafish (Parsons et al., 2002). The anti-heparan sulfate antibody recognises the glycosaminoglycan moiety of the heparan sulfate proteoglycans (David et al., 1992); similarly, the anti-chondroitin sulfate antibody is immunospecific for an octasaccharide motif present in chondroitin sulfate-containing proteoglycans (Ito et al., 2005). Secondary antibodies were Alexa-Fluor 488-, 546- and 568-conjugated goat anti-mouse and goat anti-rabbit antibodies (1:200; Invitrogen).

Immunostaining on cryosections (16 μm) was performed as previously described (Devoto et al., 1996). For whole-mount immunostaining with the Laminin antibody, embryos were fixed in 4% formaldehyde overnight at 4°C and then stored in methanol, followed by several washes in PBS-Tween 0.1%, digestion in Proteinase K (10 μg/ml) for 1 minute at tail-bud stage and post-fixation in 4% formaldehyde for 20 minutes at room temperature.

For other antibody staining, embryos were fixed in 4% formaldehyde for 4 hours at 4°C followed by cold acetone treatment for 10 minutes and then blocking for 30 minutes in PBDTx (1% BSA, 1% DMSO, 0,5% Triton X-100 in PBS) – 2% goat serum. Staining was conducted in PBDTx.

For both types of staining, primary antibody was incubated overnight at 4°C and secondary antibody for 5 hours at room temperature.

Sections (mounted in DAPI-containing Vectashield, Vector, catalog number H-1200) and embryos were observed and images were acquired using a Nikon Digital Sight DS-5mc digital camera mounted on a Nikon Eclipse 90i microscope (20× and water-immersion 40× objectives).

For the lateral view on Fig. 3 and detail images on Fig. 5, Fig. 6 and Fig. S1 in the supplementary material, embryos were mounted in Vectashield (Vector, catalog number H1000) and images acquired with 40× oil-immersion objective under a Leica SP5 confocal microscope.

Quantification and statistical analysis of numbers of pioneer and MFF nuclei

To buffer the natural variability in Engrailed+ nuclei number from one somite to another, nuclei of Prox1–/Engrailed+ MFFs and Prox1+/Engrailed+ pioneers were counted over *x* somites in *n* embryos at two axial levels, trunk (somites 9-11) and tail (somites 18-22), under a Nikon Eclipse 90i microscope. The statistical significance of the difference of nuclei number distributions between two conditions were assessed using the two-tailed Mann-Whitney *U*-test (GraphPad Prism5 software).

RESULTS

Engrailed expression is absent from the entire central domain in the *sly* mutant

Pioneer and MFF precursors develop in close proximity to Laminin-rich extracellular matrices (Fig. 1A,C). In the *sly* mutant (Parsons et al., 2002; Stemple et al., 1996; Wiellette et al., 2004), Laminin
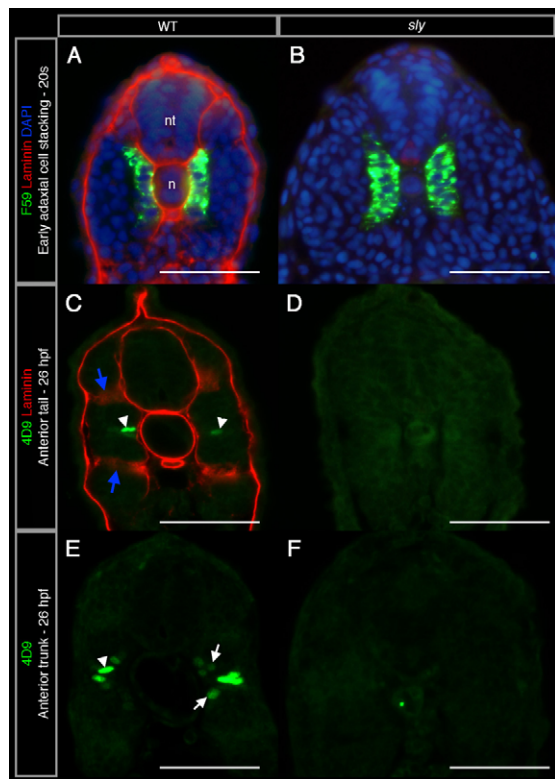
Fig. 1. Pioneer and MFF precursors develop in close proximity to Laminin-rich extracellular matrices and Engrailed expression is absent from pioneers and MFFs in *sly*. (**A**,**B**) Transverse sections of 20 s stage wild-type (A) and *sly* embryos (B) at the level of early adaxial cell stacking. Dorsal is upwards. F59 (in green) labels adaxial cells and Laminin is in red; nuclei are counterstained with DAPI (in blue). (**C-F**) Transverse sections of 26 hpf wild-type (C,E) and *sly* (D,F) embryos at the level of the anterior tail (C,D) and the anterior trunk (E,F). (C,D) Laminin is in red. Owing to the somite chevron shape, vertical myosepta of the neighbour somite (blue arrows) are visible on these transverse sections. (C-F) 4D9 (in green) labels pioneers (arrowheads, C,E) and MFFs (arrows, E) in wild-type embryos and is absent in *sly* embryos (D,F). n, notochord; nt, neural tube. Scale bar: 50 μm.
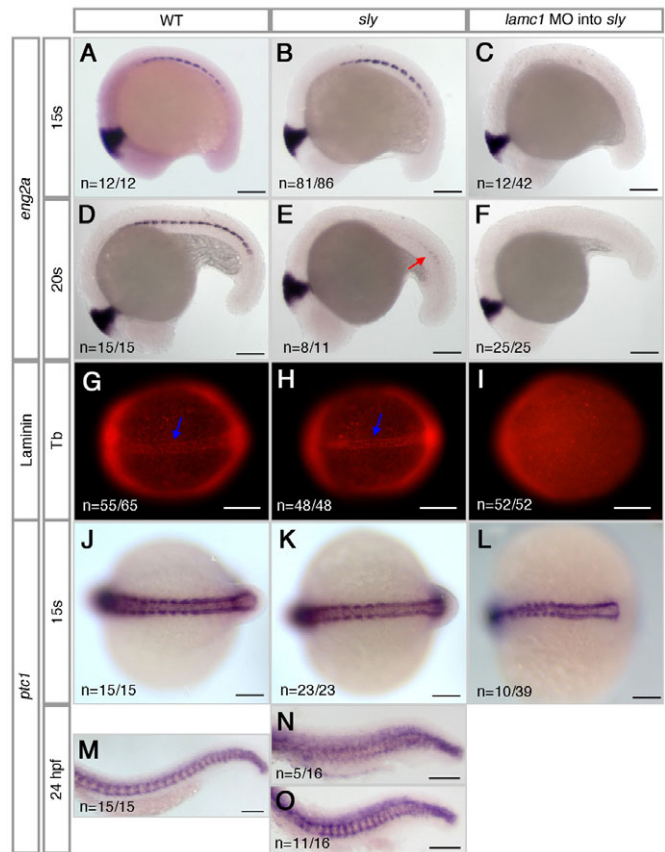

