2.2.3.3: Phusion polymerase PCR reactions

PCR amplification of long sequences was performed using Phusion Hot Start DNA Polymerase (2U/µl) (Finnzymes). Phusion requires at least 15 seconds per Kb during the elongation phases. Ultramer PCR reactions were performed in a 20µl volume:

11.7µl MilleQ H_2O
4µl Phusion HF 5x buffer
1.6µl 20mM dNTPs
0.5µl F ultramer (20µM)
0.5µl R ultramer (20µM)
1.2µl mini prep
0.5µl 2U/µl Phusion DNA polymerase (2U/µl) (Finnzymes).

1. Denaturation	98°C	5 min
2. Denaturation	98°C	1 min
3. Annealing 3°C higher than primer homology annealing temp.	1 min	
4. Elongation	72°C	90 secs
5. Repeat step 2-4	19x	
6. Final elongation	72°C	10 min
7. HOLD	10°C	

Amplification of linear targeting constructs by PCR reactions using ultramer end primers were performed in a 300µl volume (split into 15 tubes of 20µl).

193µl MilleQ H_2O
60µl Phusion HF 5x buffer
24µl 10mM dNTP mix
7.5µl ultramer end primer F (20µM)
7.5µl ultramer end primer R (20µM)
5µl mini prep of template
3µl Phusion DNA polymerase

1. Denaturation	98°C	40 secs
2. Denaturation	98°C	30 secs
3. Annealing 3°C higher than primer homology annealing temp.		45 secs
4. Elongation	72°C	2 min
5. Repeat step 2-4	19x	
6. Final elongation	72°C	10 min
7. HOLD	10°C	

2.2.4: TOPO cloning of PCR products

PCR products were ligated into pCRII-TOPO using the TOPO TA Cloning kit (Invitrogen). 1µl of PCR product was mixed with 1µl salt solution, 3µl sterile H_2O, and 1µl TOPO vector. The reaction was gently mixed, and left at room temperature for 5 minutes. It was then placed on ice for 20-30 minutes before 2µl of the cloning reaction was transformed into TOP10F' competent cells (Invitrogen). For blue/white screening of bacterial colonies, LB agar plates were spread with 40µl of 40mg/ml X-gal (Promega), and 40µl of 100µg/µl IPTG (Sigma) and left to dry before the cells are plated. White colonies were selected for restriction analysis of miniprep DNA.

2.2.5: Ligation

2.2.5.1: Vector preparation

Vector DNA was digested using the appropriate enzyme, in a volume of 20µl. 100ng of vector was used, along with 2µl 10x buffer, 2µl 10x BSA and 0.5µl enzyme. After 90 minutes incubation at 37°C, the enzyme was inactivated by incubation for 15 minutes at 65°C.

To prevent vector self-ligation, Shrimp Alkaline Phosphatase (SAP, USB, 1U/l µl) was used to dephosphorylate the DNA ends. SAP buffer was used at 1x, and 1µl of SAP is added to the vector. The reaction was incubated for 30 minutes at 37°C, and terminated by incubating at 65°C for 15 minutes. The appropriate amount of insert was added to the mixture, and precipitated overnight at -20°C. This was resuspended in 15µl H_2O.

2.2.5.2: Ligation reaction

Ligations were performed in a 20µl volume, using T4 DNA ligase (Invitrogen, High concentration T4 ligase). 4µl 5x ligation buffer and 1µl T4 ligase was added to the 15µl resuspended pellet. The reaction was incubated for 6 hours at room temperature and placed on ice for at least 5 minutes before their transformation into ultracompetent XL-Gold cells (Stratagene). Alternatively, the ligation reaction was performed overnight at 16°C.

2.2.6: Recovery of plasmid DNA

Filter paper containing plasmid DNA was cut up to little pieces, and soaked overnight in 1x TE pH7.0, 4°C. For transformations, 2µl of the resuspended plasmid in TE was used.

