4.2: Results

4.2.1: BAC zC34A17 incorporates the entire zebrafish *lama1* gene

Partially sequenced BAC ends that lie either side of the *lama1* gene on zebrafish chromosome 24 can be visualised using the Ensembl Genome Browser (*Figure 4.1*). I selected two BAC ends: zC34A17.za and zC34A17.ya, corresponding to BAC zC34A17 from the zC CHOR211 library (*Table 4.1*). The total size of BAC zC34A17 is 172,709 bases, and the *lama1* genomic sequence insert comprises 160,061 bases of this. BAC zC34A17 contains the entire *lama1* gene, plus 39,759 bases upstream and 26,741 bases downstream of the *lama1* gene.

BAC end	Length (bases)	Position
zC34A17.za	737	Chr24: 43410018 - 43410753
zC34A17.ya	731	Chr24: 43569637 - 43570078

Table 4.1: The length and position of the two BAC ends of BAC zC34A17.

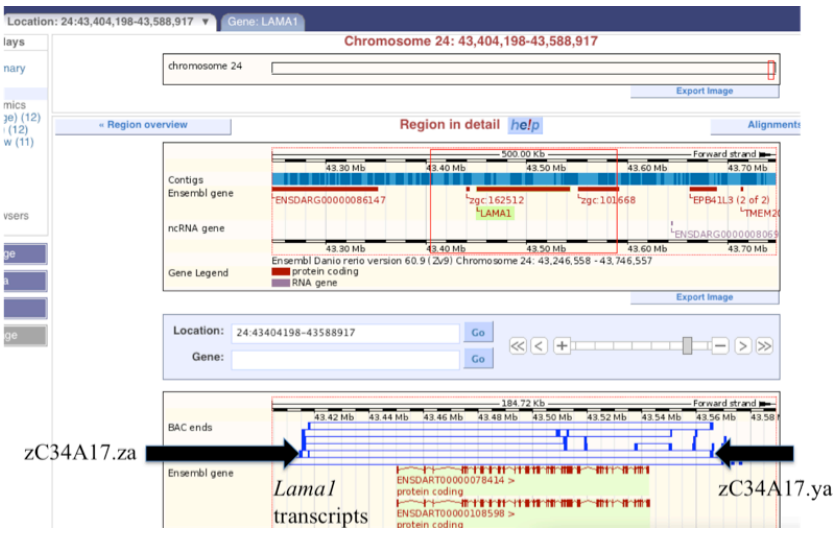

Figure 4.1: A screen shot taken from Ensembl Genome Browser, highlighting the position of the ends (black arrows) of BAC zC34A17 in relation to the *lama1* gene. The 5' end of the gene is to the left.

4.2.2: BAC zC34A17 is successfully electroporated into EL250 cells

DH10B cells containing BAC zC34A17 were streaked onto chloramphenicol plates, and individual colonies grown as mini-cultures and then mini-prepped. To confirm that the correct DNA sequence was present in BAC zC34A17, a series of PCR tests were performed to amplify specific known sequences within BAC zC34A17 and the *lama1* gene (see *Figure 4.2*). Following isolation of DH10B colonies that contained the correct BAC zC3417 DNA sequence, BAC zC34A17 midi-prep DNA was electroporated into electrocompetent EL250 cells (see section 2.2.18). The same set of PCR tests were carried out again on individual colonies to confirm a successful electroporation of BAC zC34A17 into the EL250 cells (*Figure 4.3*).

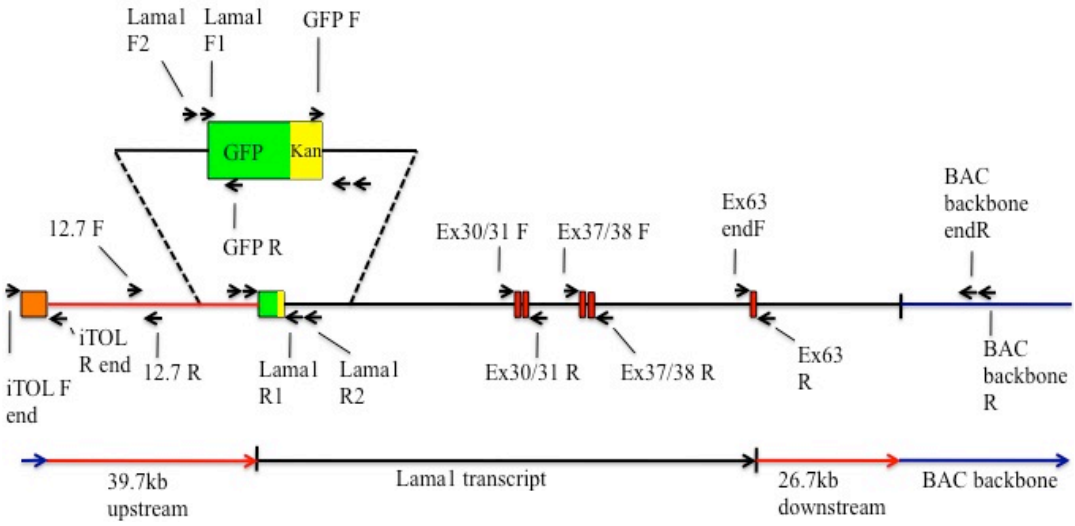

Figure 4.2: *A schematic representation of BAC zC34A17 and the positioning of primer binding sites.* Forward primers (arrows pointing to the right) and reverse primers (arrows pointing to the left) are spread throughout the *lama1* gene. Red lines indicate DNA sequence that lies upstream or downstream of the *lama1* transcript. Blue lines indicated BAC backbone vector DNA sequence. Orange box represents the iTOL insert, green box represents the GFP cassette, yellow box represents the Kanamycin resistance gene within the GFP cassette. Red rectangles represent exons (30 and 31, 37 and 38, and 63).

	1	**2**	**3**	**4**	**37/38**	**12.7**
Primer pair	Lama1 F1 Lama1 R1	Lama1 F1 Lama1 R2	Lama1 F2 Lama1 R1	Lama1 F2 R2	Ex 37/38 F Ex 37/38 R	12.7kb up F 12.7kb up R
Size (bases)	303	352	491	540	985	501

Table 4.2: *A table of primer pairs used to analyse BAC DNA*. The size of the DNA fragments generated from specific primer pairs is shown. Specific primer pairs correspond to the primer pairs used in *Figure 4.3*. The sequence of each primer can be found in *Table 2.5* and *2.6*.

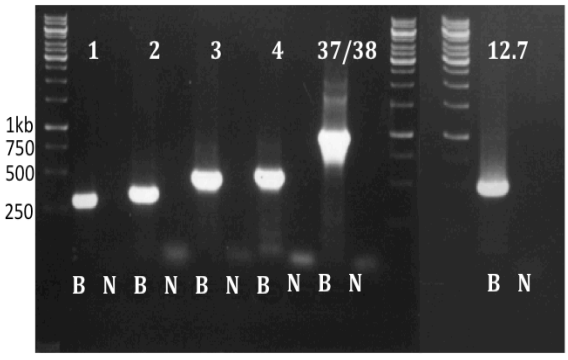

Figure 4.3: *Analysis of BAC DNA sequence extracted from EL250 cells.* BAC zC34A17 is successfully electroporated into EL250 cells, as revealed by PCR analysis and gel electrophoresis. Each DNA band generated by PCR is the expected size (bases) for each primer pair used. The data represents PCR analysis from a single BAC sample only. Primer pair used is indicated above each DNA band, and corresponds to the primer pairs listed in *Table 4.2*. Size of ladder is indicated on the left-hand side. Abbreviations: BAC DNA (B), no DNA control (N).

Primer pairs 1-4 all amplify sequences around the *lama1* transcript start site, within BAC zC34A17 (*Figure 4.2*). PCR analysis and sequencing of the products reveals that the correct DNA sequence is present. It is particularly important that this region of DNA is correct, as the *lama1* start site sequence will be the site of GFP reporter gene insertion, by the process of homologous recombineering. Primer pair 37/38 amplifies the DNA sequence between exon 37 and exon 38,

whilst primer pair 12.7 amplifies a DNA sequence that lies 12.7kb upstream of the *lama1* start site (*Figure 4.2*). Both 37/38 and 12.7 primer pairs have generated the correct DNA fragments. Therefore, sequences upstream and downstream of the *lama1* start site are present, and there is no break or degradation of the BAC DNA. It was crucial to verify this because enhancer elements controlling *lama1* gene activation may lie in these regions.

4.2.3: Homologous recombineering successfully inserts the GFP-Kan reporter gene into the *lama1* start site within the BAC

To generate the BAC construct required to do transgenesis, a GFP reporter gene was inserted exactly into the *lama1* transcript start site. This was performed using homologous recombineering (section 2.2.21).

After successful recombination and removal of the Kanamycin resistance gene from the GFP reporter cassette, the BAC zC34A17-GFP reporter construct was tested again by PCR analysis (*Figure 4.4*). This confirmed that all upstream and downstream sequences from the *lama1* start site are still present, and that the GFP reporter was inserted into the *lama1* start site.

	12.7	**37/38**	**1**	**A**	**B**	**C**
Primer pair	12.7kb up F 12.7kb up R	Ex 37/38 F Ex 37/38 R	Lama1 F1 Lama1 R1	Lama1 F1 GFP R	Lama1 F2 GFP R	Lama1 R1 GFP F
Size (bases)	501	985	1200	648	836	Should not work (651)

Table 4.3: *A table of primer pairs used to analyse BAC DNA.* The size of the DNA fragments generated from specific primer pairs is shown. Specific primer pairs correspond to the primer pairs used in *Figure 4.4*. The sequence of each primer can be found in *Table 2.5* and *2.6*.

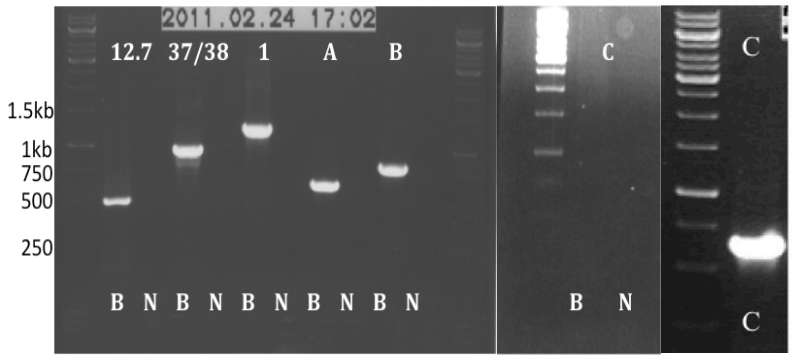

Figure 4.4: *Analysis of BAC zC34A17 DNA following homologous recombineering with GFP DNA*. The GFP reporter construct is recombined into the BAC zC34A17 construct and the Kanamycin resistance gene is successfully removed, as revealed by PCR analysis and gel electrophoresis. Each DNA band generated by PCR is the expected size (bases) for each primer pair used. The data represents PCR analysis from a single BAC sample only. Primer pairs used are indicated above each DNA band, and correspond to the primer pairs listed in *Table 4.3*. DNA used is indicated at bottom of the gel. Size of ladder is indicated on the left-hand side. Abbreviations: BAC DNA (B), no DNA control (N), control DNA (C).

Primer pairs 12.7 and 37/38 amplify a DNA sequence that lies 12.7kb upstream of the *lama1* start site, and a DNA sequence that encompasses exon 37, intron 37, and exon 38, respectively. These sequences have been successfully amplified, demonstrating that upstream and downstream DNA sequences have not been altered during the process of inserting the GFP reporter cassette by homologous recombineering. Primer pair 1 amplifies a sequence that now lies either side of the GFP reporter cassette, and now generates a larger fragment size of 1200bp (instead of 303bp in the absence of GFP) confirming that GFP has been inserted into the *lama1* start site. Primer pairs A and B generate DNA fragments that span from just upstream of the GFP start site to within the GFP reporter gene. The correct size and sequences of the DNA fragments generated confirm that the GFP reporter cassette has been inserted correctly, and has not been altered during homologous recombination and Kanamycin removal. Primer pair C consists of a reverse primer located just downstream of the GFP reporter cassette and a forward primer that was designed against the Kanamycin resistance gene in the GFP reporter cassette (*Figure 4.2*). Prior to Kanamycin removal, primer pair C generates a fragment that is 651bp when using control DNA (C) (*Figure 4.4*). After successful removal of Kanamycin from the BAC zC34A17-GFP reporter construct, this PCR product should not be generated. Indeed, *Figure 4.4* reveals that Kanamycin has been successfully removed from the BAC zC34A17-GFP reporter construct, as shown by the lack of a DNA fragment with primer pair C.

4.2.4: Early *lama1* GFP expression recapitulates the endogenous *lama1* mRNA expression pattern

Prior to the generation of a transgenic stable line of *lama1* GFP zebrafish, the expression of *lama1* GFP was assessed in embryos that had undergone transient injection of BAC zc34A17-GFP report construct. This produces a mosaic expression pattern of *lama1* GFP, and crucially it reveals whether *lama1* GFP is expressed in temporal and spatial pattern that recapitulates that of the endogenous *lama1* mRNA. *lama1* mRNA is expressed in the zebrafish embryo as maternal transcripts at the four-cell stage (*Figure 4.5*A). However, it is unknown when zygotic *lama1* transcripts are first synthesised, although zygotic genes are often first expressed around 3hpf at the 1000-cell stage (Aanes et al. 2011). I hypothesised that BAC zC34A17-GFP (*lama1* GFP) should begin to be transcribed at about 3hpf and thus GFP fluorescence will be detected just after 3hpf, allowing time for the translation of the protein. I predicted that the GFP fluorescence would be detected as a mosaic pattern similar to that of endogenous *lama1* mRNA, which is ubiquitous throughout the blastoderm during epiboly (*Figure 4.5*C), and then accumulates in the chordamesoderm/mesodermal midline and neural plate (*Figure 3.1*L,M). By the 6-somite stage, I expected that *lama1* GFP would be expressed in the ventral neural tube, notochord, paraxial mesoderm and PSM, matching the expression of *lama1* (*Figure 4.5*E,Ei).

GFP fluorescence is first detected at about the 50% epiboly stage of zebrafish development. By 75% epiboly, GFP is expressed ubiquitously throughout the blastoderm of the embryo (n =8/8) (*Figure 4.5*B), and later becomes restricted to the chordamesoderm/mesodermal midline and the forming notochord by the 3-somite stage (n = 16) (*Figure 4.5*). At the 6-somite stage, GFP is expressed more widely and more intensely as distinctive tissues form (*Figure 4.14ab*). GFP expression intensifies in the notochord, neural tube, neural plate and forming anterior CNS, and is also detected in the paraxial mesodermal tissues in the prospective slow and fast muscle domains (n = 27) (*Figure 4.5*F,Fi).

