Regulation of *Sonic hedgehog* (*Shh*) expression in the zebrafish retina

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submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

Presented by
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Thesis Defense: **18.05.2007**

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The research described in this thesis is carried out at the Institute of Toxicology and Genetics, Forschungszentrum Karlsruhe, Germany under the supervision of Prof. Dr. Uwe Straehle.

Dedicated to

My Dear parents, Vino and Rathi

Abbreviations

Shh	Sonichedgehog
Fgf	Fibroblast growth factor
Twhh	Tiggy winkle hedgehog
Ehh	Echidna hedgehog
hpf	hours post fertilisation
RetE	retinal enhancer
GCL	Ganglion Cell Layer
INL	Inner Nuclear Layer
Ar-A	activating region A
Ar-B	activating region B
Ar-C	activating region C
MOs	morpholinos
syu	*sonic-you*
smu	*slow muscle omitted*
GFP	Green Fluorescent Protein
PTU	Phenyl Thio Urea
Tk	Thymidine kinase
HYB	Hybridisation buffer
ETS	Erythroblastoma Twenty Six
ERM	Ets Related Molecule
PEA3	Polyoma Enhanced Virus 3

Summary

Sonic hedgehog (*Shh*) a vertebrate homolog of the *Drosophila melanogaster* gene hedgehog, is a secreted protein that controls numerous differentiation processes during vertebrate development. In the vertebrate retina, *Shh* controls neurogenesis and its expression spreads in a wave like manner.

The *shh* locus has been well characterised in vertebrates and the regulatory regions driving the expression in the notochord and the midline of the CNS were identified. In zebrafish, *shh* transgenes were established that drive GFP reporter expression very similar to that of the endogenous *shh* gene in the central nervous system including the retina. The cis-regulation behind *shh* expression in the retina was not known. This prompted me to map the region responsible for retina expression. I found it to be distinct from the previously described enhancers controlling expression in the midline of the neural tube and in the notochord. This novel regulatory region mediates expression of a GFP reporter cassette in the ganglion cell layer (GCL) and inner nuclear layer (INL) of the retina. The expression is initiated in a ventronasal patch and later spreads across the GCL and INL in a wave like pattern.

A deletion approach identified a 300 bp region to be sufficient and necessary for driving expression in the retina. By a second series of mutations across this region using a linker scanning approach, a minimal 40 bp core important for expression was identified. While one clusters of point mutation impaired expression in both GCL and INL, another region was mapped that affected expression exclusively in the GCL. Thus, expression in the two layers can be separated.

Pea3 and Erm factors were predicted to bind to the retinal enhancer. *In vitro* protein binding and morpholino studies prove that they are key regulatory factors responsible for driving expression in the retina. Pea3 and Erm are factors known to act downstream of Fgf signals. Earlier studies have demonstrated that Fgf signalling from the optic stalk is responsible for initiation of retinal neurogenesis. Moreover, *shh* was shown to be required for the spread of the *shh* expression wave in an auto-regulatory manner. Pharmacological inhibition and morpholino studies were performed to study the the role of Fgfs and Shh in the propagation of the expression. My results suggest that several distinct Fgfs and Hedgehogs act sequentially at several levels and that the propagation of the expression wave is dependent on a co-operation between FGF and Shh signalling pathways. This is reminiscent to the spread of the expression of the Drosophila *hedgehog* gene through the eye imaginal disc.

Zusammenfassung

Sonic hedgehog (*Shh*) ist ein Wirbeltier-Homolog des Drosophila melanogaster Gens Hedgehog. Es ist ein sekretiertes Protein, das zahlreiche Differenzierungsprozesse während der Wirbeltierentwicklung kontrolliert. In der Retina von Wirbeltieren kontrolliert *Shh* die Neurogenese und seine Expression breitet sich in wellenähnlicher Art aus.

Der *Shh* Lokus wurde in Wirbeltieren ausführlich beschrieben und die regulatorischen Elemente, die die Expression in der Chorda und der ventralen Mittellinie des ZNS steuern, wurden identifiziert. Im Modelorganismus Zebrafisch wurden transgene Tiere erzeugt, die Reporter-GFP in einem Muster exprimieren, das dem des endogenen *Shh* im Zentralnervensystem sehr ähnlich ist, inklusive der Retina. Dieses Cis-regulatorische Element des *Shh* Locus war bis dahin nicht bekannt. Dies veranlasste mich dazu, die Region, die für die Expression in der Retina verantwortlich ist, zu kartieren. Ich fand heraus, dass sie sich von zuvor beschriebenen Enhancern unterscheidet, welche die Expression in der Mittellinie des Neuralrohrs und in der Chorda kontrollieren. Diese neue regulatorische Region vermittelt die Expression einer GFP-Reporterkassette in der Ganglienschicht (GCL) und der inneren nuklearen Schicht (INL) der Retina. Die Expression wird in einem ventronasalen Fleck initiiert und breitet sich später wellenartig über die GCL und die INL aus.

Durch Deletionen konnte eine 300 bp lange Region identifiziert werden, die hinreichend und notwendig ist, um die Expression in der Retina zu steuern. Eine zweite Serie von Mutationen in dieser Region mittels der „linker scanning" Methode führte zur Identifizierung einer minimalen 40 bp Kernsequenz, die wichtig für die Expression ist. Eine Anhäufung (Cluster) von Punktmutationen beeinträchtigte die Expression sowohl in der GCL als auch in der INL und es konnte eine weitere Region kartiert werden, die die Expression ausschließlich in der GCL beeinflusste. Somit kann die Expression in diesen zwei Schichten aufgetrennt werden.

Es wurde vorhergesagt, dass Pea3/Erm Faktoren an den retinalen Enhancer binden. In vitro Proteinbindungsexperimente und Morpholino-Injektionen bewiesen, dass sie Schlüsselfaktoren für die Regulation der Expression in der Retina sind. Es ist bekannt, dass Pea3 und Erm stromabwärts von FGF Signalen wirken. Frühere Studien zeigten, dass FGF Signale vom Augenstiel verantwortlich für die Initiation der retinalen Neurogenese sind. Weiterhin wurde gezeigt, dass *Shh* auf autoregulatorische Weise für die Ausbreitung der *Shh* Expressionswelle notwendig ist. Es wurden pharmazeutische Hemmung und Morpholino-Studien angewendet, um die Rolle von FGFs und *Shh* in der Ausbreitung der Expression zu untersuchen. Meine Ergebnisse lassen darauf schließen, dass mehrere verschiedene FGFs und Hedgehogs aufeinanderfolgend auf mehreren Stufen wirken und dass die Ausbreitung der Expressionswelle abhängig von der Kooperation zwischen FGF- und *Shh*-Signalwegen ist. Dies erinnert an die Ausbreitung der Expression des Drosophila Hedgehog Gens über die Imaginalscheibe des Auges.

Introduction

1. The Cis regulatory network

Metazoan genes contain highly structured regulatory elements that direct complex patterns of expression in the different cell types during development. For proper expression of a gene, a 5' promoter element with a proper transcriptional start site is required to assemble a protein complex for RNA synthesis. Most of the genes require additional elements located immediately upstream or downstream of this minimal promoter called the regulatory promoters for the robust expression of the gene (Butler and Kadonaga, 2002). Apart from these promoter elements, some genes require certain other cis-regulatory elements for proper gene expression in a spatio-temporal manner. Some cis-regulatory elements function as enhancers, but they also function as silencers and insulators (Dillon and Sabbattini, 2000) (Fig 1).

An enhancer is an element that physically interacts with the core promoter via protein-protein interactions through the transcription factors bound to it to enhance the activity of the promoter (Dillon and Sabbattini, 2000). A typical gene is likely to contain several enhancers that can be located in 5' and 3' regulatory regions as well as introns. Each enhancer can be responsible for a subset of the total gene expression pattern; they frequently mediate expression only within a specific tissue or cell type. A typical enhancer is something around 100-500 bp in length and contains many protein binding sites. For example the seven stripes of the *even-skipped* expression in the Drosophila embryo depend on five separate enhancers; two located on the 5' of the transcription site and three located 3' of the gene (Fujioka et al., 1999; Small et al., 1993). These enhancers function in an autonomous fashion owing to short range repression: sequence-specific repressors bound to one enhancer do not interfere with the activities of neighbouring enhancers. These repressors must bind within 50-100 bp of an upstream activator region or the core promoter in order to inhibit expression (Mannervik et al., 1999). A silencer exhibits similar structural features of an enhancer but suppresses the promoter activity (Cook, 2003).

Insulators also called boundary elements are known to restrict the activities of enhancers and silencers so that they do not affect the expression of the neighbouring

genes in an unspecific manner (Bell et al., 2001). They execute their activities by blocking the enhancer/silencer-promoter interactions (Burgess-Beusse et al., 2002). Locus control regions are thought to control at the level of local chromatin environment, making it accessible to transcription factors (Wood WI, 1982). Genomic regions harbouring these regulatory elements can stretch as much as 1 Mb in either direction of the transcriptional unit (Kimura-Yoshida C, 2004) Some of these enhancers can also reside in the introns of the neighbouring genes, without interfering with their expression (Kleinjan et al., 2002; Lettice et al., 2003).

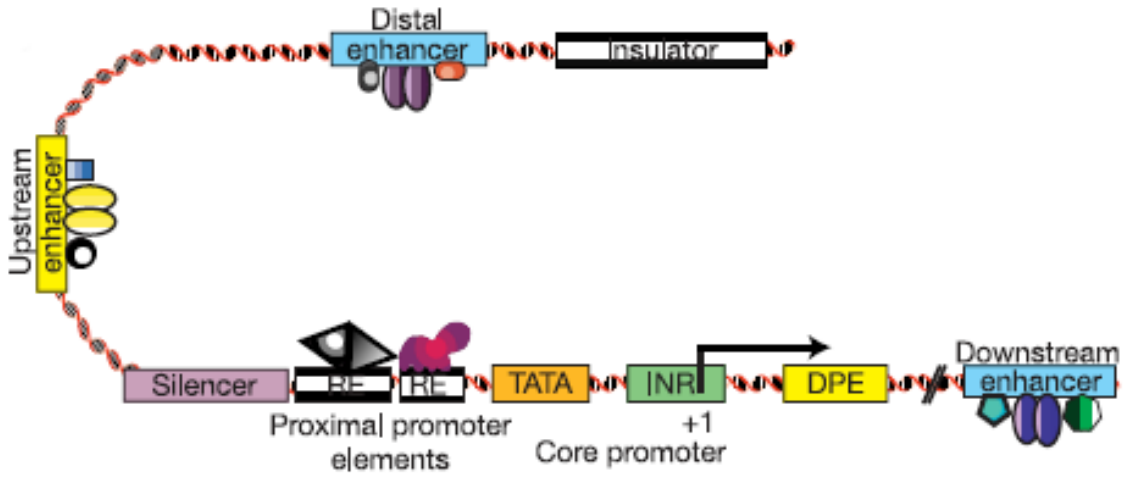


Figure 1: Representation of a typical metazoan transcription unit and its control modules that consist of multiple enhancers in combination with silencer(s) and insulators. INR and DPE represent initiator and downstream promoter elements respectively. The control module interacts with the basal transcriptional unit for proper gene expression in a spatio-temporal manner (Modified from Levine M and Tjian R 2003, Nature, 424(10):147-151).

Comparative genomics is a new branch of biology employing comparison based strategies to identify functional features of genomes (Nobrega and Pennacchio, 2004). During recent years, whole genome sequences of many vertebrates such as human, mouse, zebrafish, fugu, rat, chicken, frog and many others have become available (ENSEMBL). This will help the scientific community to access the sequences of all genes of these species (Aparicio et al., 2002; Lander et al., 2001; Venter et al., 2001). Genome comparisons are crucial tools to annotate whole-genome sequences and will provide insight into the cis-regulatory architecture of gene loci. In simple words, functional DNA would have less changed than non-functional DNA during the course of evolution (Jukes and Kimura, 1984). Comparison of distally related species can be used

to annotate conserved expressed features such as protein-coding genes and to identify regulatory elements controlling gene expression (phylogenetic footprinting) (Ahituv et al., 2004; Hardison, 2000; Muller et al., 2002; Tagle et al., 1988).