Fig. 2. *eng2a* expression in pioneers requires maternal and/or residual *lamc1*, while *ptc1* expression in the caudal part of the embryo is not affected in *lamc1*-depleted embryos. (**A-F**) Lateral views of *eng2a* expression at the 15 (A-C) and 20 s stage (D-F) in wild-type (A,D), *sly* (B,E) and morpholino-injected *sly* embryos (C,F). The fraction of embryos displaying the corresponding phenotype is shown on each panel. In 20 s stage *sly* embryos, no (*n*=3/11) to weak and transient expression (*n*=8/11) is found in only two or three caudal somites (red arrow in E). (**G-I**) Dorsal views of Laminin labelling (in red, blue arrows) at the tail bud (Tb) stage in wild-type (G), *sly* (H) and *lamc1* MO-injected *sly* embryos (I). Dorsal (**J-L**) and lateral views (**M-O**) of *ptc1* expression at the 15 s stage (J-L) and 24 hpf (M-O) in wild-type (J,M), *sly* (K,N,O) and *lamc1* MO-injected *sly* embryos (L). Scale bar: 100 μm.

staining is absent from these sites and Engrailed expression is absent from pioneers (Fig. 1B,D). We therefore assessed the presence of Engrailed in the MFFs, the other component of the central domain.

In 26 hpf wild-type embryos in the anterior somites (*n*=26/26), Engrailed is expressed at a low level in MFFs (arrows) and at a higher level in pioneers (arrowheads) (Fig. 1E and Fig. 5A-D) (this study) (Wolff et al., 2003). In 26 hpf *sly* embryos (*n*=55/55), Engrailed expression at wild-type level is absent (Fig. 1F and Fig. 5E-H). In a subset of the embryos (*n*=28/55), weak Engrailed expression is detected in two to six cells, often in the trunk and at the trunk-tail transition (data not shown). This probably corresponds to a declining level of an earlier expression of three *engrailed* genes observed in *sly* embryos up to the 15 s stage (Fig. 2A,B,D,E for *eng2a*; data not shown for *eng1a* and *eng1b*). By *lamc1* knockdown in *sly* embryos, we show that this early expression (Fig. 2C,F for *eng2a*; data not shown for *eng1b*) is due to maternal and/or residual *lamc1* expression (Fig. 2G-I) that disappears by the 5 s stage in the *sly* mutant.

The *sly* mutation therefore specifically affects Engrailed expression in both the slow and fast muscle fibre types, i.e. in the entire central domain.

Hedgehog signalling is operational in *sly* embryos

Hh signalling has been shown to regulate *engrailed* expression in both slow and fast muscle fibre types (for a review, see Ochi and Westerfield, 2007). Its spatio-temporal window of action on pioneers has been delimited to the anterior presomitic mesoderm and last formed somites (Hirsinger et al., 2004), while MFFs require Hh signalling only from the 18 s stage (Wolff et al., 2003). Ptc1 (Ptch2 – Zebrafish Information Network) is both a receptor of Hh ligands and a target of the Hh pathway (Concordet et al., 1996) and as such was used to assess Hh signalling in wild-type and *sly* embryos up to 24 hpf. *ptc1* is first expressed in adaxial cells and then in MFFs as slow muscle fibres migrate laterally (Concordet et al., 1996; Wolff et al., 2003).
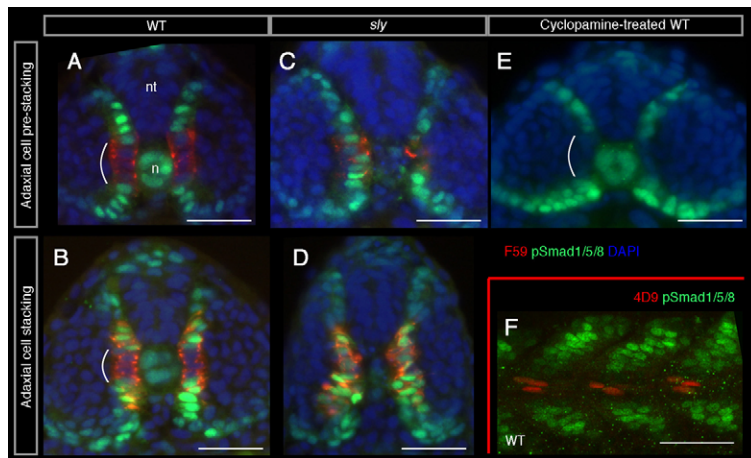
Fig. 3. pSmad expression is precisely regulated in pioneers and their precursors in wild-type, *sly* and cyclopamine-treated embryos. (A-E) Transverse sections of 18-20 s stage wild-type (A,B), *sly* (C,D) and cyclopamine-treated embryos (E) at the level of adaxial cell pre-stacking (A,C,E) and stacking (B,D). Dorsal is upwards. F59 (in red) labels adaxial cells and nuclear pSmad labelling is in green; nuclei are counterstained with DAPI (in blue). (F) Lateral view (projection of 11 confocal sections, 0,6 μm z-step) of a 18-20 s stage wild-type embryo, at the trunk level. Anterior is on the left, dorsal is upwards. 4D9 (in red) labels pioneers and nuclear pSmad labelling is in green. n, notochord; nt, neural tube. Scale bar: 50 μm.

ptc1 expression in *sly* is similar to wild-type from the 5 s stage to 24 hpf, in particular at the 15 s (*n*=23/23 embryos of a *sly* clutch), 20 s (*n*=8/8 *sly* embryos) stages and 24 hpf (*n*=16/16 *sly* embryos), even though in some 24 hpf *sly* embryos, the staining is more diffuse (*n*=5/16) (Fig. 2J,K,M-O).

