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Joseph Pickering, Vincent T. Cunliffe, Freek Van Eeden, Anne-Gaëlle Borycki

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Hegdehog signalling acts upstream of laminin lpha1 transcription in the zebrafish paraxial mesoderm

Joseph Pickering, Vincent T. Cunliffe, Freek Van Eeden, and Anne-Gaëlle Borycki*

Bateson Centre, Department of Biomedical Science, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK.

* to whom correspondence should be addressed: a.g.borycki@sheffield.ac.uk

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Keywords: Laminin, Hedgehog signalling, zebrafish, embryo.

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[1] **Abbreviations:** GFP: green fluorescent protein; CNS: central nervous system; hpf: hours post fertilization;

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Abstract

Laminin-111 ($\alpha1\beta1\gamma1$) is a member of the Laminin family of extra-cellular matrix proteins that comprises 16 members, components of basement membranes. Laminin-111, one of the first Laminin proteins synthesized during embryogenesis, is required for basement membrane deposition and has essential roles in tissue morphogenesis and patterning. Yet, the mechanisms controlling Laminin-111 expression are poorly understood. We generated a zebrafish transgenic reporter line that reproduces faithfully the expression pattern of *lama1*, the gene encoding Laminin $\alpha1$, and we used this reporter line to investigate *lama1* transcriptional regulation. Our findings established that *lama1* expression is controlled by intronic enhancers, including an enhancer directing expression in the paraxial mesoderm, anterior spinal cord and hindbrain, located in intron 1. We show that Hedgehog signalling is necessary and sufficient for *lama1* transcription in the paraxial mesoderm and identify putative Gli/Zic binding sites that may mediate this control. These findings uncover a conserved role for Hedgehog signalling in the control of basement membrane assembly via its transcriptional regulation of *lama1*, and provide a mechanism to coordinate muscle cell fate specification in the zebrafish embryo.

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1. Introduction

Laminins are proteins of the extracellular matrix that play critical roles during embryonic development and in adult tissues. As key components of basement membranes, Laminins are instrumental in defining boundaries, shaping tissues and organs, maintaining epithelial organization, and providing structural support [1-3]. They have also important signalling functions through their binding to and activation of two cell surface receptors, Integrins and Dystroglycan [4, 5].

Laminins are heterotrimeric molecules containing one alpha, one beta and one gamma chain. Five genes encoding for Laminin alpha (*Lama*), four genes encoding for Laminin beta (*Lamb*) and three genes encoding for Laminin gamma (*Lamc*) have been identified and contribute to the formation of approximately 16 distinct Laminins in vertebrates [6-8]. Amongst those, *Lamb1* and *Lamc1* encode subunits shared by many Laminin isoforms, and thus display a broad, nearly ubiquitous expression pattern [9]. In contrast, *Lama* genes have a more specific expression pattern, consistent with the restricted distribution of the subunit they encode. Laminin α subunits are also required for the progression through the secretory pathway and extracellular release of Laminin heterotrimers [10]. Thus, it is crucial to uncover how *Lama* gene expression is controlled, as it dictates the distribution of a given Laminin.

Laminin-111 ($\alpha1\beta1\gamma1$) is the first Laminin protein produced in the developing mammalian embryo, and is detected by western blot at the 16-cell stage and by immunofluorescence within the Reichert's and the embryonic basement membranes in pre-implantation mouse embryos [9, 11]. Consistent with this distribution, knockout of *Lama1*, the gene encoding Laminin $\alpha1$, results in early peri-implantation lethality (around E6.5) associated with the loss of Reichert's basement membrane [9]. During mouse embryogenesis, Laminin-□□1 is mainly incorporated in basement membranes associated with epithelia of the central nervous system, kidneys, lungs, and retina, as well as skeletal muscles [12-14]. In mouse adult tissues, Laminin $\alpha1$ is mostly restricted to epithelial organs, including kidneys, testis and the eye [15, 16]. Thus, although Laminin $\alpha1$ is abundant during embryogenesis, it is

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largely down-regulated in adulthood. This indicates that *Lama1* transcription is dynamically controlled during development.

In mouse skeletal muscles, a Laminin α1- and Laminin α5-containing basal lamina associated with embryonic muscles is gradually replaced by a Laminin α2-containing basal lamina in adult muscles [14, 17-21]. As in mouse embryos, Laminin α1 is the earliest subunit detected in the tail bud and presomitic mesoderm of zebrafish embryos, closely followed by Laminin α2 in adaxial cells and Laminin α4 and α5 in slow and fast muscle cells [8, 22-24]. In amniotes and fish skeletal muscles, muscle-associated Laminins appear to have dual roles. First, they have a structural role, anchoring muscle fibres to the basal lamina and linking the extra-cellular matrix to the cytoskeleton, which stabilises the sarcolemma during muscle contraction. Consequently, *Lama2* loss-of-function mutations result in congenital muscular dystrophy in human, mouse and zebrafish [25-27], and immobilizing *lama2*-deficient zebrafish reduces the structural damages inflicted on myofibres upon contraction [28]. Interestingly, in zebrafish and in mouse congenital muscular dystrophy models, 'embryonic' Laminin α1 can compensate for the loss of Laminin α2, as forced expression of *Lama1* in *Lama2*-deficient mice suppresses the dystrophic phenotype while loss of *lama1* (*bashful* (*bal*) mutant) in *lama2* (*candyfloss* (*caf*)) mutant zebrafish exacerbates the fibre detachment phenotype [29-31]. Second, Laminins have also a role in the specification and patterning of skeletal muscle progenitor cells. In the mouse, loss of somitic *Lama1* expression in *Sonic Hedgehog* (*Shh*)-deficient embryos impairs the assembly of the myotomal basement membrane and results in aberrant migration and differentiation of muscle progenitor cells [17]. Likewise, *lamc1* (*sleepy* (*sly*)) mutation disrupts the pre-patterning of adaxial cells and Hedgehog-mediated induction of muscle pioneer and medial fast muscle cell differentiation in zebrafish embryos [22]. Collectively, these findings suggest that Laminin α1 is a target of therapeutic interest in muscle pathologies, and its distribution and function is intricately associated with Hedgehog signalling in developing skeletal muscles; however, the nature of this relationship remains elusive.

Here, we used BAC transgenesis in the zebrafish in order to identify the regulatory elements controlling *lama1* expression during embryogenesis. We characterized an intronic enhancer sufficient to drive reporter gene expression in the embryonic central nervous system (CNS) and skeletal muscles. Further analyses showed that Hedgehog

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signalling controls both endogenous *lama1* expression and transgene expression driven from the intronic enhancer, revealing a central role for Hedgehog signalling in the regulation of *lama1* expression in mesoderm-derived and neural tissues. Given our findings and the known role of Laminin γ1 in patterning adaxial cells for Hedgehog-mediated myogenesis, we propose that Hedgehog-controlled *lama1* expression regulates spatially and temporally the deposition of Laminin-111 (α1β1γ1) and thus the timing of adaxial cell fate specification during embryonic myogenesis. Furthermore, it provides a novel line of investigations to exploit the therapeutic effects of Laminin α1 in congenital muscular dystrophies.

2. Results

2.1. The BAC transgenic line lama1:GFP recapitulates lama1 expression during zebrafish development.

The zebrafish *lama1* locus is syntenic to the human and mouse *Lama1* locus, and is characterised by the conserved presence of two genes upstream of *lama1*: *ptprma*, which encodes protein tyrosine phosphatase, receptor type, M, a, and *lrrc30*, which encodes for Leucine-rich repeat-containing protein 30 (Fig. 1A). To identify the regulatory elements controlling *lama1* expression, we used a BAC recombineering approach to generate a stable zebrafish transgenic line (*lama1:GFP*) expressing the reporter gene EGFP under the control of *lama1* regulatory sequences **[32]**. BAC zC34A17 contains the entire *lama1* locus in addition to 39.76kb and 26.74kb of upstream and downstream sequences, respectively (Fig. 1A). A EGFP cassette was inserted at the start codon of *lama1* in BAC zC34A17, and five independent founder lines were established and grown to the F3 generation. All lines showed a similar GFP expression pattern, which was assessed from the 1-cell stage to 96 hpf, and compared to the expression pattern of *lama1* (Fig. 1B-N). *Lama1* transcripts were detected in 1-cell stage through to 50% epiboly zebrafish embryos (Fig. 1B-F). However, no GFP-positive cell was observed at these stages (data not shown), indicating that as previously reported [23], *lama1* was maternally expressed or that elements controlling the early expression of *lama1* were not present in BAC zC34A17. At 75% epiboly, both *lama1* transcripts and GFP were

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expressed very broadly (Fig. 1G). However, by the 6-somite stage, *lama1* and GFP expression became restricted to the anterior ventral CNS, notochord, and the paraxial mesoderm (psm and somites) (Fig. 1H). This expression pattern was maintained at the 15-somite stage, when GFP also became detectable in the eye and otic vesicles (Fig. 1I). At 24 hpf, GFP expression was observed in the anterior CNS, including the telencephalon, the midbrain-hindbrain boundary, and the anterior spinal cord (Fig. 1J). GFP expression was also evident in both the lens and retina of the eye, and in the otic vesicles (Fig. 1J,K). In the trunk, reporter gene expression was observed in somitic muscles, the uro-genital opening, the pre-somitic mesoderm (psm) and the posterior notochord, in a pattern resembling that of endogenous *lama1* (Fig. 1J,L-N). Closer examination of 24hpf zebrafish embryos following transient transgenesis and co-labelling with antibodies specific to slow muscle fibres (F59), fast muscle fibres (F310), and to the notochord (keratan sulphate) revealed that GFP expression was present in both slow and fast muscle fibres, and within the notochord (Fig. 1O-T). Similar to endogenous *lama1*, GFP expression presented an antero-posterior gradient in the trunk with higher expression in posterior somites, posterior notochord, and in the psm (Fig. 1J). However, it was noticeable that while *lama1* expression was down-regulated in thoracic somites, GFP expression was maintained (Fig. 1J). We attributed this difference to the stability of GFP, as although observed in all somites in 24 hpf zebrafish embryos (Fig. 1J,U), *gfp* transcripts in somites declined in 48hpf embryos and by 72hpf were no longer detectable (Fig. 1V-Y and data not shown), indicating that the persistence of GFP in 24 hpf somites was transient and likely due to the higher stability of *gfp* compared to *lama1* transcripts. Notable sites of expression in 48hpf embryos included the telencephalon, the eye, the midbrain-hindbrain boundary, the otic vesicles, the branchial arches, and the pectoral fins (Fig. 1V-Y). Thus, the transgenic line *lama1:GFP* recapitulates the expression pattern of endogenous *lama1*, suggesting that all regulatory elements necessary for *lama1* expression during zebrafish embryogenesis are contained within BAC zC34A17.