To derive insights into central biological processes through comparative genomic analysis, the first challenge is the choice of species to compare. Comparison of genomes of related species was shown to provide a possible shortcut in the identification of regulatory regions as they form islands of partially conserved sequence in non-coding regions (Hardison, 2000; Pennacchio et al., 2006; Wasserman et al., 2000). Within the vertebrate lineage, the teleost genomes such as those of pufferfish (*Takifugu rubripes*), medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*) represent promising tools to elucidate the regulatory architecture. High expectations have been put into the comparative analysis of the pufferfish (Takifugu, Tetradon). However, also comparisons of teleosts with mammalian genomes have led to the identification of several functional enhancers, for example, in paired box (*pax*) genes (Santagati et al., 2003)), homeo box (*hox*) genes (Anand et al., 2003; Aparicio et al., 1995; Marshall et al., 1994), the pro-neural gene *neurogenin1* (Blader et al., 2003), distal-less (*dlx*) genes (Ghanem et al., 2003) (Zerucha et al., 2000) and the sonic hedgehog homolog (*shh*) (Goode et al., 2003; Goode et al., 2005; Muller et al., 1999). Multi species DNA sequence comparison has become a common methodology to identify and characterise the functional elements that are strictly conserved over a large evolutionary distance.

Alignment of the sequences is the core process in comparative genomic analysis. A number of alignment programs are available to align 2 or more DNA sequences. A list of commonly used databases and tools in comparative genomics is given in the table 1 (Frazer et al., 2004; Loots and Ovcharenko, 2004; Ovcharenko et al., 2004; Wasserman and Sandelin, 2004). These websites and alignment tools can be used separately, or in combination to answer various question related to the comparative aspects of the genomics. The ones which are of particular interest are the UCSC, ENSEMBL and ECR genome browsers. The first two contain reference sequences and working draft assemblies for a large collection of vertebrate and invertebrate genomes and provide various interactive tools for the analysis of these genome data. The ECR browser on the other hand is a dynamic whole-genome navigation tool for visualizing and studying the evolutionary relationship between vertebrate and invertebrate genomes. This graphical browser presents Evolutionary Conserved Regions (ECRs) that have been mapped

within alignments of the orthologous and syntenic genomes and also depicts color coded evolutionary conserved regions in relation to known genes that have been annotated in the base genome (Fig 2).

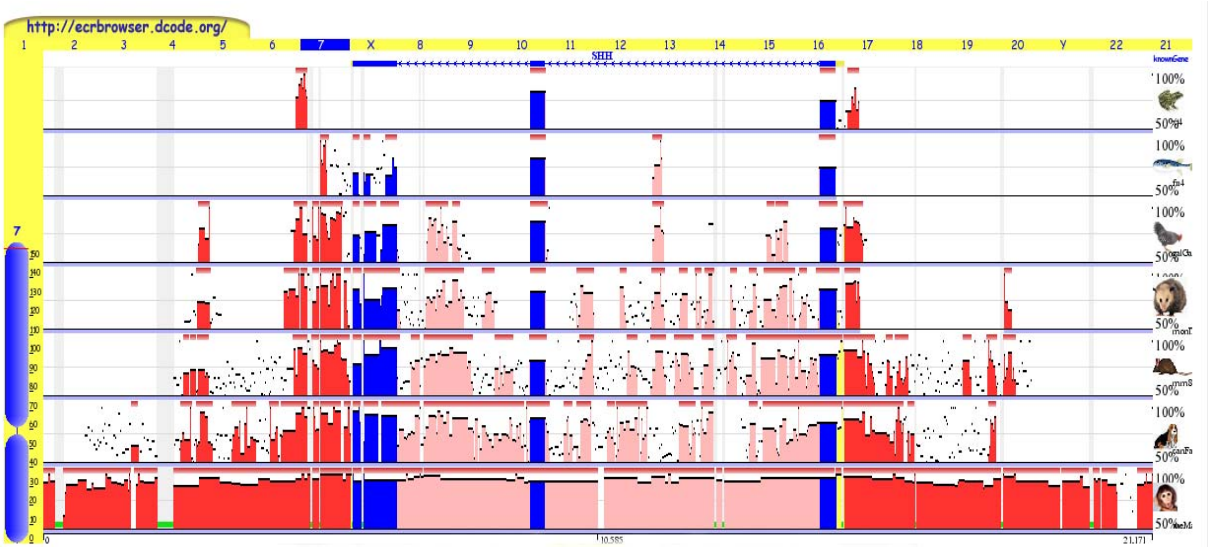


Figure 2: The ECR browser coordinates for the *shh* gene, the conservation has been shown individually between various species. Blue lines represent the known exons, yellow ones show the non-coding exons while pink/red depict non-coding, non-genic evolutionary conserved elements which represent the putative cis-regulatory elements. Width in the conservation panel tells the size, while the height shows the degree of conservation.

In addition to accessing pre-computed alignments for the available genomes, the ECR browser can also be used as an alignment tool. Pre-computed annotation of conserved transcription factor binding sites in human and mouse genomes is also available in the browser (Ovcharenko et al., 2004), which is based on the in-silico techniques of phylogenetic footprinting. The technique involves the multi-species DNA sequence comparison to a finer scale of highly conserved 6-12 bps that is comparable to the size of a single transcription factor binding module. This provides the footprints of various transcription factors binding at various elements within the genome (Gumucio et al., 1996; Kappen and Yaworsky, 2003).

Tool / Database	Website
ECR genome browser	http://www.ecr-browser.dcode.org/
ENSEMBL	http://www.ensembl.org
UCSC genome browser	http://genomeucsc.edu
PIPMAKER	http://bio.cse.psu.edu/pipmaker
VISTA	http://www.gsd-lbl.gov/VISTA/index.html
NCBI Blast	http://www.ncbi.nlm.nih.gov/BLAST/

Table 1: Websites/ databases for sequence alignment and comparative genomic tools

Identification of the regulatory elements by comparative genomic analysis seems to be less obvious than the identification of genes and exons owing to the very small size and degenerative DNA sequence of the transcription factor binding site (6-10 bp) and renders a genome wide computational analysis very difficult as not only the binding sites but the context in which it is placed, plays an important role (Nobrega and Pennacchio, 2004). However, functionality of most of the metazoan enhancers is established by a combination of multiple factors binding in a modular fashion. This results in an enhancer element being comparable to or generally even larger than individual exons. But contrary to the coding region they lack intron/exon junction features and are not expressed. These elements are coined conserved non-genic sequences (CNSs) or conserved non-genic elements (CNGs). Once a putative regulatory region has been identified by comparative genomic analysis the next step is to evaluate its biological relevance.

Recently zebrafish has emerged as a popular model system for a number of disciplines including vertebrate development and modelling human disease (Dooley and Zon, 2000; Ingham, 1997; Ishikawa, 2000; Lin, 2000; Talbot and Hopkins, 2000; Udvadia and Linney, 2003; Zon, 1999). It has a smaller genome than the human. At the same time, zebrafish is distant in evolutionary terms from mammals making them suitable for comparative genomics. More importantly, transgenic technologies are readily applicable allowing functional analysis of cis-regulatory elements in the transparent, ex-utero developing, live embryo (Amacher, 1999). The recent boom in transgenic applications in zebrafish is mainly due to two reasons: (1) the fast-growing

database of information on zebrafish promoters and other cis-regulatory elements and their increasing availability (Amacher, 1999) application of the green fluorescent protein (gfp) reporter gene, which is easily detectable *in vivo* in the transparent embryo. In addition to GFP, a whole series of colour-shifted fluorescent proteins were shown to be readily detectable in the transparent zebrafish embryo (Shaner et al., 2005). Signal from fluorescent proteins may be further enhanced using a Gal4-VP16 binary system (Koster and Fraser, 2001).

A technically undemanding protocol of micro-injection of DNA constructs with the putative regulatory region fused to a reporter such as green fluorescent protein into fertilised egg results in at least 2–5% of founders transmitting the reporter gene with detectable activity into the F1 generation (Lin, 2000). Co-injection with SceI meganuclease has become popular recently where the plasmids are designed with two flanking SceI sites. This technique increases the rate of integration with several transgenic lines generated (Thermes et al., 2002). Alternative gene transfer methods have also been attempted in fish with variable success. Among them, transposon (Urasaki et al., 2006) and retrovirus-mediated gene insertion (Ellingsen et al., 2005) methods have the potential to become alternatives to conventional microinjection of purified DNA fragments as they allow single copy integration into the recipient genome. The function of the regulatory region can then be studied very elaborately in these stable transgenic animals. In addition to the stable transgenic lines, regulatory elements can be assayed directly in micro-injected transient transgenic fish by the analysis of mosaic expression of reporter activity (Ertzer et al., 2007; Muller et al., 1999). This reduces the several months period normally required for obtaining the F1 generation of transgenic lines to a matter of days, making the system a suitably high-throughput bioassay for genes expressed in the developing embryo. Also the use of antisense oligonucleotides morpholinos for knock down of a gene is very effective in zebrafish (Nasevicius and Ekker, 2000).

2. Sonic hedgehog gene

The original hedgehog gene, identified in Drosophila (Nusslein-Volhard and Wieschaus, 1980) is named after its mutant phenotype: the embryo is covered with pointy denticles resembling a hedgehog. The vertebrate hedgehog family is represented by at least three members: Desert hedgehog (*Dhh*), Indian Hedgehog (*Ihh*) and Sonic hedgehog (*Shh*) possibly resulting from two major gene duplication events during the evolution of vertebrates, the first gave rise to *Dhh* and the second produced *Ihh* and *Shh*. Both duplication occured before the emergence of vertebrates and probably before the evolution of chordates (Kumar et al., 1996; Zardoya et al., 1996). All the three have been cloned in different animals. In zebrafish, *shh*, tiggy-winkle hedgehog (*Twhh*) and echidna hedgehog (*Ehh*) have been characterised (Currie and Ingham, 1996; Ekker et al., 1995; Ertzer et al., 2007; Krauss et al., 1993; Muller et al., 2002). Tiggy winkle hedgehog is considered to have originated from a more recent duplication of the shh gene in the zebrafish. Shh is the most extensively characterised vertebrate homolog, and has multiple roles like patterning of the neural tube (Stecca and Ruiz i Altaba, 2005) axis symmetry (Sampath et al., 1997), somite patterning (Munsterberg and Lassar, 1995) eye development (Esteve and Bovolenta, 2006; Russell, 2003) and many others. It can act as both a short range, contact dependent factor and a long range diffusible morphogen (Chuang and Kornberg, 2000; Johnson and Tabin, 1997).

2.1 Secretion

Newly synthesized Hh protein undergoes a series of posttranslational modifications within the secretory pathway that lead to the presentation at the cell surface of the mature and signalling active lipid-modified Hh (Mann and Beachy, 2004; Torroja et al., 2005). Following cleavage of an aminoterminal signal sequence upon entering the secretory pathway, the Hh protein undergoes an autocatalytic processing reaction that involves internal cleavage between Gly–Cys residues (Bumcrot and McMahon, 1995; Lee et al., 1994; Tabata and Kornberg, 1994). The amino-terminal product of this cleavage receives a covalent cholesteryl adduct (Porter et al., 1996a) giving rise to the active signalling molecule (Lee et al., 1994; Marti et al., 1995; Porter et al., 1996a; Pringle

et al., 1996; Tabata and Kornberg, 1994)(Fig. 3). The carboxy-terminal domain of the Hh precursor, which has no known additional function, mediates the autoprocessing reaction (Lee et al., 1994; Porter et al., 1995). The importance of autocatalytic processing in the biogenesis of active Hh proteins is revealed by missense mutations in the Drosophila Hh gene (Porter et al., 1995) and in the human *shh* gene, here associated with holoprosencephaly (Roessler et al., 1996). These mutations can be classified as alterations in the amino-terminal signalling domain, which affect either the secretion or activity of the signalling domain, or in the carboxy-terminal processing domain, demonstrating the requirement for processing in the release of the active signal. The second lipophilic modification of the Hh signalling protein is a fatty acid, palmitate, in the aminoterminal cysteine, which is exposed by signal sequence cleavage (Chamoun et al., 2001; Pepinsky et al., 1998). The cholesterol moiety is thought to direct Hedgehog protein traffic in the secretory cell (Ingham, 2000; Lewis et al., 2001; McMahon, 2000; Porter et al., 1996b).

