3.2: Results

3.2.1: *lama1* maternal transcripts are present in the zebrafish embryo

To test the hypothesis that zebrafish *lama1* is expressed in a conserved pattern, I used a DIG-labelled RNA probe to perform whole-mount in situ hybridisation experiments. I designed a probe that is 929 bases in length, consisting of 121 bases upstream of the 5'UTR, and 808 bases into the open reading frame. This region of DNA sequence does not contain homology with any other Laminin genes in the zebrafish, revealed using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

I first examined the expression pattern of *lama1* in wild-type zebrafish at the earliest stages of development. Previously, others have first detected *lama1* transcript expression from 3-8hpf, encompassing the 1000-cell stage to the 75% epiboly stage of gastrula, by in situ hybridisation (Pollard et al. 2006) and RT-PCR (Zinkevich et al. 2006). I observed that *lama1* is expressed throughout the blastoderm as maternal transcripts, as early as the two-cell stage (*Figure 3.1*A,C,E,F). This expression is specific, as shown by the absence of labelling when using a *lama1* sense control probe (*Figure 3.1*B,D). Zebrafish zygotic gene expression is initiated around 3hpf, correlating with the 1000-cell stage (Kane and Kimmel 1993; Aanes et al. 2011). At this stage, *lama1* is still expressed strongly and remains ubiquitous throughout the epiboly stages (*Figure 3.1*G,I,J,K) until the tail bud stage, at which point it starts to become restricted to the midline and the forming chordamesoderm and neural plate (*Figure 3.1*L,M). A *lama1* sense control probe reveals that this restriction of *lama1* expression is specific (*Figure 3.1*N).

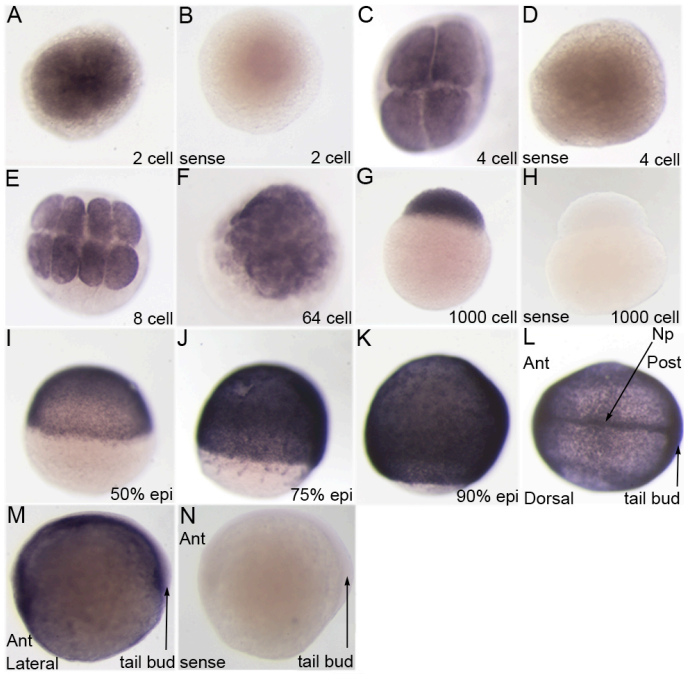

Figure 3.1: *Analysis of lama1 expression by whole-mount in situ hybridisation in the zebrafish embryo from the 2-cell stage to tail bud stage (10hpf) using a DIG-labelled lama1 antisense RNA probe (A,C,E,F,G,I-M) or control sense probe (B,D,H,N).* *lama1* is expressed from the 2-cell stage stage (A) as a maternal transcript. Expression is ubiquitous at the 4-cell stage (C), 8-cell stage (E), 64-cell stage (F), 1000-cell stage (G), 50% epiboly stage (I), 75% epiboly stage (J), and 90% epiboly stage (K). By the tail bud stage, *lama1* is restricted to the neural plate and tail bud (L,M). *lama1* staining is specific, as shown by the lack of staining in embryos assessed by whole-mount in situ hybridisation using a control *lama1* sense probe (B,D,H,N). Dorsal view (A,B,C,D,E,F,L) and lateral view (G,H,I,J,K,M,N) images taken at 100x magnification. Anterior of lateral and dorsal view images is to the left (L,M,N). Abbreviations: anterior (Ant), posterior (Post), neural plate (Np).

3.2.2: *lama1* is expressed in the PSM, paraxial mesoderm, chordamesoderm, and the forming anterior CNS during early segmentation stages

lama1 is expressed in the forming anterior CNS, eye, neural tube, somites and the pre-somitic mesoderm (PSM) in E9.5 mouse embryos. If *lama1* expression is conserved during evolution, I would expect to observe *lama1* expression in these tissues in the zebrafish embryo. Indeed, using in situ hybridisation, I observed that *lama1* is strongly expressed in the ventral neural tube and throughout the somites, PSM, and the notochord by the 6-somite stage (*Figure 3.2*A,Ai,B,C), but not throughout the anterior CNS. By the 9-somite stage, *lama1* is expressed more diffusely in the developing anterior CNS, but continues to be expressed throughout the somites, PSM, and the notochord (*Figure 3.2*D,Di,Dii). Within the neural tube, intense *lama1* expression marks the distinctive floor plate region (*Figure 3.2*Di). From the 12-somite stage onwards, *lama1* begins to be down-regulated within the somites, particularly the forming fast muscle domain, but is maintained in the adaxial cells adjacent to the notochord (*Figure 3.2*Ei). The notochord also displays a down-regulation of *lama1*, although *lama1* remains strongly expressed in the posterior notochord and PSM (*Figure 3.2*Eii). An increase in expression occurs in the anterior CNS and forming eye region (*Figure 3.2*E). This up-regulation continues at the 15-somite stage, when *lama1* is now also detected in the diencephalon and forming optic tectum of the forebrain, in addition to the expression within the forming eye and otic placode (*Figure 3.2*F). At the 15-somite stage, further down-regulation of *lama1* in the somites and notochord is observed,

although adaxial cells and the PSM maintain strong expression (*Figure 3.2*G,Fi). Floor plate expression of *lama1* is clearly visible at this stage (*Figure 3.2*F,Fi).

Altogether, these observations indicate that during the early somitic stages of zebrafish development, *lama1* expression is very dynamic. This suggests that precise temporal and spatial regulatory mechanisms operate to control *lama1* expression in the early zebrafish embryo.

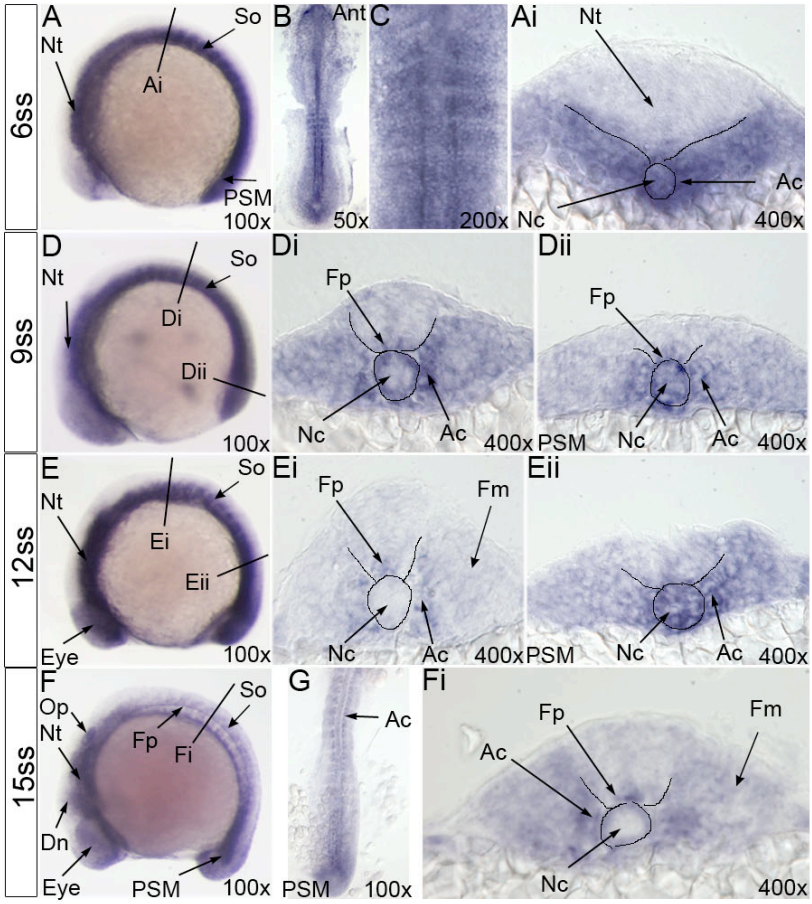

Figure 3.2: *Analysis of lama1 expression in the zebrafish embryo from the 6-somite stage to the 15-somite stage by whole-mount in situ hybridisation* (A-G). A: Lateral view of a 6-somite stage embryo, at 100x magnification. Ai: 15µm transverse cryosection of a 6-somite stage embryo, at 400x magnification. B and C: Dorsal flat-mount images of a 6-somite stage embryo at 50x and 200x magnification, respectively. *lama1* is expressed in the PSM (A,B), adaxial cells (Ai,B,C), and throughout the somite (Ai,C). Expression is also observed in the notochord and the forming neural tube, particularly within the floor plate (Ai). D: Lateral view of a 9-somite stage embryo, at 100x magnification. Di and Dii: 15µm transverse cryosections of a 9-somite stage embryo, at 400x magnification. At the 9-somite stage, expression expands in the anterior CNS (D) but begins to be down-regulated within the somites and notochord (Di), although it is maintained in the adaxial cells and the floor plate (Di,Dii). Notochord expression strongly persists at the level of the PSM (Dii). E: Lateral view of a 12-somite stage embryo, at 100x magnification. Ei and Eii: 15µm transverse cryosections of a 12-somite stage embryo, at 400x magnification. By the 12-somite stage there is a further down-regulation in the somite and notochord (Ei), although notochord *lama1* expression remains strong in posterior regions (Dii,Eii). Anterior CNS and eye expression is also up-regulated at the 12-somite stage (E). F: Lateral WISH of a 15-somite stage embryo, at 100x magnification. Fi: 15µm transverse cryosection of a 15-somite stage embryo, at 400x magnification. G: Dorsal flat-mount image of a 15-somite stage embryo at 100x magnification. *lama1* expression in the floor plate can be seen in lateral whole-mount images in 15-somite stage embryos, which have a reduced expression of *lama1* in the somites and the notochord (F,Fi). Expression remains in the adaxial cells (Fi,G). Expression is also observed in the otic placode and developing diencephalon and optic tectum (F). Anterior of whole-mount images is to the left. Positions of transverse cryosections along the antero-posterior axis are indicated by straight lines on the lateral whole-mount images. Abbreviations: anterior (Ant), neural tube (Nt), notochord (Nc), adaxial cell (Ac), floor plate (Fp), fast muscle (Fm), pre-somitic mesoderm (PSM), otic placode (Op), optic tectum (Ot), diencephalon (Dn).