In anti-*lamc1* morpholino-injected *sly* embryos (*n*=10/39 embryos of a *sly* clutch), despite a slight reduction in the most anterior somites, *ptc1* is normally expressed in the rest of the somites and presomitic mesoderm at the 15 s stage (Fig. 2L).

Based on *ptc1* expression, Hh signalling is not affected in *sly* embryos.

Pioneers, MFFs and their precursors are unresponsive to Bmp, in a Hedgehog-independent manner

A candidate to mediate the *sly* phenotype is Bmp. BMP sources are located dorsally and ventrally to the myotome; receptors and intracellular transducers of the Bmp pathway are dynamically expressed in the somite and, in particular, in adaxial cells and sclerotome (Dick et al., 1999; Mintzer et al., 2001; Muller et al., 1999; Nikaido et al., 1999; Patterson et al., 2010; Rissi et al., 1995; Schmid et al., 2000) (http://zfin.org, expression pattern database).

To learn whether Bmp signals to muscle precursors, wild-type embryos were immunolabelled for phosphorylated Smad1/5/8 (pSmad), the active form of the Bmp intracellular transducer (for a review, see Massague, 1998).

pSmad expression was assessed at the 18-20 s stage with respect to Engrailed expression in pioneers. At the adaxial cell pre-stacking level, where slow muscle precursors are committing to their fates, none of the adaxial cells expresses pSmad (*n*=22/22) (Fig. 3A, bracket). pSmad is, however, present in a ventral population, probably the sclerotome, and in a medial dorsal population, probably future fast muscle fibres (data not shown). Just anteriorly, where adaxial cells undergo stacking, adaxial cells that translocated dorsally and ventrally show pSmad labelling while adaxial cells that stayed centrally are still pSmad-negative (*n*=22/22) (Fig. 3B, bracket). These central cells, by their position and number (two to five cells per somite), are probable pioneer precursors. The pSmad-positive dorsal and ventral adaxial cells become slow muscle fibres and migrate laterally as they downregulate pSmad expression (data not shown). At a further anterior level, where pioneers are fully differentiated and express Engrailed, pSmad labelling is mutually exclusive from Engrailed labelling (*n*=16/16) (Fig. 3F).

Remarkably, the interruption in pSmad labelling matches the region where high levels of notochord-derived Hh signalling act on adaxial cells. In view of the antagonistic interactions between Hh and Bmp pathways (for reviews, see Guo and Wang, 2009; Hirsinger et al., 2000), we asked whether Hh signalling was responsible for this interruption. pSmad expression was thus assessed at the 18-20 s stage in embryos treated with cyclopamine from the blastula stage.

This treatment has previously been shown to result in a total loss of Hh signalling in the myotome (Hirsinger et al., 2004; Wolff et al., 2003) and we indeed confirmed the absence of Engrailed expression in cyclopamine-treated embryos at 26 hpf (*n*=17/17; data not shown). However, the interruption in pSmad labelling is similar to wild-type (*n*=10/10) (Fig. 3E, bracket), despite the expected abnormal morphology of the somite. This aspect of the early pattern of Bmp-responding cells is therefore independent of the Hh signalling.

pSmad expression was then assessed with respect to Engrailed expression in MFFs at 26 hpf. At that stage, Engrailed expression appears in MFFs in the trunk. MFFs occupy the entire mediolateral extent of the central domain. Caudally, the pSmad-positive domain is restricted to a medial one cell-thick line interrupted at the level of the notochord (*n*=24/24) (Fig. 4A, horizontal lines). Before the onset of Engrailed expression in MFFs, while slow muscle fibres migrate, the pSmad-positive domain transiently expands laterally in the fast muscle population, but a pSmad-negative central domain is maintained (*n*=24/24) (Fig. 4B, horizontal lines). After completion of slow muscle fibre migration, Engrailed-positive MFFs (Fig. 4C, arrow) develop in this central domain that stays devoid of pSmad labelling (*n*=21/21) (Fig. 4C, horizontal lines). Notably, pSmad expression is restricted again to a medial one-cell thick line of fast muscle fibres.

These data show that Bmp signalling in the myotome is tightly regulated in space and time, independently of Hh. It is specifically absent from pioneers, MFFs and their precursors throughout their development.

Response to Bmp signalling is ectopically activated in pioneer and MFF precursors in *sly*

We next addressed the pattern of Bmp responsiveness in *sly* where Engrailed is not expressed. Unlike wild-type and cyclopamine-treated embryos, pSmad labelling is detected in the entire adaxial cell population at both levels of pre-stacking (*n*=5/9) and stacking (*n*=6/8) in 18-20 s stage *sly* embryos (Fig. 3C,D). As the strength
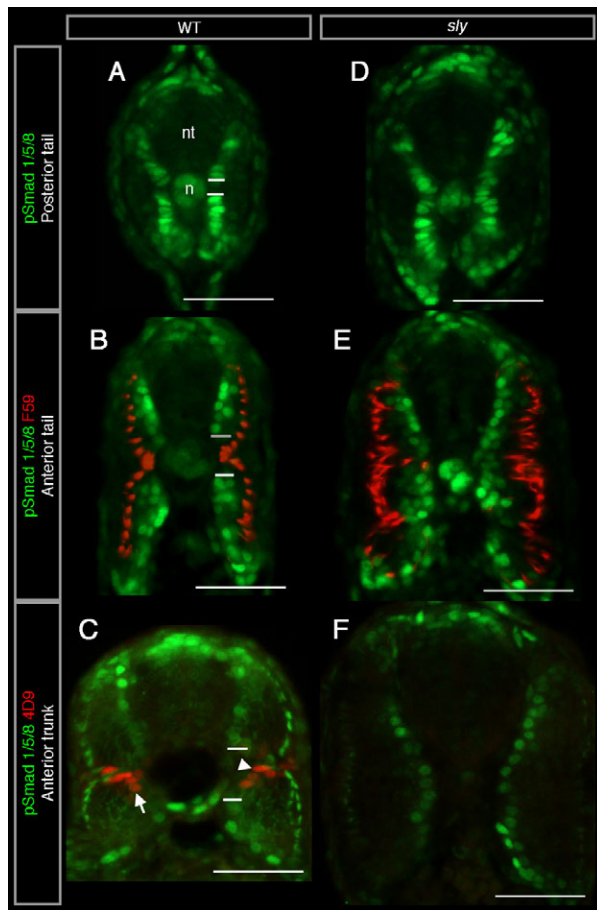
Fig. 4. pSmad expression in MFFs and their precursors is regulated in a similar way to that in pioneers. (**A-F**) Transverse sections of 26 hpf wild-type (A-C) and *sly* (D-F) embryos at the level of the posterior tail (A,D), the anterior tail (B,E) and the anterior trunk (C,F). Dorsal is upwards. Nuclear pSmad labelling is in green. Anti-pSmad antibody decorates myofibrils in differentiating slow and fast muscle fibres (C,F). (B,E) F59 (in red) labels migrating slow muscle fibres. (C,F) 4D9 (in red) labels pioneers (arrowhead) and MFFs (arrow). The horizontal white lines delimitate the pSmad-negative central domain. n, notochord; nt, neural tube. Scale bar: 50 μm.