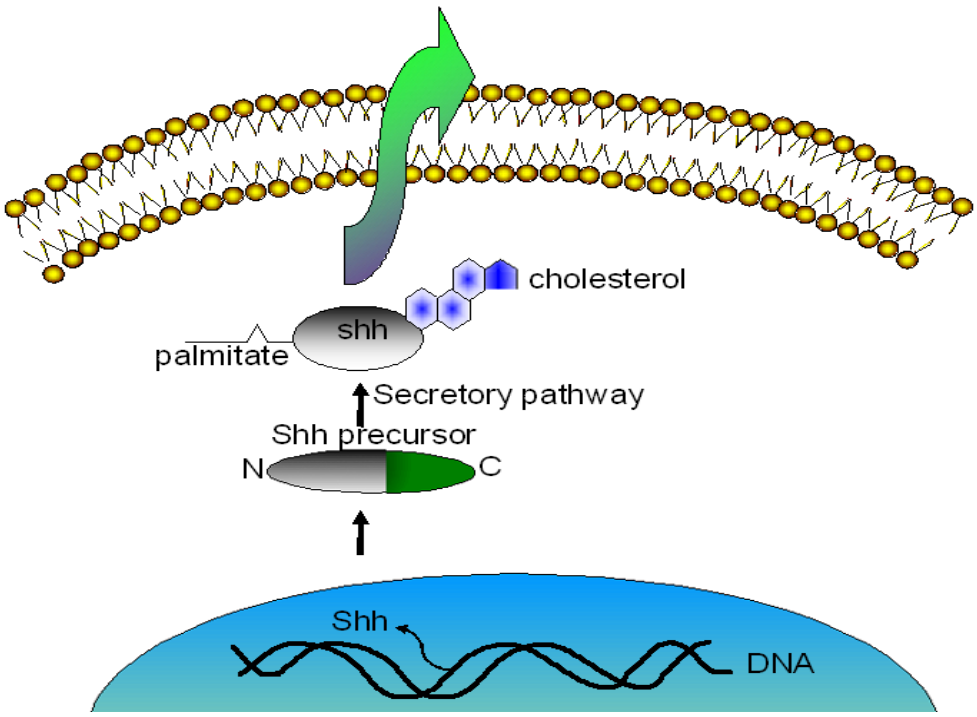


Figure 3: *Shh* secretion: The *shh* precursor is processed to generate the N-*shh* morphogen, to which two modifications are introduced; a cholesterol group at the C-terminal and a palmitate moiety at the N-terminal (Modified from Marti & Bovolenta 2002).

It has been demonstrated that the N-terminal 19 kD peptide stays tightly associated with the surface of cells in which it was synthesised, while the C –terminal peptide is freely diffusible (Lee et al., 1994; Marti et al., 1995; Porter et al., 1995; Roelink et al., 1995). This close association with the cell surface is mediated by the covalent association of the 19 kD form to cholesterol. By tethering the 19 kD peptide to the surface, a high local concentration of N-terminal Hh peptide is generated on the surface of the Hh expressing cells. From a number of studies, it is clear that the N-terminal peptide is the active molecule which is responsible for short and long range Hh signalling activities in Drosophila and vertebrates (Marti et al., 1995; Porter et al., 1995; Roelink et al., 1995). Very long range signalling is possible because the association with the cell membrane may not be so strong and low levels of the 19kD form may diffuse away from the secreting cell.

Vertebrate embryonic development utilises both short and long range mechanisms of *shh* signalling. Short range signalling by *shh* is apparent during floorplate induction by the notochord within the neural tube (Johnson and Tabin, 1995). Long range signalling by *shh* occurs during motor neuron formation in the neural tube, sclerotome induction and proliferation in the somites, and limb patterning along the anterior-posterior axis (Chuang and Kornberg, 2000; Goetz et al., 2002; Zeng et al., 2001).

2.2 *Shh* signalling

The *shh* signalling cascade is conserved from invertebrates to mammals (Goodrich et al., 1996; Ingham and McMahon, 2001; Marigo et al., 1996; Nybakken et al., 2002) with some recently reported divergences (Huangfu and Anderson, 2006; Varjosalo et al., 2006). *Shh* signalling involves the two multiple-pass, transmembrane proteins Patched (Ptc) and Smoothened (smo) that can interact with one another at the cell surface. Ptc is a twelve pass transmembrane protein that binds the ligand (Marigo et al., 1996; Pepinsky et al., 1998) and Smo is a seven transmembrane protein that acts as a signal transducer (Alcedo et al., 1996; Ingham et al., 2000). In the absence of Hh, Ptc inhibits the positive signalling activity of Smo and further downstream targets of Hh signalling. The binding of Hh to Ptc regulates Smo trafficking to the plasma membrane and it has also been established that in the absence of Hh, Ptc downregulates the activity of Smo (Alcedo et al., 2000) (Denef et al., 2000; Ingham et al., 2000). Within the nucleus of the *shh*

responding cell, zinc finger transcription factors of the Ci (Cubitus interruptus) family act at the last step of the Hh signal transduction pathway (Altaba, 1999 ; Hynes et al., 1997; Ruiz i Altaba, 1999).

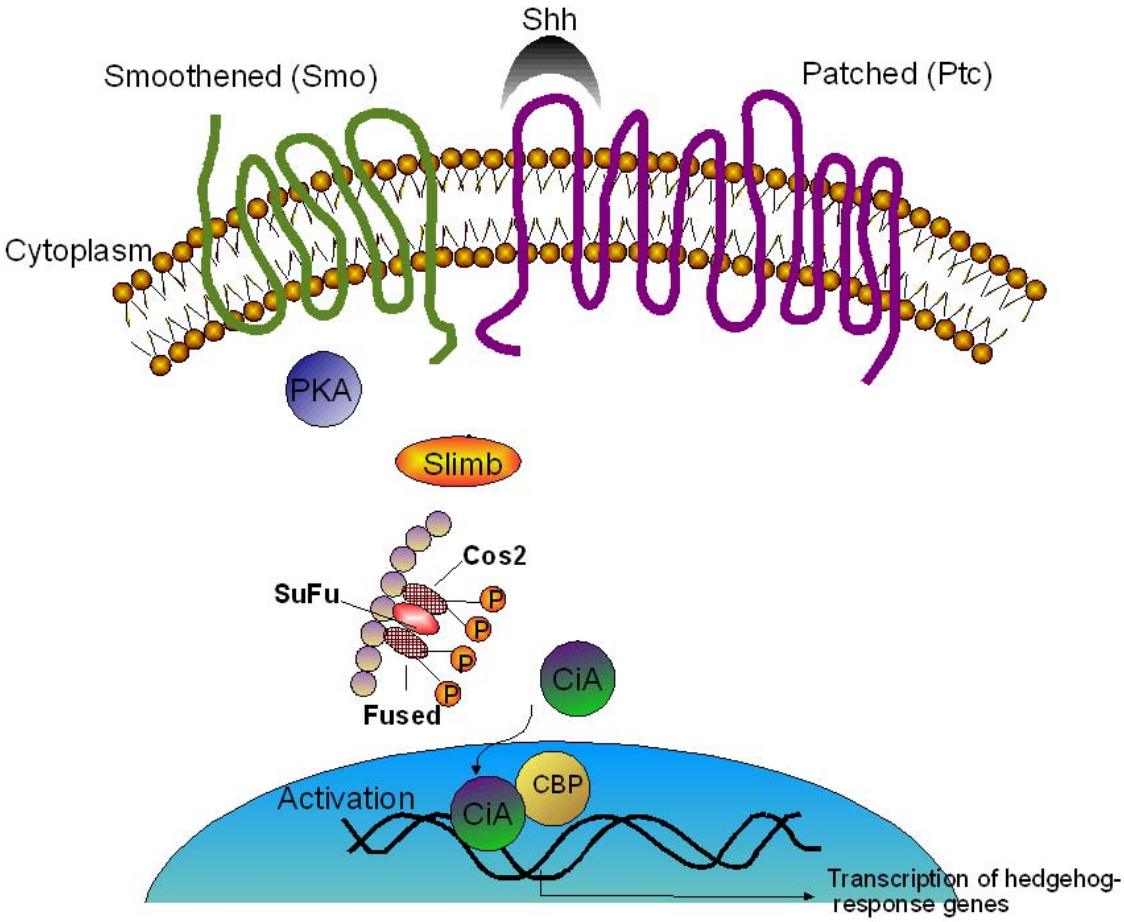


Figure 4: In the presence of Hh, Ptc inhibition of Smo is relieved, smo then signals through unknown mechanisms to the Fu/Cos2/Ci complex, causing hyperphosphorylation of Fu and Cos2 and causing the complex to loosen its hold on microtubules. This leads to the stabilization of full length Ci, which can then travel to the nucleus and function as a transcriptional activator, upregulating transcription of Hh target genes (Modified from Nybakken & Perrimon 2002).

Upon secretion, Hh binds to Ptc and relieves the inhibitory effects that Ptc normally has on Smo. Active Smo regulates the bifunctional transcription factor Ci. Full-length Ci protein is stabilised in response to Hh to become a transcriptional activator (CiA). In the absence of Hh ligand, Ci is proteolytically processed into a shorter form (CiR) that acts as a transcriptional repressor of target genes. Both the proteolytic processing and the nuclear translocation of Ci are tightly regulated processes that involve a microtubular protein complex containing the atypical kinesin protein Costal 2 (Cos2), the serine

threonine kinase Fused (Fu) and the novel protein Suppressor of fused (Sufu). Cos2 regulates the production of both CiR and CiAct (Lum et al., 2003; Sisson et al., 1997; Wang et al., 2000b; Wang and Holmgren, 2000).

In order to activate the Hh pathway, Smo has to be stabilized at the plasma membrane (Zhu et al., 2003). Recent data suggest that mouse Smo is localized to cilia in response to Hh signalling (Corbit et al., 2005). Signal transmission from the membrane to cytoplasm proceeds through recruitment, by Smo, of Cos 2, which routes pathway activation by interaction with other components of the cascade (Jia et al., 2003; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003). The mechanism of sorting and stabilization of Smo at the plasma membrane is still unclear.

In the absence of Hh signalling, Cos2 forms a complex with Fu, Sufu and the Ci transcription factor and they promote cleavage of the full-length Ci to CiR and keep full-length Ci out of the nucleus. In response to low levels of Hh, the Smo-Cos2 complex is recruited to the membrane, and this relieves the inhibitory effect of Cos2 on Ci, which may lead to dissociation of Ci from the Smo-Cos2 complex (Ruel et al., 2003). However, Ci is not fully activated and cannot enter the nucleus, because Ci is tethered by Sufu in a complex that also includes Cos2. At high levels of Hh signalling, this final restriction is removed, and CiA can move into the nucleus to activate the pathway to a high level (Methot and Basler, 2001; Wang and Holmgren, 2000).

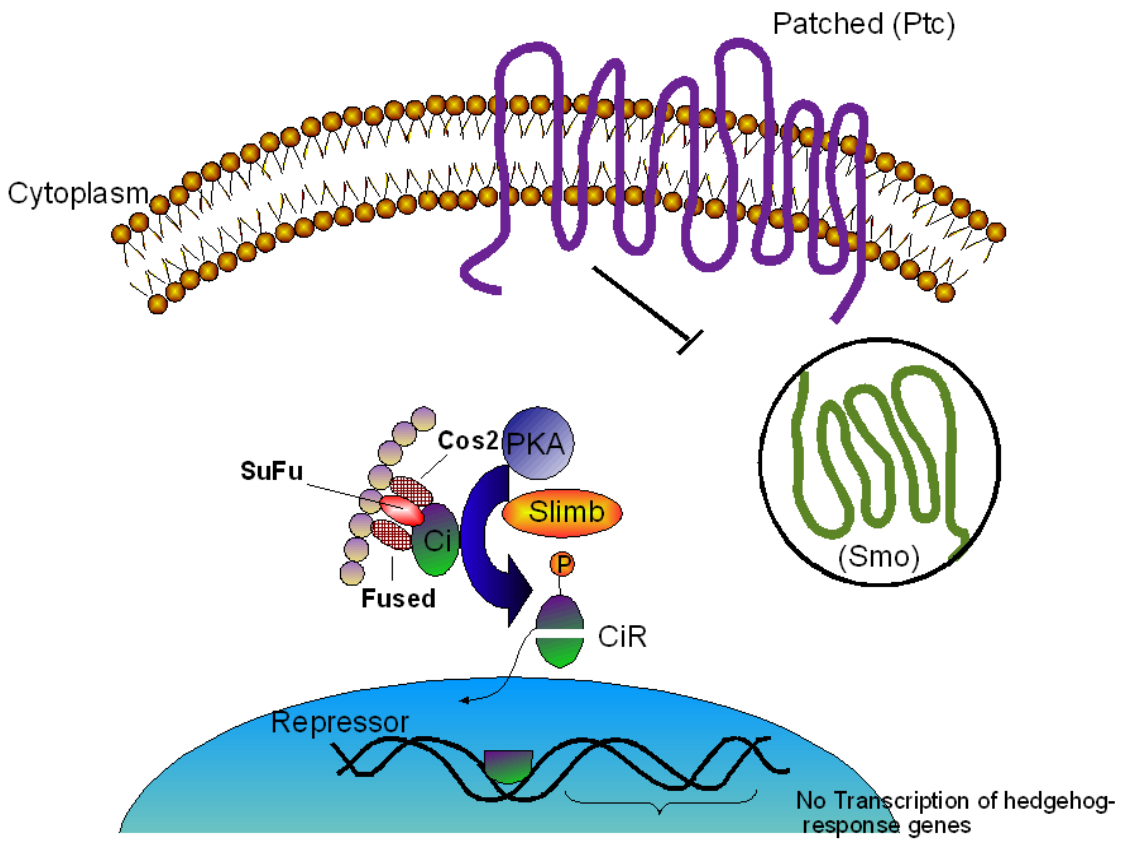


Figure 5: In the absence of Hh signalling, the multiple-pass, transmembrane proteins Patched (Ptc) inhibits the positive signalling activity of Smoothened (Chen et al.). The costal2 (Cos2), Fused (Fu), and Cubitus interruptus (Ci) proteins are bound together in a high molecular weight complex which is attached to microtubules, PKA phosphorylates Ci and it is then cleaved into the repressor form CiR which moves to the nucleus and represses Hh target genes (Modified from Nybakken & Perrimon 2002).