3.2.3: *lama1* expression is down-regulated in maturing somites and up-regulated in the developing anterior CNS between the 19-somite stage and 28hpf

At day E9.5, the mouse shows expression of *Lama1* in the gut, pro-nephric tubules, and the uro-genital region (Miner et al. 2004; Anderson et al. 2009). In zebrafish, the gut-forming endodermal cells are located on the ventral side of the embryo, and are organised as a single stripe of cells by the 20-somite stage (Ober et al. 2003). After somitogenesis, these cells will form a rod-like structure, a process requiring cellular migration and cellular rearrangements (Horne-Badovinac et al. 2001). The pro-nephric tubules are also located on the ventral side of the embryo, and consist of two epithelial tubules on either side of the midline which will fuse and exit at the uro-genital region (Kimmel et al. 1995). The uro-genital region lies posterior to the yolk sac extension. In addition to the sites of expression already described, I hypothesised that *lama1* expression will also be conserved in the forming zebrafish gut, pro-nephric tubules, and the uro-genital region.

From the 19-somite stage, a complex pattern of specific *lama1* expression emerges, as revealed by in situ hybridisation, compared to a *lama1* sense control probe (*Figure 3.3*B). Continued down-regulation of somitic *lama1* mRNA occurs, and this happens to a greater extent within the more anterior mature somites (*Figure 3.3*A). This results in a posteriorised expression in the somites whereby weak somitic *lama1* expression is observed anteriorly and stronger expression is observed posteriorly (*Figure 3.3*A). Interestingly, the remaining *lama1* expression within the somite is now restricted to the adaxial cells and the slow muscle cells that will migrate out to the superficial surface of the somite (*Figure 3.3*Ai).

At the posterior of the embryo, strong expression of *lama1* is maintained in the PSM. *lama1* mRNA is also maintained throughout the anterior CNS, diencephalon, eye and otic vesicle (*Figure 3.3*A).

Expression in the notochord is now abolished, except for the most posterior notochord at the level of the PSM (*Figure 3.3*A). Co-inciding with the down-regulation in the notochord is the initiation of *lama1* in the hypochord, which underlies the notochord (*Figure 3.3*A,C). This is a transient endodermal derived rod-like structure, which plays a role in the patterning of the ventrally located dorsal aorta (Cleaver et al. 2000; Eriksson and Lofberg 2000). *lama1* transcripts in the hypochord and the floor plate of the neural tube are detected along the entire antero-posterior axis of the zebrafish (*Figure 3.3*A). This is particularly evident at the 23-somite stage (*Figure 3.3*C). *lama1* mRNA is not associated with the dorsal aorta, as observed using in situ hybridisation (*Figure 3.3*Ai). However, it is strongly expressed more ventrally in other domains of the vasculature system and in the pro-nephric tubules at the 19-23-somite stage (*Figure 3.3*A,Ai,C). Some of the expression characterised as the ventral vasculature region may also include endodermal cells that contribute to the formation of the gut. Indeed, the forming gut and pro-nephric tubules converge to the adjacent anal opening and uro-genital region, which also lie next to the blood island (Kimmel et al. 1995).

Therefore, at both the 19 and 23-somite stage, zebrafish embryos display high levels of *lama1* expression in the ventral vasculature, pro-nephric tubules and the uro-genital region.

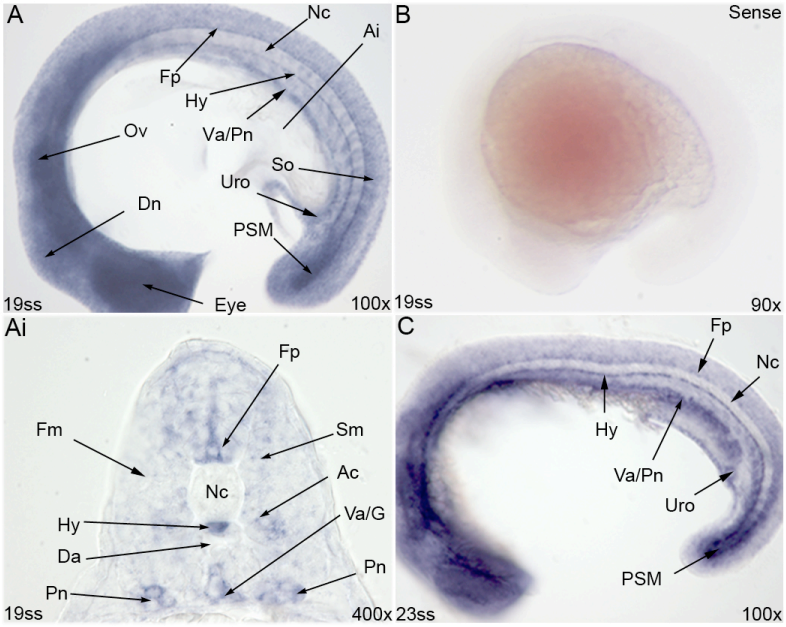

Figure 3.3: *Analysis of lama1 expression in the zebrafish embryo at the 19-somite (A,Ai) and 23-somite (C) stages by whole-mount in situ hybridisation*. *lama1* expression at the 19-somite stage in a whole-mount embryo at 100x magnification (A), and in a 15µm transverse cryosection at 400x magnification (Ai). *lama1* is specifically expressed in the PSM, posterior somites, uro-genital region, neural tube, notochord, hypochord, otic vesicles and the eye and anterior CNS (A,Ai). *lama1* is also detected in the ventral vasculature/forming gut region, and the pro-nephric tubules (Ai). In contrast, embryos hybridised with a *lama1* sense control probe lack staining (B). C: Lateral view of a 23-somite stage embryo at 100x magnification. *lama1* expression in somites is further down-regulated (C). Position of transverse cryosection along the antero-posterior axis is indicated by a straight line on the lateral whole-mount image (A). Anterior of whole-mount images is to the left. Abbreviations: notochord (Nc), floorplate (Fp), hypochord (Hy), otic vesicle (Ov), somite (So), diencephalon (Dn), pre-somitic mesdoderm (PSM), slow muscle (Sm), adaxial cell (Ac), fast muscle (Fm), dorsal aorta (Da), vasculature (Va), gut (G), pro-nephric tubules (Pn).

By 25hpf, *lama1* expression is weak within all the somites, although it is still clearly present within the PSM (*Figure 3.4*A,Aii,Aiii). By 28hpf however, there is a further reduction of *lama1* expression within the PSM (*Figure 3.4*D). Cross sections of 25hpf embryos maintained in staining solution reveal a lack of specific expression within the somitic compartments in the trunk and tail, although strong hypochord expression is detected in the posterior of the embryo (*Figure 3.4*Aiii). By 28hpf, expression of *lama1* is also down-regulated in the hypochord in an anterior to posterior direction (*Figure 3.4*D). This is in line with the timing of degeneration of the transient hypochord (Eriksson and Lofberg 2000). *lama1* expression is observed throughout the neural tube with an accumulation of *lama1* transcripts detected at the ventricular zone (*Figure 3.4*Aii). In addition, *lama1* mRNAs are observed in the lateral line organs that lie on the superficial surface of the somite (*Figure 3.4*Aii). At both 25hpf and 28hpf, *lama1* expression in the anterior CNS is maintained and can be detected in identifiable structures including the telencephalon, the epiphysis, the optic tectum, the cerebellum, the diencephalon, the eye and the otic vesicle (*Figure 3.4*A,Ai,C,D). Between 25hpf and 28hpf, *lama1* mRNA expression levels in the anterior CNS decrease as the expression becomes more defined and less widespread (*Figure 3.4*A,D). As a result, expression within the otic vesicle is more obvious. *lama1* mRNA is also maintained in the

uro-genital region (*Figure 3.4*A,D). *lama1* sense control probe embryos lack specific stain (*Figure 3.4*B,E).

Thus, after the completion of somitogenesis, the expression of *lama1* is rapidly down-regulated within the somites, and then decreased within the PSM. This is in line with the expression of *Lama1* in mouse muscle fibres, which only express *Lama1* during the embryonic stages of development (Miner et al. 1997; Patton et al. 1997).