Early GFP expression therefore matches endogenous *lama1* mRNA expression pattern as hypothesised. In agreement with my prediction, GFP fluorescence is detected shortly after the time at which zygotic gene transcription is initiated.

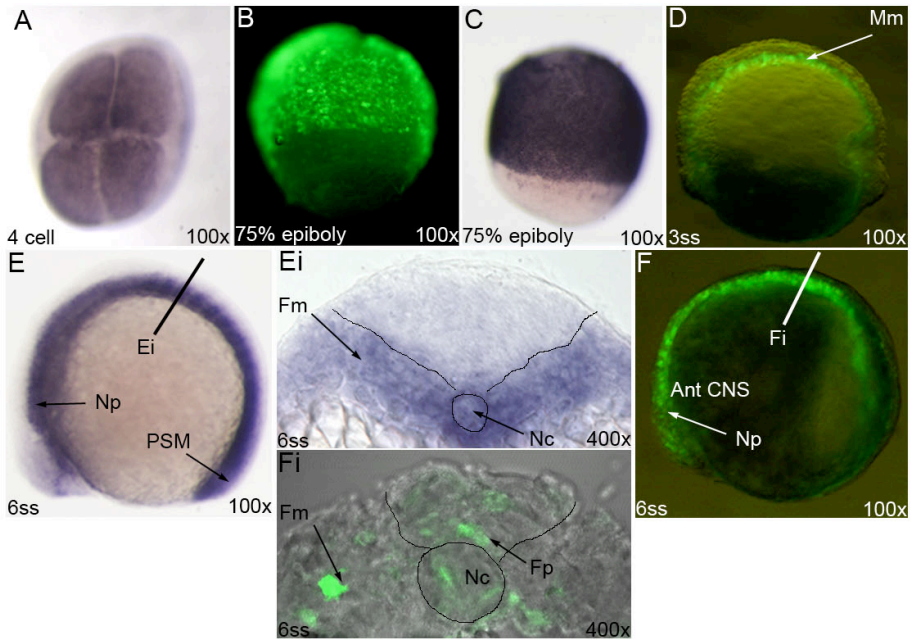

Figure 4.5: *Analysis of GFP expression in the zebrafish embryo from the 4-cell stage to the 6-somite stage using fluorescence microscopy.* 50pg *lama1* GFP BAC DNA was injected at the one-cell stage. GFP fluorescence was analysed at 75% epiboly (B), 3-somite (D), and 6-somite (F) stages and compared to *lama1* expression assessed by WISH at 4-cell (A), 75% epiboly (C) and 6-somite (E) stages. *lama1* GFP expression is initiated at 75% epiboly, throughout the blastoderm (B), matching the pattern of *lama1* expression shown by WISH at 75% epiboly (C). At the 3-somite stage, GFP becomes restricted to the neural plate and mesodermal midline (D). At the 6-somite stage, GFP expression recapitulates the *lama1* in situ hybridisation pattern (E). GFP is expressed in the anterior CNS and somites. A 15µm transverse crysosection taken from a 6-somite stage WISH embryo (Ei) or from a 6-somite stage embryo expressing *lama1* GFP (Fi), at 400x magnification. *lama1* mRNA and *lama1* GFP are expressed in the neural tube, notochord, and throughout the somite (Ei,Fi). The position of transverse sections along the antero-posterior axis are indicated by straight lines on the lateral whole-mount images (E,F). All whole-mount images were captured at 100x magnification. Abbreviations: mesodermal midline (Mm), neural plate (Np), pre-somitic mesoderm (PSM), fast muscle (Fm), notochord (Nc), anterior (Ant), floor plate (Fp).

By the 15-somite stage, I expected that *lama1* GFP would be detected in the forming anterior CNS and eye, the neural tube, throughout the forming somites, the PSM, and weakly in the notochord (*Figure 4.6*A,Ai).

At the 15-somite stage, GFP fluorescence is widely distributed throughout the embryo, in all sites of endogenous *lama1* mRNA expression. Strong GFP fluorescence is observed throughout the anterior CNS including the eye (43% embryos) and diencephalon (*Figure 4.6*B), the floor plate of the neural tube (43% embryos) (*Figure 4.6*Bi,Bii), the notochord (45% embryos) (*Figure 4.6*Bii), throughout the somites (78% embryos) (*Figure 4.6*Biii), and the PSM (63% embryos) (n = 58) (*Figures 4.6*B, *4.14*ab, *4.17*A and *Table 4.5*A).

Thus, GFP distribution recapitulates *lama1* mRNA expression pattern, with GFP initiated in the eye and anterior CNS in addition to the tissues previously characterised at the 15-somite stage. However, the intensity of GFP fluorescence in the notochord is slightly stronger than expected, when compared to the expression of *lama1* mRNA in this structure at the 15-somite stage (*Figure 4.6*Ai).

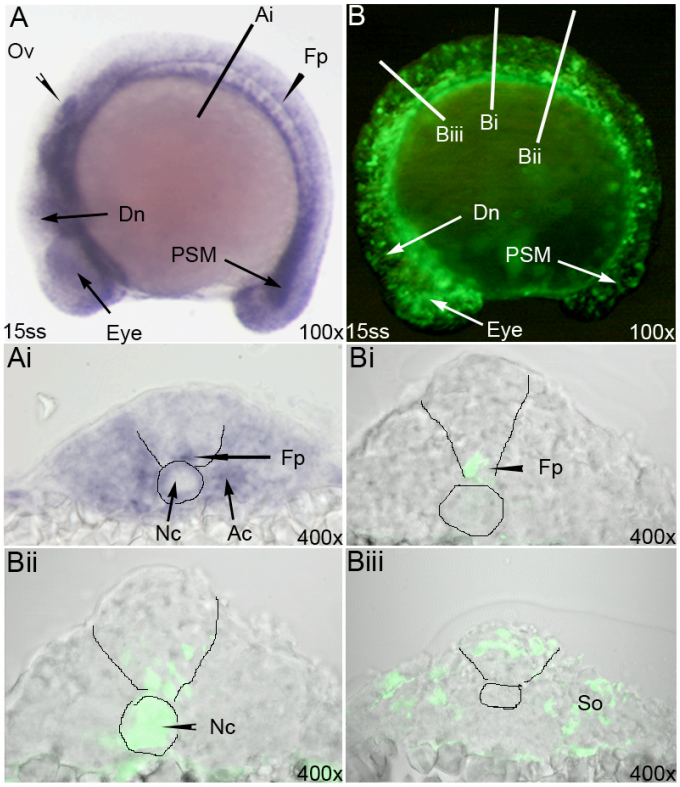

Figure 4.6: *Analysis of GFP expression in the 15-somite stage zebrafish embryo using fluorescence microscopy.* 50pg *lama1* GFP BAC DNA was injected at the one-cell stage. GFP fluorescence was analysed in a whole-mount embryo at 100x magnification (B) and in 15µm transverse cryosections at 400x magnification (Bi,Bii,Biii), and compared to *lama1* expression assessed by WISH in a whole-mount embryo (A) and a 15µm transverse cryosection (Ai). GFP expression fully recapitulates the *lama1* mRNA pattern (A,B). GFP is expressed in the eye, diencephalon and anterior CNS (B), floor plate (Bi), notochord (Bii), somites (B,Biii) and the PSM (B). Positions of transverse sections along the antero-posterior axis are indicated by straight lines on the lateral whole-mount images (A,B). Abbreviations: diencephalon (Dn), otic vesicle (Ov), floor plate (Fp), pre-somitic mesoderm (PSM), notochord (Nc), adaxial cell (Ac), somite (So).

If the BAC reporter construct contains all regulatory elements for *lama1* expression, the intensity of GFP fluorescence should decrease in anterior somites at the 19-somite stage, but be maintained in adaxial cells and the PSM (*Figure 3.3*A,Ai and *4.7*B). GFP expression should also increase in the anterior CNS, eye, and ventral vasculature and pro-nephric tubules. By 25hpf, I would expect that GFP fluorescence would be down-regulated throughout the somite but maintained in the anterior CNS, eye, otic vesicle, PSM and weakly in the neural tube (*Figure 4.7*D,Di). No notochord expression would be expected at 25hpf.

Consistent with my predictions, GFP expression is observed in the anterior CNS, eye, floor plate and the neural tube, otic vesicle, ventral vasculature, uro-genital region, and the PSM (30% embryos at the 19-somite stage, 21% embryos at 25hpf), at both the 19-somite stage (n = 30) and at 25hpf (n = 289) (*Figures 4.7*A,C,Cii,E and *4.8*A,C,Ci,E, and *Figures 4.14ab* and *4.17*B,C and *Tables 4.5*B and *4.6*C). Within the anterior CNS at 25hpf, GFP is detected in the olfactory placode, telencephalon, epiphysis, optic tectum, cerebellum and the rhombencephalon (*Figures 4.7*C and *4.8*A). GFP expression in the eye is observed in the lens and retina (44% embryos) (*Figure 4.8*B). In the ventral vasculature, I observed individual GFP-positive cells travelling around the vasculature system in live embryos. However, even at 25hpf strong GFP continues to be detected in the notochord (n = 87/289 embryos, 30%) and the somitic muscle fibres (n = 220/289 embryos, 76%) (*Figures 4.7*Ci,Cii and *4.8*D,E and *Figures 4.14ab* and *4.17*C and *Table 4.6*C), despite the fact that *lama1* mRNA has been down-regulated in these tissues at this point (*Figure 4.7*D). GFP is also detected in the yolk at both the 19-somite stage (23% embryos) and at 25hpf (24% embryos), and in the epidermis at 25hpf (13% embryos) (*Figure 4.14b*), although *lama1* is not expressed in these tissues. It is possible that GFP labelling in the yolk corresponds to migrating cells involved in vasculature formation (*Figure 4.12*G). Alternatively, it could be due to auto-fluorescence from the globular yolk structure. It is also possible that *lama1* is expressed in the epidermis, but its detection is obscured by the high levels of mRNA expression in the somites and pre-somitic mesoderm.

Within the somite, GFP is detected in both fast and slow muscle fibres (*Figures 4.9*B,E and *4.10*B), and is confirmed by antibody co-localisation analysis using F310 and F59 antibodies, respectively. Keratan sulphate antibody also confirms expression of GFP in the notochord (*Figure 4.11*B,E).

Overall, the mosaic pattern of GFP expression does recapitulate the pattern of *lama1* expression. However, GFP is maintained in the notochord and somites beyond the stage of *lama1* down-regulation in these tissues. The continuing strong GFP fluorescence in the notochord and somites at 25hpf could be due to stability of the GFP.

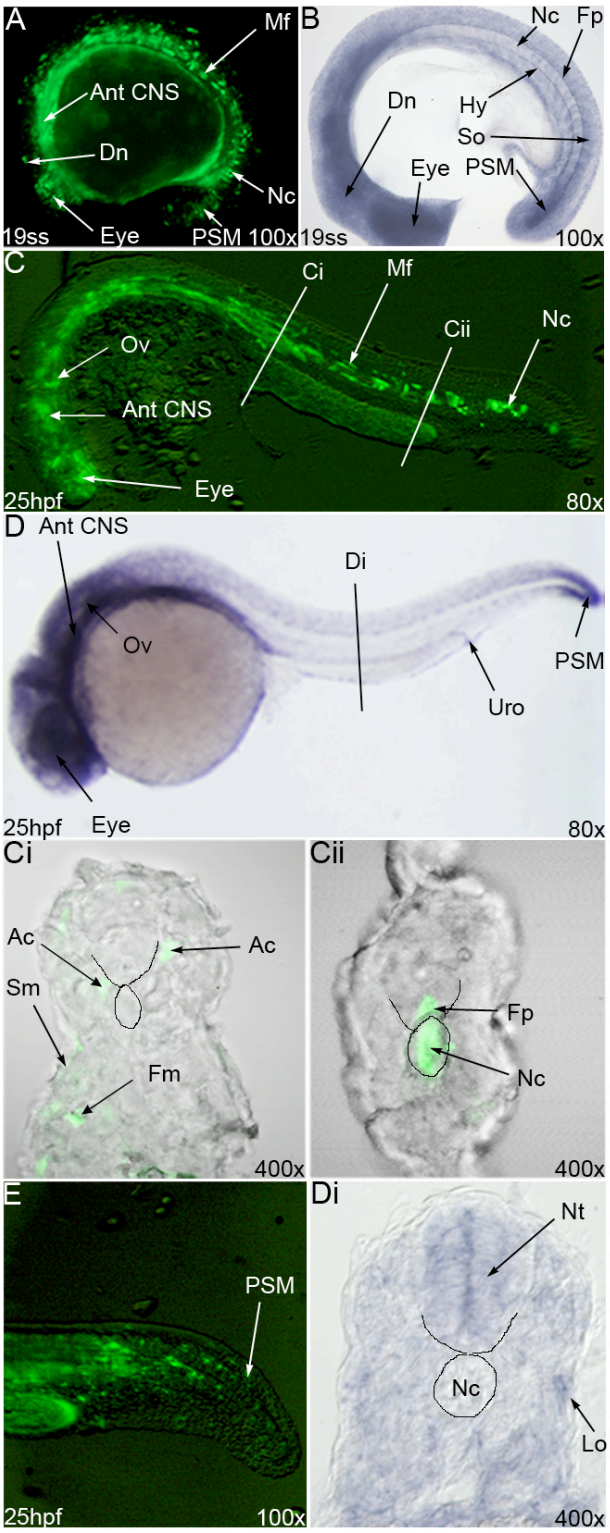

Figure 4.7: *Analysis of GFP expression in the zebrafish embryo at the 19-somite stage and at 25hpf using fluorescence microscopy*. 50pg *lama1* GFP BAC DNA was injected at the one-cell stage. GFP fluorescence was analysed at 19-somite (A) and 25hpf (C,Ci,Cii,E) stages and compared to *lama1* expression assessed by WISH at 19-somite (B) and 25hpf (D,Di) stages. GFP expression fully recapitulates *lama1* expression (A-D), with GFP expression detected in the eye, diencephalon and anterior CNS (A), otic vesicle (C), floor plate (Cii), notochord (Cii), somites (A,C,Ci) and the PSM (A,E) at both of these stages of development. At 25hpf, there is only low levels of *lama1* expression in the somites (D), although GFP persists in both the fast and slow muscle fibres (C,Ci). The positions of transverse cryosections along the antero-posterior axis are indicated by straight lines on the lateral whole-mount images (C,D). Anterior of whole-mount images is to the left. Magnification is indicated on each image. Abbreviations: diencephalon (Dn), muscle fibre (Mf), notochord (Nc), pre-somitic mesoderm (PSM), hypochord (Hy), somite (So), floor plate (Fp), otic vesicle (Ov), uro-genital region (Uro), slow muscle (Sm), adaxial cell (Ac), fast muscle (Fm), neural tube (Nt), lateral line organ (Lo).