of the *sly* phenotype is variable in terms of Engrailed expression levels, the embryos where pSmad expression is not significantly ectopically activated may correspond to mutant embryos with residual levels of Engrailed expression.

At a later stage, in 26 hpf *sly* embryos, pSmad expression is ectopically activated and maintained in the central domain from which MFFs emerge (n=21/21) (Fig. 4D,E,F). Notably, the lateral expansion and then restriction of pSmad expression in the fast muscle population occurs as in wild-type embryos (n=21/21) (Fig. 4E).

Response to Bmp signalling is therefore ectopically activated in *sly* pioneer and MFF precursors.

Knockdown of Bmp signalling in *sly* rescues Engrailed expression in pioneers and MFFs

We tested whether inhibition of Engrailed expression in *sly* is due to an ectopic response to the Bmp signalling. Treatment with dorsomorphin blocks Smads1/5/8 phosphorylation and therefore specifically knocks down Bmp signalling (Yu et al., 2008). *sly*

embryos were treated from the 6 s stage and Engrailed expression was assessed at 26 hpf when MFFs have developed in the trunk.

Compared with *sly* embryos (Fig. 5E-H) that do not show any Engrailed labelling, treated *sly* embryos (Fig. 5I-L) show full rescue of Engrailed expression in both pioneers (arrowheads) and MFFs along the entire axis (n=41/41). Numbers of pioneer and MFF nuclei were counted and statistically analysed (Table 1). Except for a lower number of MFF nuclei in dorsomorphin-treated *sly* somites (statistically significant at P=0.01), the numbers of trunk and tail pioneer nuclei in treated *sly* somites are similar to those in untreated wild-type somites. Furthermore, the intensities of the Engrailed labelling and the nuclei shape and cell body positions are similar between normal and rescued cells.

In addition, Engrailed expression is activated at the correct time in pioneers while slightly delayed in MFFs. Wild-type Engrailed expression starts in somites 23-25 for pioneers (n=4) and in somites 14-15 for MFFs (n=4). In treated *sly* embryos, pioneers start to express Engrailed in somites 23-24 (n=3) and MFFs in somites 12-14 (n=3).

Bmp signalling has been shown to delay myogenesis in zebrafish somites (Patterson et al., 2010). However, we find that Myosin heavy chain expression is synchronously activated within slow or fast muscle populations irrespective of pSmad normal or ectopic expression. Furthermore, Engrailed expression is not detected in older *sly* embryos (30 hpf) (data not shown). This shows that absence of Engrailed expression in *sly* is not linked to delayed myogenesis. It is likely that the indirect activation of Bmp signalling in *sly* (our study) is mild compared with its direct activation via strong overexpression of Bmp2b in the somites (Patterson et al., 2010). Fine-tuning of the levels of Bmp signalling may indeed be crucial to trigger myogenesis delay as wild-type pSmad-positive cells enter myogenesis on time (our study) (Patterson et al., 2010).

The *sly* phenotype is therefore imputable to ectopic activation of the response to Bmp signalling in pioneer and MFF precursors, leading to Engrailed repression in these cells.

Knockdown of Bmp signalling in wild-type increases the number of Engrailed-positive pioneers and MFFs

To test whether Bmp signalling also inhibits Engrailed expression in the wild-type context, a similar dorsomorphin treatment was conducted on wild-type embryos and analysed at 26 hpf (Fig. 5M-P).

Numbers of pioneer and MFF nuclei were counted and statistically analysed (Table 1). In the trunk, treated somites (Fig. 5M-P) show additional pioneers and MFFs compared with untreated somites (statistically significant at P=0.01). In the tail, however, the

Table 1. Quantification of pioneer and MFF nuclei in the dorsomorphin treatment experiments

	Untreated WT	Treated *sly*	Treated WT
Trunk pioneers	2-5	2-4	3-7*
	x=15; n=5	x=15; n=5	x=15; n=5
Trunk MFFs	9-15	2-12*	17-27*
	x=15; n=5	x=14; n=5	x=15; n=5
Tail pioneers	2-6	2-5	3-6
	x=24; n=5	x=13; n=3	x=24; n=5