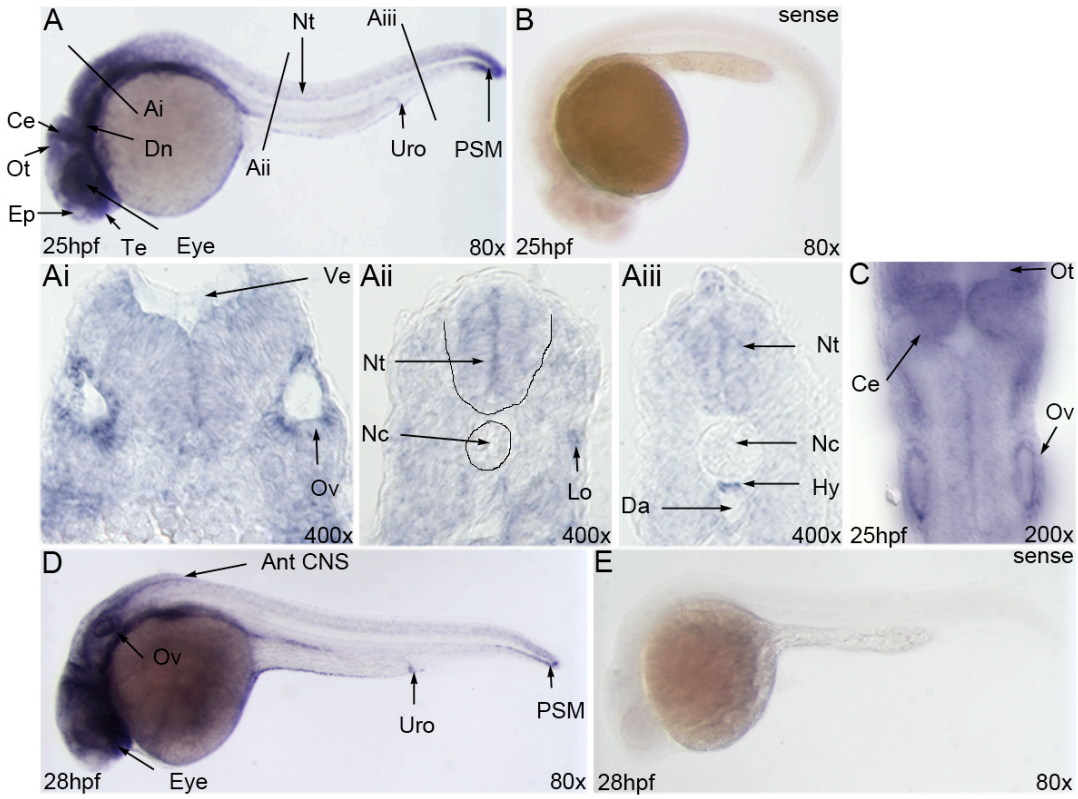

Figure 3.4: *Analysis of lama1 expression in the zebrafish embryo at 25hpf and 28hpf, by whole-mount in situ hybridisation using a DIG-labelled lama1 antisense RNA probe (A,Ai,Aii,Aiii,C,D) or control sense probe (B,E).* *lama1* expression in a 25hpf embryo assessed using an antisense RNA probe (A) or a sense RNA probe (B), at 80x magnification. *lama1* is expressed specifically and strongly in the eye, anterior CNS, PSM and the uro-genital region (A). Within the anterior CNS, expression is observed in the telecephalon, the epiphysis, the optic tectum and the cerebellum (A). In somites, *lama1* expression is greatly reduced (A). Ai, Aii, Aiii: 15µm transverse cryosections through a 25hpf embryo, at 400x magnification. *lama1* is expressed in the otic vesicles and the anterior CNS around the 4th ventricle of the brain (Ai). At the level of the trunk, *lama1* expression is observed in the neural tube and the lateral line organ, although it is reduced in the somitic compartments and the notochord (Aii). More posteriorly, *lama1* is expressed in the hypochord in addition to the neural tube (Aiii). C: Dorsal flat-mount of a 25hpf embryo at 200x magnification. *lama1* is strongly expressed in the otic vesicles and the optic tectum and cerebellum. *lama1* expression in a 28hpf embryo assessed using an antisense RNA probe (D) or a sense RNA probe (E), at 80x magnification. *lama1* is expressed in a similar pattern to that of a 25hpf embryo, with specific expression in the eye, anterior CNS, PSM and uro-genital region. *lama1* expression in the PSM has considerably reduced. Positions of transverse cryosections along the antero-posterior axis are indicated by straight lines on the lateral whole-mount image (A). Anterior of whole-mount images is to the left. Abbreviations: cerebellum (Ce), optic tectum (Ot), epiphysis (Ep), neural tube (Nt), diencephalon (Dn), uro-genital (Uro), pre-somitic mesoderm (PSM), 4th brain ventricle (Ve), otic vesicle (Ov), notochord (Nc), lateral line organ (Lo), hypochord (Hy), dorsal aorta (Da), anterior (Ant).

3.2.4: *lama1* expression in somites is extinguished by 49hpf, but is initiated in the pectoral fin and jaw muscles

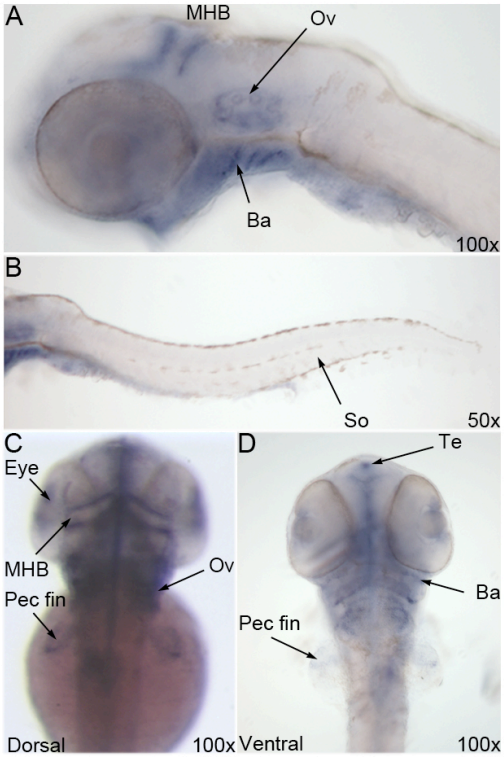

Figure 3.5: *Analysis of lama1 expression in the zebrafish embryo at 49hpf by whole-mount in situ hybridisation* (A-D). A: Lateral view of *lama1* expression in the head at 100x magnification. *lama1* expression is restricted to the midbrain-hindbrain boundary, the otic vesicle, and the forming branchial arches. B: Lateral view of the trunk and tail at 50x magnification. *lama1* expression in the somites and PSM is fully down-regulated. C: Dorsal view of the head and trunk at 100x magnification. Expression is observed in the otic vesicle and midbrain-hindbrain boundary. Expression is initiated in the pectoral fin. D: Ventral view of the head and trunk at 100x magnification. *lama1* is expressed in the branchial arches and a small patch in the telencephalon. Anterior of whole-mount lateral images is to the left. Abbreviations: midbrain-hindbrain boundary (MHB), otic vesicle (Ov), branchial arches (Ba), somite (So), telencephalon (Te), pectoral fin (Pf).

The decrease in *lama1* expression observed in the zebrafish PSM between the 25hpf and 28hpf stages suggests that *lama1* mRNA may be absent from the PSM by 49hpf. I hypothesised that by the 49hpf stage, remaining expression will be within the anterior CNS and eye. Within the anterior CNS, it is likely that the expression levels will be further decreased and restricted to specific organs, namely the eye and otic vesicle. This hypothesis is supported by data showing that *lama1* is required for normal eye development in the mouse and zebrafish (Falk et al. 1999; Libby et al. 2000; Semina et al. 2006). I also hypothesised that *lama1* expression will be initiated within the forming pectoral fins, analogous to the expression detected in the mouse limb bud at E11.5 (unpublished data, Kalin Narov thesis).

By 49hpf, there has been a dramatic down-regulation of *lama1* expression in the anterior CNS, and no expression is detected in the somites, tail or neural tube at this stage (*Figure 3.5*A,B). In the anterior CNS, expression remains at the midbrain-hindbrain boundary, otic vesicle, telencephalon, and very weakly in the eye (*Figure 3.5*A,C,D). As predicted, expression is initiated in the forming pectoral fin, but also in the forming branchial arches, which will later contribute to the musculature of the jaw (*Figure 3.5*A,C,D). These later stages of zebrafish development therefore also show conservation of *lama1* expression relative to the mouse.

At 74hpf, *lama1* expression displays a similar pattern to that observed at 49hpf, with *lama1* mRNA detected at the midbrain-hindbrain boundary, the otic vesicle, the pectoral fins, weak expression in the eye, and the muscles that are derived from the branchial arches (*Figure 3.6*A,B,E,F). More specifically, I observed *lama1* expression in the dorsal pharyngeal wall, adductor operculi, levator operculi, and dilator operculi muscles of the jaw (*Figure 3.6*B). *lama1* is also detected in the heart at 72hpf (*Figure 3.6*E). Expression in the otic vesicle is lost by the 97hpf stage, although pectoral fin and weak midbrain-hindbrain boundary and jaw muscle expression remains (*Figure 3.6*C,D,G).

Figure 3.6: *Analysis of lama1 expression in 74hpf and 97hpf zebrafish embryos by whole-mount in situ hybridisation.* A-E: Lateral views of 72hpf (E), 74hpf (A,B) and 97hpf (C,D) embryos at 50x (A,C) and 100x (B,D,E) magnification. *lama1* expression is absent from the somites (A,C) but is maintained in the midbrain-hindbrain boundary and jaw muscles at both stages (A,B,D,E). At 72hpf, *lama1* is also detected in the otic vesicles, (B) but is down-regulated in the otic vesicles by 97hpf (D). Expression is also observed in the heart (E). F and G: Dorsal views at 74hpf (F) and 97hpf (G) at 100x magnification. *lama1* expression is maintained in the pectoral fins at both stages of development. Anterior of lateral and dorsal views is to the left or top, respectively. Abbreviations: midbrain-hindbrain boundary (MHB), otic vesicle (Ov), dorsal pharyngeal wall (Dpw), adductor operculi (Ao), levator operculi (Lo), dilator operculi (Do), heart (He), pectoral fin (Pf).