Further regulators of the pathway which act at the surface of cells responding to *shh* have been identified in the vertebrate CNS. Hedgehog interacting protein (Hip) is a type 1 transmembrane protein that attenuates *shh* signalling by binding N-*shh* with an affinity similar to that of Ptc (Coulombe et al., 2004; Jeong and McMahon, 2005; Olsen et al., 2004) whereas vitronectin, an extracellular matrix glycoprotein, enhances *shh* activity during motor-neuron differentiation, also by binding *shh* directly (Pons and Marti, 2000).

In Drosophila, Ci is phosphorylated by several kinases: the cAMP-dependent protein kinase (PKA), Shaggy Sgg/GSK3 — the Drosophila Glycogen Synthase Kinase3 (GSK3) homolog and Casein Kinase 1(CK1) (Chen et al., 1999; Jia et al., 2002; Jiang and Struhl, 1998; Price and Kalderon, 1999; Wang et al., 1999). These kinases phosphorylate Ci at multiple sites in three clusters in its C-terminal region (Zhang et al., 2005) and each

phosphorylation site contributes to the proteolysis of Ci. Moreover, it has been recently shown that Costal-2 recruits these kinases to efficiently phosphorylate Ci and it has been proposed that Hh inhibits Ci phosphorylation by specifically interfering with kinase recruitment (Zhang et al., 2005).

Fully phosphorylated Ci can be recognized by Slimb, a component of the SCF ubiquitin ligase that allows proteasome-dependent processing of Ci (Price and Kalderon, 1999). Recent studies show that PKA is also responsible for phosphorylation of smo at multiple sites and this is necessary for Hh signal transduction (Apionishev et al., 2005; Jia et al., 2004; Zhang et al., 2004). It has been demonstrated that PKA antagonizes hedgehog signalling by partly regulating the Ci mediated transcription of *shh* target genes (Concordet et al., 1996).

Cyclopamine, modified Vitamin D3 and Forskolin are potent inhibitors of Hh signalling acting at different levels of the cascade. Cyclopamine interacts directly with Smo and blocks signalling (Chen et al., 2002), (Taipale et al., 2000) and this has been shown in zebrafish and mammals (Chen et al., 2002; Neumann and Nuesslein-Volhard, 2000) Vitamin D3 acts similarly (Bijlsma et al 2006). Another pharmaceutical compound, Forskolin, stimulates adenyl cyclase and thereby leads to the increase of cellular cAMP levels which in turn negatively regulates Hh signalling in zebrafish through PKA (Barresi et al., 2000; Masai et al., 2005).

Vertebrates have two Ptc homologs Ptc1 and Ptc2 and has been characterised (Carpentar et al., 1998; (Motoyama et al., 1998; Pearse et al., 2001). Ptch1 appears to be the major receptor during mouse embryonic development (Goodrich et al., 1997) (Wolff et al., 2003). Zebrafish ptc2 mutants (*lepre-chaun*) have a relatively mild phenotype (Koudijs et al., 2005). There are three Ci homologs Gli1, Gli2 and Gli3 in vertebrates each with distinct function and a single smo protein that mediates all Hh signalling by regulating the three homologs (Bai et al., 2004; Motoyama et al., 2003). Gli genes from chicken, zebrafish, Xenopus and mouse have been isolated (Marigo et al., 1996) (Karlstrom et al., 2003; Lee et al., 1997; Platt et al., 1997). In the neural tube and lymph buds, Gli and Ptc are expressed in similar domains (Platt et al., 1997). A gradient of gli activity mediates *shh* signalling in the neural tube (Jacob and Briscoe, 2003; Stamataki et al., 2005). Ectopic expression of *shh* induces Gli1 transcription and widespread expression of Gli1 that results in the ectopic differentiation of floor plate cells and ventral neurons within the neural tube (Lee et al., 1997).

Like Ci, all three vertebrate Gli proteins have five highly conserved zinc finger DNA binding domains and C-terminal activation domains, with Gli2 and Gli3 also having N-terminal repressor domains (Dai et al., 1999; Sasaki et al., 1999). *Shh* signalling regulates Gli2 transactivation by suppressing its processing and degradation (Pan et al., 2006). Like Ci, Gli3 can be processed to form a repressor isoform, and this proteolytic processing is inhibited by Hh signalling (Litingtung et al., 2002; Wang et al., 2000a). In zebrafish, Gli1 is the major activator of Hh target genes while Gli2 and Gli3 play both activator and repressor roles in different regions of the embryo (Karlstrom et al., 2003; Tyurina et al., 2005). Zebrafish Gli1 (*detour*) and Gli2 (*you-too*) mutants show disrupted Hh signalling (Karlstrom et al., 2003). These suggest that Gli is a target of *shh* and at the same time an intracellular mediator of *shh* signal in target cells.

2.3 *Shh* in vertebrate development

The Hh family of secreted proteins regulates many developmental processes in both vertebrates and invertebrates (McMahon et al., 2003). *Shh* acts as a morphogen and has multiple roles during neural development (Ashe and Briscoe, 2006; Briscoe and Therond, 2005; Fuccillo et al., 2006). It is involved in the determination of cell fate and embryonic patterning during early vertebrate development. The *shh* signalling pathway functions throughout development. During early vertebrate embryogenesis *shh* is mainly expressed in the node, notochord, floor plate and limb (Chang et al., 1994; Johnson et al., 1994); (Marti et al., 1995; Riddle et al., 1993). During embryonic development, *shh* is expressed in many epithelial tissues, like the teeth, hair follicles, lung epithelium, in the endoderm during early stages of gut formation, in the retina, in dorsal CNS cortical structures like cerebral cortex, optic tectum, cerebellar cortex (Iseki et al., 1996; Neumann and Nuesslein-Volhard, 2000; Traiffort et al., 2001; Urase et al., 1996; Wall and Hogan, 1995).

The best characterised function of *shh* is the neuronal specification in the ventral CNS (Jessell, 2000; Marti and Bovolenta, 2002; McMahon et al., 2003; Patten and Placzek, 2000; Stecca and Ruiz i Altaba, 2005). Signalling by a *shh* gradient establishes distinct progenitor domains in the neural tube by regulating the expression of a set of homeodomain proteins that comprises members of the Pax, Nkx, Dbx and Irx families (Briscoe and Ericson, 2001; Pierani et al., 1999). By induction or repression of these

transcription factors *shh* defines five progenitor domains in the neural tube through the mediation of Gli activity (Stamataki et al., 2005). *Shh* also plays a later role in the ventral CNS in the specification of oligodendrocytes (Davies and Miller, 2001; Orentas et al., 1999; Poncet et al., 1996; Pringle et al., 1996; Trousse et al., 1995) and subsequently is required for specification of optic nerve oligodendrocyte precursors (Gao and Miller, 2006; Orentas et al., 1999).

Shh from Purkinje cells is essential for the growth of the external germinal layer (EGL) in the cerebellum (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). The level of *shh* signalling regulates the complexity of cerebellar foliation (Corralis et al 2006). The *Shh* pathway also independently controls patterning, proliferation and survival of neuroepithelial cells by regulating Gli activity (Cayuso and Marti, 2005).

Hh signalling has also been implicated in patterning the left right (LR) axis asymmetry (Pagan-Westphal and Tabin, 1998; Sampath et al., 1997; Schilling et al., 1999). In the chick embryo, asymmetric expression of *shh* in the node is required for the development of left-right asymmetry of the heart (Levin et al., 1995).

In vertebrates, *Shh* signalling is required for patterning of mesodermal structures like the limbs and somites. *Shh* signalling from the zone of polarizing activity is involved in establishing the antero-posterior patterning of the limb (Riddle et al., 1993). *Shh* secreted from the notochord controls determination of sclerotome and somite patterning (Bumcrot and McMahon, 1995; Lassar and Munsterberg, 1996; Munsterberg and Lassar, 1995).

Loss of activity of the Hh signalling in human embryos can cause severe ventral CNS developmental anomalies, including holoprosencephaly, polydactyly, craniofacial defects and skeletal malformations (McMahon et al., 2003; Muenke and Beachy, 2000; Roessler et al., 1996; Zhang et al., 2006). Inappropriate activation of Hh signalling is responsible for nearly all basal cell carcinomas, some medulloblastomas and rhabdomyosarcomas and has been implicated in other tumors (Bale and Yu, 2001; Marino, 2005; Pasca di Magliano and Hebrok, 2003; Stecca and Ruiz i Altaba, 2005). In addition, recent findings about the relationship between primary cilia and the mouse Hh pathway (Huangfu et al., 2003) suggest that Hh signalling may be affected in human syndromes caused by defects in cilia, including Bardet-Biedl syndrome, Kartagener syndrome, polycystic kidney disease and retinal degeneration (Pan et al., 2005).

Mice with *shh* mutations are cyclopic and lack motor neurons, floor plate and ventral forebrain (Chiang et al., 1996). By contrast, zebrafish *sonic-you* (*syu*, an ortholog of mammalian *shh*) mutants develop normal medial floor plate and motorneurons and have relatively normal ventral forebrain patterning (Schauerte et al., 1998), although they lack lateral floor plate. A possible explanation is that in zebrafish, additional Hh family members, *twhh* (Ekker et al., 1995) and *ehh* (Currie and Ingham, 1996) may act redundantly with *shh* to pattern the ventral neural tube (Nasevicius and Ekker, 2000; Zardoya et al., 1996).

3. Vertebrate eye development

The development of the vertebrate eye is first seen as a pair of bilateral depressions called optic pits in the developing forebrain (Kumar, 2001) (Fig. 6). These pits eventually become pouches called optic vesicles (OV; step 1). Subsequently, they bend ventrally and rotate slightly in an anterior direction. These changes serve to bring the primordia from a horizontal to a more vertical orientation in relation to the embryonic neural axis. The optic lumen and optic stalk are formed at this stage. As the overlying lens placode (LP) invaginates (step 2) to form the lens vesicle (LV; step 3) (and ultimately the lens (L); step 4), the underlying outer surface of the optic vesicle also invaginates to form the optic cup (OC) that now has two closely apposed layers; the inner layer becomes the neural retina and the outer layer forms the retinal pigmented epithelium. The overlying cornea is derived from the surface ectoderm and neural crest cells. The developing neural retina (step 5) undergoes an ordered series of births and migrations of individual cell types, ultimately giving rise to an adult retina (Fig 4) organized into several layers.

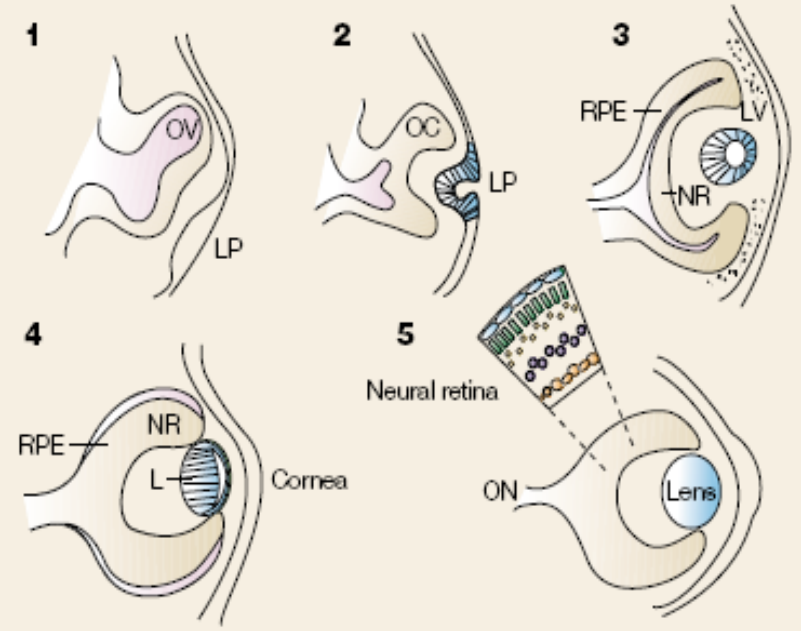


Figure 6: The development of the vertebrate retina shown in series (Adapted from Kumar 2001).