2.2.7: Transformation

1µl of plasmid (10-100ng/ml) or ligation reaction was added to 40µl of chemically competent *E.coli* cells (ultracompetent XL-Gold cells, Stratagene) on ice. Cells were mixed gently and incubated on ice for 20-30 minutes. Cells were heat shocked at 42°C for 40 seconds, and immediately placed on ice. 1ml SOC medium was added, followed by a 1 hour incubation at 37°C in a shaking incubator (250rpm). 20-100 µl of cells were plated on pre-warmed LB agar plates, which contained the appropriate antibiotic (50µg/ml Ampicillin or Kanamycin), and incubated at 37°C overnight.

2.2.8: Plasmid DNA

Cell cultures were grown overnight at 37°C in a shaking incubator (250rpm) in 5ml or 100ml LB containing the appropriate antibiotic, for either a mini (<20µg DNA) or midi prep (<100µg DNA), respectively. The culture was centrifuged at 6000rpm for 5 minutes (x2) for a mini prep, or for 15 minutes (x2) for a midi prep. DNA was isolated from the pelleted cells using the Qiaprep columns (Qiagen), following the spin protocols provided in the QIAGEN Plasmid Purification Handbook. Midi prep DNA was resuspended in 100-200µl milleQ H_2O.

2.2.9: Estimation of DNA or RNA concentration

1µl, 0.1µl, 0.01µl and 0.001µl of DNA or RNA was run on a 1% Agarose/TAE or Agarose/TBE gel with 3µl 10mM Ethidium Bromide, respectively, along with 3µl of GeneRuler 1Kb Ladder (Fermentas). DNA or RNA concentration was estimated by comparison of samples intensity to the ladder bands of a known concentration. Alternatively, DNA or RNA concentration was determined using the Nanodrop spectrophotometer (NanoDrop).

2.2.10: Restriction digestion

Restriction digests were performed using restriction enzymes from either Roche, Promega, or New England Biolabs, along with their corresponding buffers. 2µl mini prep DNA was digested in a total volume of 20µl, with 2µl 10x buffer, 2µl 10x BSA, and 0.5µl enzyme at 37°C for 1 hour. 5µl of the reaction was run on a 1% Agarose/TAE gel. For RNA probe synthesis, 10µg of midi prep DNA was digested in a total volume of 200µl containing 20µl 10x buffer, 20µl 10x BSA and 2µl of enzyme.

2.2.11: Gel extraction

DNA was extracted from agarose gels using QIAquick columns (Qiagen), following the spin protocols provided in the QIAGEN QIAquick Spin Handbook.

2.2.12: Phenol/Chloroform extraction

The initial volume of DNA solution is taken as 1 volume. To this, 0.5 volume of phenol and 0.5 volume of chloroform/isoamylic alcohol (24:1) were added, and vortexed for 30 minutes. This was then centrifuged at 13,000 RPM for 10 minutes, and the upper aqueous phase transferred to a new tube. 1 volume of chloroform/isoamylic alcohol (24:1) was added and vortexed for 10 minutes, before being spun at 13,000RPM for another 10 minutes. The upper aqueous phase containing the DNA was recovered. The DNA was then precipitated and resuspended to the desired concentration.

2.2.13: DNA/RNA precipitation

DNA was precipitated by adding 1/10 volume of 3M sodium acetate (pH 5.3), 2 volumes 100% ethanol and 1µl glycogen, and incubated overnight at -20°C, or for 1 hour at -80°C. RNA was precipitated by adding 1/20 volume of 4M LiCl, 1µl glycogen, and 2.5 volume or 3 volume ethanol for an overnight or 1 hour incubation respectively. DNA and RNA was pelleted by centrifugation at 13,000RPM for 30 minutes at 4°C, washed with 70% ethanol (RNA grade for RNA precipitation), and centrifuged for 10 minutes at 13,000RPM at 4°C. Supernatant was removed and the pellet left to air-dry, before being resuspended at the desired concentration in milleQ or DEPC-treated H_2O.