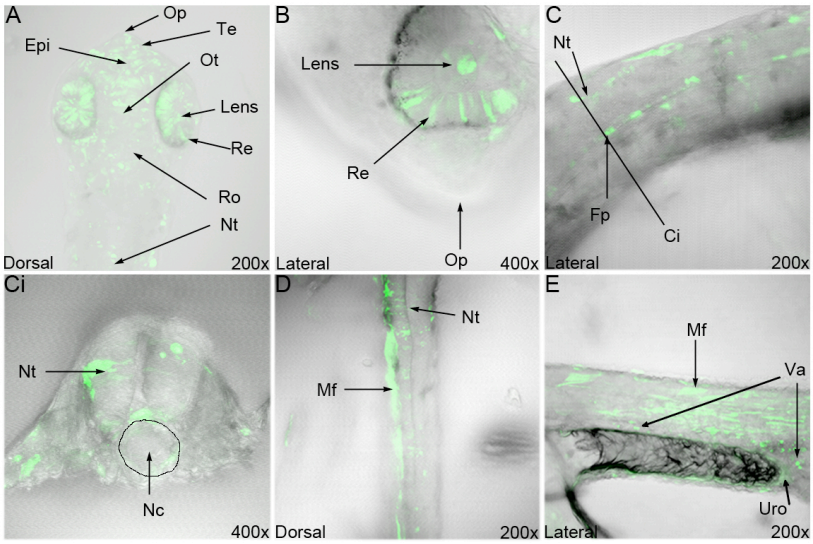

Figure 4.8: *Analysis of lama1 GFP expression in the 25hpf zebrafish embryo using fluorescence microscopy*. 50pg *lama1* GFP BAC DNA was injected at the one-cell stage. GFP expression was analysed in dorsal views of the head (A) and neural tube (D) at 200x magnification, and in lateral views of the head at 400x magnification (B), anterior trunk at 200x magnification (C), and the somites at 200x magnification (E). Expression was also analysed in a 15µm thick transverse cryosection (Ci) at 400x magnification. GFP is expressed in the anterior CNS, olfactory placode, telencephalon, epiphysis, optic tectum and the rhombencephalon (A), in addition to the retina and lens of the eye (B). In the neural tube, GFP is expressed in the floor plate of the neural tube, as well as in more dorsal locations within the neural tube (C,Ci,D). In the trunk of the zebrafish, GFP is detected in the muscle fibres of the somite and also in individual cells of the ventral vasculature, and in the uro-genital region. The position of the transverse cryosection along the antero-posterior axis is indicated by a straight line on the lateral whole-mount image (C). Anterior of lateral view images is to the left. Abbreviations: olfactory placode (Op), telencephalon (Te), epiphysis (Epi), optic tectum (Ot), rhombencephalon (Ro), neural tube (Nt), retina (Re), floor plate (Fp), notochord (Nc), muscle fibre (Mf), vasculature (Va), uro-genital (Uro).

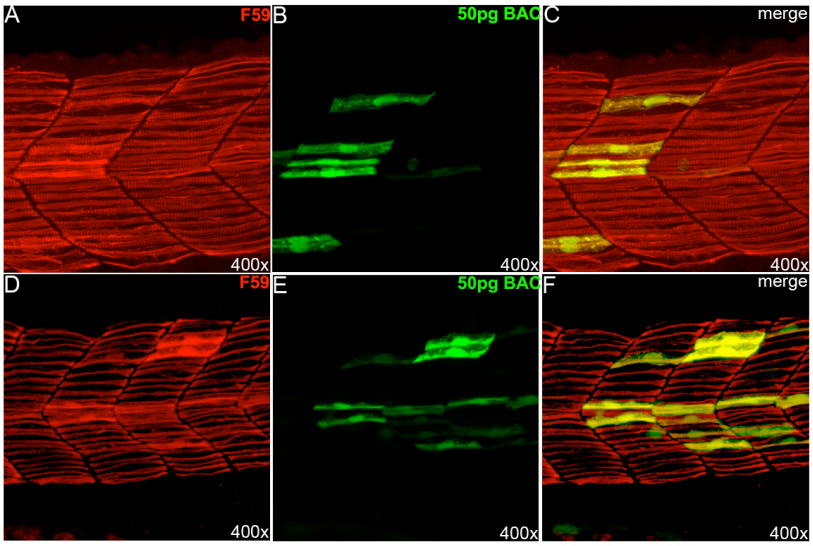

Figure 4.9: *Analysis of GFP expression in slow muscle fibres at 25hpf using fluorescence microscopy*. 50pg *lama1* GFP BAC DNA was injected at the one-cell stage. Lateral view images show expression of F59 antibody (A,D) and GFP (B,E) in the slow muscle fibres of two different embryos at 400x magnification, confirmed through merging of the red F59 and green GFP channels (C,F). Anterior is to the left.

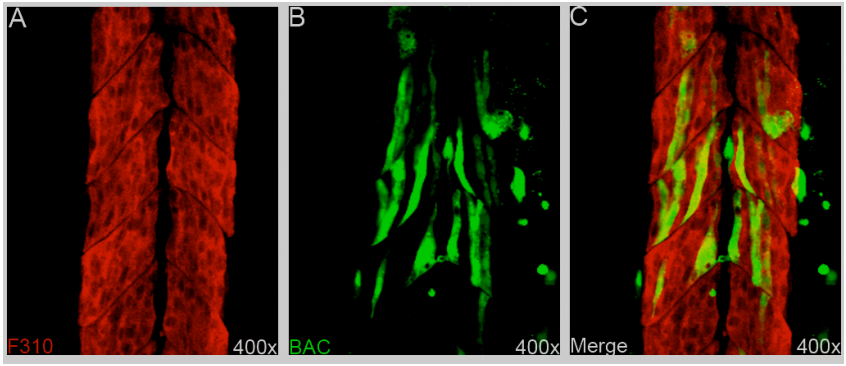

Figure 4.10: *Analysis of GFP expression in the fast muscle fibres at 25hpf using fluorescence microscopy*. 50pg *lama1* GFP BAC DNA was injected at the one-cell stage. Lateral view images show expression of F310 antibody (A) and GFP (B) in the fast muscle fibres at 400x magnification, confirmed through merging of the red F310 and green GFP channels (C). The non-parallel organisation of GFP-expressing muscle fibres also indicates that expression is within the fast muscle fibres Anterior is to the top.

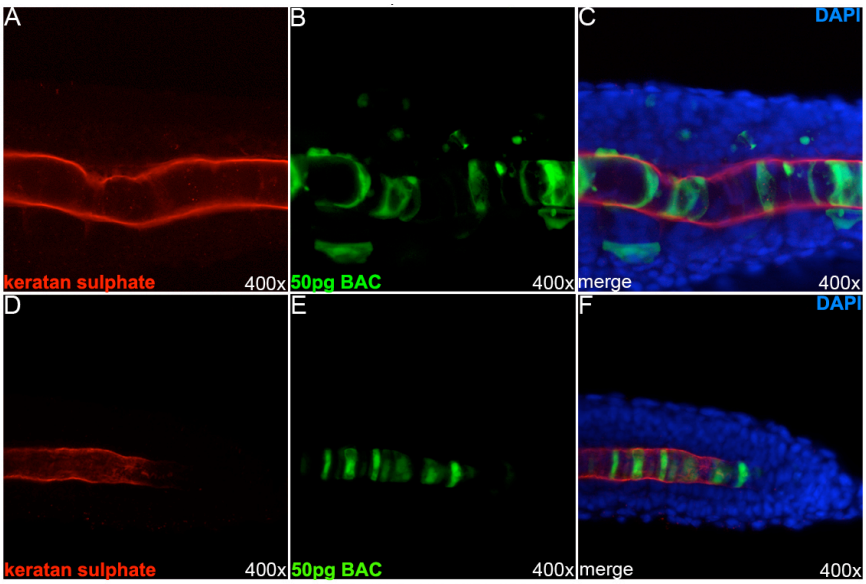

Figure 4.11: *Analysis of GFP expression in the notochord at 25hpf using fluorescence microscopy*. 50pg *lama1* GFP BAC DNA was injected at the one-cell stage. Lateral view images show expression of Keratan sulphate antibody (A,D) and GFP (B,E) in the notochord of two different embryos at 400x magnification, confirmed through merging of the red Keratan sulphate and green GFP channels (C,F). Anterior is to the left.

4.2.5: GFP persists in muscle fibres and anterior CNS in 49hpf zebrafish

By 49hpf, *lama1* expression is not detected in somites, PSM and the notochord, and is down-regulated in the anterior CNS (*Figure 4.12*C,D). Thus, GFP expression should also be extinguished in the somites, PSM and notochord at 49hpf. Although mostly down-regulated in the anterior CNS, GFP should be maintained within the midbrain-hindbrain boundary, telencephalon, otic vesicle and the eye, which are all sites of *lama1* expression (*Figure 4.12*C,D). I also expect GFP expression to initiate in the pectoral fins, and the branchial arches (*Figure 4.12*D,F).

At 49hpf (n = 108), GFP is detected in the midbrain-hindbrain boundary, telencephalon, otic vesicle, heart, and the eye (*Figure 4.12*A,B), recapitulating the endogenous *lama1* expression pattern. GFP expression is also initiated in the branchial arches and the pectoral fins (n = 5/108 embryos, 5%) (*Figure 4.12*B,E). Fluorescence is also observed in the vasculature of the yolk sac, and in the epidermis (18% embryos) (*Figure 4.12*A,G). Despite the lack of *lama1* mRNA in the anterior CNS (not including the telencephalon or midbrain-hindbrain boundary), neural tube, notochord, muscle fibres, and the PSM at 49hpf, GFP is clearly visible in these tissues. Strong GFP is detected in the muscle fibres (n = 54/108 embryos, 50%), the horizontal myoseptum, and the floor plate (n = 8/108 embryos, 7%) (*Figure 4.12*A,Ai,B,Bi). Expression is also observed in the vasculature and uro-genital region in some embryos (n = 3/108 embryos, 3%) (*Figure 4.12*A,G). However, the number of 49hpf embryos (n = 108) with GFP-labelled cells in the anterior CNS (32% 49hpf vs 67% 25hpf embryos), eye (21% 49hpf vs 44% 25hpf embryos), otic vesicle, neural tube (7% 49hpf vs 12% 25hpf embryos), notochord (2% 49hpf vs 30% 25hpf embryos), yolk sac, muscle fibres (50% 49hpf vs 76% 25hpf embryos), PSM (20% 49hpf vs 21% 25hpf embryos), vasculature and uro-genital region (3% 49hpf vs 11% 25hpf embryos) (*Figure 4.17*D and *Table 4.6*D) is lower compared to younger embryos such as at 25hpf (n =289) (*Figure 4.14a,b*). There is also an increased number of embryos that do not have any GFP-labelled cells at the 49hpf stage (n = 50/108 embryos, 46%) compared to the 25hpf stage (n = 66/289 embryos, 23%) (*Figure 4.14b*). Thus, despite the persistent GFP expression in 49hpf embryos, GFP expression is down-regulated as indicated by the decreased numbers of embryos with tissue specific expression. Importantly, GFP expression is initiated on schedule in the pectoral fins and branchial arches.

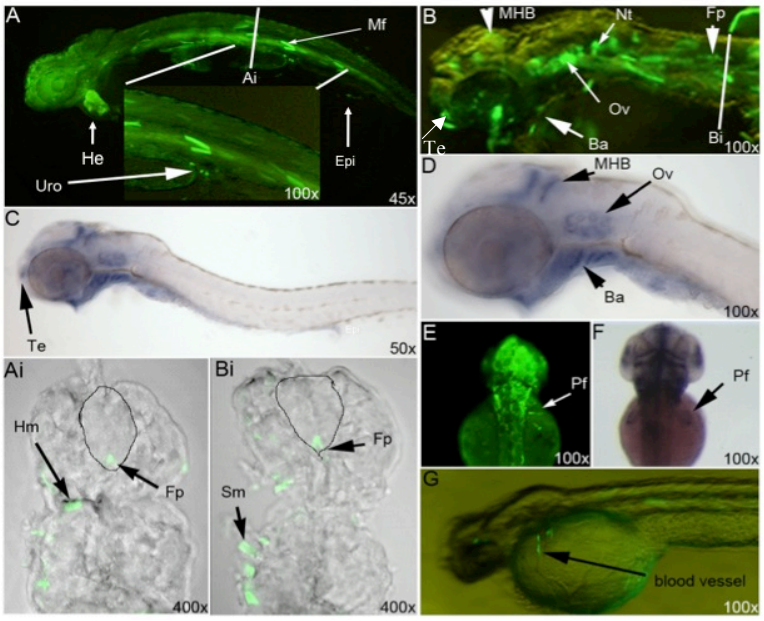

Figure 4.12: *Analysis of GFP expression in 49hpf zebrafish embryos using fluorescence microscopy*. 50pg *lama1* GFP BAC DNA was injected at the one-cell stage. GFP fluorescence was analysed in lateral view images (A,B,G) and dorsal images (E) and compared to *lama1* expression assessed by WISH (C,D,F). GFP is detected in the muscle fibres and epidermis (A), in addition to the uro-genital region (inset image in A), whilst *lama1* is absent from the somites and uro-genital region (C). GFP is also observed in the blood vessels of the yolk sac (G). Within the head, GFP is expressed in the eye, telencephalon, midbrain-hindbrain boundary, otic vesicle, branchial arches, and floor plate of the neural tube (B). *lama1* is also expressed in the telencephalon, midbrain-hindbrain boundary, otic vesicle and the branchial arches, but is absent in the neural tube, notochord and PSM. Dorsal view images reveal GFP (E) and *lama1* (F) expression in the pectoral fin, at 100x magnification. 15µm thick transverse cryosections show expression of GFP in the floor plate and horizontal myoseptum (Ai), and the superficial slow muscle (Bi). The positions of the transverse sections along the antero-posterior axis are indicated by straight lines on the lateral whole-mount images (A,B). Anterior of lateral images is to the left. Magnification of images is indicated. Abbreviations: uro-genital region (Uro), heart (He), muscle fibre (Mf), epidermis (Epi), midbrain-hindbrain boundary (MHB), branchial arches (Ba), otic vesicle (Ov), neural tube (Nt), floor plate (Fp), telencephalon (Te), slow muscle (Sm), horizontal myoseptum (Hm), pectoral fin (Pf).