The vertebrate retina consists of six major classes of neurons and one class of glial cells, which are organized into distinct layers (Wawersik and Maas, 2000; Zucker and Dowling, 1987). The lineage of the cells in the retina is known: all of the cells, except for astrocytes, are derived from multipotential retinal precursor cells (RPC) (Holt et al., 1988; Livesey and Cepko, 2001; Turner et al., 1990; Wetts and Fraser, 1988). The different cell types are generated in an invariant sequence, with retinal ganglion cells (RGCs), cone photoreceptors, horizontal cells and the majority of the amacrine cells generated first, followed by bipolar cells, Müller glia and the remaining amacrine cells generated in a second wave of histogenesis, which extends into the postnatal period (Young, 1985). Rod photoreceptors are generated throughout the retina development.

4. Development of zebrafish retina

The development of the zebrafish retina is similar to that of other vertebrates Fig. 7. Early eye morphogenesis in the zebrafish occurs between 12 and 36 hours postfertilization (hpf), and is well studied (Chuang and Raymond, 2001; Kimmel et al., 1990; Li et al., 2000; Schmitt and Dowling, 1994; Woo and Fraser, 1995). During

gastrulation, the developing eye is a single eyefield that separates into two lateral optic vesicles (Varga et al., 1999; Woo and Fraser, 1995). The next step is the formation of the optic primordia that evaginate from the forebrain as solid masses of cells (step A). After initial evagination (6-7 somite stage [SS]), the optic primordia take on a wing-like shape (8-9 SS, step B) (Rubenstein and Beachy, 1998; Schmitt and Dowling, 1994; Varga et al., 1999) Subsequently, they bend ventrally and rotate slightly in an anterior direction (10-12 SS, step C) as in other vertebrates. The choroid fissure forms by an involution along the anterior region of the eyecup (18-20 SS, step D). By 24 hours postfertilization the eyecups are well formed. Between 24 and 36 hpf, the eyes rotate further in relation to the axis of the embryo, and this repositions the choroid fissure to a typical ventral location by 36 hpf (step E). Because of the two rotations of the eye during early morphogenesis, particularly the later one, the anterior-posterior orientation of the emerging optic primordium ultimately becomes the ventral-dorsal axis of the completed eyecup. Axonal outgrowth are seen around 36 hpf (step F).

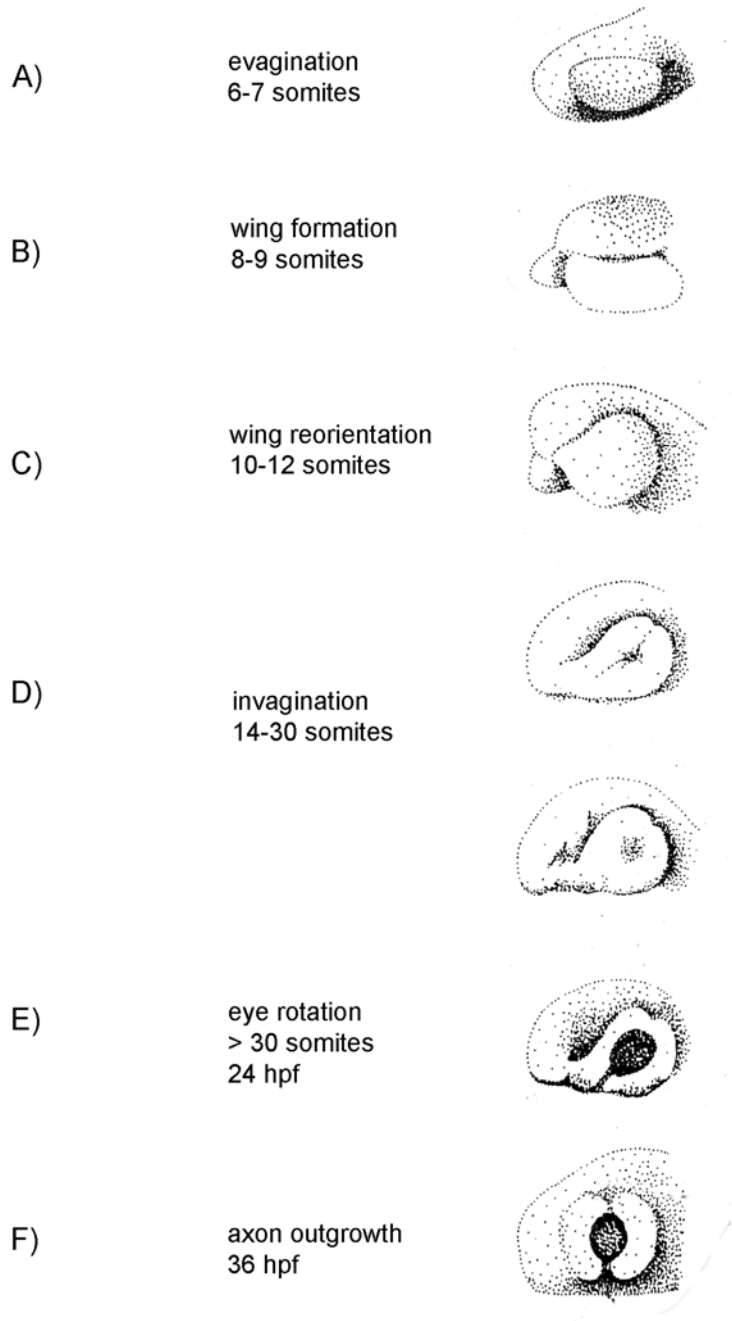


Figure 7: Eye morphogenesis in zebrafish embryo between 12-36 hpf (hours post fertilisation) involving six major steps as shown above (Adapted fom Schmitt & Dowling 1994).

The zebrafish eye is well developed around 72 hpf and is highly laminated (Fig.8) into layers, the ganglion cell layer (GCL) consisting of retinal ganglion cells (RGC), the inner nuclear layer (INL) consisting of amacrine cells, horizontal cells, bipolar cells and Müller glial cells, the outer nuclear layer (ONL) or the photoreceptor layer consisting of rods and cones and the retinal pigmented epithelium. The axons of the retinal neurons also form the plexiform layers in between these layers.

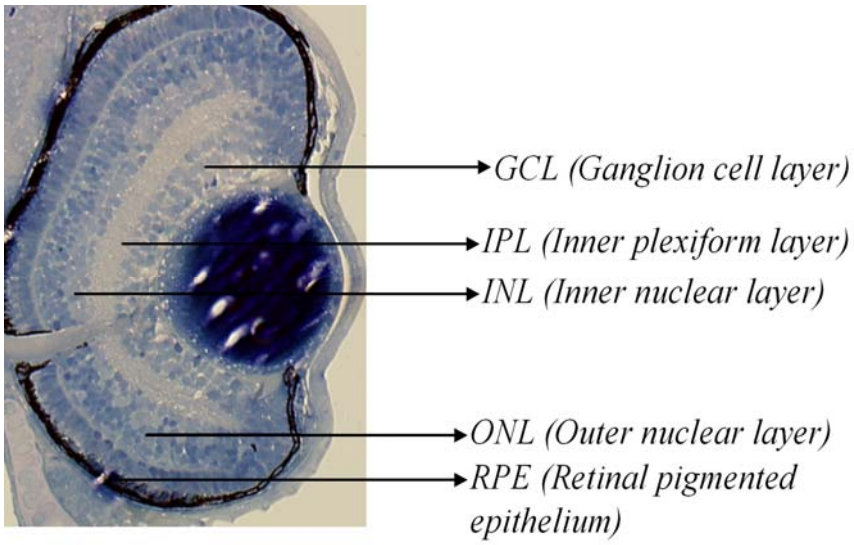


Figure 8: Section of a zebrafish larval eye at 76 hpf, showing the characteristic lamination of a vertebrate retina.

Several signalling pathways have been implicated in instructing different uncommited cell types to proliferate, differentiate and organise into tissue-specific lineages in the eye. These include the *hedgehog* (*Hh*), *wingless* (*Wnt*), Fibroblast growth factors (Fgf), bone morphogenetic protein (BMP), Nodal and the Transforming growth factor β (TGF- β) families (Cavodeassi et al., 2005; Dominguez and Hafen, 1997; Martinez-Morales et al., 2005; Moore et al., 2004; Neumann and Nuesslein-Volhard, 2000; Yang, 2004). Members of these families activate specific intracellular cascades that control gene transcription, ultimately determining the behaviour of the responding cell. Especially Hh and Fgfs play an important role in eye development (Esteve and Bovolenta, 2006; Russell, 2003) and their functions are cited in detail next.

4.1 *Shh* and retinal neurogenesis

Shh plays an important role as a morphogen in vertebrate eye development (Esteve and Bovolenta, 2006; Russell, 2003). The earliest known function of *shh* secreted from the ventral midline is the splitting of the eye field into two lateral optic primordia followed by induction of optic stalk tissue at the expense of neural retina (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003; Take-uchi et al., 2003). *Shh* mutations cause severe cyclopia in mice and humans (Belloni et al., 1996; Chiang et al., 1996; Roessler et

al., 1996). Slightly later, neurogenesis is initiated in the retina by a signal originating from the optic stalk (Masai et al., 2000)

Hh and retinal neurogenesis has been well studied in Drosophila (Dominguez and Hafen, 1997). The photoreceptors differentiate in a wave that moves from posterior to anterior, beginning adjacent to the stalk of the eye imaginal disc (Fig. 9). Expression of the proneural gene *atonal* moves anterior to the wave of differentiation and is required for neurogenesis in the Drosophila eye. Hh is expressed slightly posterior to *atonal* and diffuses ahead of *atonal* expression to promote *atonal* expression and therefore the forward movement of the wave of differentiation (Heberlein and Treisman, 2000; Jarman, 2000).

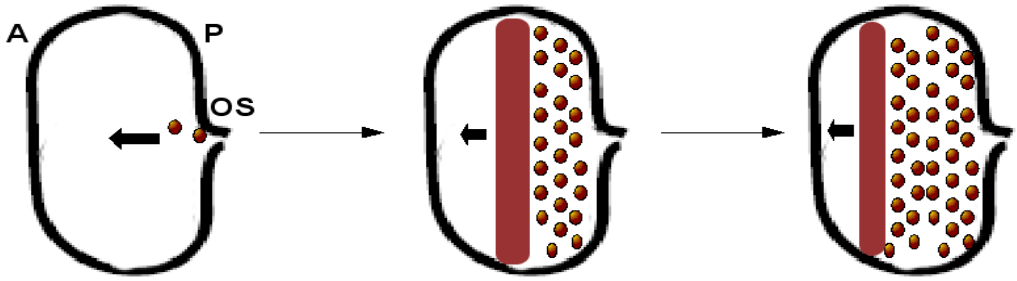


Figure 9: Hh's and progression of the neurogenic wave in the eye. In Drosophila, a wave of *atonal* expression precedes Hh expression, and neurogenesis begins after *atonal* expression. Hh signalling is perceived in front of the wave and promotes *atonal* expression, causing it to spread into the undifferentiated epithelium (Modified from Russell 2003).

In zebrafish, the retinal differentiation process is strikingly similar. The first patch of post mitotic neurons is found close to the optic stalk (Fig. 10). Two waves of gene expression spread from these newly formed RGCs: a wave of *ath5* and a wave of *shh*. *Ath5* is an *atonal* homologue that transiently sweeps across the differentiating retinoblasts and then is maintained in the periphery of the retina, where cells keep differentiating (Masai et al., 2000). *Shh* expression is initiated first in differentiated RGCs and then extends as their differentiation proceeds (Neumann and Nuesslein-Volhard, 2000). Both *shh* and *ath5* waves are necessary for the propagation of neurogenesis: blocking the Hh cascade with cyclopamine results in a severe arrest of retinal differentiation (Neumann and Nuesslein-Volhard, 2000) and *lakritz* embryos that

completely lack *ath5* proteins do not develop RGCs (Kay et al., 2001). Interactions between Hh signalling and *ath5* expression have been recently investigated by blocking Hh signals at different developmental stages in zebrafish embryos (Stenkamp and Frey, 2003).