2.2. An intronic enhancer drives lama1 expression in the CNS and skeletal muscles.

To characterize the sequences required for *lama1* expression, we performed a deletion series on the *lama1:GFP* transgene, and analysed the transcriptional output of deleted constructs by transient transgenesis in the zebrafish. Removal of sequences up to 3.3kb

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upstream (*Δ-3.3lama1:GFP*) and all downstream sequence (*Δ-3.3lama1:GFPΔex63*) of *lama1* had no effect on reporter gene expression (Fig. 2A,B, and compare Fig. 2C and D). Likewise, deletion of intragenic sequences downstream of exon 4 did not affect GFP expression (*Δ-3.3lama1:GFPΔex4*) (Fig. 2A,B). This suggested that the enhancer(s) responsible for *lama1* expression in the embryo are located either within 3.3kb upstream of the *lama1* start site or in intragenic sequences within the first 4 exons. We cloned the 3.3kb upstream sequence with EGFP into the TOPO vector and tested several deletions to identify the minimal sequence required for basal transcription and found that a 0.8kb sequence upstream of *lama1,* predicted by bioinformatics to act as a minimal promoter, drove weak GFP expression in 2/55 embryos at 15-somite stage and in 2/76 embryos at 24 hpf (Fig. 2A,B). The 3.3kb upstream sequence tested in isolation in a GFP reporter construct yielded weak GFP expression in 10/58 embryos, which was significantly enhanced when intragenic sequences comprised between exons 1-4 were included (*Δ-3.3lama1:GFPΔex4*), indicating that specific enhancers for *lama1* expression are likely to be intragenic. Thus, all constructs tested subsequently contained the minimal promoter (0.8kb) upstream of the reporter gene GFP. We assessed a 10kb sequence spanning exon 1 to 2 (*Δ-0.8lama1:GFPΔex2*), which was sufficient to drive the complete pattern of *lama1* expression at the 15-somite and 24 hpf stages (Fig. 2A,B,E).

Transient transgenic embryos carrying a transgene with further deletion into intron1 removing the 3' half of intron 1 (*Δ-0.8lama1:GFPΔ4497*) revealed good levels of GFP expression in the trunk musculature and somewhat lower GFP expression in the anterior spinal cord and CNS at 24hpf (Fig. 2A,B,F). A similar expression pattern was observed in 19-somite and 49hpf embryos (data not shown). This suggested that this latest construct contained the enhancer(s) for mesoderm-derived (psm and axial mesoderm) and neuroectoderm-derived (neural tube, eye, brain) tissue expression. To further define the boundaries of the regulatory sequence(s) driving embryonic *lama1* expression, we generated a construct containing sequences of intron 1 up to +2373 (*Δ-0.8lama1:GFPΔ2373*), which activated, but did not maintain reporter gene expression (Fig. 2A,B,G,H). Further deletions into intron 1 until +1575bp (*Δ-0.8lama1:GFPΔ1575*) showed loss of reporter gene expression at both 15-somite stage and 24hpf (Fig. 2A,B,I), suggesting that no regulatory element is present within the 5' end of intron 1. We concluded that the enhancer(s) driving *lama1* expression in embryogenesis must be

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situated between +1575 and +4497. Furthermore, as the construct *Δ–0.8lama1:GFPΔ2373* did not maintain reporter expression and a construct *Δ-0.8lama1:GFP1551-*2324 was not sufficient to activate or maintain reporter expression (data not shown), we hypothesized that the 3' half of the intronic sequence of *Δ-0.8lama1:GFPΔ4497* contains the enhancer(s) for *lama1*. Indeed, both a fragment containing sequence between +2318 and +3226 (*Δ-0.8lama1:GFP2318-3226*) and a fragment containing sequences between +2205-2922 (*Δ-0.8lama1:GFP2205-2922*) were sufficient to activate and maintain reporter gene expression (Fig. 2A,B,J,K).

To confirm our findings, embryos injected with *Δ-0.8lama1:GFPΔ4497* and with *Δ-0.8lama1:GFP2205-2922* were raised to adulthood and screened for transmission to the germ line. While the stable line for *Δ-0.8lama1:GFP2205-2922* had very low levels of expression, suggesting that this short sequence was not sufficient for robust, stable expression (data not shown), the stable line containing the construct *Δ-0.8lama1:GFPΔ4497* displayed strong GFP expression at the 10-somite stage in the paraxial and axial mesoderm, and in the CNS (Fig. 2L,M). In 24hpf embryos, GFP expression levels were high in the psm and posterior somites, labelling both slow and fast muscle fibres (Fig. 2N,P). GFP was also observed in the anterior CNS, including in the telencephalon, tegmentum, cerebellum and rhombencephalon, and in sensory structures such as the olfactory placodes (Fig. 2N,O). Overall, the expression pattern displayed by *Δ-0.8lama1:GFPΔ4497* embryos mimics closely that of *lama1:GFP* embryos, although *Δ-0.8lama1:GFPΔ4497* embryos did not present GFP expression in the eye nor in the otic vesicles (compare Fig. 2N,O with Fig. 1J,K). This indicates that enhancers controlling *lama1* expression in the paraxial mesoderm and most of the anterior CNS are situated between positions +1-4497 of intron 1.

2.3. Hedgehog signalling is necessary and sufficient to activate lama1 expression in the zebrafish paraxial mesoderm.

Given our previous findings that Shh signalling is required for *Lama1* expression in the mouse paraxial mesoderm and neural tube [17], we asked whether Hedgehog signalling was necessary for *lama1* expression in the zebrafish embryo.

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We first analysed *smu*^{hi1640} mutant embryos, which carry a misense mutation in Smoothened, a transmembrane protein essential for Hedgehog signalling [33]. Consistent with previous reports [33], the absence of Hedgehog response in *smu*^{hi1640} mutant embryos resulted in the down-regulation of *ptc1* transcripts in the anterior CNS, the eye and the paraxial mesoderm at 28hpf (Fig. 3A,B). Interestingly, there was also a down-regulation of endogenous *lama1* expression in the psm and urogenital opening of *smu*^{hi1640} mutant embryos (Fig. 3C,D). Although *lama1* expression was slightly reduced in the anterior CNS, expression in the spinal cord and vasculature/pronephric duct did not appear to be affected in *smu*^{hi1640} mutant embryos (Fig. 3C,D). Similarly, when Hedgehog signalling was inhibited by applying cyclopamine, a potent inhibitor of Smoothened activity [34], to wild-type zebrafish embryos, we observed a decrease in *lama1* expression, concomitant with a down-regulation in *ptc1* expression, in the paraxial mesoderm and urogenital opening of treated compared to control embryos (Fig. 3E-H). Expression of *lama1* in the anterior CNS, neural tube and the vasculature/pronephric duct was weakly reduced, as in *smu*^{hi1640} mutant embryos (Fig. 3G,H). Together, these observations indicate that Hedgehog signalling is necessary for *lama1* expression in the paraxial mesoderm and urogenital opening, and contributes, but is not essential, to *lama1* expression in the CNS. Finally, *lama1* expression in the eye appears independent of Hedgehog signalling.

We next asked whether Hedgehog signalling was sufficient to regulate endogenous *lama1* expression. *ptc1*^{hu1062} and *ptc2*^{lep} mutant zebrafish carry a splice donor mutation in *ptc1* and a nonsense mutation in *ptc2*, respectively [35, 36]. While single homozygous embryos display a mild phenotype, *ptc1*^{hu1062}*ptc2*^{lep} double mutant embryos exhibit aberrant activation of the Hedgehog signalling pathway characterised by an up-regulation of the Hedgehog target gene *ptc1* in the paraxial mesoderm, neural tube, anterior CNS and the eye at 15-somite stage and 28hpf (Fig. 4A-D). Aberrant Hedgehog signalling caused also an increase in *lama1* expression in the neural tube, psm, hypochord, uro-genital region and the ventral vasculature and pro-nephric duct at 28hpf (Fig. 4E-H). Interestingly, *lama1* expression was not up-regulated in somites at 28hpf, although we consistently observed increased *lama1* expression in somites of 15-somite stage *ptc1*^{hu1062}*ptc2*^{lep} embryos (Fig. 4E-H), suggesting distinct temporal regulatory mechanisms in somites. Constitutive activation of the Hedgehog pathway can also be achieved through the injection of dominant negative protein kinase A (*dnPKA*) transcripts in zebrafish embryos [37-39]. Thus, one-cell stage zebrafish embryos were injected with

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dnPKA mRNA and analysed at the 15-somite stage and at 28hpf. As reported previously, dnPKA caused constitutive activation of Hedgehog signalling, which was evidenced by a robust up-regulation of *ptc1* expression in treated compared to control embryos at 15-somite stage and at 28hpf (Fig. 4I-L). Endogenous *lama1* expression was increased in the eye, anterior CNS, spinal cord and paraxial mesoderm (psm and somites) of *dnPKA*-injected embryos at the 15-somite stage, and in the anterior CNS, spinal cord and psm, but not somites at 28hpf (Fig. 4M-P).

Together, these findings demonstrate that Hedgehog signalling has a differential effect on embryonic tissues that express *lama1* in the zebrafish embryo: it is necessary and sufficient to drive expression in the zebrafish tailbud, pre-somitic and paraxial mesoderm where *lama1* expression appears to be subject to a temporal regulation as Hedgehog signalling drives *lama1* expression in early (15-somite stage), but not late (28hpf), somitogenesis. Finally, in other sites expressing *lama1* such as the anterior CNS, the spinal cord and the eye, Hedgehog signalling is sufficient but not necessary for *lama1* expression. These observations suggest that the spatial and temporal expression pattern of *lama1* may result from the combinatorial input of different signalling pathways, including the Hedgehog pathway, acting upon a single enhancer or distinct enhancers.