4.2.6: GFP expression persists in 74hpf zebrafish embryos

By 74hpf, *lama1* expression pattern is very similar to that observed at 49hpf (*Figures 3.5* and *3.6*). I predicted that GFP expression in the somites, PSM, notochord and neural tube would be extinguished, although it would remain in the midbrain-hindbrain boundary, eye, otic vesicles, jaw muscles, and the pectoral fins (*Figure 3.6*).

At 74hpf (n = 239), GFP expression mimics *lama1* expression (*Figures 4.13*B, *3.6*C), indicating that the enhancers which maintain *lama1* expression in the midbrain-hindbrain boundary region (11% embryos) (*Figure 4.13*F), eyes (14% embryos) (*Figure 4.13*C,H,J), otic vesicles (*Figure 4.13*H), jaw muscles (*Figure 4.13*C,E) and the pectoral fins (6% embryos) (*Figure 4.13*C), are present in BAC zC34A17. Although GFP recapitulates *lama1* expression even at these later stages of embryonic development, aberrant expression is detected in muscle fibres (n = 100/239 embryos, 42%) (*Figures 4.13*D,Di,K compared to *Figure 4.13*A), throughout the anterior CNS (n = 33/239 embryos, 14%), the neural tube (5% embryos) (*Figure 4.13*F,G,Gi), the notochord (1% embryos) *(Figure 4.13*G), the ventral vasculature and uro-genital region (4% embryos) (*Figure 4.13*K,L), the tail (14% embryos) and epidermis (11% embryos) (*Figure 4.13*D,L), and the yolk and heart (18% embryos) (*Figures 4.13*C,I, *4.14*ab, *4.17*E and *Table 4.7*E). Therefore, even at 74hpf (and 97hpf, *Figures 4.14ab* and *4.16*), *lama1* GFP continues to be expressed in all sites of

previous expression. However, the number of embryos that express GFP in these tissues is reduced compared to younger embryos (*Figure 4.15*). The number of zebrafish embryos expressing GFP within the pectoral fin, eye muscles, jaw muscles and the heart increases with embryo maturation (*Figure 4.14ab*). These tissues all form during late embryogenesis. For example, the pectoral fins and jaw muscles have not developed at 24hpf, but are pronounced by 48hpf. The late formation of these tissues can account for the later expression of GFP in these specific cells.

In tissues with an early onset of formation during embryogenesis, the number of GFP expressing cells decreases as zebrafish develop. There is a significant reduction in the number of GFP-expressing cells in muscles (** p-value = 0.0013), anterior CNS (* p-value = 0.0121) and the eye when comparing 26hpf to 76hpf embryos (*Figure 4.16*). On average, a 25hpf embryo (n = 14) has 44 GFP-expressing muscle fibres, whilst 76hpf embryos (n = 33) have about 14 GFP-expressing muscle fibres per embryo (*Figure 4.16*). Analysis of the number of GFP-expressing cells at 97hpf (n = 20) shows a significant increase in the number of GFP-expressing cells in the heart (** p-value = 0.0011, compared to 76hpf) but not in other tissues. Limited variations in the number of GFP-expressing cells in 97hpf compared to 26hpf or 76hpf embryos may be attributed to the larger size of tissues, leading to increased chance of integrating the GFP BAC construct.

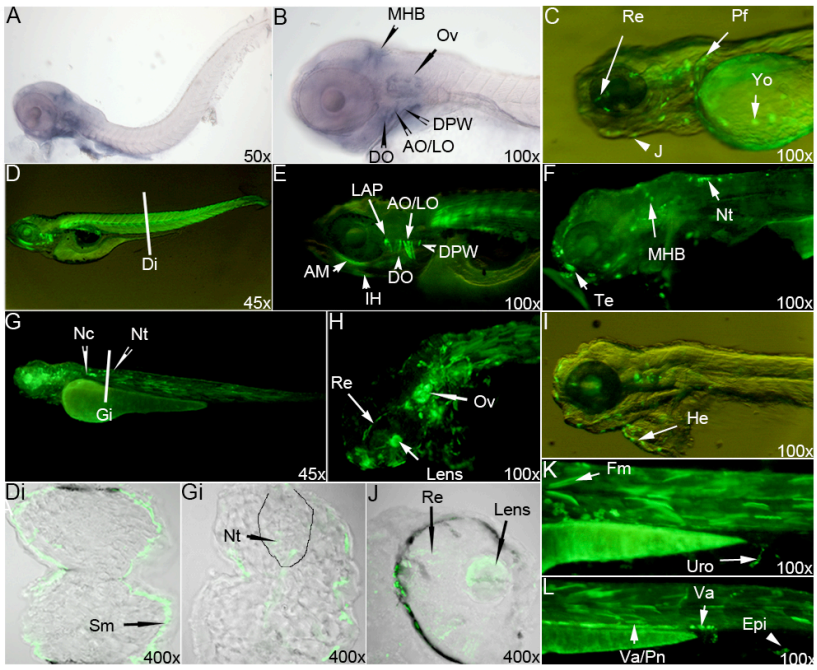

Figure 4.13: *Analysis of GFP expression in 74hpf zebrafish embryos using fluorescence microscopy*. 50pg BAC DNA was injected at the one-cell stage. GFP fluorescence (C-L) was analysed and compared to *lama1* expression (A,B). Similar to *lama1* (A,B), GFP is expressed in the eye (C,H,J), telencephalon (F), midbrain-hindbrain boundary (F), otic vesicle (H), jaw muscles (C,E), and the pectoral fins (C). GFP is also detected in the neural tube (F,G,Gi), notochord (G), and the heart (I). Strong GFP expression is observed in the fast and slow muscle fibres (D,Di,K), the vasculature and pro-nephric tubules (L), and the uro-genital region (K), despite absence of *lama1* in these tissues at this developmental stage (A). GFP is also detected in the epidermis (L). 15µm thick transverse cryosections confirm that GFP is expressed in the slow muscle (Di), neural tube (Gi), and the lens and retina of the eye (J). Positions of transverse cryosections (Ci,Ei) along the antero-posterior axis are indicated by straight lines on the lateral whole-mount images. Anterior of whole-mount images is to the left. Magnification is indicated on images. Abbreviations: telencephalon (Te), midbrain-hindbrain boundary (MHB), otic vesicle (Ov), dilator operculi (DO), adductor operculi (AO), levator operculi (LO), dorsal pharyngeal wall (Dpw), levator arcus palatini (LAP), adductor mandibulae (AM), interhyoideus (IH), jaw (J), notochord (Nc), neural tube (Nt), heart (He), retina (Re), slow muscle (Sm), fast muscle (Fm), uro-genital (Uro), vasculature/pro-nephric tubules (Va/Pn), yolk (Yo), pectoral fin (Pf), epidermis (Epi).

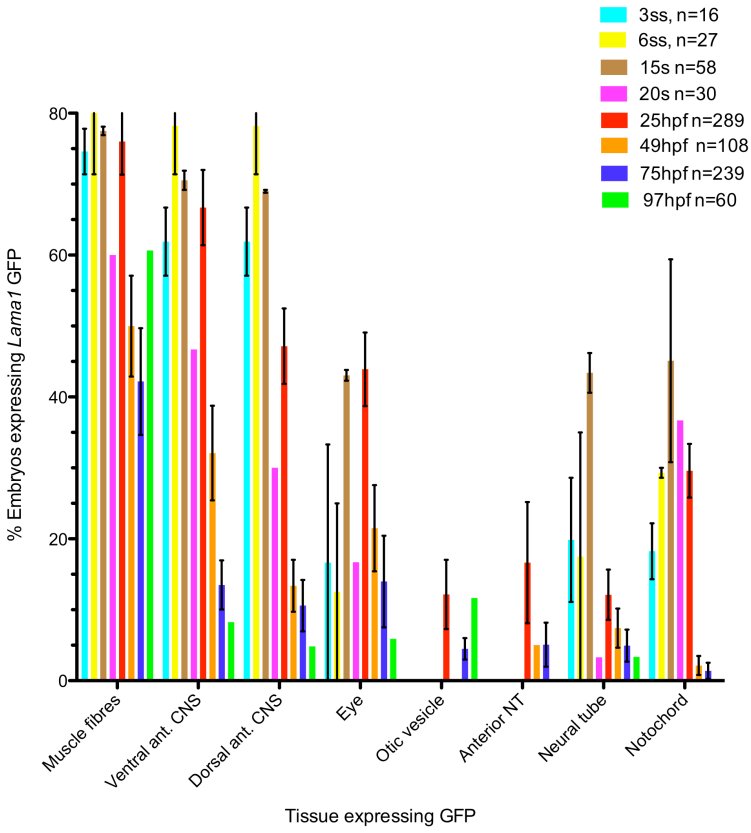

Figure 4.14a: *A comparison of GFP expression at differing stages of embryonic development.* 50pg BAC DNA was injected at the one-cell stage. The number of embryos that express GFP in muscle fibres, anterior CNS, eye, neural tube and notochord is greater in embryos younger than 25hpf. At 49hpf and 75hpf, less embryos express GFP in these tissues, demonstrating a down-regulation of *lama1* GFP. Within the anterior CNS, GFP is expressed more in ventral regions, compared to dorsal regions. GFP expression is detected by 25hpf, although expression is more obvious in later developmental stages, probably due to the increased size of the otic vesicle structure. Experimental variation is likely to account for the large number of embryos expressing GFP in muscle fibres of 97hpf zebrafish. n = number of zebrafish analysed. Standard errors of the means are shown on the graph where possible. Abbreviations: somite stage (ss), anterior CNS (ant. CNS), neural tube (NT), pre-somitic mesoderm (PSM).

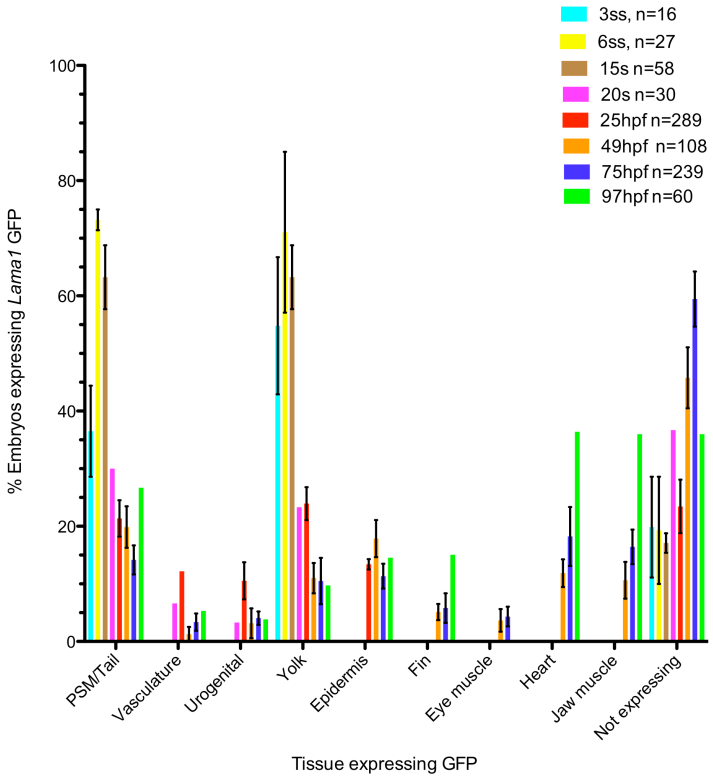

Figure 4.14b: *A comparison of GFP expression at differing stages of embryonic development.* 50pg BAC DNA was injected at the one-cell stage. The number of embryos that express GFP in the PSM/tail region and ventral vasculature is greatest from the 3-somite stage to 25hpf and the 20-somite stage to 25hpf stage, respectively. A peak in the number of embryos that express GFP in the uro-genital region is observed at 25hpf. A large number of embryos that are 49hpf to 97hpf do not express any GFP compared to younger embryos ranging from the 3-somite stage to 25hpf. An increase in embryo age is also associated with a decreased amount of GFP fluorescence on the yolk, but an increased amount of embryos that express GFP in the pectoral fin, eye and jaw muscles, and the heart. n = number of embryos analysed. Standard errors of the means are shown on the graph where possible.

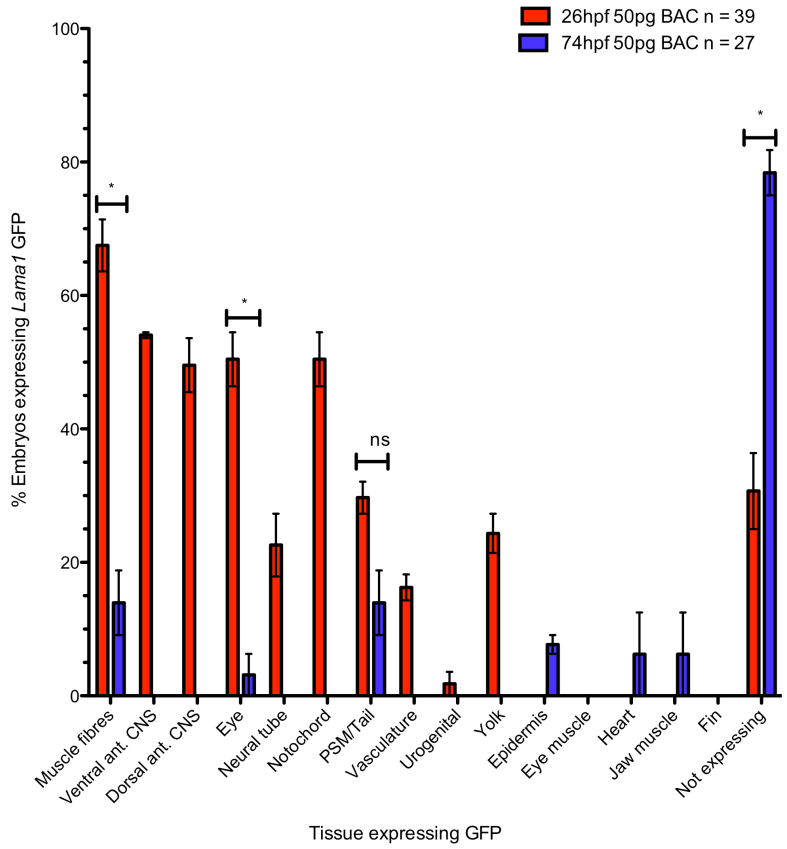

Figure 4.15: *A comparison of GFP expression in 25hpf and 74hpf zebrafish embryos.* 50pg BAC DNA was injected at the one-cell stage, and half of the batch of injected zebrafish was fixed at 25hpf whilst the other half of the same batch was fixed at 74hpf. The number of embryos that express GFP in specific tissues decreases between 25hpf and 74hpf. There is a statistically significant decrease in the number of embryos expressing GFP in the muscle fibres (p-value = 0.0132) and the eyes (p-value = 0.0116) between these two developmental stages, and no GFP expression was detected in the anterior CNS, neural tube, notochord, vasculature, uro-genital region, yolk, or the pectoral fin of these 74hpf embryos. GFP expression is not detected in the eye or jaw muscle or pectoral fin at 25hpf because these structures have not yet formed. The number of embryos that do not express any GFP significantly increases at the 74hpf stage (p-value = 0.0188). Statistical test used was the un-paired, two-tailed t-test. n = the number of zebrafish. ns = not significant. Standard errors of the means are shown on the graph. Abbreviations: anterior (ant), pre-somitic mesoderm (PSM).