2.2.14: Visualisation and image capture of agarose gels

Gels contained 3µl Ethidium Bromide (10mg/ml) per 50ml, and were viewed under U.V. light. Images were captured using Uvidoc system (Uvitec).

2.2.15: Bacterial artificial chromosome (BAC) selection

Ensembl website was used to identify a BAC encompassing the zebrafish *lama1* locus (*Table 2.7*) (*Figure 4.1*).

Gene	BAC	BAC library	Vector	Antibiotic resistance	Host cell
lama1	zC34A17	zC CHOR211	pTARBAC2.1	Chloramphenicol	DH10B

Table 2.7: Zebrafish *lama1* gene is contained in BAC zC34A17, cloned into the pTARBAC2.1 vector.

2.2.16: BAC mini prep

The desired BAC in DH10B cells was streaked onto 12.5µg/ml Chloramphenicol (Cm) plates and grown over night at 37˚C. Colonies were picked and grown in 3ml LB broth overnight at 37˚C. 1.6ml of bacteria culture was pelleted at 13,000rpm for 10 minutes at room temperature, and the supernatant was removed. Cells were resuspended in 100µl buffer P1 (Qiagen), and the cells lysed in 200µl buffer P2 (Qiagen). Following the addition of 150µl ice cold buffer P3 (Qiagen), lysed cells were spun at 13,000rpm for 10 minutes at room temperature. 1ml of EtOH was added to the supernatant, which was placed at -80˚C for 1 hour. BAC DNA was pelleted by centrifugation at 13,000RPM for 30 minutes at 4°C, washed with 70% ethanol, and centrifuged for 10 minutes at 13,000RPM at 4°C. Supernatant was removed and the pellet left to air-dry, before being resuspended in 50µl milleQ H_2O. PCR tests confirmed the presence of the correct BAC DNA sequence (see results section 4.2.2).

2.2.17: BAC midi prep

Mini cultures were used to inoculate 100ml midi cultures containing the appropriate antibiotic. BAC DNA was isolated following the User-Developed Protocol on the Qiagen website (http://www.qiagen.com/literature/default.aspx), and resuspended in 100-200µl milleQ H_2O.

2.2.18: BAC electroporation into EL250 cells

300ng of BAC DNA was mixed with 40µl of EL250 cells that contain genes essential for recombineering under the control of a temperature sensitive repressor (Lee et al., 2001; Yu et al., 2000). After incubation on ice for 30-60 seconds, the cell/DNA mixture was pipetted into an ice-cold cuvette (gene pulser 0.1cm, Bio-rad) and electroporation was carried out using the Ec1 programme (1.3kV and 200 Ohms) of a Bio-rad electroporator. Cells were then shaken for 90 minutes at 32˚C, plated onto selective agar plates (Cm), and grown overnight at 32˚C. Colonies were picked and grown in 3ml LB broth cultures containing the appropriate antibiotic overnight at 32˚C. PCR tests were performed using BAC mini culture or BAC mini prep samples, to confirm the presence of intact BAC DNA within the EL250 cells (see results section 4.3).

2.2.19: Generating the target reporter construct

The target reporter construct is a cassette containing an eGFP ORF with its own kozak sequence and Kanamycin (KAN) resistance gene flanked with FRT sites, cloned into a TOPO vector (K5 construct) (*Table 2.8*) (*Figure 2.1*). The homology arms were added using ultramers (Integrated DNA Technologies), which were designed to have about 80 nucleotide homology with the BAC DNA, and 20 nucleotide homology with the eGFP cassette (*Table 2.6*) (*Figure 2.1*). The forward ultramer has homology with the BAC DNA sequence directly upstream of the gene (*lama1*) start site whilst the reverse ultramer has homology with the BAC DNA sequence immediately

downstream of the gene ATG. The ATG of the gene of interest (*lama1*) should align with the ATG of the eGFP.