2.4. Gli/Zic binding sites within the lama1 intronic enhancer form specific transcriptional complexes

Given the control of mesodermal *lama1* expression by Hedgehog signalling and the identification of zebrafish *lama1* regulatory sequences within intron 1, we performed *in silico* analyses of *lama1* first intron to identify transcriptionally active regions and putative Hedgehog response elements. We searched for mono and tri-methylation on histone H3 at lysine 4 (H3K4me1 and H3K4me3) that are often associated with active chromatin [40, 41]. Data mining of a genome-wide study of histone modification marks found in 24hpf zebrafish embryos revealed a sequence marked by H3K4me3 overlapping with the transcriptional start site of zebrafish *lama1* [42], consistent with the known association of H3K4me3 histone modifications with promoter elements and the presence of the *lama1* promoter immediately upstream of exon 1 [43] (Fig. 5A). Of particular interest was the presence of a sequence marked by H3K4me1, a modification often associated with enhancer elements, located at position +2324-3283, a region situated within the 4497bp

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transgene that contains most *lama1* regulatory sequences (Fig. 5A,B). Interestingly, the domain marked by H3K4me1 overlapped only partially with the sequence *Δ-0.8lama1:GFP2205-2922* tested in transgenesis (Fig. 5B), providing a possible explanation for the reporter expression observed in transient, but not in stable transgenic fish (Fig. 2K). Additional H3K4me1 marks present in intron 1 fall in regions, which did not display reporter gene expression when tested by transgenesis (Fig. 5A). Based on these analyses, we hypothesized that the enhancer driving *lama1* expression in the paraxial mesoderm, urogenital region and anterior CNS is located at the site marked by H3K4me1 within the *Δ-0.8lama1:GFPΔ4497* transgene. We performed a search for binding sites for Gli proteins, the mediators of Hedgehog signalling, and for Zic proteins, which are zinc finger transcription factors of the Gli superfamily forming heterodimers with and modulating Gli proteins (Fig. 5C,D) [44]. Notably, we identified two alternative Zic binding sites (Zic sites 1 and 3) and one site with similarities to both conventional Zic and Gli binding sites (Gli/Zic site 2) within the putative enhancer region marked by H3K4me1 (Fig. 5C,D). No other putative Gli binding site was present within the rest of the *Δ-0.8lama1:GFPΔ4497* construct. We tested whether proteins from zebrafish embryo nuclear extracts could form complexes on the Gli and Zic binding sites by electromobility shift assay (EMSA) using radioactively labeled oligonucleotides encompassing sites 1–3. The oligonucleotides containing sites 1-3 formed specific complexes in the presence of nuclear extract from 24hpf zebrafish embryos (black arrowheads in Fig. 5E). Binding to sites 1-3 was efficiently competed by an excess of non-radiolabelled oligonucleotides (competitor), indicating that the complexes observed were specific (Fig. 5E). Furthermore, the addition of an excess of unlabelled oligonucleotides in which the Zic binding site was mutated was unable to compete complex formation at site 1, and to a lesser extent at site 3, suggesting that the high-molecular complexes detected by EMSA form at the Zic binding sites (black stars in Fig. 5E). Mutations within the Gli binding site of site 2 did not abolish competition by excess unlabelled oligonucleotides suggesting that either the complexes observed do not involve Gli, or more likely that the mutations introduced in the Gli/Zic site 3 were not sufficient to fully abolish binding. Combined, these findings suggest that *lama1* transcriptional control by Hedgehog signalling may operate from a region located within the 5' half of intron 1, and could involve a direct control via binding to at least one binding site with a consensus sequence for Zic proteins.

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2.5. Hedgehog signalling controls the transcriptional activity of the lama1 reporter lines

Given the presence of Gli/Zic binding sites within the *lama1* intronic enhancer, we next investigated whether Hedgehog signalling regulates *lama1* expression at the transcriptional level through its control of the intronic enhancer activity. We manipulated Hedgehog signalling and assessed the effect on reporter gene expression from the two stable lines we generated, *lama1:GFP* and *Δ-0.8lama1:GFPΔ4497.* Decreased Hedgehog signalling was achieved by growing *lama1:GFP* and *Δ-0.8lama1:GFPΔ4497* zebrafish embryos in the presence of cyclopamine, and aberrant Hedgehog signalling was achieved by injecting *lama1:GFP* and *Δ-0.8lama1:GFPΔ4497* with *dnPKA* transcripts (Fig. 6). Consistent with the fact that *lama1:GFP* reporter gene expression mirrors *lama1* expression pattern, we observed that *gfp* expression was down-regulated in the psm, urogenital region and anterior CNS of cyclopamine-treated compared to control *lama1:GFP* embryos (Fig. 6A,B). In contrast, there was a lesser effect of cyclopamine on the eye, midbrain-hindbrain junction, and the vasculature/pronephric duct (Fig. 6A,B). Conversely, *dnPKA*-injected *lama1:GFP* embryos displayed an up-regulation of *gfp* in most tissues expressing *lama1*, including the psm, somites, urogenital region, and anterior CNS at 15-somite stage and 24hpf (Fig. 6C-F). These data confirm that the *lama1:GFP* line mimics closely endogenous *lama1* and that the Hedgehog response elements are contained within the BAC sequence of *lama1:GFP.* We next asked whether the 5kb intronic region of *Δ-0.8lama1:GFPΔ4497* was Hedgehog responsive. We applied cyclopamine to *Δ-0.8lama1:GFPΔ4497* embryos, and observed a strong down-regulation of reporter gene expression in the psm and a moderate decrease of reporter gene expression in the anterior CNS at 24hpf (Fig. 6G,H). Conversely, reporter gene expression was up-regulated in the psm of 15-somite stage and 24hpf *Δ-0.8lama1:GFPΔ4497* embryos with *dnPKA* mRNA at the 1-cell stage compared to control embryos (Fig. 6I-L). As observed with endogenous *lama1* and with the *lama1:GFP* transgene, reporter gene expression was transiently up-regulated at the 15-somite stage, but not at 24hpf, in somites of *dnPKA*-injected embryos (Fig. 6I-L), confirming the temporal effect of Hedgehog signalling on *lama1* expression in somites. We conclude that Hedgehog signalling-mediated control of *lama1* in the psm and somites maps to the intronic enhancer present in the *Δ-0.8lama1:GFPΔ4497* transgene.

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3. Discussion

In this study, we report on the generation of a stable transgenic zebrafish line recapitulating the endogenous expression pattern of *lama1*. Through deletion analyses, we further established that *lama1* expression in the anterior CNS and paraxial mesoderm is driven by an enhancer located within intron 1. Furthermore, we demonstrate that both endogenous *lama1* expression and reporter gene expression driven by the intronic enhancer is controlled by Hedgehog signalling, possibly through Gli/Zic binding sites within the *lama1* intron 1 enhancer, in the paraxial mesoderm. We propose a model whereby the dual control of *lama1* and muscle cell fate determinants by Hedgehog signalling coordinates the temporal and spatial specification of muscle pioneer and medial fast muscle cells in the zebrafish embryo.

3.1. Conservation of lama1 expression pattern in vertebrates.

Lama1 has a complex expression pattern with near ubiquitous expression in early zebrafish embryogenesis, which becomes restricted over time to tissues of mesodermal and ectodermal origin, including the neural tube, anterior CNS, most sensory organs (eye, otic vesicles and olfactory placodes), somitic and limb muscles, urogenital tissues and notochord [8, 23, 24, 45]. Thus, as in mammals, zebrafish *lama1* is temporally regulated to be expressed broadly in early embryogenesis and down-regulated in late embryogenesis. This expression pattern overlaps with the distribution of Laminin-111, the main Laminin α1-containing isoform in embryos, and is consistent with defects in the development of the notochord, cerebellum and the eye in zebrafish *bal* mutant and conditional *Lama1* knockout mice [23, 45-50]. Furthermore, the emergence of *LAMA1* mutations associated with developmental defects in the cerebellum and retina in humans suggests a conversed expression pattern and function for Laminin α1 during evolution [51].

Our stable transgenic line *lama1:GFP* recapitulates faithfully the temporal and spatial expression pattern of *lama1* in the zebrafish embryo, providing a useful resource to

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monitor the transcription of *lama1*. Overall, the expression pattern of *lama1* reported in this study and by others in the zebrafish embryo shows a high degree of similarity with the expression pattern observed in chick and mouse embryos, indicating that *lama1* expression is conserved during vertebrate evolution. However, there are notable differences between species in *lama1* expression in the notochord and the paraxial mesoderm. In the zebrafish embryo, *lama1* is transiently transcribed in the chordamesoderm and notochord between the tail bud stage and the 19-somite stage, and is likely to contribute to the deposition of Laminin-111 around the notochord [23, 24]. In contrast, no *Lama1* expression has been observed in the chick and mouse notochord, although Laminin-111 is deposited in the peri-notochord basement membrane [14, 17, 52]. Likewise, the spatial distribution of *lama1* transcripts in the paraxial mesoderm differs between zebrafish and mouse embryos. Although deposited in the myotomal basement membrane assembled at the surface of muscle progenitor cells, Laminin α1 is transcribed by sclerotomal, and not myogenic cells in the mouse embryo [17]. In contrast, *lama1* is transcribed by adaxial, slow and fast muscle cells in the zebrafish embryo and contributes to the somitic basement membrane and myotendinous junction [23, 24, 53]. The molecular basis for these differences may reside in the existence of different regulatory elements controlling *lama1* expression in these domains. Alternatively, it could be due to differences in the availability of specific combinations of transcription factors between mouse and fish tissues.

3.2. Lama1 expression in the zebrafish embryo is controlled by distinct intronic regulatory elements

The expression pattern of *lama1:GFP* demonstrates that it contains all necessary regulatory elements for *lama1* transcription and involves the activity of a 5' intronic element driving expression in the paraxial mesoderm and anterior CNS and a 3' intronic element driving expression in the neural tube, uro-genital opening, notochord and sensory organs, contained within intron 1 (Fig. 7A,B). Our findings point to the existence of one or two enhancers within a 1kb region of the 5' intronic element controlling *lama1* expression in the paraxial mesoderm and anterior CNS: a) this genomic region drives robust reporter gene expression in transient transgenesis; b) it is marked by the histone methylation H3K4me1 associated with active enhancers*;* c) Gli/Zic binding sites are

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concentrated in the 1kb region labelled by H3K4me1, suggesting a possible direct control of *Δ−0.8lama1:GFPΔ4497* transcription by Hedgehog signalling. At present, we cannot rule out the existence of two distinct enhancers within the 1kb region, given that loss of Hedgehog signalling affects differently reporter gene expression in the paraxial mesoderm and the anterior CNS.