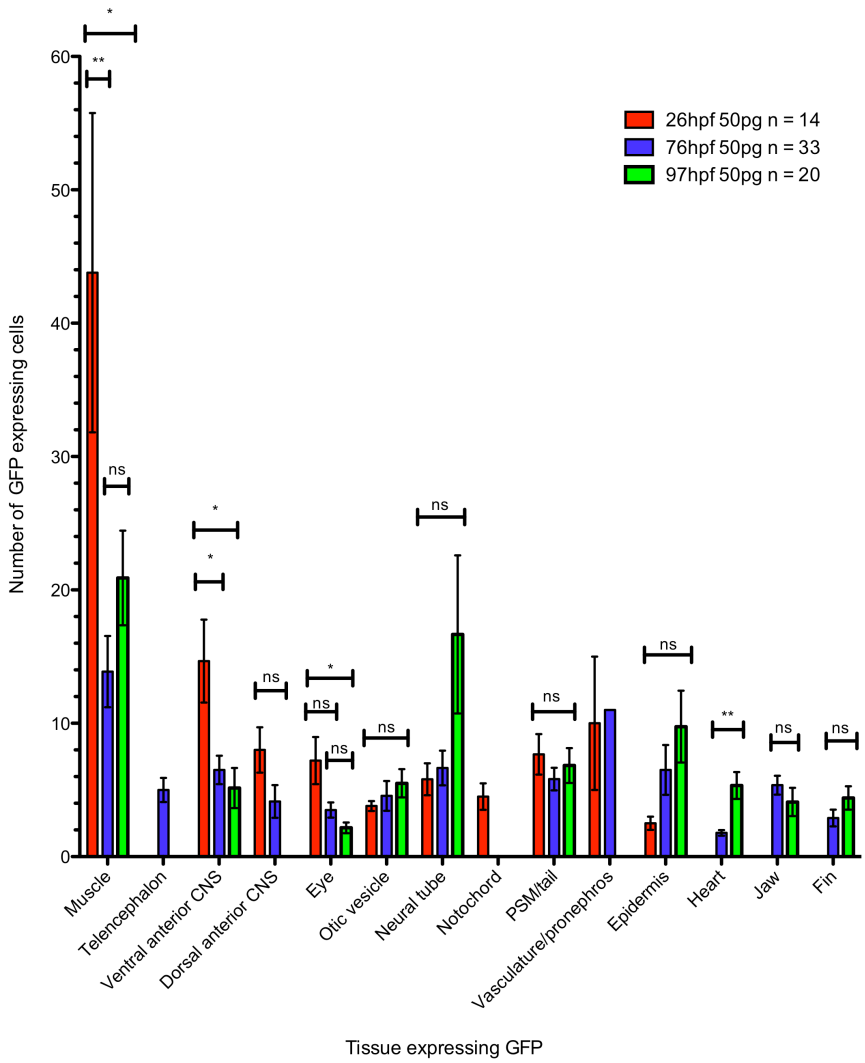

Figure 4.16: *A comparison of the number of GFP-expressing cells at different stages of zebrafish development.* 50pg BAC DNA was injected into the zebrafish embryo at the one-cell stage. The number of cells that express *lama1* GFP decreases as the zebrafish embryo develops. 26hpf embryos have the largest number of GFP-expressing cells in muscle fibres, PSM, anterior CNS, eye, and the notochord. The ventral CNS contains more GFP-expressing cells compared to the dorsal CNS. At both 76hpf and 97hpf, there is a statistically significant decrease in the number of muscle fibres (p-value = 0.0013 at 76hpf, p-value = 0.0423 at 97hpf) and ventral anterior CNS cells (p-value = 0.0121 at 76hpf, p-value = 0.0249 at 97hpf) that express GFP, compared to at 26hpf. There is also a significant reduction in the number of GFP positive cells within the eye between 26hpf and 97hpf (p-value = 0.0491). No significant difference was detected in the number of GFP-expressing cells within the otic vesicle or PSM/tail. No GFP was detected in the dorsal anterior CNS, notochord, or ventral vasculature region at 97hpf. At 97hpf, more neural tube cells express GFP compared to the 26hpf and 76hpf stages, although this difference is not significant (p-value = 0.0566 for 26hpf). There is a significant increase in the number of cells expressing GFP in the heart at 97hpf (p-value = 0.0011). Results are dependent upon injection conditions and the integration of the BAC DNA into the genome. Statistical test used was the un-paired, two-tailed t-test. n = the number of zebrafish. ns = not significant. Standard errors of the means are shown on the graph where possible. Abbreviations: pre-somitic mesoderm (PSM).

Tissue colour	% embryos expressing GFP highlighted tissue
Purple	0-10%
Pink	11-20%
Dark blue	21-30%
Light blue	31-40%
Green	41-50%
Red	51-60%
Yellow	61-70%
Orange	71-80%

Table 4.4: The % embryos that express GFP in a specific tissue correlate to the colour indicated in the table.

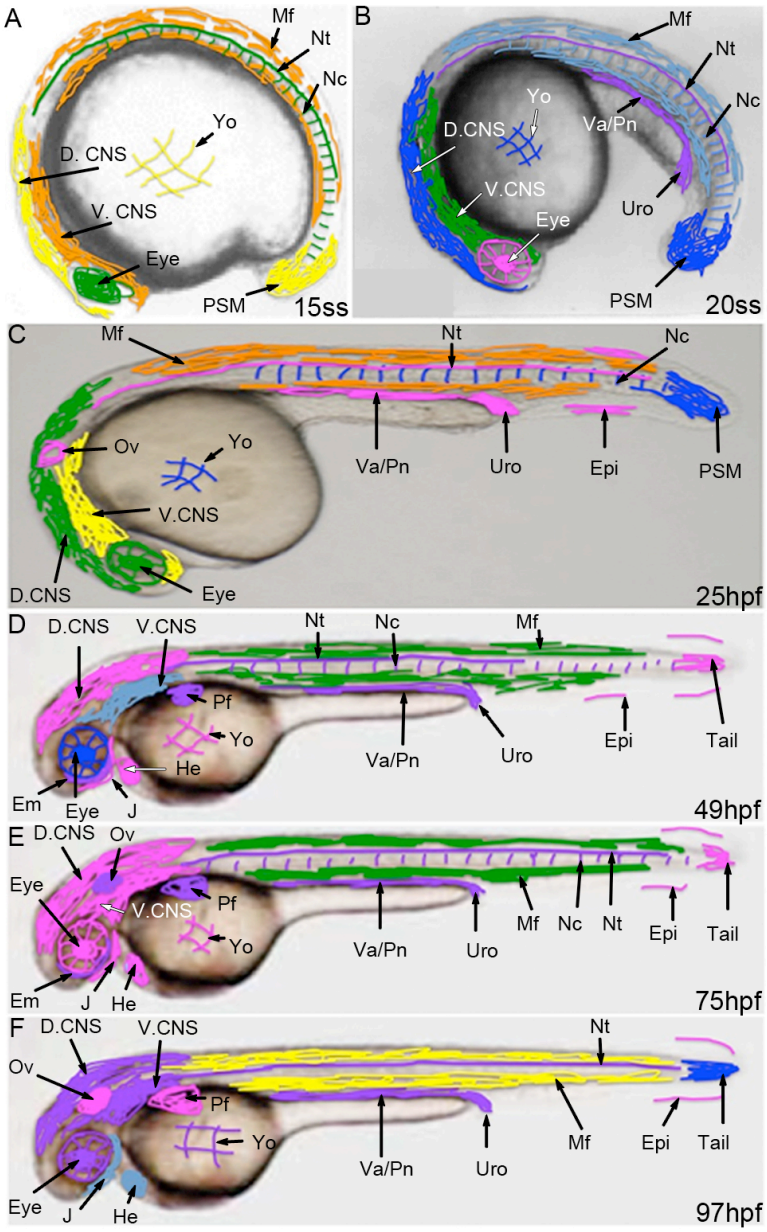

Figure 4.17: *A schematic representation of the GFP expression pattern at different stages of development.* 78% and 76% of embryos express GFP in muscle fibres at the 15-somite stage (A) and at 25hpf (C), respectively, as shown by orange highlighting. Only 37% of embryos express GFP in muscle fibres at the 20-somite stage (B), although this could be due to experimental variance, as only one batch of 20-somite stage zebrafish embryos were recorded. By 49hpf (D) and 75hpf (E), only 50% and 42% embryos express GFP in muscle fibres, respectively, representing a possible down-regulation of GFP expression. Only one batch of 97hpf zebrafish embryos (F) was recorded. Expression of GFP in the anterior CNS and eye is greatest at the 15-somite stage to 25hpf, in comparison to embryos that are 49hpf to 97hpf. Expression of GFP in the notochord and PSM/tail is most likely at the 15 and 20-somite stage. Anterior of is to the left. Abbreviations: muscle fibres (Mf), neural tube (Nt), notochord (Nc), yolk (Yo), otic vesicle (Ov), ventral anterior CNS (V.CNS), dorsal anterior CNS (D.CNS), vasculature/pro-nephric tubules (Va/Pn), uro-genital region (Uro), epidermis (Epi), pre-somitic mesoderm (PSM), heart (He), jaw (J), pectoral fin (Pf), eye muscle (Em).

A) 15-somite stage zebrafish, n = 58		
Colour	**Tissue**	**% Embryos expressing GFP in this tissue**
Orange	Ventral anterior CNS (V.CNS)	71
Yellow	Dorsal anterior CNS (D.CNS)	69
Green	Eye	43
	Otic vesicle (Ov)	-
Green	Neural tube (Nt)	43
Orange	Muscle (Mf)	78
Green	Notochord (Nc)	45
Yellow	PSM	63
	Epidermis (Epi)	-
Yellow	Yolk (Yo)	63

B) 20-somite stage zebrafish, n = 30		
Colour	**Tissue**	**% Embryos expressing GFP in this tissue**
Green	Ventral anterior CNS (V.CNS)	47
Dark blue	Dorsal anterior CNS (D.CNS)	30
Pink	Eye	17
	Otic vesicle (Ov)	-
Purple	Neural tube (Nt)	3
Light blue	Muscle (Mf)	37
Purple	Vasculature/pro-nephros (Va/Pn)	7
Purple	Uro-genital (Uro)	3
Light blue	Notochord (Nc)	37
Dark blue	PSM	30
	Epidermis (Epi)	-
Dark blue	Yolk (Yo)	23

Table 4.5: The % embryos that express GFP in a specific tissue at the 15-somite stage (A) and the 20-somite stage (B), and the colour used to represent this tissue expression in *Figure 4.17*.

C) 25hpf zebrafish, n = 289		
Colour	**Tissue**	**% Embryos expressing GFP in this tissue**
Yellow	Ventral anterior CNS (V.CNS)	67
Green	Dorsal anterior CNS (D.CNS)	47
Green	Eye	44
Pink	Otic vesicle (Ov)	12
Pink	Neural tube (Nt)	12
Orange	Muscle (Mf)	76
Pink	Vasculature/pro-nephros (Va/Pn)	12
Pink	Uro-genital (Uro)	11
Dark blue	Notochord (Nc)	30
Dark blue	PSM	21
Pink	Epidermis (Epi)	13
Dark blue	Yolk (Yo)	24

D) 49hpf zebrafish, n = 108		
Colour	**Tissue**	**% Embryos expressing GFP in this tissue**
Light blue	Ventral anterior CNS (V.CNS)	32
Pink	Dorsal anterior CNS (D.CNS)	47
Dark blue	Eye	21
	Otic vesicle (Ov)	-
Purple	Neural tube (Nt)	7
Green	Muscle (Mf)	50
Purple	Vasculature/pro-nephros (Va/Pn)	1
Purple	Uro-genital (Uro)	3
Purple	Notochord (Nc)	2
Pink	Tail	20
Pink	Epidermis (Epi)	18
Pink	Jaw (J)	11
Purple	Pectoral fin (Pf)	5
Pink	Heart (He)	12
Purple	Eye muscle (Em)	4

Table 4.6: The % embryos that express GFP in a specific tissue at 25hpf (C) and 49hpf (D), and the colour used to represent this tissue expression in *Figure 4.17*.

E) 74hpf zebrafish, n = 239		
Colour	**Tissue**	**% Embryos expressing GFP in this tissue**
Pink	Ventral anterior CNS (V.CNS)	14
Pink	Dorsal anterior CNS (D.CNS)	11
Pink	Eye	14
Purple	Otic vesicle (Ov)	5
Purple	Neural tube (Nt)	5
Green	Muscle (Mf)	42
Purple	Vasculature/pro-nephros (Va/Pn)	3
Purple	Uro-genital (Uro)	4
Purple	Notochord (Nc)	1
Pink	Tail	14
Pink	Epidermis (Epi)	11
Pink	Jaw (J)	16
Purple	Pectoral fin (Pf)	6
Pink	Heart (He)	18
Purple	Eye muscle (Em)	4
Pink	Yolk (Yo)	11

F) 97hpf zebrafish, n = 60		
Colour	**Tissue**	**% Embryos expressing GFP in this tissue**
Purple	Ventral anterior CNS (V.CNS)	8
Purple	Dorsal anterior CNS (D.CNS)	5
Purple	Eye	6
Pink	Otic vesicle (Ov)	12
Purple	Neural tube (Nt)	3
Yellow	Muscle (Mf)	61
Purple	Vasculature/pro-nephros (Va/Pn)	5
Purple	Uro-genital (Uro)	4
	Notochord (Nc)	0
Dark blue	Tail	27
Pink	Epidermis (Epi)	15
Light blue	Jaw (J)	36
Pink	Pectoral fin (Pf)	15
Light blue	Heart (He)	36
	Eye muscle (Em)	-
Purple	Yolk (Yo)	10

Table 4.7: The % embryos that express GFP in a specific tissue at 74hpf (E) and 97hpf (F), and the colour used to represent this tissue expression in *Figure 4.17*.