Values represent the range of numbers of nuclei per somite.
x, the number of somites counted; n, the number of embryos counted.
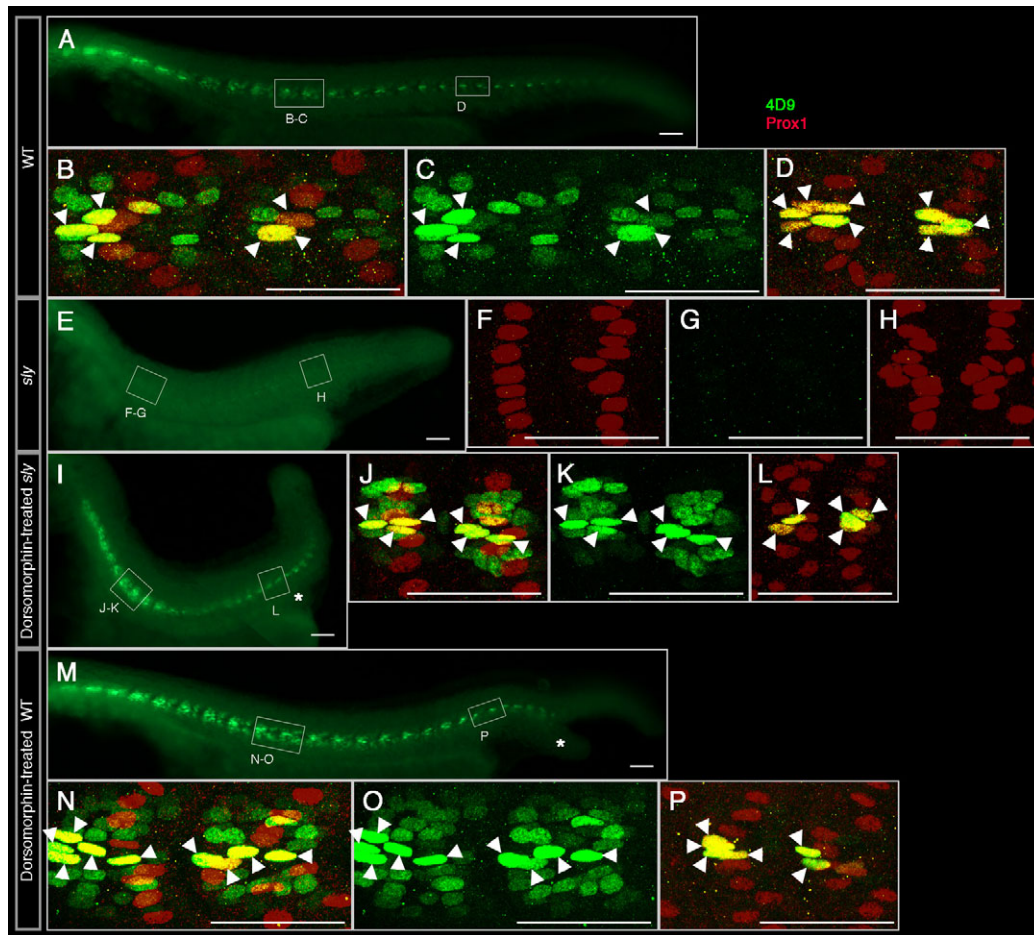Asterisks indicate values that are significantly different (P=0.01) from the untreated wild-type value.

Fig. 5. Knockdown of Bmp signal rescues Engrailed expression in *sly* pioneers and MFFs and ectopically activates Engrailed expression in wild-type slow and fast lineages. (A,E,I,M) Lateral views of wild-type (A), *sly* (E), dorsomorphin-treated *sly* (I) and dorsomorphin-treated wild-type embryos (M) at 26 hpf. Anterior is leftwards, dorsal is upwards. (**B-D,F-H,J-L,N-P**) Magnification views (projection of confocal sections) of the regions around somite 10 (B-C,F-G,J-K,N-O) and somite 18 (D,H,L,P) for each type of embryo. (A-P) 4D9 (in green) labels pioneer (arrowheads) and MFF nuclei; Prox1 (in red) labels slow muscle fibre nuclei, including pioneers. Because of the projection, some nuclei artificially overlap. All pioneer nuclei are therefore identified by arrowheads. (A,C,G,I,K,O) 4D9 labelling only. The asterisk locates ectopic tails in dorsomorphin-treated embryos. Scale bar: 50 μm.

number of pioneers are not statistically different between treated and untreated somites. The relative intensities of Engrailed labelling between pioneers and MFFs and the shape of the nuclei are similar between ectopic and normal cells. Nonetheless, the domain where MFFs are found is expanded dorsoventrally.

In addition, unlike in pioneers, Engrailed expression is prematurely activated in MFFs. Wild-type Engrailed expression starts in somites 23-25 for pioneers and 14-15 for MFFs (*n*=4). In dorsomorphin-treated wild types, pioneers start to express Engrailed in somites 24-26; MFFs do so in somites 15-18 (*n*=5), which corresponds to a three-somite posterior shift.

These results show that Bmp signalling inhibits Engrailed expression in the wild-type context as well.

The presence of heparan sulfate proteoglycans is affected in *sly* embryos

No direct interaction between laminins and Bmp has been described. However, the extracellular matrix-associated heparan sulfate proteoglycans (HSPGs) have been shown to affect BMP bioavailability (for a review, see Umulis et al., 2009). We therefore assessed the presence of HSPGs in wild-type and *sly* embryos.

At the 18-20 s stage, at the level of adaxial cell pre-stacking (data not shown) and stacking (Fig. 6A), in wild-type embryos (*n*=9/9), HSPGs are found around somitic cells but at a much weaker level around adaxial cells. In *sly* embryos (*n*=6/6), HSPG labelling is very weak (Fig. 6B).

At 26 hpf, at the axial level posterior to the emergence of Engrailed-positive MFFs, in wild-type embryos (*n*=9/9), HSPGs are still present around somitic cells and are now strongly present in the basement membrane around the notochord and the ventral neural tube (Fig. 6C). In *sly* embryos (*n*=8/8), HSPGs are absent from these sites (Fig. 6D).

HSPGs are therefore at the right place at the right time to potentially regulate BMP bioavailability and their presence is disrupted in *sly* embryos.