In human and mouse, *Lama1* transcription operates from a conserved proximal promoter containing essential Sp1/3 and Kruppel-like factor (KLF) binding sites [54, 55]. In the zebrafish, our deletion analyses coupled with bioinformatics have also identified that *lama1* transcription is initiated from a short proximal promoter of 800bp. However, the zebrafish promoter shares no sequence homology with the mouse or human *Lama1* promoter (data not shown). Likewise, the zebrafish intronic enhancer identified in the *Δ−0.8lama1:GFPΔ4497* construct does not appear to be conserved in mammals (data not shown). This is perhaps not surprising given that a number of functionally active enhancers, while driving similar expression patterns, are not conserved at the sequence level between distant vertebrates (such as mouse and zebrafish) [56]. Instead, these non-coding regulatory elements tend to be found in syntenic regions and share conserved transcription factor binding sites or sequence blocks, albeit in a different order [56]. In mammals, the regulatory elements controlling *Lama1* expression in the embryo have not been identified yet, although an enhancer responsible for *Lama1* expression in the parietal endoderm located 3kb upstream of *Lama1* and controlled by Sox7 and Sox17 has been previously characterized [57, 58]. Further work is required to determine whether *lama1* regulation in vertebrates is controlled by functionally conserved enhancers.

3.3. Hedgehog signalling controls lama1 expression in the paraxial mesoderm

Hedgehog signalling is necessary and sufficient for *lama1* expression in the paraxial mesoderm and the uro-genital region, and is sufficient to up-regulate expression in the anterior CNS and spinal cord. In line with these observations, it is worth noting that in addition to the Gli and Zic binding sites present in the region controlling *lama1* expression in the anterior CNS and paraxial mesoderm, we identified two further Gli binding sites in the region controlling uro-genital and spinal cord expression (Fig. 7A). Therefore,

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although indirect control by Hedgehog signalling cannot be fully ruled out at this stage, we favour the possibility that, at least in the paraxial mesoderm, Hedgehog signalling acts directly on *lama1* transcription through its control of the intronic enhancer(s) identified in this study. First, Hedgehog signalling has a similar effect on the transcriptional output from the *lama1:GFP* and *Δ−0.8lama1:GFPΔ4497* reporter lines than on endogenous *lama1* expression. Second, the putative Gli/Zic binding sites identified within the paraxial mesoderm intronic enhancer form specific transcriptional complexes in the presence of zebrafish embryo nuclear extracts. The close proximity of Gli and Zic binding sites is a common feature of several enhancers of developmentally important genes, and is consistent with the known synergistic transcriptional activity and nuclear transport control of Gli by Zic proteins [59-61]. Both *gli* and *zic* genes are expressed in the paraxial mesoderm. *zic2b* and *zic3* are of particular interest, given their expression in the paraxial mesoderm and down-regulation during somitogenesis [62, 63], and the essential roles their mammalian counterparts play in patterning and differentiation of somite derivatives [64, 65]. Combined with the well-established function of hedgehog signalling in the specification and differentiation of zebrafish adaxial cells [34], these observations support a dual control of *lama1* by Gli and Zic proteins.

Together with our previous report that Hedgehog signalling is required for *Lama1* expression in the mouse embryo [17], our observations suggest that *Lama1* control by Hedgehog signalling is conserved in vertebrates, albeit with some differences between mouse and zebrafish embryos. Hedgehog signalling is differentially required for *lama1* expression in the pre-somitic mesoderm and spinal cord between zebrafish and mouse embryos [17]. Shuffling of Hedgehog response elements between tissue-specific enhancers may underlie these differences [66]. Alternatively, the differential requirement for Hedgehog signalling between zebrafish and mouse embryos may reflect a spatial and temporal difference in Hedgehog response, as suggested by the expression pattern of *Ptc1*, a generally accepted readout of Hedgehog signalling, in the pre-somitic and somitic mesoderm of zebrafish and amniote embryos [67, 68].

The conservation of a Hedgehog-mediated control of *lama1* regulation during evolution evokes a selective pressure and suggests a functional role for the tandem Hedgehog signalling and *lama1* in the developing paraxial mesoderm. Consistent with this possibility, we previously showed that by controlling both *Lama1* and *Myf5* expression in the developing mouse myotome, Sonic Hedgehog signalling coordinates myogenic cell

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fate determination and muscle patterning [17, 19, 69]. A similar relationship between Hedgehog signalling and *lama1* may be at play in the control of zebrafish muscle cell fate specification. Indeed, muscle cell fate specification is impaired in the absence of Laminin γ1, the γ chain associated with Laminin α1 in zebrafish embryos [22, 70] (Fig. 7C). Laminin-111 (α1β1γ1) deposition at the surface of adaxial cells pre-patterns the paraxial mesoderm by establishing a BMP-free zone and creating a permissive environment for Hedgehog signalling-mediated induction of muscle pioneer and medial fast muscle cell differentiation [22, 70]. As Laminin α□ has a leading function in the secretion of Laminin-111 heterotrimers [10], we propose that Hedgehog-mediated control of *lama1* expression in the zebrafish pre-somitic mesoderm provides a mechanism to coordinate spatially and temporally the inductive activity of Hedgehog signalling on muscle pioneer and medial fast muscle cell differentiation (Fig. 7C). Given that other embryonic tissues displayed Hedgehog-mediated control of *lama1* expression and are patterned by Hedgehog signalling, we predict that the tandem Laminin-111/Hedgehog is likely to be re-iterated in distinct contexts and to operate in a similar manner in other tissues.

4. Experimental procedures

Maintenance of zebrafish lines

Wild-type, *smu*^{hi1640} (Chen et al., 2001), *ptc1*^{hu1062}/*ptc2*^{lep} [35, 36], *Δ–0.8lama1:GFPΔ4497* and *lama1:GFP* zebrafish lines were maintained at 28°C on a 14-hour light/10-hour dark cycle and staged following standard methods [71]. Pigmentation was prevented by immersion in 0.2mM N-phenylthiourea. All procedures involving any experimental animals were performed in compliance with local animal welfare laws, guidelines and policies. n numbers are indicated in figure legends and correspond to the ratio of embryos displaying the phenotype illustrated over the total number of embryos analysed.

Cyclopamine treatment of embryos

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Cyclopamine (Calbiochem) dissolved in 100% ethanol was added to E3 medium to a final concentration of 100µM, and added to zebrafish embryos at stage 80% epiboly. Control fish were treated with ethanol dissolved in E3 medium.

dnPKA micro-injection in embryos

dnPKA RNA was synthesised from pCS2MT vector using Not1/Sp6 RNA polymerase and the Sp6 mMESSAGE mMACHINE Kit (Ambion). Embryos were injected with 1-2nl of RNA solution containing phenol red at the 1 cell stage using a micromanipulator.

Bacterial artificial chromosome and promoter constructs

eGFP was inserted at the *lama1* ATG start site in BAC zC34A17 by using an eGFP-SV40pA-FRT-Kn-FRT recombineering targeting cassette, as previously published [32]. Deletions were generated by homologous recombineering using ultramers with homology to either sides of the BAC DNA sequence to be deleted. To delete sequences present upstream of the *lama1* start site, an iTol2-Amp-iTol2 cassette was inserted, whereas a Kanamycin resistance gene was inserted into the BAC to delete sequences downstream of the *lama1* start site. Deletions were confirmed by PCR analysis. The *Δ-3.3lama1:GFP* and *Δ-0.8lama1:GFP* constructs were generated by Phusion Hot Start DNA Polymerase (2U/µl) (Finnzymes) PCR using *lama1:GFP* DNA as the template. PCR products were cloned into pCRII TOPO vector (Invitrogen). Intronic enhancer sequences were amplified by PCR and Kpn1/BamH1 cloned downstream of GFP in the *Δ-0.8lama1:GFP* vector, following the addition of Kpn1 and BamH1 linkers.

In silico analyses

Promoter prediction was performed using McPromoter and Transcription factor binding sites were identified using MatInspector provided by Genomatix (http://www.genomatix.de/). Histone methylation marks were extracted from the ChIP-Seq data performed by Aday et al. on 24hpf zebrafish embryos, mapped on the zebrafish genome (zv7) and uploaded on the UCSD genome browser server [42].

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In situ hybridisation

In situ hybridisation was performed as previously described [72]. Digoxygenin labelled *lama1* antisense RNA was transcribed (T7 polymerase/HindIII) from a 929bp DNA template generated by RT-PCR, using RNA extracted from 19-somite stage zebrafish embryos and the following primers: forward 5'- GAAGTTTGAA TGAGAGAAAGACGGAC-3' and reverse 5'- CCTTGATGGAGTAATAATACCGTCG-3.' The *gfp* RNA probe was amplified from a pBluescript vector containing the GFP sequence (from S. Elworthy, University of Sheffield) using the primers: forward 5'-GCCTCGGTGAGTTTTCTCCTTC-3' and reverse 5'-GATGCCGTTCTTCTGCTTGTCG and the probe generated using BamH1 and T7. The *ptc1* antisense RNA probe was generated from a pGEM-T-Easy vector, using ApaI/Sp6 polymerase. Whole-mount embryo images were captured under a MZ12.5 stereomicroscope (Leica) using a SPOT INSIGHT Colour camera, and SPOT Advanced digital image capture software (Diagnostic Instruments).

Immunofluorescence

Methanol-fixed embryos were permeabilised in 0.05% Trypsin-EDTA (Gibco) at 37^{o}C. Embryos were then incubated overnight at 4^{o}C in 0.1% Tween-20, 2% heat inactivated goat serum, 0.2% BSA in PBS, and primary antibody. Primary antibodies were F59 (DSHB at 1:10), F310 (DSHB at 1:50), and Keratan sulphate (DSHB at 1:100). Secondary antibody was Alexa 594 goat anti mouse (Invitrogen at 1:500). Embryos were photographed using either a Leica MZ160F fluorescence stereomicroscope (Leica), with a DFC300FX camera (Leica) with Leica FireCam Mac V3.1.0 (Leica), an Olympus FV-1000 confocal microscope, with Olympus FluoView FV-1000 ASW 1.6 confocal software, or an Apotome.2 microscope (Zeiss) with an Imager.Z1 camera (Zeiss) and Zen2 software.

Electro-mobility shift assay (EMSA)

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Electro-mobility shift assay was carried out as described previously [73] using oligonucleotides described in Table S1. Nuclear extracts were prepared from 24hpf wild-type zebrafish embryos.