Ultramers were added to the GFP-Kanamycin resistance gene fragment (amplified from the K5 construct) following the PCR procedures listed in section 2.2.3.3. PCR products were purified using the using the QIAGEN MinElute kit, with the following modification: 750µl of 35% guanidine hydrochloride aqueous solution was added to the column after the DNA binding step, and centrifuged for 1 minute. PCR purification then carried on as described in the manufacturer's protocol. Elution was performed in 10µl milleQ H_2O. Following gel extraction using MinElute columns (section 2.2.11), 3µl of DNA was mixed with 3µl of 2x BioMix Red (Bioline), and incubated at 72°C for 15 minutes. DNA PCR product was then cloned into TOPO pCRII vector (Invitrogen) (section 2.2.4).

BAC insert/(deletion)	Vector used for ultramer PCR	Reference for vector
GFP-FRT-KAN-FRT	TOPO pCRII K5	(Renshaw et al., 2006)
Tol2-AMP	TOPO pCRII iTOL2	Gift from S. Elworthy
Tol2-AMP (Δ3.3kb)	TOPO pCRII iTOL2	Gift from S. Elworthy
KAN (ΔEx63)	TOPO pCRII	Invitrogen
KAN (ΔEx4)	TOPO pCRII	Invitrogen
KAN (ΔEx2)	TOPO pCRII	Invitrogen
KAN (ΔEx1)	TOPO pCRII	Invitrogen
KAN (Δ5kb)	TOPO pCRII	Invitrogen
Tol2-AMP (Δmin.prom)	TOPO pCRII iTOL2	Gift from S. Elworthy

Table 2.8: Vector/insert construct used to generate a linear target construct by PCR.

2.2.20: Generating a linear target construct

A linear targeting construct was generated by PCR using specific ultramer end primers (for PCR conditions see section 2.2.3.3). The TOPO DNA template was removed from the PCR product by addition of 5µl DpN1 enzyme and incubation for 2 hours at 37˚C. PCR product was then purified and gel extracted using QIAGEN MinElute columns and the protocols listed in the QIAGEN PCR purification and gel extraction handbook. The linear targeting construct was resuspended in 20µl milleQ H_2O.

2.2.21: Recombineering of the target construct with the BAC

A 20ml culture of the EL250 BAC-containing cells was prepared from a 1ml starter culture, and grown at 32˚C until a density of 0.5 at OD600 was achieved. Cells were then shaken at 42˚C for 15 minutes, followed by shaking on ice for 20 minutes. Cells were then spun down to a pellet at 13,000rpm for 30 seconds, pooled together and resuspended in 1ml ice-cold milleQ H_2O. This was repeated twice more, using 44µl of ice-cold milleQ H_2O for the final resuspension. 300ng of linear target construct was mixed and electroporated into the cells, as in section 2.2.18. Following shaking at 32˚C for 90 minutes, 50-200µl cells were plated onto 4 or 5 agar plates containing the antibiotic that the linear target construct has resistance to. PCR tests on BAC mini preps confirmed correct recombineering had taken place and the presence of target construct insert.