3.2.5: A role for Hh in the regulation of *lama1* expression

The expression pattern of *lama1* in the zebrafish closely resembles that of the mouse, with the main sites of expression including somites, PSM, neural tube, anterior CNS, eye, otic vesicle, pro-nephric ducts and uro-genital region, and the pectoral fins, analogous to the mouse limb buds. This suggests that conserved mechanisms are likely to operate to regulate the initiation and maintenance of *lama1* expression in these tissues. Previous investigations into the regulation of *Lama1* expression have been performed in vitro, using F9 embryonal carcinoma cells and embryoid bodies (Wang et al. 1985; Aumailley et al. 2000). These have identified RA, FGF, TGF-β, and Integrin β1 signalling pathways that are capable of up-regulating *Lama1* expression (Wang et al. 1985; Aumailley et al. 2000; Li et al. 2001; Li et al. 2002). As I mentioned already, previous work in the lab has demonstrated that Shh signalling is required for *Lama1* expression in the somites and neural tube, but not the PSM, in E9.5 mouse embryos (Anderson et al. 2009). This raises the interesting possibility that somitic and neural tube expression of *lama1* in the zebrafish is also regulated by Shh. Thus, I hypothesised that *lama1* expression in the zebrafish somites and neural tube is regulated by Shh signalling. Supporting this, these tissues in the zebrafish are already known to be associated with Shh expression and signalling, as are most of the other sites of *lama1* expression in the zebrafish.

To test this hypothesis, I have examined the expression pattern of *lama1* by in situ hybridisation in zebrafish embryos with mutations in Smoothened (*smu* zebrafish), or in zebrafish embryos that have been treated with cyclopamine, an inhibitor of Smoothened. 100μM of cyclopamine was applied to the culture medium of 80% epiboly zebrafish embryos and embryos were maintained in this medium until their harvest. This concentration of cyclopamine fully down-regulates the Hh signalling pathway, as assessed by *ptc1* transcript levels (Wolff et al. 2003). As both *smu* zebrafish and cyclopamine-treated zebrafish are unable to activate the Hh signalling pathway (Wolff et al. 2003), I should expect a down-regulation or loss of *lama1* expression in the somites and neural tube if *lama1* is controlled by Hh signalling in the zebrafish. I have also assessed the expression pattern of *lama1* in zebrafish embryos with mutations in Patched1 and Patched2 (*ptc1/2*) (Koudijs et al. 2008), and in zebrafish embryos injected with dominant negative PKA (dnPKA) mRNA at the one-cell stage (Hammerschmidt et al. 1996). Both approaches are known to result in an up-regulation of Hh signalling (Hammerschmidt et al. 1996; Koudijs et al. 2008). An up-regulation of *lama1* expression in the somites and neural tube of *ptc1/2* or dnPKA zebrafish embryos is predicted if Hh signalling regulates *lama1* expression. To ascertain that the Hh signalling pathway is affected as predicted in these zebrafish embryos, I have used a DIG-labelled RNA probe to assess the expression of the Hh-target gene *ptc1* (*Figures 3.7* and *3.16*) (Wolff et al. 2003).

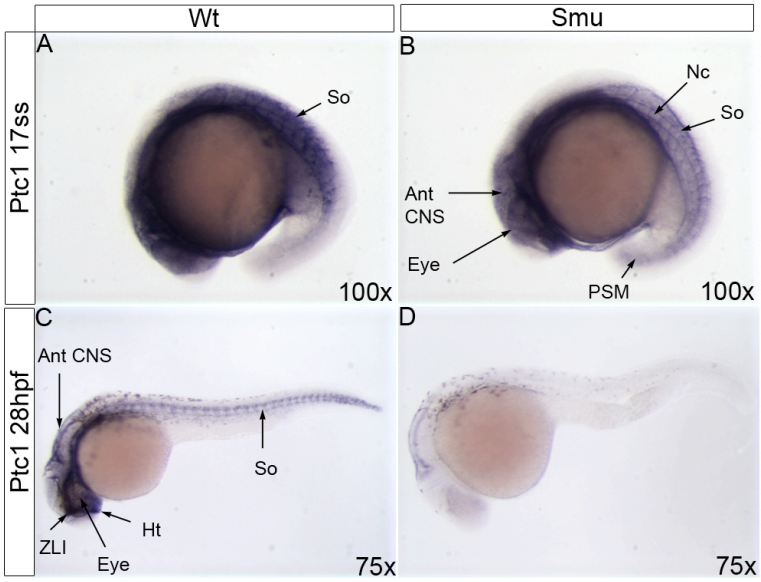

Figure 3.7: *Analysis of ptc1 expression in smu zebrafish embryos at the 17-somite stage and at 28hpf by whole-mount in situ hybridisation* (A,D). *ptc1* expression in a 17-somite stage wild-type embryo (A) and a *smu* embryo (B) at 100x magnification. *ptc1* expression is reduced in *smu* embryos compared to wild-type embryos. *ptc1* expression in a 28hpf wild-type (C) and *smu* embryo (D) at 75x magnification. By 28hpf, *ptc1* expression is nearly abolished in *smu* embryos compared to wild-type embryos (C,D). Anterior is to the left. Abbreviations: somite (So), pre-somitic mesoderm (PSM), zona limitans intrathalamica (ZLI), hypothalamus (Ht).

3.2.5.1: Hh signalling is not required for early *lama1* expression

17% (9/52) of 17-somite stage embryos obtained from a $smu^{+/-}$ cross display reduced *ptc1* expression in the somites compared to wild-type embryos (*Figure 3.7*A,B). In agreement with mendelian rule, these embryos are predicted to be *smu* embryos. In *smu* zebrafish embryos, some *ptc1* expression is retained within the anterior CNS, somites and PSM (*Figure 3.7*B), suggesting that residual Hh signalling may still be occurring at this stage of development. *lama1* expression remains unchanged in 15-somite stage *smu* zebrafish embryos (*Table 3.1*), and strong expression is detected in all previously characterised *lama1* expressing tissues such as the somites, PSM and the anterior CNS (*Figure 3.8*B). Earlier identification of *smu* zebrafish is not possible without genotyping, as the U-shaped somite phenotype is not obvious before this stage. However, no change in *lama1* expression was observed in any of the embryos obtained from crossing heterozygous *smu* zebrafish at the 6-somite stage and 12-somite stages (*Table 3.1*).

To confirm this finding, I carried out cyclopamine treatments and analysed *lama1* expression in embryos deficient in Hh signalling. In cyclopamine-treated zebrafish, *ptc1* expression is reduced in the forming anterior CNS, the PSM, and in somites (n = 7/12) at the 12-somite stage (*Figures 3.*9A,B and *3.10*). However, as in *smu* embryos, *ptc1* expression is not extinguished, and is normal in 42% of embryos (n = 12) (*Figure 3.10*). There is no difference in *lama1* expression in cyclopamine-treated zebrafish (*Figure 3.9*D,Di) at the 12-somite stage, compared to EtOH-treated control zebrafish (*Figures 3.9*C,Ci and *3.10*). Normal *lama1* expression is observed in 85% and 86% of EtOH-treated and cyclopamine-treated embryos, respectively. The small proportion of embryos that show a down-regulation of *lama1* expression upon cyclopamine treatment is

therefore likely due to natural variation in *lama1* expression, or due to the physical process during in situ hybridisation.

Stage of development	Total number of embryos analysed	Number of embryos with slight reduction of expression
6-somite	101	0
12-somite	59	0
15-17-somite	60	6

Table 3.1: The number of zebrafish embryos obtained from a cross between heterozygous *smu* embryos that display a slight reduction in *lama1* expression. Statistically, 25% of embryos are expected to be homozygote *smu* embryos.

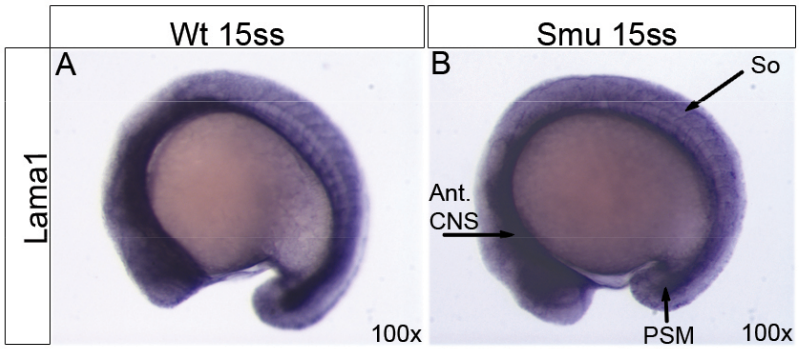

Figure 3.8: *Analysis of lama1 expression in smu zebrafish embryos at the 15-somite stage by whole-mount in situ hybridisation*. *lama1* expression in a 15-somite stage wild-type embryo (A) and a *smu* embryo (B), at 100x magnification. *lama1* expression is unaffected in *smu* zebrafish. Strong expression remains in the anterior CNS, somites and the PSM. Anterior is to the left. Abbreviations: somite (So), pre-somitic mesoderm (PSM).