4.2.7: Persistent GFP expression is not due to the stability of GFP proteins

I observed that GFP continues to be expressed in several tissues at developmental stages when *lama1* expression is down-regulated or extinguished. Three hypotheses may account for this persistent GFP expression:

1) Persistent GFP expression could be the result of the transient DNA injection into the one-cell stage embryo. Transient reporter gene expression in the injected F0 zebrafish generation does not always recapitulate that of F1 generation stable lines (Clark et al. 1994; Bonifer et al. 1996; Cranston et al. 2001). Consistent with this possibility, a stable GFP line created from F0 founders does indeed present an expression pattern more similar to that of *lama1* transcripts that that of the F0 transiently-injected zebrafish embryos (see section 4.2.9).

2) Alternatively, GFP expression may be persisting at late stages of development because some DNA regulatory elements required for the temporal control of *lama1* expression are missing in BAC zC34A17. In the absence of these regulatory elements, GFP transcription is initiated, but not switched off at the appropriate developmental stage.

3) Finally, persistent GFP fluorescence may be detected due to the stability of the GFP protein. In this case, even though GFP is no longer transcribed, the stable GFP protein product continues to be observed in the embryo. To test this possibility, I have performed an in situ hybridisation experiment using a DIG-labelled RNA probe to GFP to test whether transcription of the GFP reporter gene is still occurring at developmental stages when *lama1* expression is down-regulated. GFP transcripts should be detected in all sites of endogenous *lama1* mRNA expression, and thus should be first observed at the 50-75% epiboly stage when GFP is first detected. Thereafter, GFP mRNA should be down-regulated and extinguished in a pattern that matches that of *lama1* transcripts. Therefore, GFP transcripts should only be observed in the otic vesicle, midbrain-hindbrain boundary, telencephalon, forming jaw musculature and the pectoral fin at 49hpf.

GFP transcripts are observed in a few cells as early as the 1000-cell stage (*Figure 4.18*A,B). The number of cells expressing GFP gradually increases through the epiboly stages of gastrula (*Figure 4.18*C), and by the shield stage, GFP is expressed in a mosaic fashion throughout the blastoderm (*Figure 4.18*D). The first GFP transcripts are therefore observed at the time zygotic transcription begins (Aanes et al. 2011), at about 3hpf. This is just over 2 hours from the time that GFP fluorescence is first detected at 50-75% epiboly (*Figure 4.5*B). This time lag may reflect the time necessary to transcribe and translate GFP. It is also possible that GFP fluorescence is not detected before this stage of development because Laminin α1 protein may not be synthesised until around 50% epiboly, despite the presence of *lama1* mRNA transcripts. Unfortunately, I have been unable to test this because the rat Laminin α1 antibody does not work in the zebrafish.

By 49hpf (n = 11), GFP transcripts are strongly detected throughout the zebrafish, in all sites of GFP fluorescence, including muscle fibres, floor plate, otic vesicles, midbrain-hindbrain

boundary, heart and anterior CNS (*Figure 4.18*E,F). This expression pattern is maintained at 74hpf (n = 8), with GFP mRNA also detectable in the eye and the pectoral fin (*Figure 4.18*G,H,I). There is no significant difference in the number of cells that express GFP mRNAs within a given tissue between the 49hpf and 76hpf stages of development, except for within the heart tissue which shows a slight increase at 76hpf (* p-value = 0.0275) (*Figure 4.19*). This may reflect an increase in the number of cells that form the heart tissue between these two stages.

Comparing the number of GFP-positive cells with the number of GFP mRNA-expressing cells at 76hpf (*Figure 4.20*) also reveals no statistically significant difference between GFP mRNA and protein levels within these tissues. The heart is an exception again, as higher GFP mRNA levels are detected in comparison to GFP protein (*** p-value = 0.0001) (*Figure 4.20*). Post-translational modifications may result in rapid degradation of GFP protein specifically within the heart tissue, which could account for the difference between GFP mRNA and protein levels.

Taken together, my results indicate that the GFP reporter gene is still transcriptionally active at 49hpf and 76hpf. This is likely to account for the persistent GFP fluorescence detected in several tissues at later stages of development. As the number of cells that express GFP mRNA does not vary between 49hpf and 76hpf, it supports the idea that GFP mRNA is still synthesised, and is not degraded faster than its production rate between these two developmental stages. Further confirmation that the GFP reporter gene is transcriptionally active at 76hpf comes from the analysis of the number of GFP mRNA expressing and GFP-positive cells. No difference is observed, suggesting that the amount of GFP detected correlates to the amount of GFP mRNA synthesised. Thus, it is unlikely that persistent GFP expression is due to GFP protein stability.

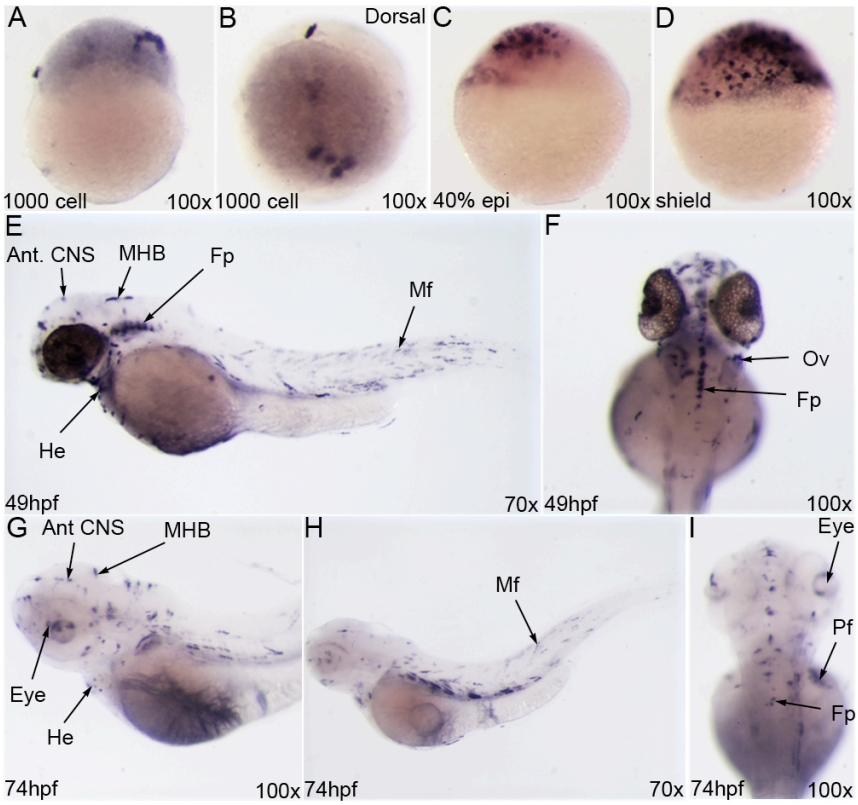

Figure 4.18: *Analysis of GFP mRNA expression in the zebrafish embryo by whole-mount in situ hybridisation (WISH).* 50pg *lama1* BAC GFP DNA was injected at the one-cell stage. GFP mRNA expression was analysed at the 1000-cell (A,B), 40% epiboly (C), shield (D), 49hpf (E,F), and 74hpf (G,H,I) stages. At the 1000-cell stage, GFP mRNA is detected in a few cells (A,B), and the number of cells expressing GFP mRNA increases at the 40% epiboly (C) and shield stage (D). By 49hpf, GFP mRNA is detected in the telencephalon, anterior CNS, eye, midbrain-hindbrain boundary, floor plate, heart, muscle fibres and otic vesicle (E,F). At 74hpf, expression persists within the eye, heart, anterior CNS, midbrain-hindbrain boundary and the muscle fibres. A dorsal view also reveals GFP mRNA expression in the pectoral fin, in addition to the floor plate of the neural tube (I). Anterior of whole-mount lateral images (E,G,H) is to the left. Magnification of each image is indicated. Abbreviations: anterior (Ant), midbrain-hindbrain boundary (MHB), floor plate (Fp), muscle fibre (Mf), heart (He), otic vesicle (Ov), pectoral fin (Pf).

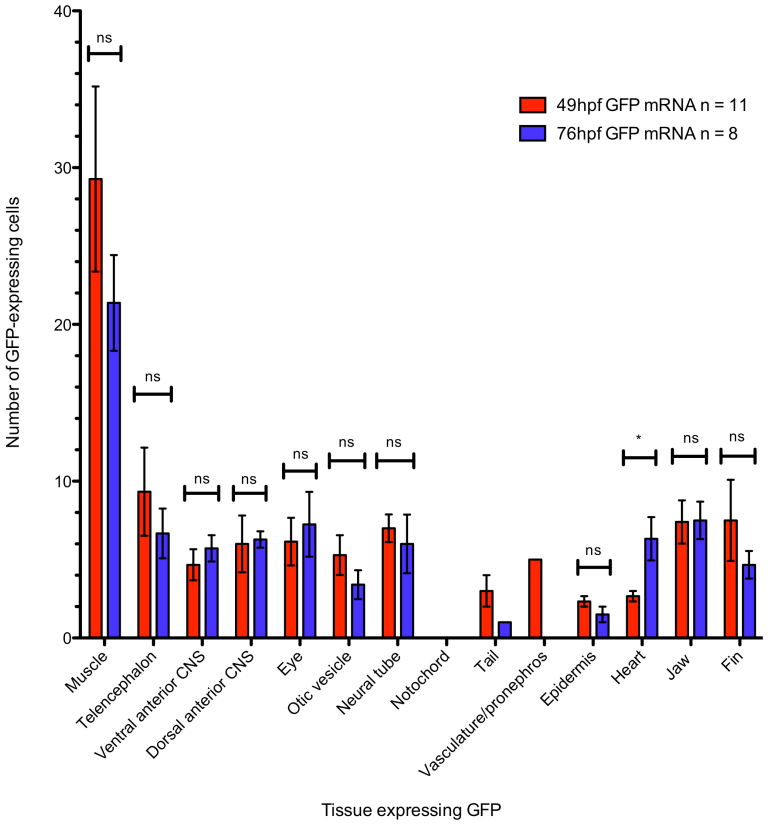

Figure 4.19: *Comparison of the number of GFP mRNA expressing cells in 49hpf and 76hpf zebrafish embryos.* 50pg BAC DNA was injected at the one-cell stage, and half of the batch of injected zebrafish was fixed at 49hpf whilst the other half was fixed at 76hpf. Similar numbers of cells express GFP mRNA at both 49hpf and 76hpf in all tissue types, except the vasculature/pro-nephric tubules which show no GFP mRNA expression in this batch of 76hpf embryos, and the heart. There is a significant increase in the number of cells which express GFP mRNA in the heart in 76hpf embryos compared to embryos at the 49hpf stage (p-value = 0.0275). Statistical test used was the un-paired, two-tailed t-test. n = the number of zebrafish. ns = not significant. Standard errors of the means are shown on the graph where possible.

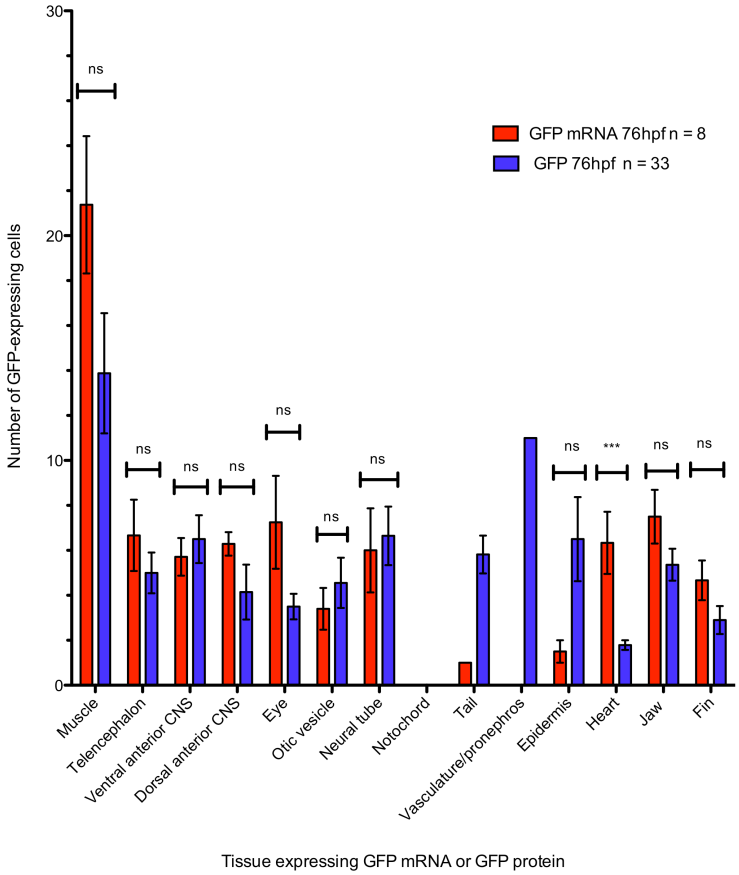

Figure 4.20: *A comparison of the number of cells that express GFP mRNA or GFP protein at 76hpf.* 50pg BAC DNA was injected at the one-cell stage, and half of the batch of injected zebrafish was collected for in situ hybridisation whilst the other half was collected for GFP fluorescence analysis. There is no significant difference between the number of cells expressing GFP mRNA or the number of GFP-positive cells in any tissue type at 76hpf, except for the heart. The heart shows a significant decrease in the number of are GFP-positive cells (p-value = 0.0001). The vasculature/pro-nephric tubules lacked GFP mRNA, despite the presence of GFP-positive cells in the vasculature of the other half of the batch. One embryo expressed GFP mRNA in the tail. Statistical test used was the un-paired, two-tailed t-test. n = the number of zebrafish. ns = not significant. Standard errors of the means are shown on the graph where possible.

4.2.8: Hh signalling regulates the expression of *lama1* GFP in the neural tube and uro-genital region

lama1 expression is modified by manipulation of the Hh signalling pathway (sections 3.2.5.1 and 3.2.5.2). The most pronounced changes occur when the levels of Hh signalling are increased, such as in *ptc1/2* embryos or in dnPKA mRNA-injected embryos. In these embryos, there is a dramatic increase in *lama1* expression in the neural tube, PSM, ventral vasculature and uro-genital region at 25hpf (*Figures 3.20* and *3.22*). A slight increase in *lama1* expression is also observed in the anterior CNS of dnPKA mRNA-injected embryos (*Figure 3.20*D).