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References

1. P.D. Yurchenco, P.S. Amenta, and B.L. Patton, Basement membrane assembly, stability and activities observed through a developmental lens, Matrix Biol 22(2004) 521-538.
2. P.D. Yurchenco, Basement membranes: cell scaffoldings and signaling platforms, Cold Spring Harb Perspect Biol 3(2011).
3. J.H. Miner and P.D. Yurchenco, Laminin functions in tissue morphogenesis, Annu Rev Cell Dev Biol 20(2004) 255-284.
4. R. Nishiuchi, J. Takagi, M. Hayashi, H. Ido, Y. Yagi, N. Sanzen, T. Tsuji, M. Yamada, and K. Sekiguchi, Ligand-binding specificities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant alpha3beta1, alpha6beta1, alpha7beta1 and alpha6beta4 integrins, Matrix Biol 25(2006) 189-197.
5. M. Bozzi, S. Morlacchi, M.G. Bigotti, F. Sciandra, and A. Brancaccio, Functional diversity of dystroglycan, Matrix Biol 28(2009) 179-187.
6. M. Aumailley, L. Bruckner-Tuderman, W.G. Carter, R. Deutzmann, D. Edgar, P. Ekblom, J. Engel, E. Engvall, E. Hohenester, J.C. Jones, H.K. Kleinman, M.P. Marinkovich, G.R. Martin, U. Mayer, G. Meneguzzi, J.H. Miner, K. Miyazaki, M. Patarroyo, M. Paulsson, V. Quaranta, J.R. Sanes, T. Sasaki, K. Sekiguchi, L.M. Sorokin, J.F. Talts, K. Tryggvason, J. Uitto, I. Virtanen, K. von der Mark, U.M. Wewer, Y. Yamada, and P.D. Yurchenco, A simplified laminin nomenclature, Matrix Biol 24(2005) 326-332.
7. H. Colognato and P.D. Yurchenco, Form and function: the laminin family of heterotrimers, Dev Dyn 218(2000) 213-234.
8. T. Sztal, S. Berger, P.D. Currie, and T.E. Hall, Characterization of the laminin gene family and evolution in zebrafish, Dev Dyn 240(2011) 422-431.

ACCEPTED MANUSCRIPT

9. J.H. Miner, C. Li, J.L. Mudd, G. Go, and A.E. Sutherland, Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation, Development 131(2004) 2247-2256.
10. P.D. Yurchenco, Y. Quan, H. Colognato, T. Mathus, D. Harrison, Y. Yamada, and J.J. O'Rear, The alpha chain of laminin-1 is independently secreted and drives secretion of its beta- and gamma-chain partners, Proc Natl Acad Sci U S A 94(1997) 10189-10194.
11. A.R. Cooper and H.A. MacQueen, Subunits of laminin are differentially synthesized in mouse eggs and early embryos, Dev Biol 96(1983) 467-471.
12. L.M. Sorokin, F. Pausch, M. Durbeej, and P. Ekblom, Differential expression of five laminin alpha (1-5) chains in developing and adult mouse kidney, Dev Dyn 210(1997) 446-462.
13. G. Klein, M. Ekblom, L. Fecker, R. Timpl, and P. Ekblom, Differential expression of laminin A and B chains during development of embryonic mouse organs, Development 110(1990) 823-837.
14. J.H. Miner, B.L. Patton, S.I. Lentz, D.J. Gilbert, W.D. Snider, N.A. Jenkins, N.G. Copeland, and J.R. Sanes, The laminin alpha chains: expression, developmental transitions, and chromosomal locations of alpha1-5, identification of heterotrimeric laminins 8-11, and cloning of a novel alpha3 isoform, J Cell Biol 137(1997) 685-701.
15. P. Ekblom, P. Lonai, and J.F. Talts, Expression and biological role of laminin-1, Matrix Biol 22(2003) 35-47.
16. M. Falk, M. Ferletta, E. Forsberg, and P. Ekblom, Restricted distribution of laminin alpha1 chain in normal adult mouse tissues, Matrix Biol 18(1999) 557-568.
17. C. Anderson, S. Thorsteinsdottir, and A.G. Borycki, Sonic hedgehog-dependent synthesis of laminin alpha1 controls basement membrane assembly in the myotome, Development 136(2009) 3495-3504.
18. C. Anderson, S.J. Winder, and A.G. Borycki, Dystroglycan protein distribution coincides with basement membranes and muscle differentiation during mouse embryogenesis, Dev Dyn 236(2007) 2627-2635.
19. A.G. Borycki, The myotomal basement membrane: Insight into laminin-111 function and its control by Sonic hedgehog signaling, Cell Adh Migr 7(2013) 72-81.
20. B.L. Patton, J.H. Miner, A.Y. Chiu, and J.R. Sanes, Distribution and function of laminins in the neuromuscular system of developing, adult, and mutant mice, J Cell Biol 139(1997) 1507-1521.
21. F. Schuler and L.M. Sorokin, Expression of laminin isoforms in mouse myogenic cells in vitro and in vivo, Journal of cell science 108 (Pt 12)(1995) 3795-3805.
22. M. Dolez, J.F. Nicolas, and E. Hirsinger, Laminins, via heparan sulfate proteoglycans, participate in zebrafish myotome morphogenesis by modulating the pattern of Bmp responsiveness, Development 138(2011) 97-106.
23. M.J. Parsons, S.M. Pollard, L. Saude, B. Feldman, P. Coutinho, E.M. Hirst, and D.L. Stemple, Zebrafish mutants identify an essential role for laminins in notochord formation, Development 129(2002) 3137-3146.
24. S.M. Pollard, M.J. Parsons, M. Kamei, R.N. Kettleborough, K.A. Thomas, V.N. Pham, M.K. Bae, A. Scott, B.M. Weinstein, and D.L. Stemple, Essential and overlapping roles for laminin alpha chains in notochord and blood vessel formation, Dev Biol 289(2006) 64-76.
25. V.A. Gupta, G. Kawahara, J.A. Myers, A.T. Chen, T.E. Hall, M.C. Manzini, P.D. Currie, Y. Zhou, L.I. Zon, L.M. Kunkel, and A.H. Beggs, A splice site mutation in

ACCEPTED MANUSCRIPT

laminin-alpha2 results in a severe muscular dystrophy and growth abnormalities in zebrafish, PloS one 7(2012) e43794.
26. H. Xu, P. Christmas, X.R. Wu, U.M. Wewer, and E. Engvall, Defective muscle basement membrane and lack of M-laminin in the dystrophic dy/dy mouse, Proc Natl Acad Sci U S A 91(1994) 5572-5576.
27. F.M. Tome, T. Evangelista, A. Leclerc, Y. Sunada, E. Manole, B. Estournet, A. Barois, K.P. Campbell, and M. Fardeau, Congenital muscular dystrophy with merosin deficiency, Comptes rendus de l'Academie des sciences. Serie III, Sciences de la vie 317(1994) 351-357.
28. M. Li and A. Arner, Immobilization of Dystrophin and Laminin alpha2-Chain Deficient Zebrafish Larvae In Vivo Prevents the Development of Muscular Dystrophy, PloS one 10(2015) e0139483.
29. T.E. Sztal, C. Sonntag, T.E. Hall, and P.D. Currie, Epistatic dissection of laminin-receptor interactions in dystrophic zebrafish muscle, Human molecular genetics 21(2012) 4718-4731.
30. K. Gawlik, Y. Miyagoe-Suzuki, P. Ekblom, S. Takeda, and M. Durbeej, Laminin alpha1 chain reduces muscular dystrophy in laminin alpha2 chain deficient mice, Human molecular genetics 13(2004) 1775-1784.
31. J.E. Rooney, J.R. Knapp, B.L. Hodges, R.D. Wuebbles, and D.J. Burkin, Laminin-111 protein therapy reduces muscle pathology and improves viability of a mouse model of merosin-deficient congenital muscular dystrophy, The American journal of pathology 180(2012) 1593-1602.
32. E.C. Lee, D. Yu, J. Martinez de Velasco, L. Tessarollo, D.A. Swing, D.L. Court, N.A. Jenkins, and N.G. Copeland, A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA, Genomics 73(2001) 56-65.
33. W. Chen, S. Burgess, and N. Hopkins, Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity, Development 128(2001) 2385-2396.
34. C. Wolff, S. Roy, and P.W. Ingham, Multiple muscle cell identities induced by distinct levels and timing of hedgehog activity in the zebrafish embryo, Current biology : CB 13(2003) 1169-1181.
35. M.J. Koudijs, M.J. den Broeder, E. Groot, and F.J. van Eeden, Genetic analysis of the two zebrafish patched homologues identifies novel roles for the hedgehog signaling pathway, BMC Dev Biol 8(2008) 15.
36. M.J. Koudijs, M.J. den Broeder, A. Keijser, E. Wienholds, S. Houwing, E.M. van Rooijen, R. Geisler, and F.J. van Eeden, The zebrafish mutants dre, uki, and lep encode negative regulators of the hedgehog signaling pathway, PLoS Genet 1(2005) e19.
37. Y. Chen, N. Gallaher, R.H. Goodman, and S.M. Smolik, Protein kinase A directly regulates the activity and proteolysis of cubitus interruptus, Proc Natl Acad Sci U S A 95(1998) 2349-2354.
38. M.A. Price and D. Kalderon, Proteolysis of cubitus interruptus in Drosophila requires phosphorylation by protein kinase A, Development 126(1999) 4331-4339.
39. G. Wang, B. Wang, and J. Jiang, Protein kinase A antagonizes Hedgehog signaling by regulating both the activator and repressor forms of Cubitus interruptus, Genes Dev 13(1999) 2828-2837.