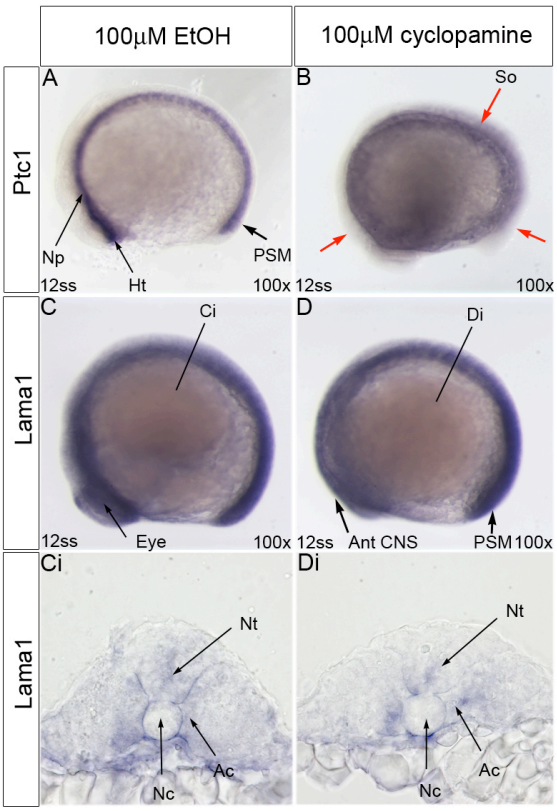

Figure 3.9: *Analysis of lama1 expression in 12-somite stage cyclopamine-treated zebrafish embryos by whole-mount in situ hybridisation (WISH).* 80% epiboly embryos were treated with 100µM cyclopamine (B,D,Di) or EtOH (A,C,Ci). Embryos were then analysed by WISH using a *ptc1* probe (A,B) or a *lama1* probe (C,D), 100x magnification. A slight reduction in *ptc1* expression occurs in cyclopamine-treated embryos (n= 7/12) (B) compared to EtOH-treated embryos (A), marked by red arrows. Ci and Di: 15µm transverse cryosections taken from the embryos in panels C and D respectively, at 400x magnification. Little difference in *lama1* expression is observed between EtOH (C,Ci) and cyclopamine-treated (D,Di) embryos. Expression persists in the anterior CNS, eye, PSM, adaxial cells of the somite, and the neural tube. Positions of transverse cryosections along the antero-posterior axis are indicated by straight lines on the lateral whole-mount images (C,D). Anterior is to the left. Abbreviations: neural plate (Np), hypothalamus (Ht), pre-somitic mesoderm (PSM), somite (So), notochord (Nc), neural tube (Nt), adaxial cell (Ac).

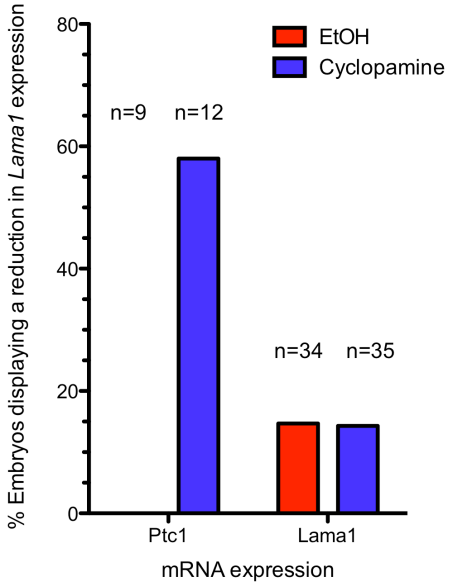

Figure 3.10: *Quantification of lama1 expression in cyclopamine-treated embryos at the 12-somite stage.* *ptc1* expression is slightly reduced in 58% of cyclopamine-treated (blue) embryos but not in ethanol-treated (EtOH) embryos (red). *lama1* expression was equally reduced (14% and 15%, respectively) in cyclopamine-treated and EtOH-treated embryos. n is the number of embryos analysed. Error bars are not shown because the experiment was only performed once.

3.2.5.1.1: Hh signalling is partially required for *lama1* expression in the PSM in 24hpf zebrafish

Analysis performed at later developmental stages reveals specific defects in *lama1* expression in the absence of Hh signalling. Indeed, a significant down-regulation of *lama1* expression is observed in the PSM (*** p-value = <0.0001) (43% of embryos, n = 111), and a more moderate effect is observed within the uro-genital region (19% of embryos, n = 47, p-value = ns) of *smu* zebrafish (*Figures 3.11*B, *3.12*D,F and *3.14*) and cyclopamine-treated zebrafish (91% embryos with PSM and uro-genital reduction) (*Figures 3.13*D and *3.15*). Cyclopamine-treated zebrafish have also a slight down-regulation of *lama1* expression in the anterior CNS (*Figures 3.13*D and *3.15*). However, no significant difference was observed in *smu* zebrafish (*Figure 3.14*).

By comparison, *ptc1* expression is almost abolished from the somites, neural tube and anterior CNS in 100% of *smu* zebrafish (n = 6) (*Figure 3.7*D) and 100% of cyclopamine-treated zebrafish (n = 12) (*Figures 3.13*B and *3.15*), indicating that by 25-28hpf all Hh signalling is abolished. Despite this, *lama1* expression is maintained in the neural tube and somites (97% of embryos, n = 39) of *smu* zebrafish (*Figures 3.12*D,Di,Dii,E and *3.14*) and cyclopamine-treated embryos (91% of embryos, n = 23) (*Figures 3.13*D and *3.15*). *lama1* expression is also maintained in the eye, otic vesicle and the hypochord (*Figures 3.11*B and *3.12*D,Di,Dii).

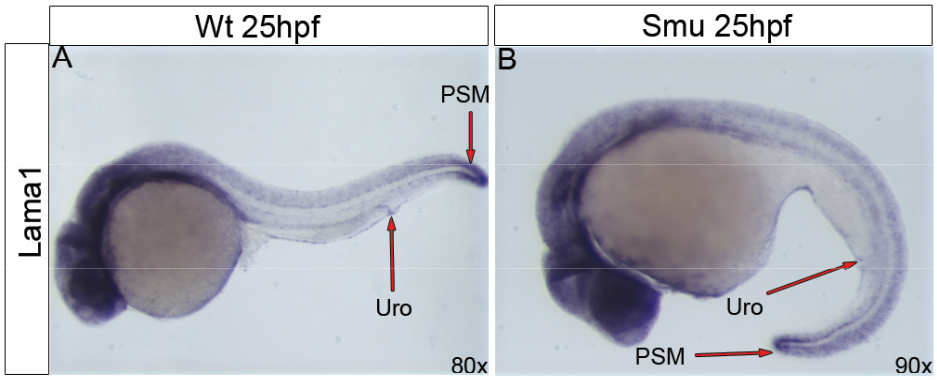

Figure 3.11: *Analysis of lama1 expression in smu zebrafish embryos at 25hpf by whole-mount in situ hybridisation (WISH). lama1* expression in a 25hpf wild-type embryo (A) and a *smu* embryo (B) at 80x magnification. *lama1* expression is reduced in the PSM and uro-genital region of *smu* embryos, marked by red arrows. Strong expression remains in the eye and anterior CNS. There is no detectable difference in *lama1* expression in the somites or neural tube. Anterior is to the left. Abbreviations: uro-genital region (Uro), pre-somitic mesoderm (PSM).

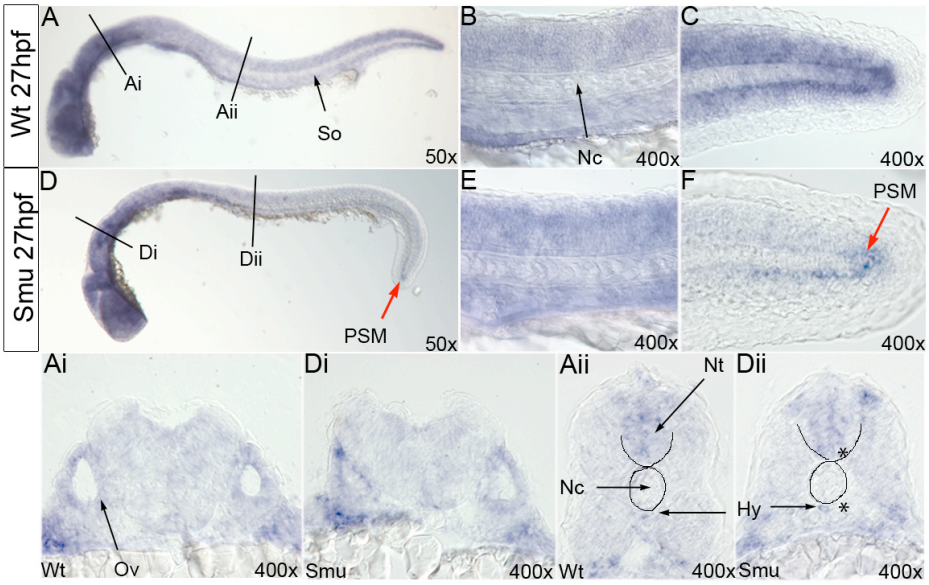

Figure 3.12: *Analysis of lama1 expression in 27hpf wild-type (Wt A-C,Ai,Aii) and smoothened mutant (smu D-F,Di,Dii) zebrafish embryos by whole-mount in situ hybridisation*. Lateral views of a wild-type embryo at 50x magnification (A) and 400x magnification (B,C). Lateral views of *smu* embryo at 50x magnification (D) and 400x magnification (E,F). There is no change in *lama1* expression in somites between wild-type and *smu* zebrafish embryos (A,B,D,E). However, *lama1* is down-regulated in the PSM of the *smu* zebrafish (indicated by red arrows) (C,F). Ai,Aii,Di,Dii are 15µm transverse cryosections at 400x magnification, and their positions along the antero-posterior axis are indicated by straight lines on the lateral whole-mount images (A,D). *lama1* expression in the otic vesicles and anterior CNS is similar between wild-type (Ai) and smu (Di) embryos. As in the wild-type (Aii), *lama1* expression also persists in the neural tube and hypochord of the *smu* zebrafish embryo (Dii), marked by asterisks. Anterior of whole-mount images is to the left. Abbreviations: somite (So), notochord (Nc), pre-somitic mesoderm (PSM), otic vesicle (Ov), neural tube (Nt), hypochord (Hy).