Heparinase III treatment, but not Chondroitinase ABC treatment, affects Engrailed and pSmad expression in a manner similar to the *sly* mutant

To test whether altered presence of HSPGs mediate the effect of *sly* on Bmp signalling, the effect of HSPGs removal from wild-type embryos was assessed on Engrailed and pSmad expression.
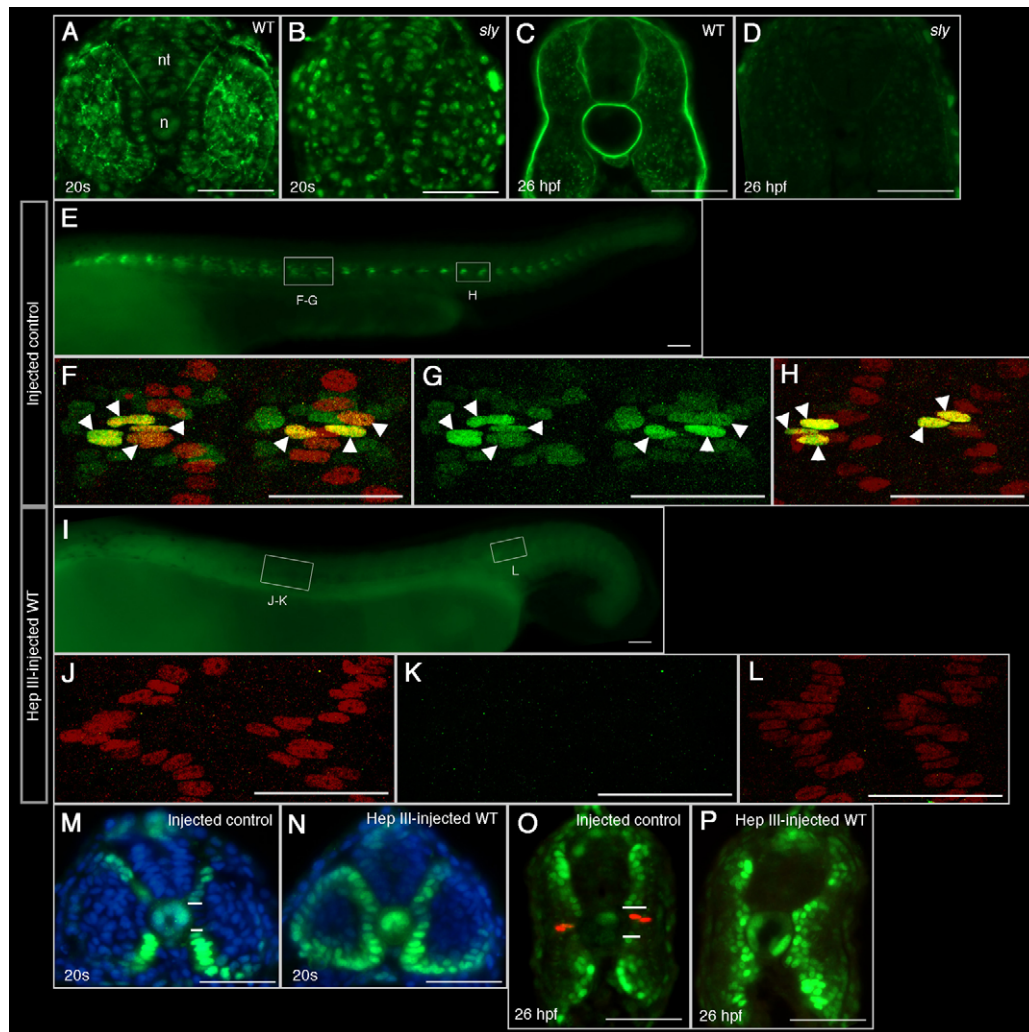
Fig. 6. Heparan sulfate proteoglycans are absent in *sly* embryos and Heparinase III treatment affects Engrailed and pSmad expression in a manner similar to the *sly* mutant. (**A-D**) Transverse sections of wild-type (A,C) and *sly* (B,D) embryos at the 20 s stage (A,B, adaxial cell stacking level) and 26 hpf (C,D, anterior tail level). Dorsal is upwards. In addition to its extracellular signal, anti-HS antibody (in green) labels all nuclei. (**E,I**) Lateral views of vehicle solution-injected wild-type (E) and Heparinase III-injected wild-type (I) embryos at 26 hpf. Anterior is leftwards, dorsal is upwards. (**F-H,J-L**) Magnification views (projection of confocal sections) of the regions around somite 10 (F-G,J-K) and somite 18 (H,L) for each type of embryo. (E-L) 4D9 (in green) labels pioneer (arrowheads) and MFF nuclei; Prox1 (in red) labels slow muscle fibre nuclei, including pioneers. Because of the projection, some nuclei artificially overlap. All pioneer nuclei are therefore identified by arrowheads. (E,G,I,K) 4D9 labelling only. (**M-P**) Transverse sections of vehicle solution-injected wild-type (M,O) and Heparinase III-injected wild-type (N,P) embryos at the 20 s stage (M,N, adaxial cell level) and 26 hpf (O,P, anterior tail level). Dorsal is upwards. (M-P) Nuclear pSmad labelling is in green. (M,N) Nuclei are counterstained with DAPI (in blue). (O,P) 4D9 (in red) labels pioneers. The horizontal white lines delimitate the pSmad-negative central domain. n, notochord; nt, neural tube. Scale bar: 50 μm.

Heparinase III cleaves HS chains, thereby eliminating their activity. Vehicle solution-injected (control) embryos showed normal labelling of HSPGs (*n*=6/6) (see Fig. S2A in the supplementary material), Engrailed (*n*=8/8) (Fig. 6E-H) and pSmad (*n*=8/8) (Fig. 6M,O). HSPGs are efficiently removed 10 hours after Heparinase III injection (*n*=13/13) (see Fig. S2A-D in the supplementary material).

After Heparinase III injection at the 10 s stage, Engrailed expression was assessed at 26 hpf (i.e. 12 hours post-injection). The majority of the embryos show a total absence of Engrailed expression at both trunk and tail levels (*n*=24/35) (Fig. 6I-L), while a minority show weaker Engrailed expression or fewer Engrailed-positive nuclei (*n*=11/35) (data not shown). The intrinsic variability between injections (volume and location) is likely to account for the different effects that we observe.

pSmad expression was then assessed at the 20 s stage at the adaxial cell pre-stacking and stacking levels after injection at the 6 s stage (i.e. 5 hours post-injection). pSmad is ectopically activated in the central domain in a subset of the injected embryos (*n*=4/10) (Fig. 6N). The lower penetrance of the effect on pSmad compared with that of the Engrailed could be due to the shorter lapse of time between injection and analysis.