ACCEPTED MANUSCRIPT

40. H. Santos-Rosa, R. Schneider, A.J. Bannister, J. Sherriff, B.E. Bernstein, N.C. Emre, S.L. Schreiber, J. Mellor, and T. Kouzarides, Active genes are tri-methylated at K4 of histone H3, Nature 419(2002) 407-411.
41. N.D. Heintzman, R.K. Stuart, G. Hon, Y. Fu, C.W. Ching, R.D. Hawkins, L.O. Barrera, S. Van Calcar, C. Qu, K.A. Ching, W. Wang, Z. Weng, R.D. Green, G.E. Crawford, and B. Ren, Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome, Nature genetics 39(2007) 311-318.
42. A.W. Aday, L.J. Zhu, A. Lakshmanan, J. Wang, and N.D. Lawson, Identification of cis regulatory features in the embryonic zebrafish genome through large-scale profiling of H3K4me1 and H3K4me3 binding sites, Dev Biol 357(2011) 450-462.
43. D. Shlyueva, G. Stampfel, and A. Stark, Transcriptional enhancers: from properties to genome-wide predictions, Nature reviews. Genetics 15(2014) 272-286.
44. R. Houtmeyers, J. Souopgui, S. Tejpar, and R. Arkell, The ZIC gene family encodes multi-functional proteins essential for patterning and morphogenesis, Cellular and molecular life sciences : CMLS 70(2013) 3791-3811.
45. N.S. Zinkevich, D.V. Bosenko, B.A. Link, and E.V. Semina, laminin alpha 1 gene is essential for normal lens development in zebrafish, BMC Dev Biol 6(2006) 13.
46. E.V. Semina, D.V. Bosenko, N.C. Zinkevich, K.A. Soules, D.R. Hyde, T.S. Vihtelic, G.B. Willer, R.G. Gregg, and B.A. Link, Mutations in laminin alpha 1 result in complex, lens-independent ocular phenotypes in zebrafish, Dev Biol 299(2006) 63-77.
47. J.D. Paulus and M.C. Halloran, Zebrafish bashful/laminin-alpha 1 mutants exhibit multiple axon guidance defects, Dev Dyn 235(2006) 213-224.
48. N. Ichikawa-Tomikawa, J. Ogawa, V. Douet, Z. Xu, Y. Kamikubo, T. Sakurai, S. Kohsaka, H. Chiba, N. Hattori, Y. Yamada, and E. Arikawa-Hirasawa, Laminin alpha1 is essential for mouse cerebellar development, Matrix Biol 31(2012) 17-28.
49. M.M. Edwards, D.S. McLeod, R. Grebe, C. Heng, O. Lefebvre, and G.A. Lutty, Lama1 mutations lead to vitreoretinal blood vessel formation, persistence of fetal vasculature, and epiretinal membrane formation in mice, BMC Dev Biol 11(2011) 60.
50. C. Heng, O. Lefebvre, A. Klein, M.M. Edwards, P. Simon-Assmann, G. Orend, and D. Bagnard, Functional role of laminin alpha1 chain during cerebellum development, Cell Adh Migr 5(2011) 480-489.
51. K.A. Aldinger, S.J. Mosca, M. Tetreault, J.C. Dempsey, G.E. Ishak, T. Hartley, I.G. Phelps, R.E. Lamont, D.R. O'Day, D. Basel, K.W. Gripp, L. Baker, M.J. Stephan, F.P. Bernier, K.M. Boycott, J. Majewski, G. University of Washington Center for Mendelian, C. Care4Rare, J.S. Parboosingh, A.M. Innes, and D. Doherty, Mutations in LAMA1 cause cerebellar dysplasia and cysts with and without retinal dystrophy, American journal of human genetics 95(2014) 227-234.
52. N. Zagris, A.E. Chung, and V. Stavridis, Differential expression of laminin genes in early chick embryo, The International journal of developmental biology 44(2000) 815-818.
53. C.J. Snow and C.A. Henry, Dynamic formation of microenvironments at the myotendinous junction correlates with muscle fiber morphogenesis in zebrafish, Gene expression patterns : GEP 9(2009) 37-42.
54. T. Niimi, Y. Hayashi, K. Sekiguchi, and Y. Kitagawa, The Sp family of transcription factors regulates the human laminin alpha1 gene in JAR choriocarcinoma cells, Biochimica et biophysica acta 1759(2006) 573-579.

ACCEPTED MANUSCRIPT

55. S.A. Piccinni, A.L. Bolcato-Bellemin, A. Klein, V.W. Yang, M. Kedinger, P. Simon-Assmann, and O. Lefebvre, Kruppel-like factors regulate the Lama1 gene encoding the laminin alpha1 chain, The Journal of biological chemistry 279(2004) 9103-9114.
56. S. Yang, N. Oksenberg, S. Takayama, S.J. Heo, A. Poliakov, N. Ahituv, I. Dubchak, and D. Boffelli, Functionally conserved enhancers with divergent sequences in distant vertebrates, BMC genomics 16(2015) 882.
57. T. Niimi, Y. Hayashi, and K. Sekiguchi, Identification of an upstream enhancer in the mouse laminin alpha 1 gene defining its high level of expression in parietal endoderm cells, The Journal of biological chemistry 278(2003) 9332-9338.
58. T. Niimi, Y. Hayashi, S. Futaki, and K. Sekiguchi, SOX7 and SOX17 regulate the parietal endoderm-specific enhancer activity of mouse laminin alpha1 gene, The Journal of biological chemistry 279(2004) 38055-38061.
59. Y. Koyabu, K. Nakata, K. Mizugishi, J. Aruga, and K. Mikoshiba, Physical and functional interactions between Zic and Gli proteins, The Journal of biological chemistry 276(2001) 6889-6892.
60. K. Mizugishi, J. Aruga, K. Nakata, and K. Mikoshiba, Molecular properties of Zic proteins as transcriptional regulators and their relationship to GLI proteins, The Journal of biological chemistry 276(2001) 2180-2188.
61. C.L. Winata, I. Kondrychyn, V. Kumar, K.G. Srinivasan, Y. Orlov, A. Ravishankar, S. Prabhakar, L.W. Stanton, V. Korzh, and S. Mathavan, Genome wide analysis reveals Zic3 interaction with distal regulatory elements of stage specific developmental genes in zebrafish, PLoS Genet 9(2013) e1003852.
62. D.L. Drummond, C.S. Cheng, L.G. Selland, J.C. Hocking, L.B. Prichard, and A.J. Waskiewicz, The role of Zic transcription factors in regulating hindbrain retinoic acid signaling, BMC Dev Biol 13(2013) 31.
63. A.T. Garnett, T.A. Square, and D.M. Medeiros, BMP, Wnt and FGF signals are integrated through evolutionarily conserved enhancers to achieve robust expression of Pax3 and Zic genes at the zebrafish neural plate border, Development 139(2012) 4220-4231.
64. T. Inoue, M. Ota, K. Mikoshiba, and J. Aruga, Zic2 and Zic3 synergistically control neurulation and segmentation of paraxial mesoderm in mouse embryo, Dev Biol 306(2007) 669-684.
65. H. Pan, M.K. Gustafsson, J. Aruga, J.J. Tiedken, J.C. Chen, and C.P. Emerson, Jr., A role for Zic1 and Zic2 in Myf5 regulation and somite myogenesis, Dev Biol 351(2011) 120-127.
66. R. Sanges, E. Kalmar, P. Claudiani, M. D'Amato, F. Muller, and E. Stupka, Shuffling of cis-regulatory elements is a pervasive feature of the vertebrate lineage, Genome biology 7(2006) R56.
67. L.V. Goodrich, R.L. Johnson, L. Milenkovic, J.A. McMahon, and M.P. Scott, Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog, Genes Dev 10(1996) 301-312.
68. K.E. Lewis, J.P. Concordet, and P.W. Ingham, Characterisation of a second patched gene in the zebrafish Danio rerio and the differential response of patched genes to Hedgehog signalling, Dev Biol 208(1999) 14-29.
69. A.G. Borycki, B. Brunk, S. Tajbakhsh, M. Buckingham, C. Chiang, and C.P. Emerson, Jr., Sonic hedgehog controls epaxial muscle determination through Myf5 activation, Development 126(1999) 4053-4063.

ACCEPTED MANUSCRIPT

70. A.K. Maurya, H. Tan, M. Souren, X. Wang, J. Wittbrodt, and P.W. Ingham, Integration of Hedgehog and BMP signalling by the engrailed2a gene in the zebrafish myotome, Development 138(2011) 755-765.
71. C.B. Kimmel, W.W. Ballard, S.R. Kimmel, B. Ullmann, and T.F. Schilling, Stages of embryonic development of the zebrafish, Dev Dyn 203(1995) 253-310.
72. C. Thisse and B. Thisse, High-resolution in situ hybridization to whole-mount zebrafish embryos, Nat Protoc 3(2008) 59-69.
73. S. Coy, J.H. Caamano, J. Carvajal, M.L. Cleary, and A.G. Borycki, A novel Gli3 enhancer controls the Gli3 spatiotemporal expression pattern through a TALE homeodomain protein binding site, Molecular and cellular biology 31(2011) 1432-1443.

Figure legends

Figure 1: A BAC transgene recapitulates *lama1* expression during zebrafish development. (A) Genomic organization of the zebrafish *lama1* locus on chromosome 24. **(B-N)** GFP reporter expression in the stable *lama1:GFP* transgenic line compared to *lama1* expression at the 1-cell (B), 4-cell (C), 8-cell (D), 64-cell (E), 1000-cell (F), 75% epiboly (G), to 6-somite stage (H), 15-somite stage (I), and at 24 hpf (J-N). Panels K-N are high magnification of the transgenic fish shown in J as indicated by white boxes. In B,C, and F, antisense and sense probes for *lama1* are shown. **(O-T)** Confocal microscopy images of transient 24 hpf transgenic *lama1:GFP* zebrafish embryos analysed by immunofluorescence using antibodies against GFP and slow myosin heavy chain (F59, red) (O,P), fast myosin heavy chain (F310, red) (Q,R), and keratan sulphate (red) (S,T). White arrowheads points to the co-localization of GFP with the relevant antibody. **(U-Y)** Comparison of endogenous *lama1* (V,X) and *gfp* expression pattern in *lama1:GFP* zebrafish embryos (U,W,Y) at 24hpf and 48hpf. Note the transient maintenance of *gfp* transcripts in the paraxial mesoderm at 24hpf, which is down-regulated at 48hpf. All embryos are shown with anterior to the left, except in panels X and Y where anterior is up. ptprma: protein tyrosine phosphatase, receptor type Ma; lrcc30: Leucine-rich repeat-containing protein 30; kan: kanamycin; ba: branchial arches; bp: blood pool; CNS: central nervous system; fp: floor plate; mhb: midbrain-hindbrain boundary; mf: muscle fibre; nc: notochord; ov: otic vesicle; pf: pectoral fin; psm: pre-somitic mesoderm; som: somite; te: telecephalon; uro: uro-genital organs; Va/pn: vasculature/pronephros.

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Figure 2: A BAC deletion analysis identifies an intronic enhancer controlling reporter gene expression in skeletal muscles. (A) Schematic representation of the deletion constructs tested by transient transgenesis in the zebrafish. Exons are represented as red boxes, introns are represented as red lines, BAC sequences are shown in blue, GFP is shown as a green box. **(B)** Transient reporter assay to assess skeletal muscle-specific reporter gene expression. Data are shown for the 15-somite and 24hpf stages. Presence (+) or absence (-) of GFP-positive skeletal muscle cells is indicated. Number of GFP expressing fish compared to the total number of injected fish is shown in parentheses. * indicates very weak GFP and few positive fibres observed. **(C-K)** GFP expression in representative transient transgenic zebrafish embryos injected with the deletion constructs indicated. Embryos are shown at the 24 hpf stage except in (H), which shows a 19-somite stage embryo. Skeletal muscle expression is indicated by red arrows; notochord expression is indicated by white arrowheads; anterior CNS expression is indicated by yellow arrows. **(L-P)** GFP expression in the *Δ−0.8lama1:GFPΔ4497* stable zebrafish line at the 10-somite stage (L,M), 21hpf (N), and 24hpf (O,P). Embryos are shown with anterior to the left. ce: cerebellum; ha: heart anlage; me: mesencephalon; op: olfactory placodes; ot: optic tectum; rh: rhombocephalon; sm: slow muscles; so: somites; te: telencephalon; tg: tegmentum.