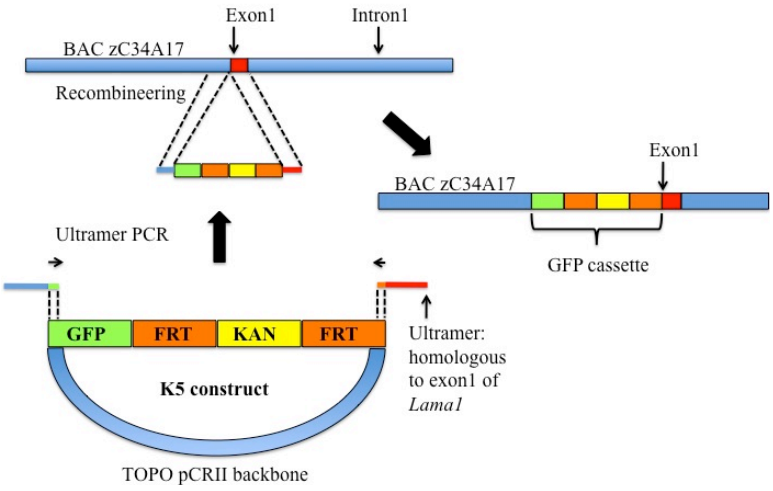

Figure 2.1: *A schematic representation of homologous recombineering using the K5 GFP reporter construct*. PCR amplifies the GFP cassette from the K5 construct using ultramer primers, which each have about 20 bases homology with the K5 construct, and 80 bases homology with BAC zC34A17 sequence. Ultramers have homology to the DNA sequence that is either side of the *lama1* ATG start site, and so after recombination, the GFP cassette is located in the position of the *lama1* ATG start site. Exon1 of *lama1* is located immediately after the GFP cassette following recombination.

2.2.22: Addition of Tol2 sites to the BAC backbone DNA sequence

A cassette containing inverted Tol2 sites and an Ampicillin resistance gene (iTOL2) (*Table 2.8*) was inserted into the BAC backbone DNA sequence using homologous recombineering (see sections 2.2.19-2.2.21) (*Figure 2.2*). Co-injection of transposase mRNA with the BAC containing Tol2 sites results in linearisation of the BAC-Tol2 DNA. Transposase catalyses integration of the excised BAC-Tol2 construct into the genome (Suster et al., 2009).

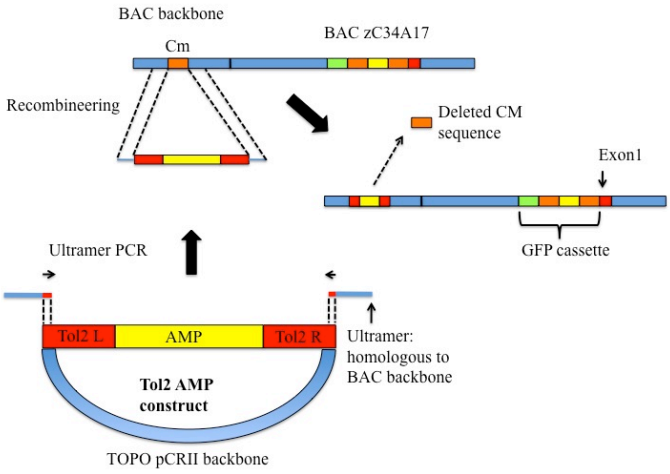

Figure 2.2: *A schematic representation of the addition of the Tol2 AMP sequence into BAC zC34A17.* Ultramers with homology to DNA sequence that is either side of the region to be deleted (Cm) in the BAC are added onto the Tol2 AMP DNA sequence by PCR. TOPO pCRII iTOL2 is used as template DNA Homologous recombineering inserts the Tol2 AMP DNA sequence into the BAC backbone sequence, in place of the Chloramphenicol (Cm) resistance gene By modifying the sequence of homology in the ultramer, the Tol2 AMP DNA sequence can insert into another location, allowing the deletion of specific regions of DNA that are between the two ultramer arms, within BAC zC34A17.

2.2.23: Removal of the Kanamycin resistance gene from the GFP reporter construct

A 100ml culture of EL250 BAC containing cells was grown overnight at 32˚C in LB broth, diluted 50x into a 20ml culture the next day, and grown to a density of 0.5 at OD600. *Flpe* genes were induced by addition of 0.1% L-arabinose (v/v) to the culture, and incubation for 1 hour at 32˚C. Cells were then diluted 1:10 in LB broth, and grown for a further 1 hour at 32˚C. 2µl of cells were plated onto an agar plate containing the appropriate antibiotic. Removal of the Kanamycin resistance gene was confirmed through PCR analysis.