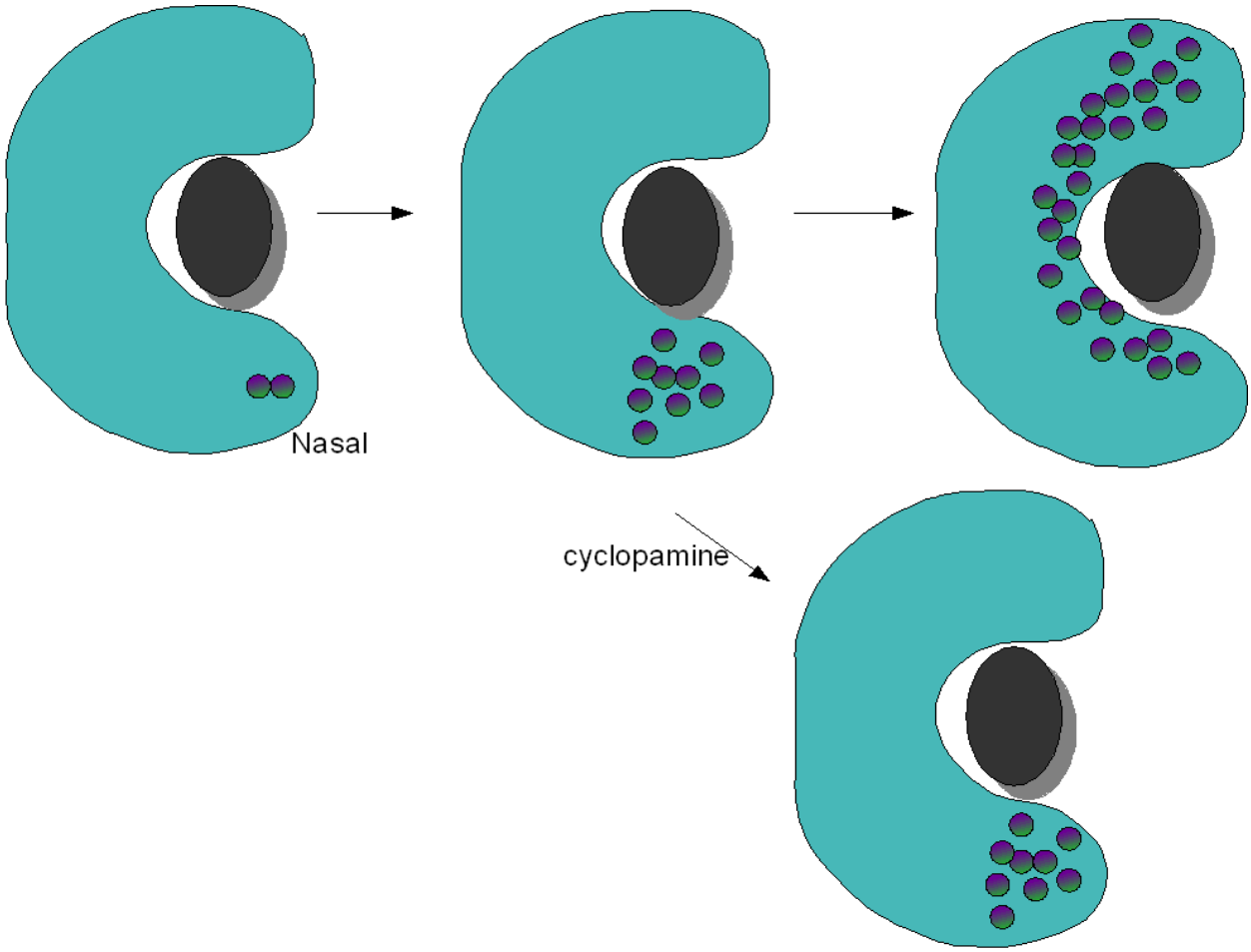


Figure 10: *Shh* wave in zebrafish. Wave is initiated in the ventral-nasal position and then spreads throughout the retina. The wave of neurogenesis is blocked by cyclopamine, but differentiation still occurs in cells that have already been exposed to *shh* (Modified from Russell 2003).

After retinal differentiation has occurred, Hh has an opposite role on RGCs; it inhibits the ultimate differentiation of retinal precursors, thereby controlling the number of RGCs (Zhang and Yang, 2001a). This process has been highlighted in chick where a dual role has been proposed for Hh signalling depending on the protein concentration: retinal progenitors that have not yet been reached by the differentiation wave receive low levels of *shh* because they are still distant from the secreting cells. At

this concentration, *shh* would stimulate the differentiation of one fraction of these progenitors as RGCs, while the other cells would remain in the progenitor state to adopt later cell fates. Behind the wave of differentiation, *shh* concentration becomes higher. This would inhibit the remaining progenitors from adopting the RGC fate, maintaining in this way a correct cell number in the RGC layer. Again, the similarities between vertebrate and Drosophila neurogenesis are remarkable. Before this dual role mechanism was characterized in chick, a work from Dominguez demonstrated that a similar mechanism also takes place in Drosophila (Dominguez and Hafen, 1997). In fact, *atonal* activation by Hh occurs at 5–7 ommatidial rows from the morphogenesis furrow (MF), the front of the differentiation wave. In contrast, at closer distances, *atonal* expression is inhibited by Hh. This response is crucial for the correct establishment of the ommatidial arrays of the Drosophila's eye. As is true in the neural tube, the fact that different retinal fates result from different Hh concentrations demonstrates that cells can read and interpret very precisely Hh protein levels.

Several other roles for *shh* in the retina has been identified. *Shh* also acts as a mitogen in the developing mammalian retina (Amato et al., 2004; Jensen and Wallace, 1997; Levine et al., 1997; Roy and Ingham, 2002) and brain (Dahmane et al., 2001). It directs cell-cycle exit by activating p57Kip2 in the zebrafish retina (Shkumatava and Neumann, 2005). Astrocyte proliferation in the rodent optic nerve is regulated by *shh* secreted from the RGCs (Wallace and Raff, 1999). *Shh* from the midline also plays an important role in regulating the growth of RGC axons and at the optic chiasm border in guiding the optic nerve across the midline (Kolpak et al., 2005; Trousse et al., 2001). Hh signalling has recently been shown to be essential for chick retinal regeneration, most likely through the stimulation of progenitor cell proliferation (Spence et al., 2004).

Shh signalling within the developing eye is required for structural maturation of Müller glia and maintainance of a properly laminated retina (Wang et al., 2002).In the zebrafish retina, *shh* secreted by amacrine cells acts as a short range signal to direct differentiation and lamination (Shkumatava et al., 2004).

In zebrafish, the initiation of neurogenesis is not dependent on *shh* signalling (Neumann and Nuesslein-Volhard, 2000; Shkumatava et al., 2004). Masai et al 2000 have reported an optic stalk signal to be the source of neurogenesis in the retina. The source could be a Fgf signal as Fgfs are expressed in the optic stalk of zebrafish (Crossley and Martin, 1995; Herzog et al., 2004; Reifers et al., 2000; Tsang et al., 2002;

Walshe and Mason, 2003) and also they play a variety of roles in eye development (Russell 2003, Yang 2004). Recently it was proven in fish that indeed Fgf is the signal emanating from the optic stalk that is responsible for species-specific initiation of retinal neurogenesis (Martinez-Morales et al., 2005).

4.2 Fgf signalling and retinal neurogenesis

The FGF family of neurotrophic signalling proteins is made up of 23 ligands, some having several isoforms (Itoh and Ornitz, 2004; Nishimura et al., 1999; Xu et al., 1999). Fgfs signal through four receptor tyrosine kinases via the MAP kinase pathway (Basilico and Moscatelli, 1992). A pharmacological compound, SU5402 inhibits Fgf signalling by blocking the tyrosine kinase activity of the Fgf receptor (Mohammadi et al., 1997).

The ETS family of transcription factor members Erm (Ets related molecule) and Pea3 (Polyoma enhanced activator virus 3) are believed to give a read out of the Fgf signalling as both genes are expressed around all early Fgf signalling sources. They are down regulated in Fgf mutant embryos in all tissues known to require Fgf function, a pharmacological inhibitor (SU5402) of the Fgf pathway completely abolishes expression of all Fgf genes, and ectopic expression of Fgf is sufficient to induce both genes (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). The Ets family of transcription factors is defined by an evolutionarily conserved 85-aminoacid ETS domain (Karim et al., 1990). Generally, these proteins activate transcription, but several members of the family are known to repress this process. DNA binding is achieved by interaction between the ETS domain and a 10-base pair sequence element termed the Ets binding site comprising a highly conserved central core sequence, 5'- GGA(A/T)-3' (Brown et al., 1998).

Fgfs are involved in many aspects of development including gastrulation, neural induction and terminal differentiation, and each member of the family has its own specific roles in different tissues, regulated both by their receptor specificity and expression profiles (Goldfarb, 1996). Fgfs are upregulated in many tumours and are associated with craniofacial abnormalities (Nie et al., 2006) and abrogation of Fgf activity leads to disrupted patterning of midline tissue between eyes, including the optic chiasma (Shanmugalingam et al., 2000). Fgf signals have been implicated in

various aspects of eye development including the segregation of neural from pigmented retina, initiation of neurogenesis, lens induction and differentiation, lens regeneration, retinal cell fate specification, photoreceptor survival, RGC axon outgrowth and axon guidance (Esteve and Bovolenta, 2006; Martinez-Morales et al., 2005; Russell, 2003; Sapieha et al., 2003; Webber et al., 2005; Yang, 2004).

It has been reported, however, that Fgf 2, 3, 5, 11, 12, 13, and 15 are all expressed in the retina of various vertebrates (Ford-Ferris et al 2001), and Fgf1 and Fgf2 in the murine lens (Govindarajan and Overbeek, 2001). Fgfr1 and 2 are expressed in the chick retina (Tcheng et al., 1994) and Fgfr1, 3 and 4 in the Xenopus retina (Launay et al., 1994). Fgf1 accelerates ganglion cell differentiation from the unpatterned epithelium in the chick retina (McCabe et al., 1999). Four zebrafish Fgfs are known to be expressed in the optic cup and/or eye (Crossley and Martin, 1995; Herzog et al., 2004; Reifers et al., 2000; Tsang et al., 2002; Walshe and Mason, 2003). These are Fgf3, Fgf8, Fgf19 and Fgf17. Zebrafish Fgf3, 8 and 17 are all expressed in the optic stalk from early stages on while Fgf19 and Fgf8 is expressed in the neural retina (Miyake et al., 2005). An optic stalk source was believed to be the source of neurogenesis (Masai et al., 2000) and the complete lack of neuronal differentiation in the retina of zebrafish Fgf3/Fgf8 double mutants suggest a crucial role for Fgf signalling in the initiation of neurogenesis (Martinez-Morales et al., 2005).

Temporal and spatial information is also provided by Fgf the onset of retinal neurogenesis. The onset and spreading of differentiation seems, however, to have species-specific spatial organization (Fig. 11).