At 26 hpf after an injection at the 10 s stage (i.e. 12 hours post-injection), pSmad is ectopically expressed in the central domain, where MFFs will emerge, in all injected embryos (*n*=12/12) (Fig. 6P).

Heparinase III treatment therefore leads to the ectopic activation of pSmad in pioneer and MFF precursors, thereby inhibiting Engrailed expression in these cells.
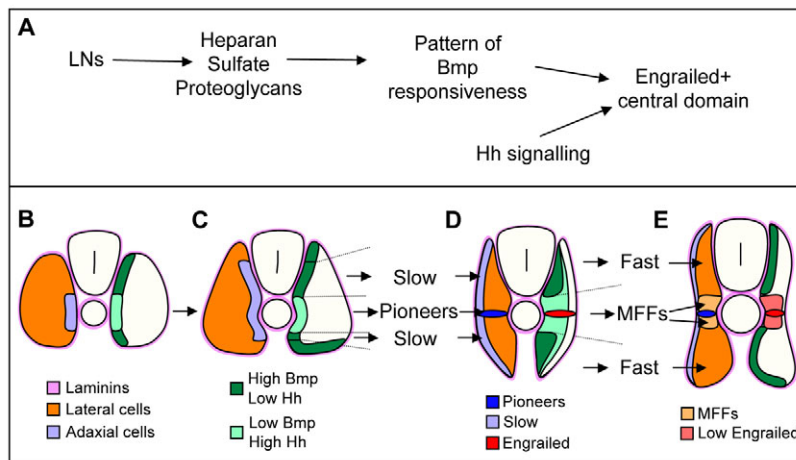
Fig. 7. Laminins, via heparan sulfate proteoglycans, participate in myotome morphogenesis by modulating the pattern of Bmp responsiveness. (**A**) Laminins (LNs) would pattern Bmp responsiveness via modulation of HSPG localisation. Combined Bmp and Hh signalling would then define an Engrailed-positive central domain. (**B-E**) Two domains can be distinguished in the medial somite. A central domain would receive low Bmp/high Hh signalling. Ventral and dorsal flanking domains would receive high Bmp/low Hh signalling. Both Engrailed-positive pioneers and MFFs develop within the central domain at successive developmental stages.

Chondroitin sulfate proteoglycans (CSPGs) are also found in sites compatible with a role in restricting BMP bioavailability (see Fig. S1A,C in the supplementary material) and at 26 hpf, CSPG labelling is weak in *sly* (see Fig. S1B,D in the supplementary material). However, Chondroitinase ABC treatment, that efficiently cleaves chondroitin sulfate chains, does not affect Engrailed expression (see Fig. S1E-L in the supplementary material), despite CSPG removal 10 hours after injection (see Fig. S2E-H in the supplementary material). CSPGs are therefore not involved in the regulation of Engrailed expression. The late decrease in CSPG labelling in *sly* could be a consequence of extracellular matrix disorganisation or altered notochord maturation.

Considering that Heparinase III treatment mimics the *sly* phenotype and that HSPG presence is affected in *sly*, our results are consistent with a role for HSPGs in mediating the effect of *sly* on Bmp.

DISCUSSION
This study shows that basement membrane laminins, via heparan sulfate proteoglycans, are instrumental in the spatial modulation of Bmp signalling. This spatial modulation is required for the patterning activity of Bmp signalling on the myotome, which, in combination with Hh signalling, leads to the definition of an Engrailed-positive central domain (see model in Fig. 7A).

Laminins, via heparan sulfate proteoglycans, modulate a subset of the pattern of Bmp responsiveness, leading to the definition of the Engrailed-positive central domain
Basement membrane disorganisation, such as the one described in *sly* (Parsons et al., 2002), has been shown to result in the disruption of proteoglycan localisation (for a review, see Li et al., 2003). This is what we observe in *sly* embryos when HSPGs and laminins colocalize in the extracellular matrices. HSPGs, unlike laminins, are also found within the somite and somitic HSPG labelling is weak in *sly*. Laminins surrounding the somite may thus play an indirect role on HSPG somitic localisation and/or synthesis.

An increasing body of evidence, including in zebrafish (Bink et al., 2003; Machingo et al., 2006; Mizumoto et al., 2009), show that the extracellular matrix-associated HSPGs have a positive or negative effect on BMP presentation to their receptors or their range of diffusion (for a review, see Umulis et al., 2009). In particular, HSPGs can restrict Bmp diffusion. We show that this occurs in the *sly* mutant. An additional mechanism whereby BMP

antagonists emanating from the notochord (Dal-Pra et al., 2006; Furthauer et al., 1999; Kinna et al., 2006) modulate Bmp signalling may also play a role in this process.

Only the central domain of the pattern of Bmp responsiveness is modulated by laminins. The lateral expansion of pSmad expression could be associated with the lateral migration of slow muscle fibres. Indeed, these two events are spatiotemporally correlated and slow muscle migration has been shown to create a morphogenetic signal that patterns fast muscle fibre elongation in its wake (Henry and Amacher, 2004).

Wild-type pSmad expression is restricted to dorsal and ventral domains. Wild-type *ptc1* expression is restricted to the central domain (Concordet et al., 1996; Wolff et al., 2003). As a consequence, two domains are set up in the medial somite (see model in Fig. 7B-E): one central domain corresponding to low Bmp/high Hh signalling flanked on either side by a ventral and a dorsal domain corresponding to high Bmp/low Hh signalling.

Initially, all adaxial cells are located in the central domain. The stacking of adaxial cells moves slow muscle precursors from the central domain to the ventral and dorsal domains. This ends their exposure to Hh, which prevents them from becoming pioneers. The central adaxial cells that stay in the central domain are exposed for a longer time to Hh and become pioneers. The combination of adaxial cell stacking and the pattern of Bmp responsiveness thus provides a mechanism to expose pioneer precursors to Hh signalling for a longer time than slow muscle precursors.

At a later stage, this medial central domain transiently expands laterally just before MFFs emerge from it. The patterning of Bmp responsiveness again defines a central domain with low Bmp/high Hh signalling and flanking domains with high Bmp/low Hh signalling. Engrailed expression is thus restricted to the central domain of the fast muscle population, allowing the MFFs to form within this domain.