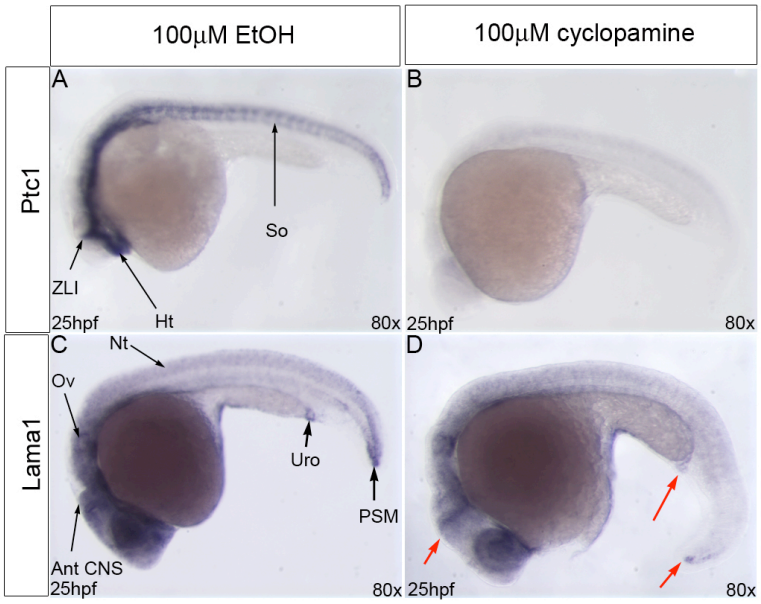

Figure 3.13: *Analysis of lama1 expression in 25hpf cyclopamine-treated zebrafish embryos by whole-mount in situ hybridisation*. 80% epiboly embryos were treated with 100µM cyclopamine (B,D) or EtOH (A,C) and analysed by whole-mount in situ hybridisation using a *ptc1* probe (A,B) and a *lama1* probe (C,D). Magnification is 80x. *ptc1* expression is down-regulated or abolished in cyclopamine-treated embryos (B), compared to EtOH-treated embryos (A). In cyclopamine-treated embryos, *lama1* expression is down-regulated in the PSM and the uro-genital region, and slightly decreased in the anterior CNS (indicated by red arrows) (D). However, *lama1* expression is unchanged in the neural tube and the somite (D), compared to EtOH-treated embryos (C). Anterior is to the left. Abbreviations: zona limitans intrathalamica (ZLI), hypothalamus (Ht), somite (So), otic vesicle (Ov), neural tube (Nt), uro-genital (Uro), pre-somitic mesoderm (PSM).

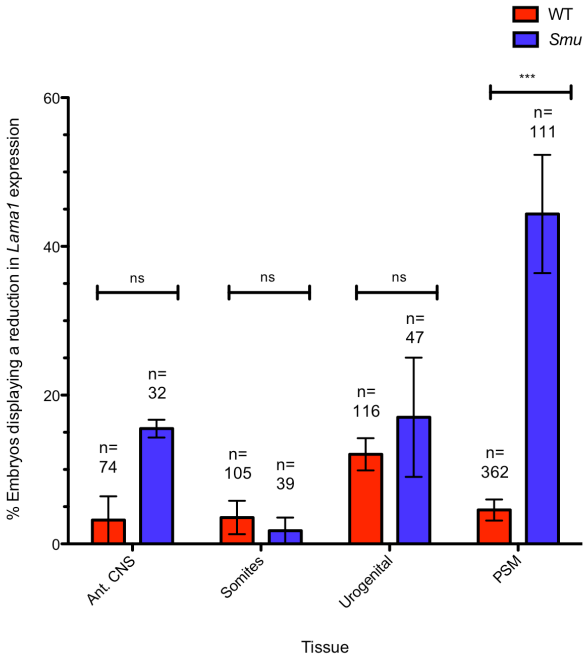

Figure 3.14: *Expression of lama1 mRNA in wild-type (WT) vs smu embryos at 25hpf.* The expression of *lama1* is significantly reduced in the PSM of *smu* zebrafish (blue) compared to WT zebrafish (red). The anterior CNS and uro-genital tissues also display some reduction in *lama1* expression, although this is not significant. *Smu* mutant embryos do not down-regulate *lama1* mRNA in the somites. An un-paired, two-tailed t-test reveals a very significant p-value (***) of <0.0001 for the reduction in PSM *lama1* expression. n is the number of embryos analysed. ns = not significant.

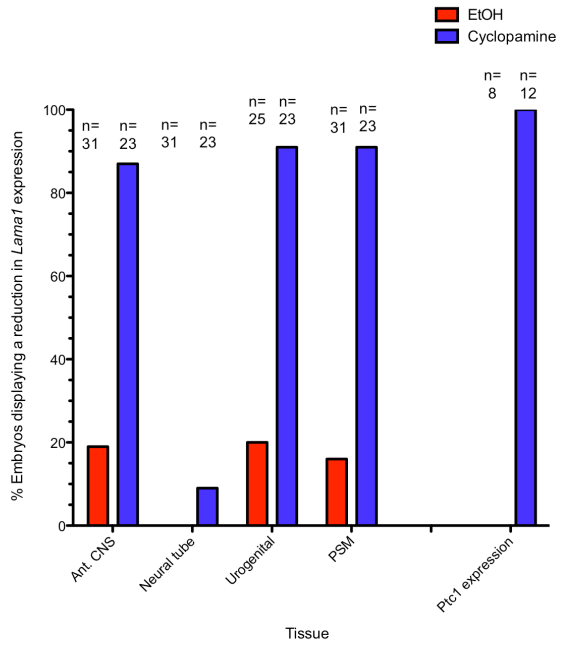

Figure 3.15: *Expression of ptc1 and lama1 mRNA in 25hpf cyclopamine-treated embryos*. *ptc1* expression is maintained in 100% of ethanol-treated embryos (EtOH) (red), but abolished in 100% of cyclopamine-treated embryos (blue), revealing an efficient shut down of the Hh signalling pathway. The expression of *lama1* is reduced in the anterior CNS, uro-genital region and PSM in 87%, 91% and 91% of embryos, respectively, that have been treated with 100µM cyclopamine. Error bars are not shown because the experiment was only performed once. n is the number of embryos analysed.

3.2.5.2: Hh signalling is sufficient for *lama1* expression in the somites, neural tube, and PSM

To assess whether Hh signalling is sufficient to induce or maintain *lama1* expression, I performed gain-of-function experiments using *ptc1/2* mutant embryos and dnPKA mRNA-injected embryos. To begin with, I tested the validity of my gain-of-function models through the analysis of *ptc1* expression. At both the 15-somite (n = 4/4 *ptc1/2* embryos, n = 22/22 dnPKA mRNA injected embryos) and 28hpf (n = 3/3 *ptc1/2* embryos, n = 44/44 dnPKA mRNA injected embryos) stages, a significant increase in *ptc1* expression is observed throughout *ptc1/2* mutant embryos (*Figure 3.16*B,D) and dnPKA mRNA-injected embryos (*Figures 3.18*B, *3.20*B, *3.22*) compared to wild-type embryos (*Figures 3.16*A,C, *3.18*A, *3.20*A, *3.22*A). In particular, somites, anterior CNS and the neural tube display strong *ptc1* expression, indicating that Hh signalling is up-regulated in these tissues.

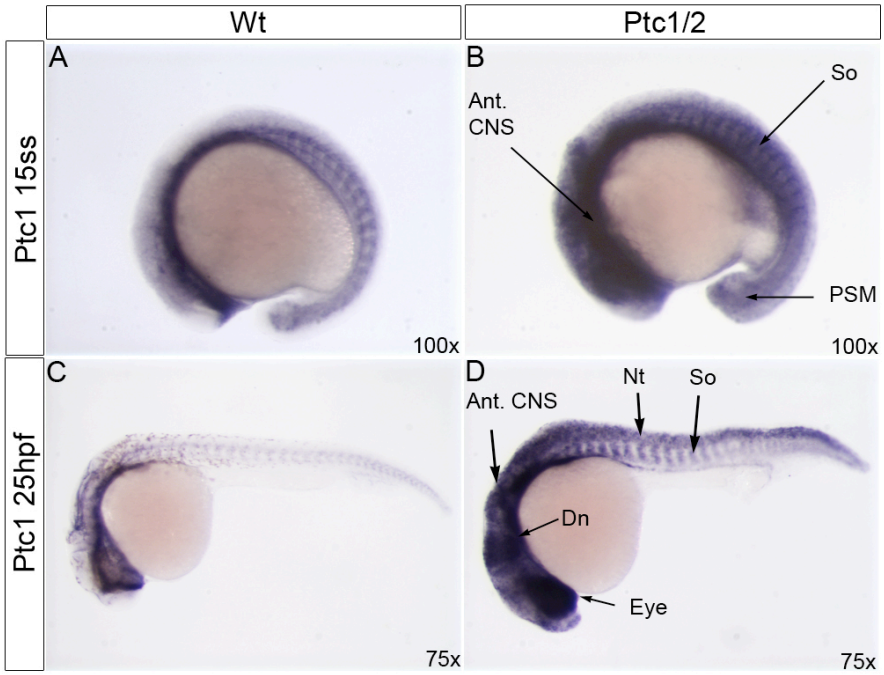

Figure 3.16: *Analysis of ptc1 expression in 15-somite and 25hpf stage ptc1/2 mutant zebrafish by whole-mount in situ hybridisation*. Lateral view of a 15-somite stage wild-type (A) and *ptc1/2* (B) embryo, using a *ptc1* probe, at 100x magnification. There is a strong increase in *ptc1* expression in the somites, anterior CNS, PSM and the neural tube in *ptc1/2* embryos. Lateral view of a 25hpf wild-type (C) and *ptc1/2* (D) embryo, using a *ptc1* probe, at 75x magnification. Increased *ptc1* expression in the *ptc1/2* mutant embryos is more pronounced at 25hp compared to the 15-somite stage, and is observed in the anterior CNS and diencephalon, eye, neural tube, somites and the PSM. Anterior is to the left. Abbreviations: somite (So), pre-somitic mesoderm (PSM), neural tube (Nt), diencephalon (Dn).