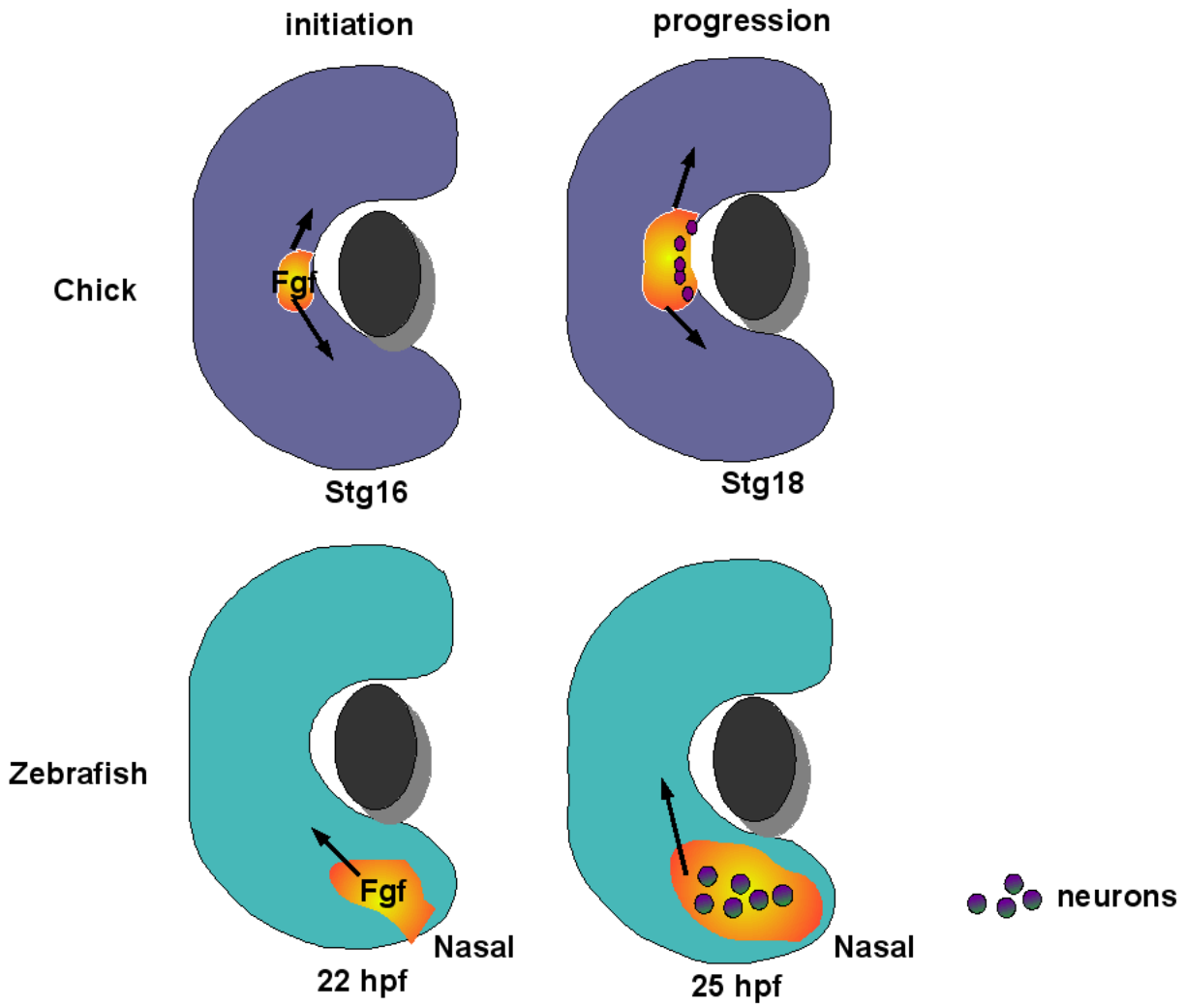


Figure 11: Fgf Signalling determines the origin of morphogenesis in the Vertebrate Retina. Fgfs released by species-specific organizing centers initiate RGC differentiation. Later in development, RGC (*ath5*) differentiation progress through the neural retina (Modified from Martinez-Morales et al 2005).

In zebrafish, the first RGCs are generated in the ventral retina close to the optic stalk and differentiation spreads in the nasodorsal direction, unfolding in a fan shaped gradient (Hu and Easter, 1999). This pattern is different in Xenopus, in which RGCs differentiation starts in a central position a little dorsal to the choroid fissure (Holt et al., 1988). In the chick, RGC appearance is initiated dorsal to the optic stalk in a central zone of the retina and differentiation spreads as an expanding circle in the centro peripheral direction (McCabe et al., 1999). The FGFs produced in a strategically localized, species-specific position provides a common molecular mechanism for the onset of RGC differentiation (Martinez-Morales et al., 2005) (Fig. 11).

5. Zebrafish Sonic hedgehog locus

The zebrafish *Sonic hedgehog* gene is a well characterised genomic locus (Chang et al., 1997; Ertzer et al., 2007; Muller et al., 1999) and therefore, suited for comparative analysis with its mouse and human orthologs (Goode et al., 2003; Goode et al., 2005; Jeong et al., 2006; Jeong and Epstein, 2003). *Shh* genes are highly conserved and have been identified within a variety of species, including human, mouse, frog, fish and chicken. Mouse and human *shh* proteins are 92% identical at the aminoacid level (Marigo et al., 1995). The importance of the *shh* gene in development renders it a prime candidate for comparative studies in order to isolate potential control elements. Examination of the zebrafish *shh* promoter region identified two retinoic acid response elements (RAREs), two HNF3β binding sites, and a putative TATA box (Chang et al., 1997). Further detailed deletion mapping of zebrafish *shh* resulted in the isolation of three intronic enhancer regions (Muller et al., 1999). Deletion mapping and Bac reporter assay have also been employed to isolate control regions in mouse *shh* locus (Epstein et al., 1999) (Jeong et al., 2006).

Enhancers that drive expression in the ventral neural tube and notochord of the developing embryo reside in the two introns and upstream sequences of both the zebrafish and the mouse *shh* gene (Epstein et al., 1999; Ertzer et al., 2007; Jeong et al., 2006; Muller et al., 1999). In a sequence comparison of human and zebrafish *shh* loci, exonic regions are clearly discernible as conserved islands (Muller et al., 2002). Apart from exons, only a few sequence stretches in the intronic regions and the upstream promoter show significant conservation. The conserved intronic regions include the enhancers *ar-A and ar-C* (ar: Activating region, see below) which were previously mapped by functional assays using transient transgenic zebrafish (Muller et al., 1999). Interestingly, both enhancer-containing regions retained sequence identity as well as orientation and relative position, suggesting importance of the position of regulatory elements within diverged intronic sequences (Ertzer et al., 2007).

In zebrafish, activating regions (*ar-A, ar-B, ar-C, ar-D*) that direct floor plate and notochord expression are located in two intronic regions of the *shh* locus (Ertzer et al., 2007). The promoter (including sequences up to -2.4 kb) drives expression in the floor plate of the midbrain, hindbrain and anterior spinal cord. Enhancers *ar-A* and *ar-B*,

which are located in the first intron, mediate expression in the notochord and the floor plate, respectively. A fourth region, *ar-C* in the second intron directed expression in the notochord, zona limitans, hypothalamus and weakly in the posterior floor plate (Ertzer et al., 2007; Muller et al., 2002) (Fig. 12).

In mouse, two floorplate enhancers, *SFPE1* and *SFPE2*, were identified that regulated reporter activity in the ventral midline of the spinal cord and hindbrain similar to the endogenous *shh* expression pattern. A third regulatory element, *Shh* brain enhancer 1 (*SBE1*) directed reporter activity to the ventral midbrain and caudal region of the diencephalon (Jeong and Epstein, 2003). By coupling Bac reporter assay with comparative sequence analysis, three novel enhancers (*SBE2, SBE3 and SBE4*) located over 400 kb from the *shh* transcription start site directing expression to the ventral forebrain were recently identified (Jeong et al., 2006) (Fig. 12). *Shh* enhancers are also capable of exerting long range activity (Lettice et al., 2003; Sagai et al., 2005; Sagai et al., 2004). The enhancer mediating expression of *shh* in the limbs lies 1 Mb away from the *shh* transcription start site and is located in the intron 5 of the Lmbr1 gene (Lettice et al., 2003; Sagai et al., 2005; Sagai et al., 2004).

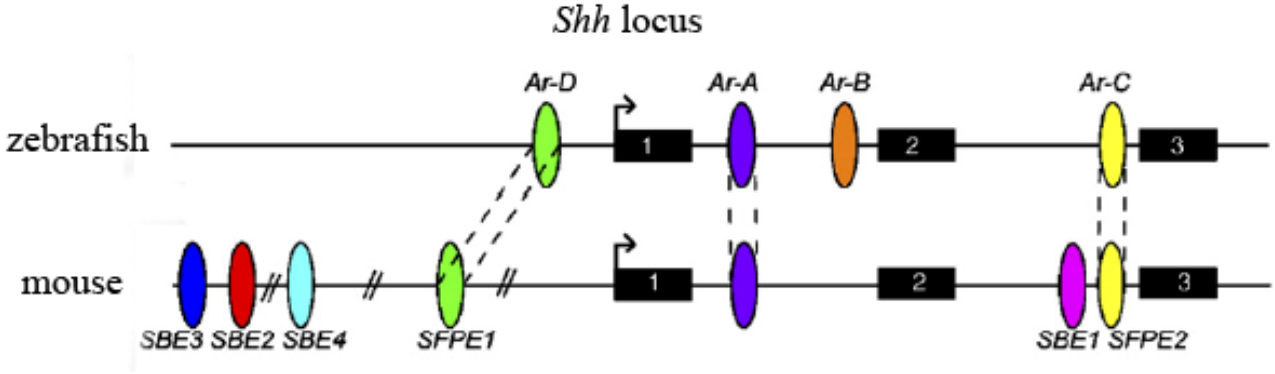


Figure 12: Scheme of the zebrafish *shh* locus. The black boxes represent the exons, circles indicate the identified activating regions in the zebrafish and mouse. Dotted lines indicate the conservation of enhancers relative to sequence and orientation in the locus. (Ar-activating region, SPFE-Shh floorplate enhancer, SBE-Shh brain enhancer). Adapted and modified from Ertzer et al 2007.

The sequences of both *ar-A and ar-C* are highly conserved in the mouse and human *shh* genes (Ertzer et al., 2007; Jeong and Epstein, 2003; Muller et al., 2002). The *ar-C* harbours a 240-bp sequence that shares sequence similarity with the SFPE2 of the mouse *shh* gene (Muller et al., 2002; Muller et al., 1999). A striking feature of *ar-C* is that

it controls expression in different structures of the zebrafish and mouse embryo (Jeong et al., 2006) despite its structural conservation. In zebrafish, *ar-C* predominantly mediates notochord expression while in mouse *SFPE2* directs floorplate expression. In contrast to zebrafish *ar-C*, intron 2 of the mouse does not have regulatory activity in the hypothalamus (Jeong et al., 2006). Instead, in the mouse *Shh* locus, regulatory sequences that reside 400 kb upstream of the promoter mediate expression in the hypothalamus (Jeong et al., 2006). The mouse *Shh* intron 1, despite its striking sequence conservation with *ar-A* (Muller et al., 2002) does not direct notochord expression in the mouse (Jeong et al., 2006). Thus, there can be dramatic changes in the tissue-specific activity of structurally conserved enhancer sequences (Ertzer et al., 2007).

6. *Shh* expression pattern in zebrafish

In zebrafish as in higher vertebrates, expression of *shh* is highly restricted to regions with organiser activity (Krauss et al., 1993; Scholpp et al., 2006; Strahle et al., 1996). In the zebrafish embryo, *shh* is expressed initially in the embryonic shield, and subsequently in the notochord, prechordal plate and the floor plate (Fig. 13). In the brain, *shh* expression is detected in the ventral midbrain, the hypothalamus, the zona limitans intrathalamica (zli) and in a small patch of cells in the telencephalon (Ertzer et al., 2007; Krauss et al., 1993). In the 2-day-old embryo, expression of *shh* is also found in the endoderm and its derivatives (Strahle et al., 1996) and in the retina (Neumann and Nuesslein-Volhard, 2000). The expression of *tiggy-winkle hedgehog* (*twhh*) is restricted to the medial floor plate and the ventral midline of the brain during early somitogenesis (Ekker et al., 1995) and in the retina (Neumann and Nuesslein-Volhard, 2000), whereas *echidna hedgehog* (*ehh*) is transcribed in the notochord exclusively (Currie and Ingham, 1996).

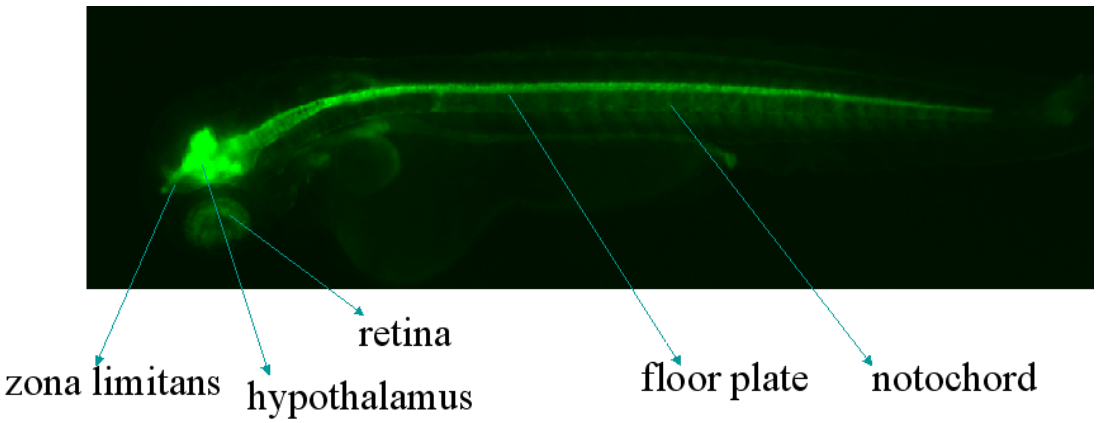


Figure 13: 48 hpf old transgenic zebrafish carrying full length *shh* gene showing expression in the zona limitans, hypothalamus, retina, floorplate and notochord (Modified from Strahle et al 2004).