As BAC zC34A17 appears to contain all the necessary enhancer elements to recapitulate the expression of *lama1*, it is likely that GFP expression will be affected by manipulation of the Hh signalling pathway. To test this, I have co-injected 100pg of dnPKA mRNA with 50pg of BAC zC34A17 DNA into wild-type embryos at the one-cell stage, and let the embryos develop until they reached 25hpf. At this point, I analysed GFP expression in control embryos injected with only BAC zC34A17 DNA, and embryos co-injected with BAC zC34A17 DNA and dnPKA mRNA. I hypothesised that increased Hh signalling caused by injection of dnPKA mRNA would increase GFP expression in the neural tube, PSM, hypochord, ventral vasculature, and the uro-genital region. This would be in line with the effects on *lama1* expression upon dnPKA mRNA injection. However, the predicted increase in GFP expression in these tissues may not be easily detectable, because the BAC zC34A17 DNA construct integrates into the genome in a mosaic fashion. Therefore, despite the presence of large amounts of Gli activator in most cells after dnPKA mRNA injection, intense ectopic GFP expression may not be clear within a given tissue, caused by a lack of BAC DNA integration into this tissue.

At 25hpf, there is a significant increase in the number of GFP-positive cells in the neural tube (8 cells compared to 2 cells, * p-value = 0.0292) and uro-genital region (* p-value = 0.0362) of BAC zC34A17 DNA and dnPKA mRNA co-injected embryos (n = 12) (*Figure 4.21*C-F,H and *4.22*), compared to BAC zC34A17 DNA injected embryos (n = 15) (*Figure 4.21*A,B and *4.22*). GFP-expressing cells in the neural tube are observed along the entire antero-posterior axis of the embryo in dnPKA mRNA injected embryos (*Figure 4.21*C-F). An increased number of GFP-expressing cells are also observed in the ventral vasculature region (*Figure 4.21*G), the PSM (*Figure 4.21*C), and the muscle fibres (*Figures 4.21*C), although the difference is not significant (*Figure 4.22*). There is also no significant difference in the number of cells that express GFP in the eye, anterior CNS, and the notochord (*Figure 4.22*).

Therefore, results suggest that BAC zC34A17, like *lama1*, is responsive to Gli activator transcription factor. Data support the finding that Hh signalling is sufficient for *lama1* expression in the neural tube and uro-genital region, at 25hpf (section 3.2.5.2).

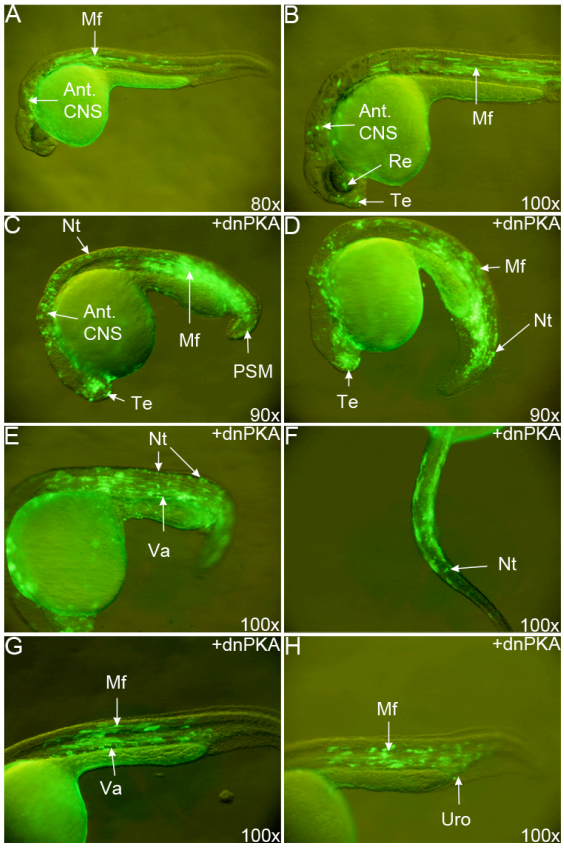

Figure 4.21: *Analysis of GFP expression in 25hpf zebrafish embryos with increased Hh signalling.* 50pg BAC DNA, or 50pg BAC DNA with 100pg dnPKA mRNA was injected at the one-cell stage. GFP expression was compared between BAC DNA injected (A,B) and BAC DNA + dnPKA mRNA co-injected embryos (C-H). In lateral view images of BAC DNA injected embryos, GFP is observed in the muscle fibres (A,B), anterior CNS (A,B), telencephalon (B), and the retina (B). In BAC DNA + dnPKA mRNA co-injected embryos, lateral view images reveal GFP expression in the telencephalon (C,D), retina (C,D), anterior CNS (C), muscle fibres (C,D,G,H), PSM (C), and the neural tube (C,E). Neural tube expression is also detected in a dorsal view image (F). There is a noticeable up-regulation of GFP expression along the entire length of the neural tube in co-injected embryos (C-F). An increased number of GFP-expressing cells are also detected in the ventral vasculature (G) and uro-genital region (H) of co-injected embryos. Anterior of lateral images is to the left. Anterior of the dorsal view image is at the top of the panel. Magnification of all images is indicated. Abbreviations: muscle fibres (Mf), anterior CNS (Ant. CNS), telencephalon (Te), retina (Re), neural tube (Nt), pre-somitic mesoderm (PSM), vasculature (Va), uro-genital region (Uro).

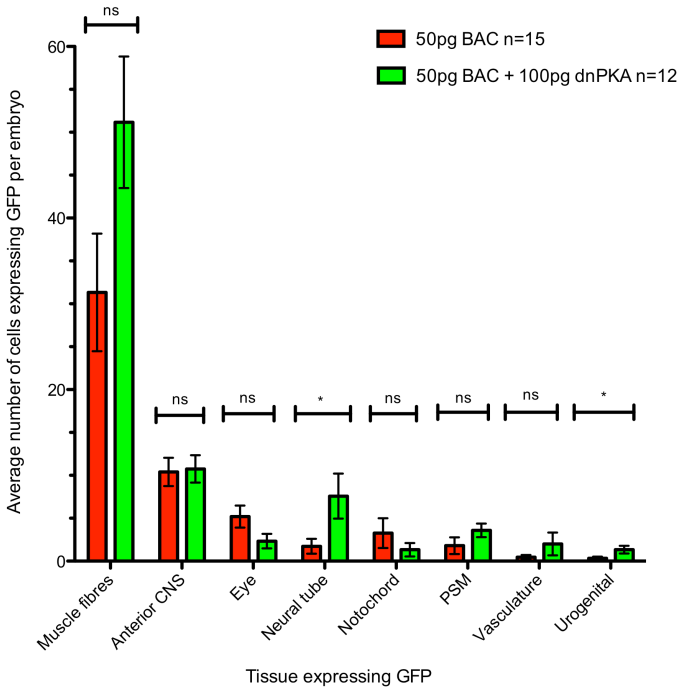

Figure 4.22: *A comparison of the number of GFP-expressing cells in 26hpf zebrafish injected with BAC DNA alone or co-injected with BAC DNA and dnPKA mRNA.* The number of GFP-expressing cells was assessed in 25hpf zebrafish embryos injected either with 50pg BAC DNA or co-injected with 50pg BAC DNA and 100pg dnPKA mRNA, at the one-cell stage. A significant increase in the number of GFP-positive cells is observed in the neural tube (* p-value = 0.0292) and uro-genital region (* p-value = 0.0362). BAC DNA-injected embryos express GFP in an average of 2 cells in the neural tube, and 0.3 cells in the uro-genital region, whilst BAC DNA and dnPKA mRNA injected embryos express GFP in an average of 8 cells in the neural tube, and 1.3 cells in the uro-genital region. No significant difference in the number of cells expressing GFP was detected in the muscle fibres, anterior CNS, eye, notochord, PSM, or vasculature. Statistical test used was the un-paired, two-tailed t-test. n = the number of zebrafish. ns = not significant. Standard errors of the means are shown on the graph. Abbreviations: pre-somitic mesoderm (PSM).

4.2.9: A stable GFP reporter line recapitulates faithfully the pattern of *lama1* expression in the zebrafish

Overall, analysis of transiently injected embryos reveals that GFP is detected in all tissues that express *lama1*, and GFP is initiated on schedule in these tissues. Despite a possible absence of regulatory elements which down-regulate *lama1* expression, BAC zC34A17 contains all the necessary DNA elements that control *lama1* expression. Therefore, GFP expression in a stable transgenic zebrafish line generated from a BAC zC34A17-injected embryo should faithfully recapitulate *lama1* expression. Comparison of GFP expression with *lama1* expression should also help to address whether BAC zC34A17 contains all necessary enhancer elements required for *lama1* expression. Analysis of GFP expression in a stable line of transgenic zebrafish also allows testing the possibility that persistent GFP expression in the F0 founder generation is the result of transient injection (section 4.2.7). These zebrafish lines will provide a useful tool for the study of *lama1* regulation as the global expression of GFP can be monitored in live embryos throughout development, and upon manipulation of candidate regulatory pathways.

By selecting for and raising GFP-positive embryos obtained from crossing the BAC injected F0 generation with wild-type zebrafish, a stable GFP transgenic line (*TgBAC(lama1:GFP)*) was created. I raised a total of 106 zebrafish embryos that had been co-injected with 50pg BAC iTOLzC34A17-GFP and 100pg transposase mRNA. Of these, five founder fish were identified, which showed germline integration of BAC zC34A17-GFP DNA, making a successful germline transmission rate of about 5%. Others have reported a transmission rate varying from 5-100% when using the iTol2 system (Bussmann and Schulte-Merker 2011).

I predicted that GFP fluorescence would be detected from about the 50-75% epiboly stage of development, and would quickly become ubiquitous throughout the blastoderm (*Figure 4.5*B). I expected that GFP expression would be initiated in all the tissues previously described to display GFP in the transient injected F0 generation embryos. Persistent GFP expression observed in the F0 transient generation suggested that regulatory elements were missing from BAC zC34A17, which functioned to down-regulate *lama1* expression. This was supported by evidence that the GFP reporter gene remained transcriptionally active in the F0 generation at 76hpf. Based on these findings, I hypothesised that a loss of regulatory elements will cause persistent GFP expression in stable transgenic *lama1:GFP* zebrafish.

All progeny from the five founder zebrafish show identical patterns of GFP expression, which therefore rules out the possibility that positional integration effects alter the expression of GFP. At the 15-16-somite stage, GFP is expressed in the eye (*Figure 4.23*B,C,E,F), otic vesicles (*Figure 4.23*B,E), telencephalon and diencephalon (*Figure 4.23*B,C,E,F), somites (*Figure 4.23*B,D,E,F), notochord (*Figure 4.23*B,E,F) and the PSM (*Figure 4.23*B,E,F). I noticed that GFP expression is not as widespread within the somites and anterior CNS in comparison to the F0

transient generation at the same developmental stage (compare *Figures 4.23*B and *4.6*B). Therefore, the GFP pattern of the stable lines resembles more closely that of *lama1* (*Figure 4.23*A) than the pattern derived from the F0 transient generation at the 15-somite stage (*Figure 4.6*B).

By 25hpf, GFP is detected in the lens and retina (*Figure 4.24*A-D), telencephalon (*Figure 4.24*A,B), diencephalon (*Figure 4.24*A,B,D,E), otic vesicles (*Figure 4.24*A,B,E), midbrain-hindbrain boundary (*Figure 4.24*A,B,D,F), vasculature and uro-genital region (*Figure 4.24*I,J), and the PSM (*Figure 4.24*K), completely matching *lama1* expression. However, unlike *lama1* expression, GFP is also detected in the fast (*Figure 4.24*H) and slow muscle (*Figure 4.24*G) fibres, and the notochord (*Figure 4.24*I,K). Interestingly, although GFP persists in the somites, vasculature and the notochord, its expression is much weaker compared to GFP detected in the anterior CNS, eye, and otic vesicle of the same zebrafish (*Figure 4.24*A), and weaker compared to transiently injected embryos at the same developmental stage (*Figure 4.7*C,E). This suggests that similar to *lama1*, GFP is being down-regulated within the these structures. In agreement with this finding, the expression of GFP mRNA was assessed in *lama1:GFP* stable line zebrafish by in situ hybridisation, which shows a progressive down-regulation within the somites between the 12-somite to 25hpf stage embryo (*Figure 4.25*E-H), but is fully maintained in the telencephalon and diencephalon, eyes, midbrain-hindbrain boundary, and the otic vesicles (*Figure 4.25*E-H). By 49hpf, a further down-regulation of GFP mRNA is observed throughout the trunk of *lama1:GFP* stable line zebrafish, and expression in the head is restricted to the eye, telencephalon, branchial arches, midbrain-hindbrain boundary, and the otic vesicles (*Figure 4.25*J). Expression is also initiated in the pectoral fin (*Figure 4.25*J,L). By 72hpf, GFP mRNA expression completely recapitulates the endogenous *lama1* expression pattern (*Figure 4.25*M,O,Q), with transcripts detectable in the telencephalon (*Figure 4.25*N,P,R), eye (*Figure 4.25*N,P,R,S), midbrain-hindbrain boundary (*Figure 4.25*N,P,R), otic vesicles (*Figure 4.25*N,R), branchial arches (*Figure 4.25*N,R), and jaw musculature (*Figure 4.25*S), heart (*Figure 4.25*N,R), and the pectoral fins (*Figure 4.25*N,P,R,S). Expression of both GFP mRNA and *lama1* is absent from the somites, uro-genital region, notochord, and the PSM (*Figure 4.25*M,N). By comparison, transiently injected embryos strongly express GFP in all of these structures at 72hpf (*Figure 4.13*).

At every stage analysed, expression of GFP mRNA is slightly stronger in comparison to *lama1*. For example, by 25hpf, *lama1* is restricted from the entire notochord (*Figure 4.25*D), whilst GFP mRNA is only down-regulated slightly in the anterior notochord (*Figure 4.25*H). Expression of GFP mRNA is also stronger in the vasculature and uro-genital region, somites, and PSM at 25hpf in comparison to *lama1* expression. (*Figure 4.25*D,H). Similarly, weak GFP mRNA expression can be detected throughout the somites at 49hpf (*Figure 4.25*J), whilst *lama1* expression is down-regulated (*Figure 4.25*I). Therefore, despite the similarity of the overall expression pattern, down-regulation of GFP mRNA is slightly delayed compared to *lama1*. This could be attributed to an

increased stability of GFP mRNA. Others have also noted increased stability of GFP transcripts (unpublished data, Stone Elworthy).