Similarly, I observed that *lama1* expression is dramatically increased in the anterior CNS, neural tube, eyes, somites and PSM in 15-somite stage *ptc1/2* mutant embryos (n = 3/3) (*Figure 3.17*B) and in 80% of embryos injected with dnPKA mRNA (n = 41, ** p-value = 0.0057) (*Figures 3.18*D,Di and *3.22*). Interestingly, expansion of *lama1* expression occurs throughout the somite, in both the prospective fast and slow muscle domains (*Figure 3.1*8Di). *lama1* expression is still excluded from the notochord at the level of the trunk but increased throughout the dorso-ventral axis of the neural tube (*Figure 3.18*Di).

However, by 25-28hpf, the effect of increasing Hh signalling on *lama1* expression differs to that observed at the 15-somite stage. In both *ptc1/2* mutant (n = 17) (*Figure 3.19*C,Cii,D), and dnPKA mRNA-injected embryos (n = 53) (*Figure 3.20*D,Di), there is no change in somitic expression of *lama1*. By contrast, there is still a significant up-regulation of *lama1* expression in the neural tube (** p-value = 0.0075 *ptc1/2*; *** p-value = 0.0004 dnPKA), PSM (** p-value = 0.0075 *ptc1/2*; * p-value = 0.0146 dnPKA), hypochord, uro-genital region, and the ventral vasculature and pro-nephric duct region (** p-value = 0.0073 *ptc1/2*) (*Figures 3.19*C,Cii,Ciii,D and *3.20*D,Di). This phenotype was observed in 100% of *ptc1/2* embryos (n = 17), whereas only few wild-type zebrafish embryos displayed an up-regulation of *lama1* in the neural tube, PSM and vasculature/pro-nephric tubules/uro-genital region (19%, 3%, and 3% of embryos, respectively) (*Figure 3.21*). As in 15-somite stage embryos, increased neural tube expression is observed along the dorso-ventral axis and is concentrated at the ventricular zone (*Figures 3.19*Cii and *3.20*Di). There may also be a slight increase in *lama1* expression in the anterior CNS in dnPKA mRNA-injected zebrafish (*Figures 3.20*D and *3.22*), although this is not obvious in *ptc1/2* mutant zebrafish (*Figure 3.19*C,Ci).

Increased Hh signalling in the zebrafish embryo therefore causes an up-regulation of *lama1* in the anterior CNS, neural tube, somites and PSM at the 15-somite stage, although the effect upon the somites does not persist by 25hpf. At 25hpf, there is also increased expression within the ventral vasculature or forming gut region, and the pro-nephric tubules and uro-genital region.

Taking into account both loss-of-function and gain-of-function analyses, these data suggest a complex spatio-temporal regulation of *lama1*. First, Hh is necessary and sufficient for *lama1* expression within the PSM, and possibly within the uro-genital region and anterior CNS. Hh signalling is also sufficient for *lama1* expression in the anterior CNS, neural tube, and somites at the 15-somite stage, although it is not necessary in these tissues. By 25hpf, Hh signalling is sufficient for *lama1* expression in the neural tube, PSM, hypochord, ventral vasculature or forming gut region, pro-nephric tubules and the uro-genital region.

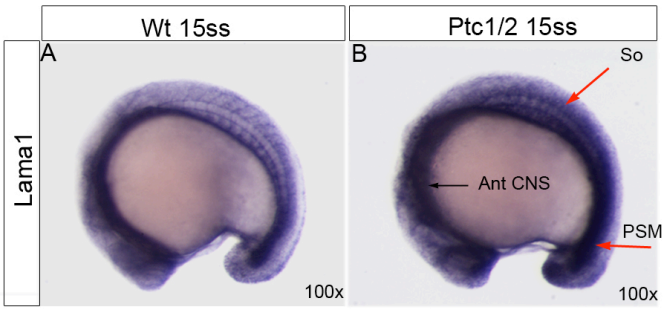

Figure 3.17: *Analysis of lama1 expression in 15-somite stage ptc1/2 mutant zebrafish embryos by whole-mount in situ hybridisation*. Lateral view of a 15-somite stage wild-type (A) and *ptc1/2* mutant (B) zebrafish embryo, using a *lama1* probe, at 100x magnification. Increased *lama1* expression is observed throughout the somites, PSM and anterior CNS of *ptc1/2* mutant zebrafish (indicated by red arrows) (B). Anterior is to the left. Abbreviations: somite (So), pre-somitic mesoderm (PSM).

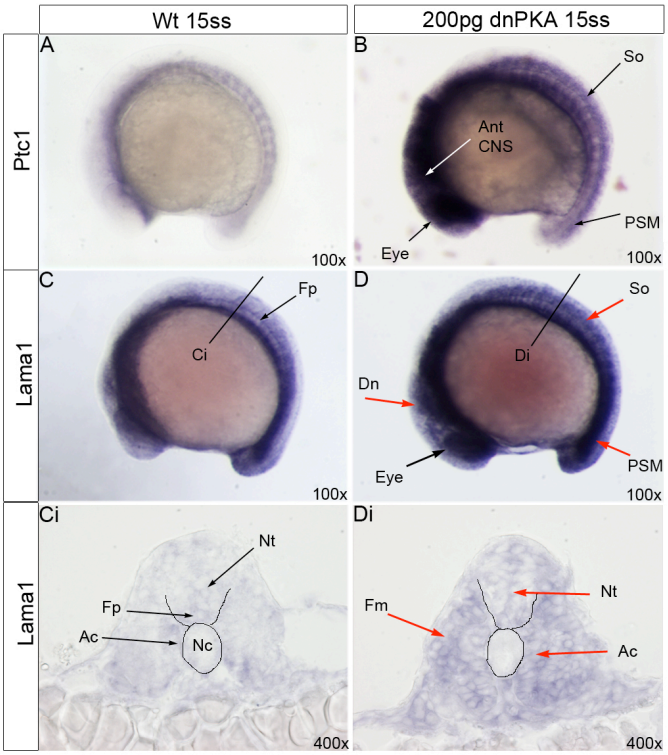

Figure 3.18: *Analysis of lama1 expression in 15-somite stage wild-type (Wt) and dominant negative PKA (dnPKA) mRNA injected zebrafish embryos by whole-mount in situ hybridisation*. Microinjection of 200pg dnPKA mRNA into the embryo was performed at the 1-cell stage. (A,B) lateral view of a wild-type (A) and dnPKA mRNA injected (B) embryo using a *ptc1* probe, at 100x magnification. *ptc1* expression is up-regulated predominantly within the anterior CNS, somites and the PSM in dnPKA mRNA injected embryos compared to wild-type embryos (A). (C,D) lateral view of a wild-type (C) and dnPKA mRNA injected (D) embryo using a *lama1* probe, at 100x magnification. Ci and Di: 15µm thick transverse cryosections taken from the embryos in panels C and D, respectively, at 400x magnification. *lama1* expression shows a similar response to that of *ptc1*, with up-regulation occurring within the somites and the PSM of dnPKA mRNA injected embryos (D) compared to wild-type embryos (C) (red arrows). There may be also a slight up-regulation within the anterior CNS (D). Increased *lama1* expression is observed throughout the somite and neural tube in dnPKA mRNA injected (Di) compared to wild-type (Ci) embryos. Positions of cryosections along the antero-posterior axis are indicated by straight lines on the whole-mount images. Anterior of whole-mount images is to the left. Abbreviations: somite (So), pre-somitic mesoderm (PSM), floor plate (Fp), diencephalon (Dn), neural tube (Nt), adaxial cell (Ac), notochord (Nc), fast muscle (Fm).