This study shows that as in amniotes (for a review, see Hirsinger et al., 2000), the combined and antagonistic patterning activities of Hh and Bmp generate distinct domains in zebrafish: a central domain, separating dorsal (epaxial) and ventral (hypaxial) domains. Despite a different location within the somite, *engrailed* expression is nonetheless regulated by a similar regulatory network in amniotes (Cheng et al., 2004) and zebrafish (this study), as it is induced by Hh and inhibited by Bmp. In cell aggregation assays, chick *engrailed-1*-expressing cells sort out from hypaxial cells, suggesting that the epaxial-hypaxial boundary may represent a true compartment boundary (Cheng et al., 2004).

Once again, this report underlines that regulatory networks involving Engrailed, BMPs and Hh in various combinations have been conserved to specify compartments (amniote limb bud, *Drosophila* wing imaginal disk).

Bmp signalling blocks cells from entering the Engrailed-positive population

The involvement of Bmp in Engrailed expression regulation is supported by the results of previous work. Knockdown of the TGFβ family member Radar expands *eng1a* expression in somites (Kawakami et al., 2005). Ectopic expression of chick Dorsalin 1 in the notochord inhibits Engrailed expression (Du et al., 1997).

In either rescued *sly* pioneers and MFFs or ectopic pioneers and MFFs, the relative intensities of Engrailed expression between pioneers and MFFs, their nuclei shapes and cell body positions are similar to wild-type cells. This suggests that Bmp signalling operates as a negative switch for the Engrailed-positive muscle fibre population. When the blockade is relieved, cells proceed and develop their characteristic morphological traits like wild-type cells. Cues other than Bmp have to instruct pioneers and MFFs to express different levels of Engrailed and exhibit distinct morphological traits. These cues may be linked to the slow or fast identity of pioneers and MFFs.

Hh has been signalling to muscle precursors for a prolonged period before it activates Engrailed expression in pioneers and MFFs (Concordet et al., 1996). Our results suggest that Bmp signalling participates in the regulation of the onset of Engrailed expression in MFFs but not in pioneers. When Bmp signalling is knocked down, ectopic MFFs appear in the dorsal and ventral myotome domains that, in wild-type, exhibit a lateral expansion of pSmad expression. Bmp signalling knockdown may affect this lateral expansion, allowing ectopic MFFs to activate Engrailed expression at an earlier stage in this domain. However, even if shifted posteriorly in knockdown experiments, Engrailed expression is not activated in MFFs at the same time as in pioneers. Fast muscle myogenesis is delayed compared with slow muscle myogenesis (for a review, see Ochi and Westerfield, 2007). In both muscle fibre types, Engrailed expression is initiated after the appearance of muscle markers such as Myosin heavy chains. Both pioneers and MFFs may thus need to reach a certain level of myogenic differentiation to activate Engrailed expression.

The roles of laminins in muscle morphogenesis and differentiation

As previously shown (Peterson and Henry, 2010), we find that Myosin heavy chain expression in the fast muscle population is shifted anteriorly by an average of four or five somites, whereas it is initiated around the correct time in slow muscle fibres. We also find that slow muscle fibre migration is delayed in *sly* (M.D., J.-F.N. and E.H., unpublished). Furthermore, delayed fast muscle fibre elongation in *sly* is linked to slow muscle fibre presence (Peterson and Henry, 2010). Considering the morphogenetic role of slow muscle migration on fast muscle fibres (Henry and Amacher, 2004), this delayed migration is likely to account for the global maturation delay of the fast muscle population in *sly*. Laminins would therefore play an indirect role on myogenesis through regulation of muscle cell behaviour. This is the case in mouse (Bajanca et al., 2006). Laminins are important for maintenance of immature muscle precursors in the dermomyotome, for proper translocation from the dermomyotome into the myotome and then for proper elongation of the differentiating fibres in the myotome.

We show that untreated and dorsomorphin-treated *sly* somites are short and straight, while Heparinase III-injected somites adopt a chevron shape, similar to wild-type somites. This differential phenotype suggests that laminins, independently from Bmp signalling, play a role in shaping the somite. This could be through two mechanisms. In *sly*, notochord cells do not vacuolate and therefore do not stretch the embryo along the anteroposterior axis (Stemple et al., 1996). The somite shortness may mechanically prevent them from adopting the chevron shape. Alternatively, pioneers, anchored in all four Laminin-rich somite boundaries, are associated with the somite chevron shape (Ochi and Westerfield, 2007; van Eeden et al., 1996). In *sly*, pioneers may not properly anchor at these sites and somites would thus not adopt a chevron shape.

The potential role in cell connectivity of the Engrailed-expressing central domain

What remains to be understood is the role of Engrailed on the myotome and surrounding tissues. The results of the Heparinase III treatment suggest that Engrailed, even though expressed in the pioneers, is not required for the chevron shape observed in the trunk somites. However considering the injection stage and location, trunk somites may experience an early and transient Engrailed expression that would be sufficient to trigger the Laminin-dependent morphogenetic program leading to proper shaping of the somite.

Muscle fibres anchor into the horizontal myoseptum to use it as a myotendinous junction (Bassett and Currie, 2003). Pioneers and the horizontal myoseptum are also a source of migrating cues for motoneuron axons (Melançon et al., 1997) and the lateral line primordium, future adult sensory organ (David et al., 2002). It is currently unclear whether this organising role is mediated by Engrailed per se and/or by pioneers.

The ancestral function of Engrailed is probably regulation of connectivity, as in axon pathfinding and targeting and synaptic specificity, through regulation of cell adhesion molecules (for a review, see Gibert, 2002). If Engrailed is directly involved, its organising role in the morphogenesis of both the myotome and surrounding tissues would be in line with its ancestral role in cell connectivity.

Along with recent studies, this work reinforces that free diffusion of morphogens triggering gene regulatory networks are not sufficient to account for the morphogenetic processes occurring in the embryo. The landscape of the extracellular matrix locally modulates the cell response to morphogens.

Competing interests statement
The authors declare no competing financial interests.

DEVELOPMENT

Supplementary material

Supplementary material for this article is available at
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