Together, results indicate that the *lama1:GFP* stable line zebrafish recapitulates faithfully endogenous *lama1* expression. This indicates that it is highly likely that BAC zC34A17 contains every necessary enhancer required to control the expression of *lama1*. The slight persistence of GFP fluorescence in the stable line can be attributed to stability of the GFP protein, in addition to the stability of GFP transcripts, rather than an absence of repressive elements. This supports the possibility that persistent GFP expression detected in the F0 transient generation is due to pseudo-expression.

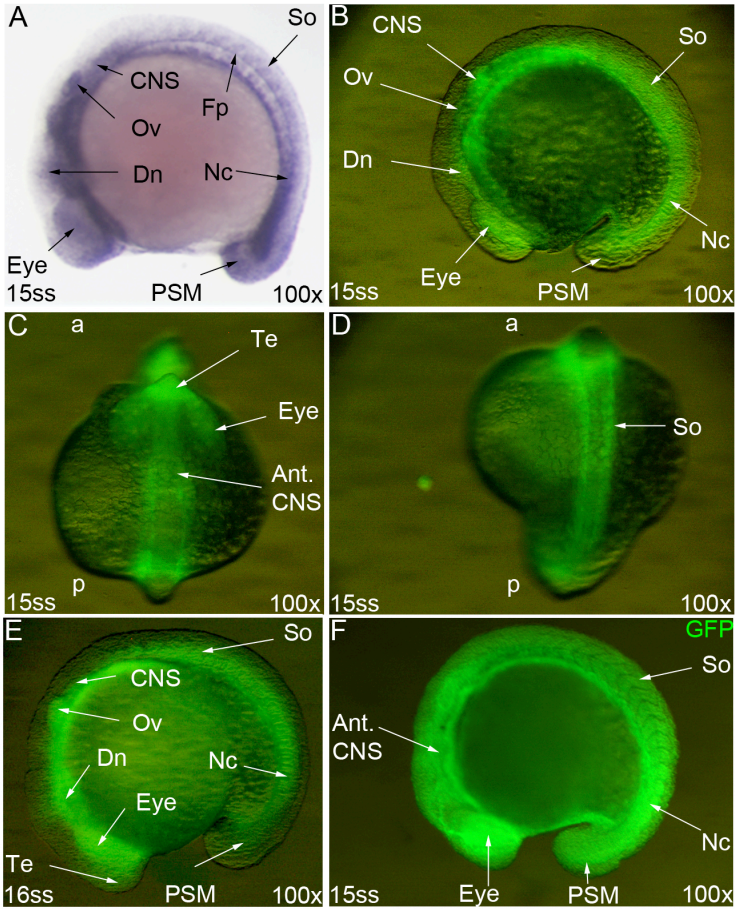

Figure 4.23: *Analysis of GFP expression in the stable transgenic lama1:GFP zebrafish embryo at the 15-16-somite stage using fluorescence microscopy.* A: Lateral view of a 15-somite stage whole-mount in situ hybridisation (WISH) embryo using a *lama1* probe at 100x magnification. B-F: Lateral (A,E,F) and dorsal views (C,D) of GFP expression at the 15 (B,C,D,F) or 16 (E)-somite stage at 100x magnification. GFP expression fully recapitulates *lama1* expression, with GFP deteced in the eye (B,C,E,F), telencephalon and diencephalon (B,C,E,F), otic vesicles (B,E), somites (B,D,E,F), notochord (B,E,F), and the PSM (B,E,F). A GFP antibody has been used to observed expression of GFP in panel F. Expression is detected throughout the embryo, with the highest intensity located in the eye, posterior notochord, and the PSM (F). Anterior of lateral views is to the left. Anterior of dorsal views is at the top of the image. Abbreviations: diencephalon (Dn), otic vesicle (Ov), central nervous system (CNS), floor plate (Fp), pre-somitic mesoderm (PSM), notochord (Nc), somite (So), telencephalon (Te), anterior (a), posterior (p).

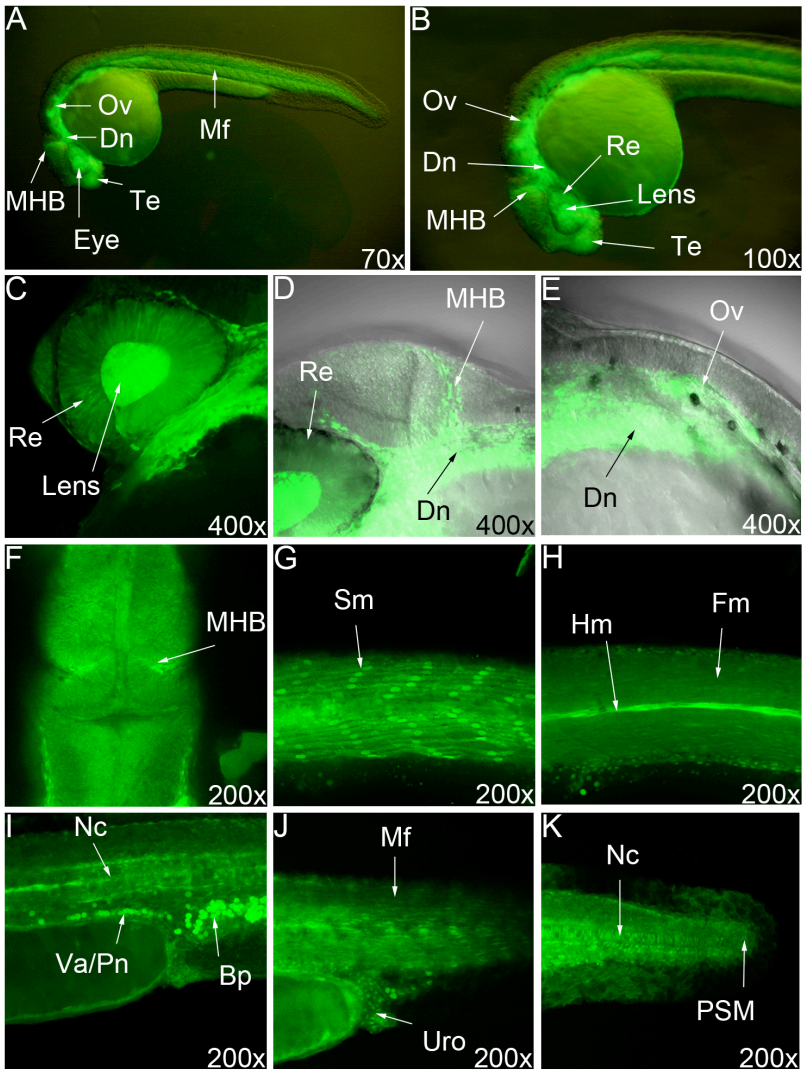

Figure 4.24: *Analysis of GFP expression in the stable transgenic lama1:GFP zebrafish embryo at 25hpf using fluorescence microscopy*. A and B: Lateral views at 70x (A) and 100x (B) magnification, showing GFP expression in the telencephalon and diencephalon, lens and retina, midbrain-hindbrain boundary, and the otic vesicle. Weaker expression of GFP is also detected in the muscle fibres. C-F: Lateral (C-E) and dorsal (F) views at 200x (F) and 400x (C-E) magnification reveal intense GFP expression in the lens and retina (C,D), diencephalon (D,E), midbrain-hindbrain boundary (D,E), and the otic vesicle (E). G and H: Lateral views at 200x magnification, show GFP expression in the slow (G) and fast (H) muscle fibres, in addition to intense expression in the horizontal myoseptum (H). Nuclei of the slow muscle fibres are noticeable (G). I-K: Lateral views at 200x magnification, show expression of GFP in and around the notochord (I,K), the vasculature/pro-nephric tubule (I) and uro-genital region (J), muscle fibres (J), and the PSM (K). GFP is also observed in the blood pool (I). Anterior of lateral views is to the left. Anterior of dorsal view is at the top of the image. Abbreviations: midbrain-hindbrain boundary (MHB), telencephalon (Te), diencephalon (Dn), otic vesicle (Ov), muscle fibres (Mf), retina (Re), slow muscle (Sm), fast muscle (Fm), horizontal myoseptum (Hm), notochord (Nc), vasculature/pro-nephric tubules (Va/Pn), blood pool (Bp), uro-genital region (Uro), pre-somitic mesoderm (PSM).

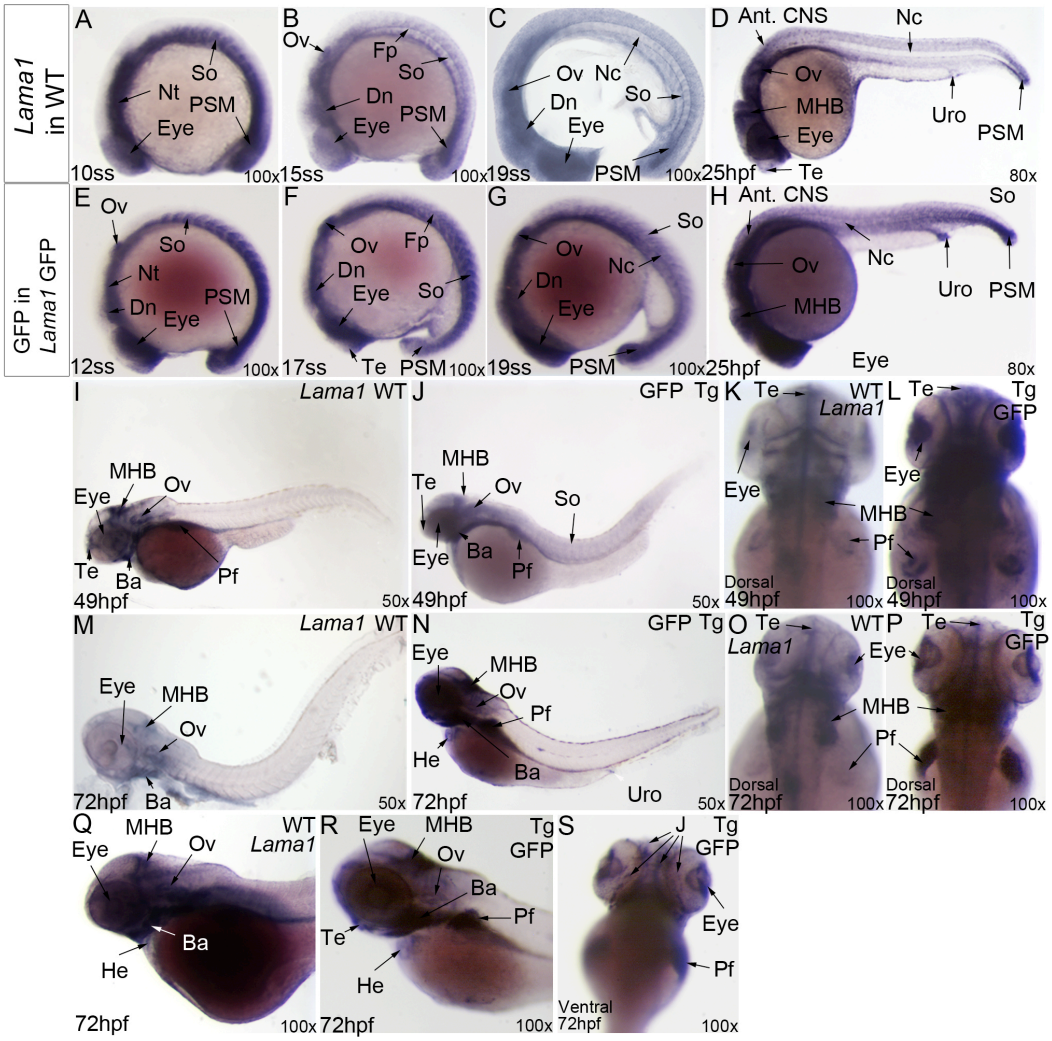

Figure 4.25: *Analysis of GFP expression by whole-mount in situ hybridisation in the stable transgenic lama1:GFP zebrafish embryo from the 12-somite stage to 72hpf.* The expression of GFP in *lama1:GFP* stable line embryos was compared to *lama1* in wild-type embryos. A-D: Lateral views of *lama1* expression at the 10 (A), 15 (B), 19 (C)-somite or 25hpf (D) stage. E-H: Lateral views of GFP expression at the 12 (E), 17 (F), 19 (G), or 25hpf (H) stage. GFP expression is similar to *lama1* expression at these stages, with GFP detected in the eye, diencephalon, anterior CNS and neural tube, otic vesicles, somites, uro-genital region (H), notochord, and the PSM. However, GFP expression is more intense, and is not down-regulated in the somites (F,G,H), notochord (H) or uro-genital region (H) to the same extent as *lama1* (B,C,D). I-L: Lateral (I,J) and dorsal views (K,L) of *lama1* (I,K) and GFP (J,L) expression at 49hpf. GFP and *lama1* expression is maintained in the telencephalon, eyes, midbrain-hindbrain boundary and otic vesicles. Both GFP and *lama1* expression are initiated in the branchial arches (J) and pectoral fins (J,L). GFP expression is down-regulated throughout the trunk, in line with the expression pattern of *lama1*. M-P: Lateral (M,N) and dorsal (O,P) views of *lama1* (M,O) and GFP (N,P) expression at 72hpf. By 72hpf, GFP expression is identical to *lama1* expression, except that it is more intense. GFP expression remains in the midbrain-hindbrain boundary, eyes, otic vesicles, branchial arches and forming jaw muscles, and the pectoral fins. Expression is also observed in the heart, and is absent from the somites, notochord and uro-genital region. Q-S: Lateral (Q,R) and ventral (S) views of *lama1* and GFP (R,S) expression at 72hpf. GFP is detected in the heart, telencephalon, eye, midbrain-hindbrain boundary, otic vesicles, branchial arches and forming jaw musculature (S), and the pectoral fins. Anterior of lateral views is to the left. Anterior of dorsal and ventral views is at the top of the image. Magnification is indicated on each image. Abbreviations: neural tube (Nt), somite (So), midbrain-hindbrain boundary (MHB), telencephalon (Te), diencephalon (Dn), otic vesicle (Ov), muscle fibres (Mf), retina (Re), slow muscle (Sm), fast muscle (Fm), horizontal myoseptum (Hm), notochord (Nc), vasculature/pro-nephric tubules (Va/Pn), blood pool (Bp), uro-genital region (Uro), heart (He), branchial arches (Ba), jaw musculature (J), pre-somitic mesoderm (PSM), pectoral fin (Pf).