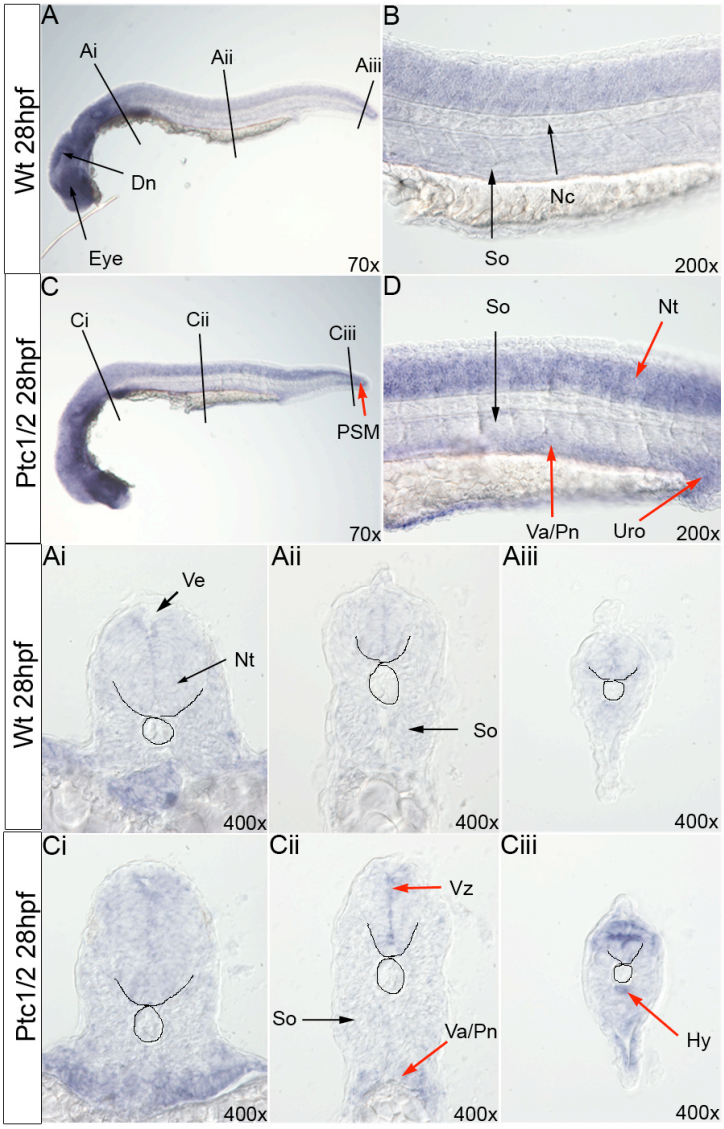

Figure 3.19: *Analysis of lama1 expression in 28hpf wild-type (Wt A,B,Ai,Aii,Aiii) and Patched 1/Patched 2 mutant (ptc1/2 C,D,Ci,Cii,Ciii) zebrafish embryos by whole-mount in situ hybridisation (WISH).* Lateral views of a wild-type embryo at 70x magnification (A) and 200x magnification (B). Lateral views of a *ptc1/2* embryo at 70x magnification (C) and 200x magnification (D). *lama1* expression is increased in the neural tube, PSM, uro-genital region, and the vasculature and pro-nephros region of the *ptc1/2* embryo (C,D) compared to the wild-type zebrafish embryo (A,B) (indicated by red arrows). Ai,Aii,Aiii,Di,Dii,Diii are 15µm transverse cryosections at 400x magnification, and their positions along the antero-posterior axis are indicated by straight lines on the lateral whole-mount images (A,C). *lama1* expression within the anterior CNS (Ci), somites (Cii) and the notochord (Ci-iii) remains unchanged in *ptc1/2* embryos compared to the wild-type embryos (Ai-iii), although an expansion of *lama1* expression occurs in the nephron primordia region (Pn) (Ci), in addition to in the pro-nephric tubules/vasculature region (Cii). The increase of *lama1* expression in *ptc1/2* mutant embryos in the neural tube occurs in the ventricular zone (Cii). *lama1* expression is also up-regulated in the PSM and the hypochord (Ciii) (indicated by red arrows). Anterior of whole-mount images is to the left. Abbreviations: diencephalon (Dn), notochord (Nc), pre-somitic mesoderm (PSM), neural tube (Nt), somite (So), vasculature (Va), pro-nephros (Pn), uro-genital region (Uro), 4th ventricle of the brain (Ve), ventricular zone of the neural tube (Vz), hypochord (Hy).

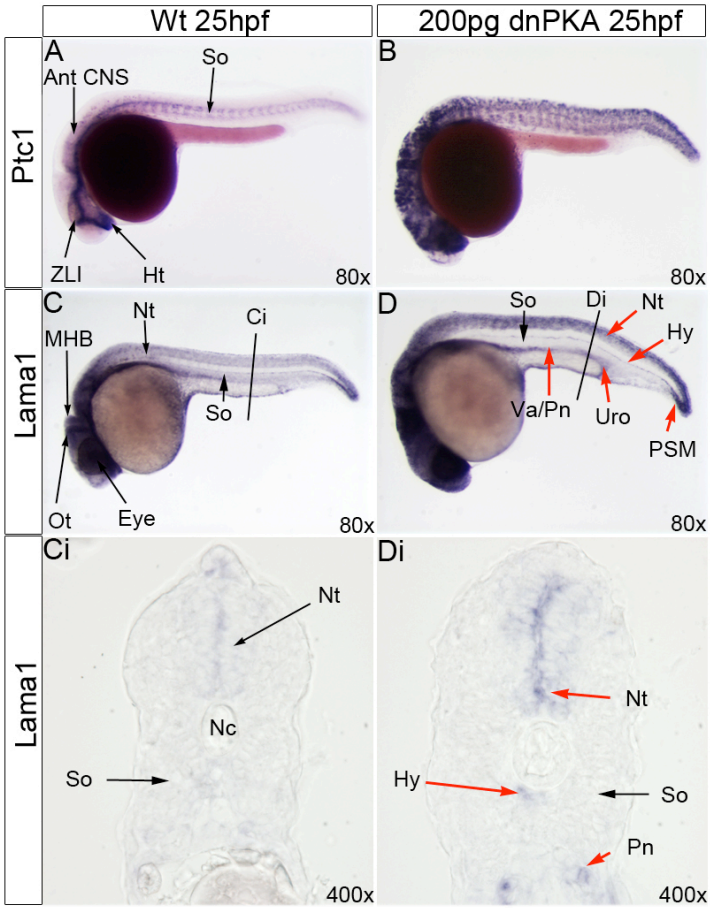

Figure 3.20: *Analysis of lama1 expression in 25hpf wild-type (Wt) and dominant negative PKA (dnPKA) mRNA injected zebrafish embryos by whole-mount in situ hybridisation (WISH).* Microinjection of 200pg dnPKA mRNA into the embryo was performed at the 1-cell stage. Lateral view of a wild-type (A) and dnPKA mRNA injected (B) embryo using a *ptc1* probe, at 80x magnification. *ptc1* expression is up-regulated throughout injected embryos, predominantly within the anterior CNS, somites and the PSM (B), compared to wild-type embryos (A). Lateral view of a wild-type (C) and dnPKA mRNA injected (D) embryo using a *lama1* probe, at 80x magnification. Ci and Di: 15µm thick transverse cryosections taken from the embryos in panels C and D, respectively, at 400x magnification. *lama1* expression is increased throughout the neural tube ventricular zone in injected embryos, as well as in the hypochord and pro-nephros region (Di) (indicated by red arrows) compared to wild-type embryos (Ci). Position of transverse cryosections along the antero-posterior axis are indicated by straight lines on the whole-mount images (C,D). Anterior of whole-mount images is to the left. Abbreviations: somite (So), zona limitans intrathalamica (ZLI), hypothalamus (Ht), midbrain-hindbrain boundary (MHB), neural tube (Nt), optic tectum (Ot), hypochord (Hy), vasculature (Va), pro-nephros (Pn), uro-genital region (Uro), pre-somitic mesoderm (PSM), notochord (Nc).

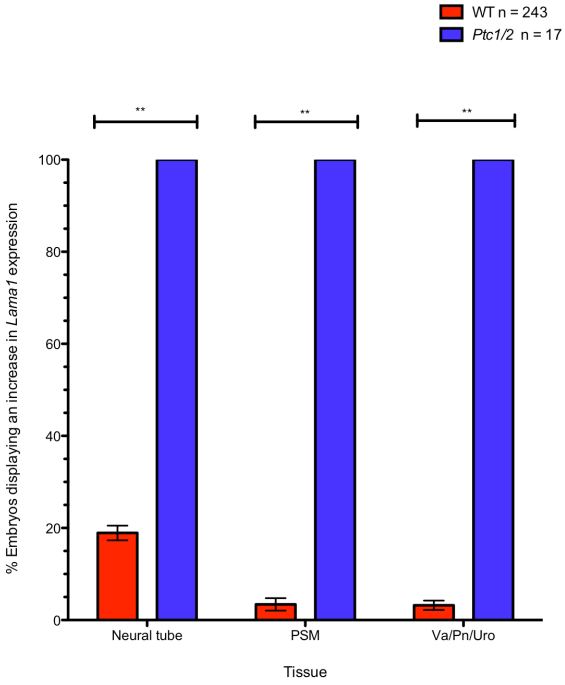

Figure 3.21: *Quantification of lama1 expression in wild-type (WT) and Patched1/2 (ptc1/2) mutant embryos at 25hpf. lama1* expression is significantly increased in the neural tube (** p-value = 0.0075), PSM (** p-value = 0.0075) and vasculature/pro-nephric tubule region/uro-genital region (** p-value = 0.0073) (Va/Pn/Uro) in 100% of *ptc1/2* mutant embryos (blue) compared to WT embryos (red). Expression characterised as the vasculature may also include the forming gut. n is the number of embryos analysed. Statistical test used was the Mann-Whitney test.

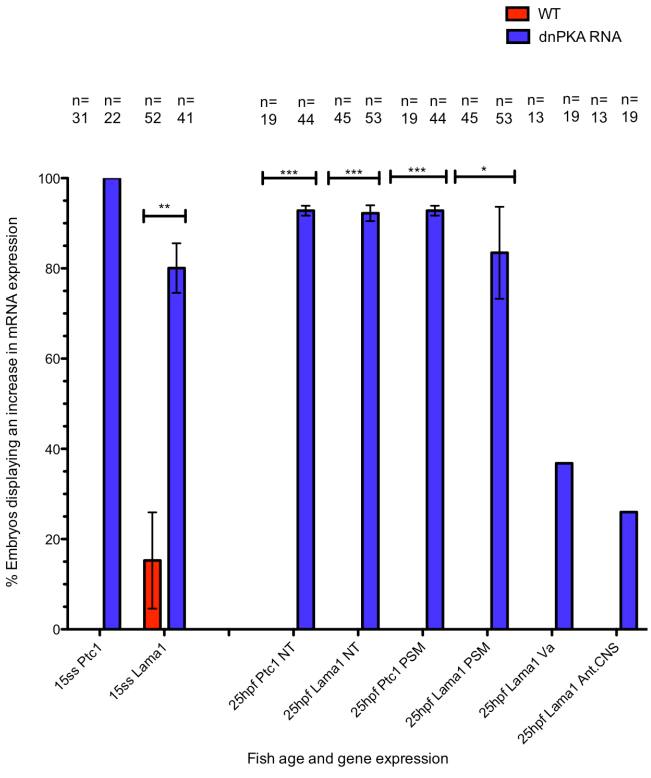

Figure 3.22: *Quantification of lama1 expression in wild-type (WT) and dominant negative PKA (dnPKA) mRNA-injected embryos, at the 15-somite and 25hpf stages. ptc1* expression is increased in 100% of embryos injected with 200pg dnPKA mRNA (blue) at the 15-somite stage, whilst the expression of *lama1* is significantly increased in 80% of embryos compared to WT embryos (red) (** p-value = 0.0057, revealed using an un-paired, two-tailed t-test). At 25hpf, *lama1* expression is significantly increased in the neural tube (NT) (*** p-value 0.0004), PSM (* p-value = 0.0146), vasculature (Va) and anterior CNS of dnPKA mRNA-injected embryos, revealed using a one-sample, two-tailed t-test). Increased *lama1* expression was not observed in any 25hpf wild-type embryos. Error bars are not shown on some columns because the experiment was only performed once. n is the number of embryos analysed.