Figure 3: Hedgehog signalling is necessary for *lama1* expression. (A-D) Impaired *lama1* expression in Smoothened mutant zebrafish. *ptc1* (A,B) and *lama1* (C,D) expression was examined by in situ hybridization in 28hpf wild-type (A,C) and *smu*^{hi1640} mutant (B,D) zebrafish embryos. n= 10/10 (A), 6/6 (B), 362/362 (C), 48/111 (D). **(E-H)** Impaired *lama1* expression in cyclopamine-treated embryos. Control (ethanol) (E,G) and cyclopamine-treated (F,H) 25hpf embryos were analysed by in situ hybridization using probes against *ptc1* (E,F) and *lama1* (G,H). n= 8/8 (E), 12/12 (F), 31/31 (G), 21/23 (H). Insert in C,D,G, and H are high magnification images of the embryo tail. Black arrows indicated expression domains affected in *smu*^{hi1640} mutant and cyclopamine-treated embryos.

Figure 4: Hedgehog signalling is sufficient to up-regulate *lama1* expression. (A-H) *Lama1* expression is up-regulated in *ptc1*^{hu1062}*ptc2*^{lep} double mutant embryos. *ptc1* (A-D)

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and *lama1* (E-H) expression was examined by in situ hybridization in 15-somite stage (A,C,E,G) and 28hpf (B,D,F,H) wild-type and *ptc1*^{hu1062}*ptc2*^{lep} double mutant zebrafish embryos. n= 12/12 (A), 15/15 (B), 4/4 (C), 3/3 (D), 23/24 (E), 243/243 (F), 3/3 (G), 17/17 (H). **(I-P)** *Lama1* expression is upregulated in *dnPKA*-injected embryos. Control (PBS) (I,J,M,N) and *dnPKA*-injected (K,L,O,P) 28hpf embryos were analysed by in situ hybridization using probes against *ptc1* (I-L) and *lama1* (M-P). n= 31/31 (I), 19/19 (J), 22/22 (K), 44/44 (L), 45/52 (M), 45 (N), 33/41 (O), 42/53 (P). Insert in F,H,N, and P are high magnification images of the embryo tail. Black arrows indicated expression domains up-regulated in *ptc1*^{hu1062}*ptc2*^{lep} double mutant and dnPKA-injected embryos.

Figure 5: Hedgehog control of *lama1* expression is mediated by Zic/Gli binding sites. (A) Distribution of H3K4me1 and H3K4me3 methylation marks within the zebrafish *lama1* locus. **(B)** Schematic representation of the two stable transgenic lines generated. Note the presence of a region rich in H3K4me1 (in blue) at +2324-3283, which is comprised within the *Δ-0.8lama1:GFPΔ4497* transgene sequence and partially overlaps with the *Δ-0.8lama1:GFP2205-2922* transgene sequence. **(C)** Schematic representation of the *lama1* intronic sequence present in *Δ-0.8lama1:GFPΔ4497* showing the position of the enhancer identified at position +2205-2922 (brown), and the position of the H3K4me1 methylated region (blue). Putative binding sites for Zic and Gli transcription factors are indicated. **(D)** Sequence comparison of the three Zic/Gli binding sites identified (in red) with consensus sequence (cons). **(E)** Electromobility shift assay (EMSA) using radiolabelled oligonucleotides encompassing sites 1-3 and nuclear extract prepared from 24hpf zebrafish embryos. Black arrowheads indicate specific complexes that are competed in the presence of unlabelled wild-type oligonucleotides. Black stars indicate specific complexes that fail to be competed in the presence of mutated oligonucleotides.

Figure 6: Hedgehog signalling controls reporter gene expression from the *lama1:GFP* and *Δ-0.8lama1:GFPΔ4497* stable lines. (A-F) The *lama1:GFP* reporter line is Hedgehog-responsive. (A,B) *gfp* expression in 24hpf control (n=48/48, A) and cyclopamine-treated (n=44/54, B) embryos. (C-F) *gfp* expression in 15-somite stage and 24hpf control (n= 17/20, C; n=47/47, D;) and *dnPKA-injected* (n=6/7, E; n=63/82, F)

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embryos. **(G-L)** The *Δ-0.8lama1:GFPΔ4497* reported line is Hedgehog-responsive. (G,H) *gfp* expression in 24hpf control (n=17/17, G) and cyclopamine-treated (n=15/24, H) embryos. (I-L) *gfp* expression in 15-somite stage and 24hpf control (n=6/7, I; n= 8/9, J) and *dnPKA-injected* (n=11/12, K; n=7/11, L) embryos. Black arrows indicate expression domains affected by Hedgehog signalling.

Figure 7: Model for Hedgehog signalling coordination of muscle cell fate specification in the zebrafish embryo. (A) Schematic representation of the modular structure of intronic enhancers for *lama1*. **(B)** Summary of the modular transcriptional control of *lama1* expression pattern by intronic enhancers. **(C)** Model for the coordinated control of cell fate specification and Hedgehog response in the zebrafish paraxial mesoderm. Hedgehog signalling induces *lama1* transcription in the pre-somitic mesoderm (①), leading to the synthesis and deposition of laminin-111 in the somitic basement membrane (in green) associated with adaxial cells (②). Laminin-111 prevents BMP signalling and the accumulation of phospho-Smad, allowing Hedgehog-mediated induction of *eng2a* and the specification of muscle pioneer and medial fast muscle cells (red area in somites) (③).

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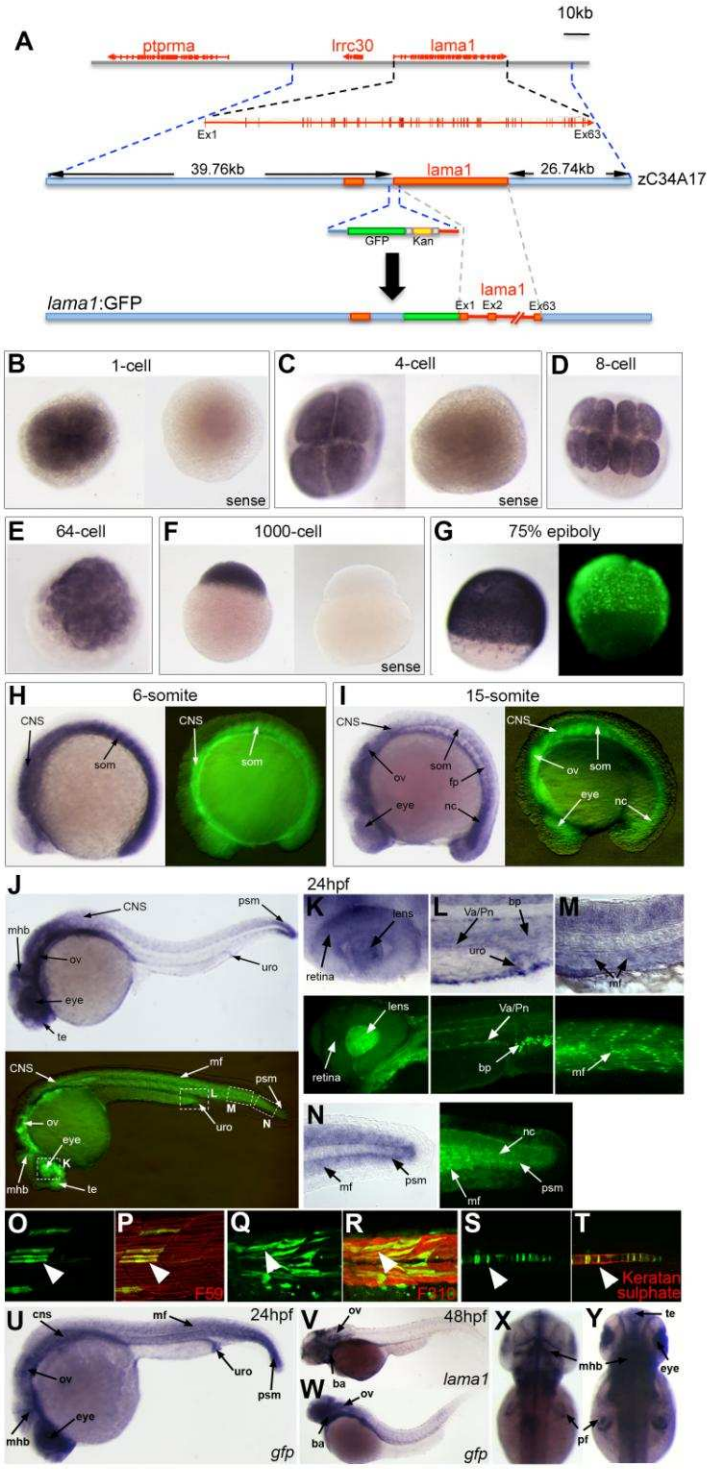