2.2.24: Deletion recombineering within the BAC

Homologous recombineering was performed to delete specific regions of BAC DNA. As in section 2.2.19, ultramers that have homology with the BAC DNA sequence directly either side of the region of DNA sequence to be deleted, were added to a selective marker cassette. To delete sequences present upstream of the *lama1* start site, the iTOL2 AMP construct was inserted (*Table 2.8*) (*Figure 2.2* and *Figure 5.3*). To delete sequences present downstream of the *lama1* start site, a Kanamycin resistance gene (from pCRII TOPO vector) was inserted into the BAC (*Table 2.8*). See *Table 5.8* for representation of BAC constructs used.

2.2.25: Micro-injection of BAC DNA

BAC DNA was purified as in section 2.2.17. 50 to 75pg of BAC DNA in a volume of 1 to 1.5nl was injected into each zebrafish embryo, at the one-cell stage. 50pg of transposase RNA was co-injected with BAC constructs containing Tol2 sites to increase integration efficiency into the genome.

2.3: In silico analysis

2.3.1: Genomic sequence retrieval

The genomic location of *lama1* and *c125* were identified in various species using the Ensembl project; www.ensembl.org. Genome sequences and locations are accurate as of Ensembl release 66, February 2012. Zebrafish sequences were obtained using Zebrafish Zv9 software on Ensembl. Sequences were also found using the National Centre for Biotechnology Information database; www.ncbi.nlm.nih.gov/gene.

2.3.2: Annotation and analyses of sequence files

Sequence files were stored, annotated and manipulated in ApE (A plasmid editor v1.10.4, http://www.biology.utah.edu/jorgensen/wayned/ape/). ApE was also used for restriction enzyme identification, plasmid sequence construction, and the viewing of chromatogram files.

2.3.3: Alignment tools

2.3.3.1: Genomic alignment tools

Genomic sequences were aligned using ECR Browser (http://ecrbrowser.dcode.org/; (Ovcharenko et al., 2004) and VISTA (http://genome.lbl.gov/vista/customAlignment.shtml) for comparative genomics. Default parameters were used. Conserved regions of interest identified were annotated onto genomic DNA sequences in ApE files (section 2.3.2).

2.3.3.2: General alignments

DNA sequences were aligned using the multiple sequence alignment software found on www.ebi.ac.uk. The following programmes were used, with default parameters: (http://www.ebi.ac.uk/Tools/msa/clustalw2/), (http://www.ebi.ac.uk/Tools/msa/tcoffee/), (http://www.ebi.ac.uk/Tools/msa/muscle/).

2.3.4: Binding site identification

To identify potential binding sites of transcription factors in specific regions of genomic sequence, the sequence was inputted into the MatInspector tool of the Genomatix server (http://www.genomatix.de; (Cartharius et al., 2005; Quandt et al., 1995).

The following parameters were used:

MatInspector library: Matrix Family Library Version 8.4 (June 2011)

Selected groups: General Core Promoters Elements, Vertebrates (0.75/optimized)

Binding sites of interest were annotated onto ApE files, whilst making reference to their positions on conserved alignments of the gene. Known binding sites (for Gli proteins) were also manually searched for in the genomic DNA sequence, and annotated onto ApE files.

2.3.5: Promoter search

To identify potential promoters in regions of genomic sequence, the genomic sequence upstream of the *lama1* start site was inputted into McPromoter software (http://tools.igsp.duke.edu/generegulation/McPromoter/), and F Prom software (http://linux1.softberry.com/berry.phtml?topic=promoter). NCBI Map viewer software (http://www.ncbi.nlm.nih.gov/projects/mapview) was also used to search for the presence of CpG islands, which could reveal potential promoter sites.

2.3.6: Blast tools

The identity of plasmid and insert DNA sequence was confirmed using the nucleotide blast tool against the nucleotide collection nr/nt (http://blast.ncbi.nlm.nih.gov/Blast.cgi). When comparing against a known sequence, a two-way blast was used (http://blast.ncbi.nlm.nih.gov/Blast/bl2seq/wblast2.cgi).