Fig. 1

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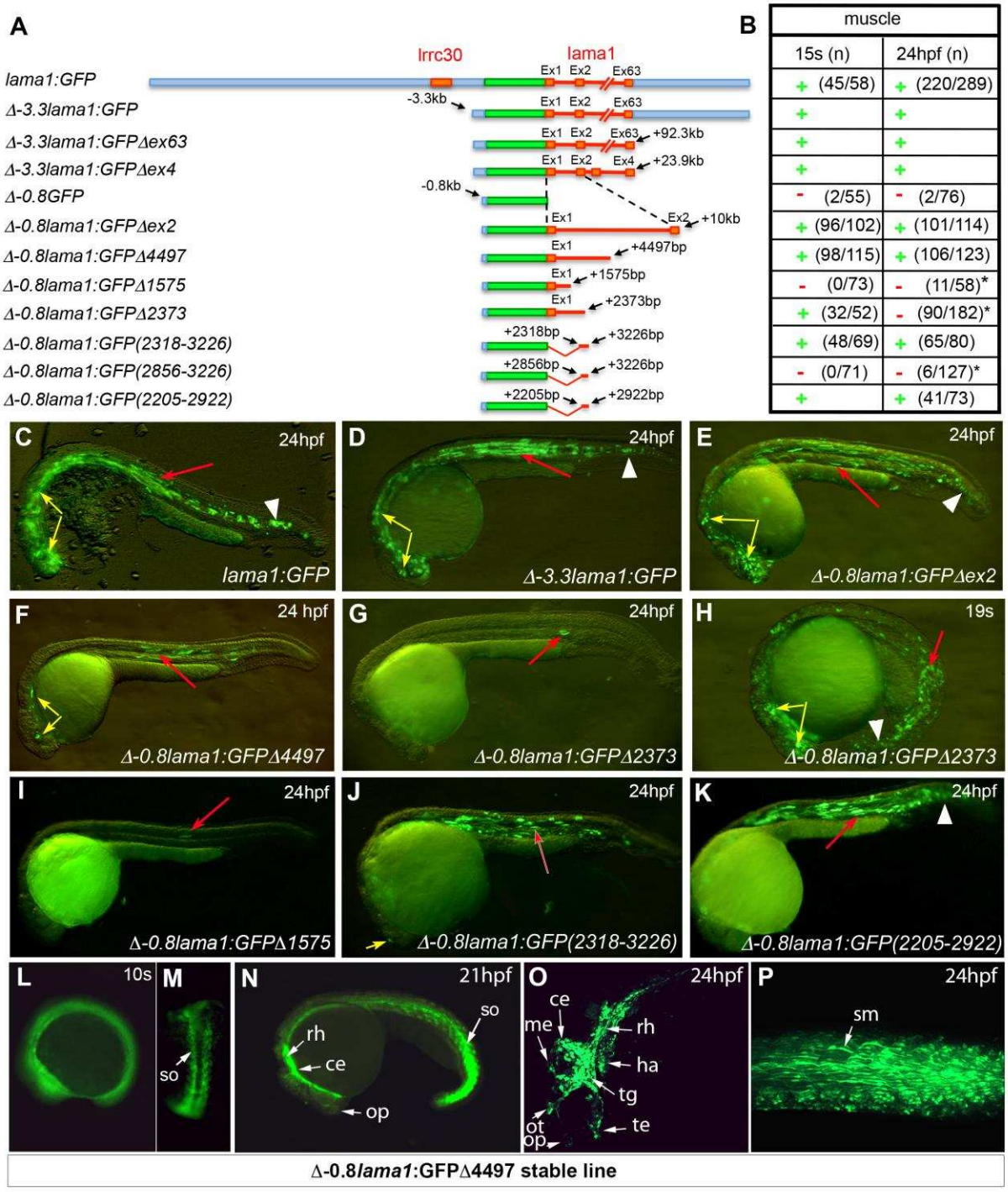

Fig. 2

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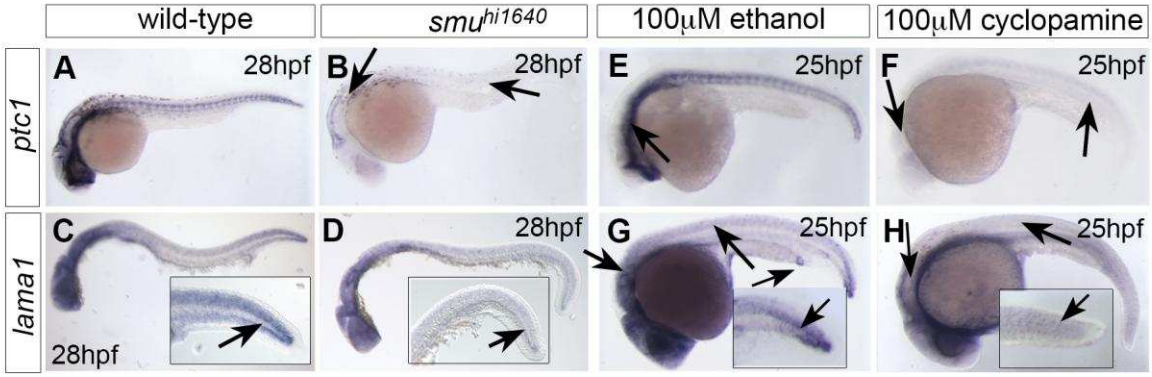

Figure 3

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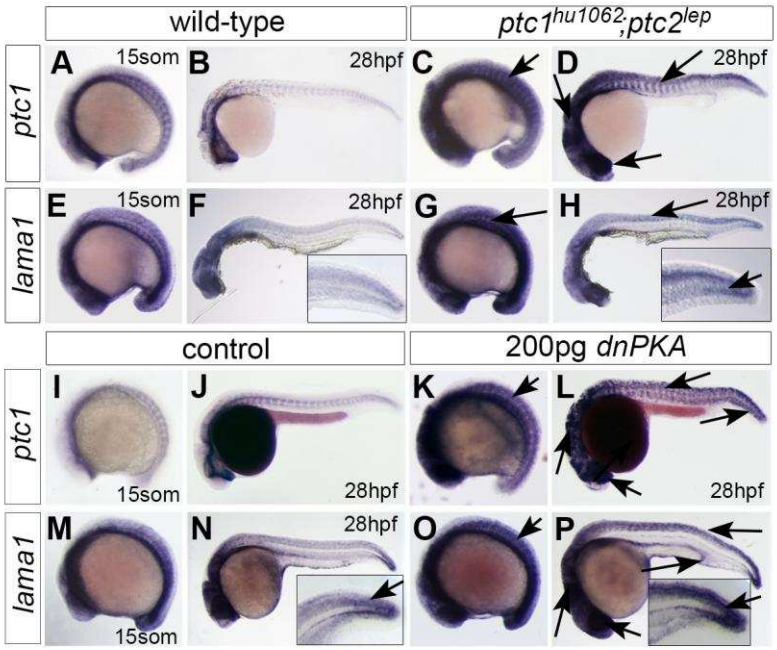

Figure 4

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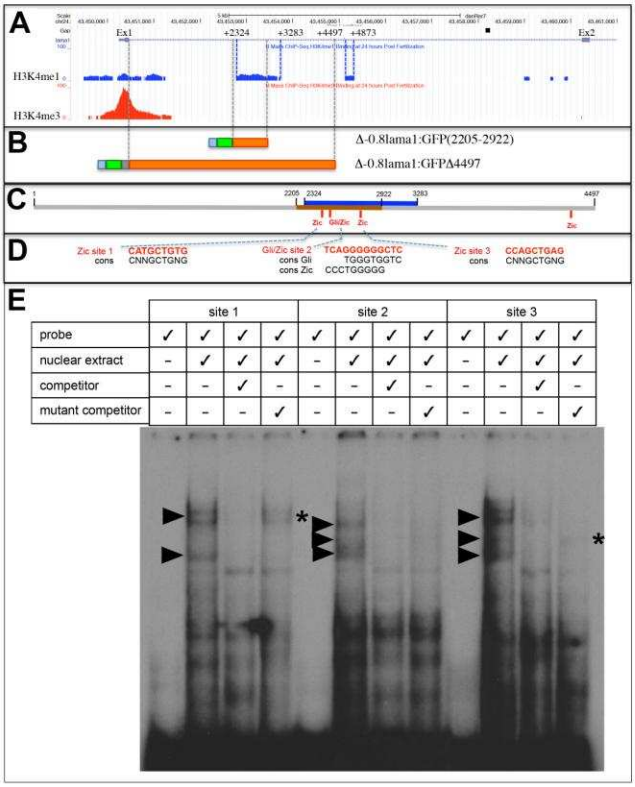

Fig. 5

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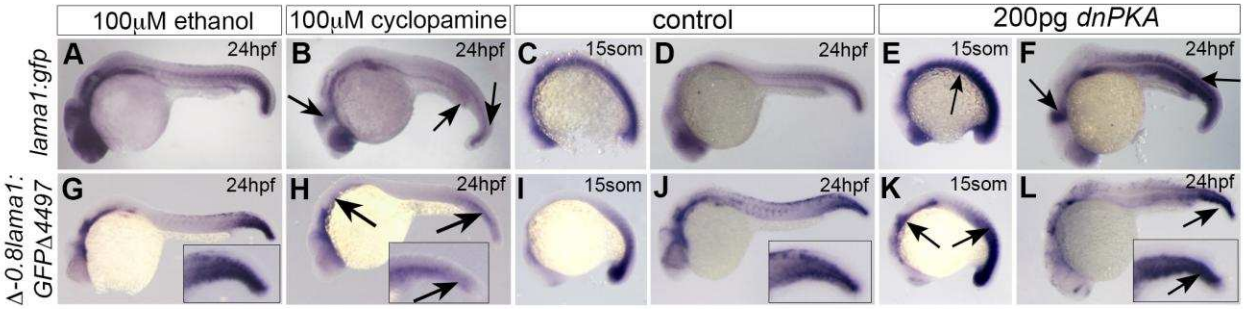

Fig. 6

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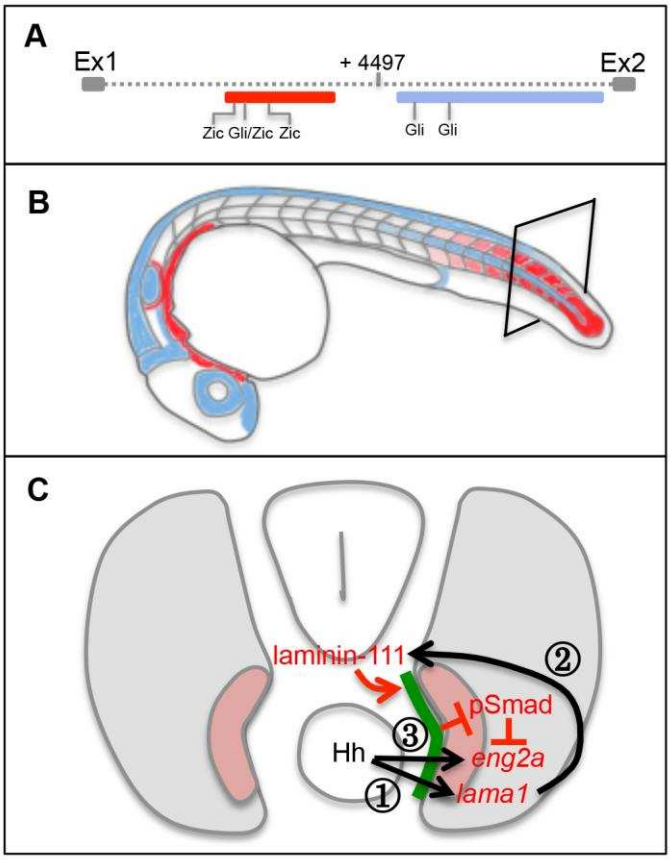

Figure 7

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Highlights

- A transgenic line, *lama1:GFP*, recapitulates the expression pattern of *lama1* in the zebrafish embryo.
- Hedgehog signalling is necessary and sufficient to drive *lama1* expression in the zebrafish paraxial mesoderm.
- An intronic enhancer controls *lama1* transcription in the paraxial mesoderm and anterior CNS.
- *lama1* intronic enhancer activity is regulated by Hedgehog signalling.

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