Figure 14: Transgenic zebrafish expressing green flouresent protein in the ganglion cell layer (GCL) and inner nuclear layer (INL) in the retina under the influence of sonic hedgehog regulatory elements (Adapted from Shukumutava et al 2004).

In the zebrafish, a *shh* wave is required for patterning of the retina (Neumann and Nuesslein-Volhard, 2000). *Shh* expression is initiated at the ventronasal position at 30 hpf and reaches the temporal region by 48 hpf (Neumann and Nuesslein-Volhard, 2000). *Shh* is expressed in the ganglion cell layer and amacrine cells of the inner nuclear layer (Fig. 14) while *twhh* is expressed only in the ganglion cell layer (Shkumatava and Neumann, 2005).

7. Scope of the project

Shh is expressed in the GCL and INL of the zebrafish retina, however the cis-regulation behind *shh* expression in the retina has not yet been unravelled. The main goal of this study is focussed at the identification and functional characterisation of the retinal enhancer. This will provide deep insights into the regulatory elements that are responsible for propagation of the *shh* wave in the zebrafish retina. Because the initiation of neurogenesis in the zebrafish retina is dependent on Fgfs secreted from the optic stalk it would be interesting to explore the effect of these Fgfs on *shh* propagation and the interplay between *Shh* and Fgf signalling in patterning the zebrafish retina.

Results

1. Two downstream enhancer regions contribute to retina expression of the *shh:gfp* transgene

Previously carried out enhancer screens in the zebrafish *shh* gene by transient co-injection experiments (Muller et al., 1999) led to the identification of four activating regions (*ar-A, B, C, D*) that direct floorplate and notochord expression. *Ar-D* was detected upstream and *ar-A*, *ar-B* and *ar-C* in two intronic regions of the *shh* locus (Fig. 15). The promoter and *ar-D* (including sequences up to -2.4kb) drove expression in the floorplate of the midbrain, hindbrain and anterior spinal cord (Chang et al., 1997); (Muller et al., 1999). Enhancers *ar-A* and *ar-B* which are located in the first intron, mediated expression in the notochord and floorplate respectively. *Ar-C* in the second intron drives expression in the notochord and weakly in the posterior floorplate. For detailed analysis of the identified regulatory regions, stable transgenic lines were generated with *shh* sequence from -2432 to +221 relative to the *shh* transcription start site inserted upstream of the green fluorescent protein (GFP) reporter gene and *shh* sequences from +549 to +5366 inserted downstream of GFP (Chang et al., 1997).

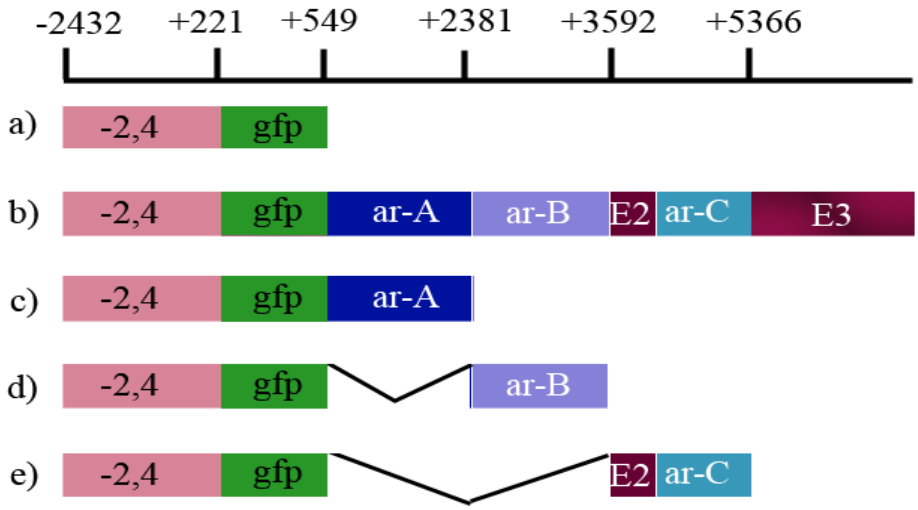


Figure 15: An outline of the enhancer constructs. The −2.4*shh:gfp* construct lacking the intronic activating regions served as a promoter control. The −2.4*shh:gfpABC* construct carrying all three activating regions *ar-A, B* and *C* represented the wildtype mini *shh* locus. Exon 1 was replaced by GFP. E2 and E3 are exons 2 and 3 respectively. A series of deletion constructs was generated to test the activating regions −2.4*shh:gfpA*, −2.4*shh:gfpB* and−2.4*shh:gfpC* individually. *Ar-D* is located within the -2.4 kb promoter sequence.

Transgenes carrying individual enhancers or combination of them were generated (Fig. 15). Expression of the transgene which contains all three intronic enhancer sequences *ar-A, B and C* is detected in the notochord, floorplate, hypothalamus, zona limitans and the retina. The enhancers responsible for *shh* expression in the midline and brain were studied elaborately (Ertzer et al., 2007) but the regulatory region specifying retina function in the zebrafish was yet to be identified. The main focus of this study is aimed at the identification and functional characterisation of the retina enhancer.

Transgenic lines that express GFP under the control of different *shh* regulatory sequences were analysed for expression in the retina. The line *-2.4shh:gfpABC* (3/3lines) drives expression in the retina in both ganglion cell layer (GCL) and in amacrine cells of the inner nuclear layer (INL) similar to the previously documented *shh:gfp* line (Neumann and Nuesslein-Volhard, 2000). Deletion of regulatory regions *ar-A, ar-B,* and *ar-C* (*-2.4shh:gfp*), resulted in complete loss of retina expression in two independent transgenic lines (Fig. 16A). Thus the same intronic sequences to which previously notochord, floorplate and brain enhancers were mapped seem to be crucial for driving expression in the retina as well.

To map the location of the retina regulatory region, deletion constructs that contained either *ar-A, ar-B* or *ar-C* alone downstream of the −*2.4shh:gfp* reporter were created (Fig .15). The transgenes *-2.4shh:gfpA* (4/4 lines) and *-2.4shh:gfpB* (2/4 lines) mediated both expression in the retina (Fig. 16C, D). The latter transgenic line expressed, however, only in the ganglion cell layer and gave more frequently mosaic expression and reduced penetrance. The *-2.4shh:gfpC* (2/2 lines) did not drive any expression in the retina (Fig. 16E). Only 2 out of the 4 stable lines of*-2.4shh:gfpB* showed GFP expression in the GCL but all the 4 lines of *-2.4shh:gfpA* showed similar expression in two layers of the retina. These findings uncovered two enhancer regions *ar-A* and *ar-B* with overlapping but different expression patterns that contribute to the expression in the retina. A*r-A* mediated more robust expression and was active in both GCL and INL, therefore a systematic analysis was further performed in the *ar-A* region.

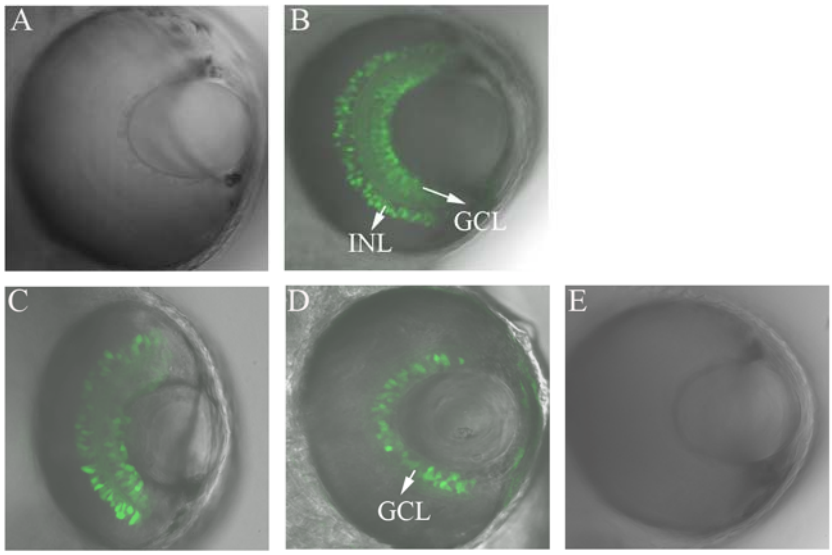


Figure 16: Expression pattern of the *shh* enhancers in the retina. Stable transgenic lines -*2.4shh:gfpABC* and -*2.4shh:gfpA* drive expression in the ganglion cell layer (GCL) and inner nuclear layer (INL) of the zebrafish retina (B, C), in the -*2.4shh:gfpB* expression is seen only in the GCL (D) while the -*2.4shh:gfp* and -*2.4shh:gfpC* (A, E) does not drive any GFP expression. Anterior to the top in all images. Confocal images taken at 72 hpf.

2. Identification of a novel retina enhancer mediating *shh:gfp* expression in the retina

The *ar-A* enhancer mediated *shh:gfp* expression in the GCL and INL of the retina. Previous studies in our lab (Ertzer et al., 2007) revealed that the *ar-A* is essential for regulating *shh* expression in the notochord. The *ar-A* enhancer is around 1.9 kb long (+549 to +2381). Sequence analysis revealed that a 350 bp (+2021 to +2381) of *ar-A* exhibited a high degree of conservation among several species and is sufficient to drive notochord expression (Muller et al., 1999) (Fig. 17A). In order to check if the 350 bp mediates retina expression , deletion constructs carrying only the 350 bp region of *ar-A* (+2021 to +2381) (Fig. 17C) and another construct carrying all the upstream intronic sequences +549 to +2021 (Fig. 17B) were cloned downstream of the -*2.4shh:gfpSce I* construct. SceI sites were introduced into the plasmid as previous reports showed a higher efficiency for obtaining stable transgenics (Thermes et al., 2002). The *ar-A* construct carrying both conserved and non-conserved sequences from +549 to +2381 was also injected as an experimental control.

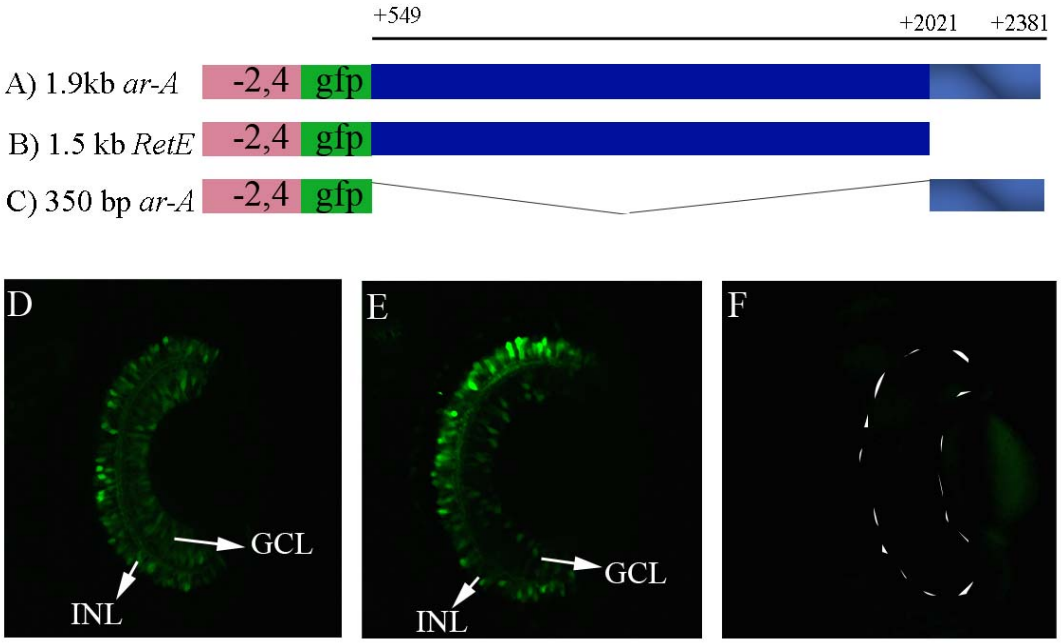


Figure 17: A scheme outlining the cloning of the plasmids to *-2.4shh:gfp*. A novel 1.5 kb retinal enhancer *RetE* drives *shh:gfp* expression in the GCL and INL of the zebrafish retina (E) similar to full length *ar-A* (D). The highly conserved 350 bp notochord enhancer of *ar-A* (light blue box) is not capable of driving any expression (F). Confocal images at 72 hpf. Anterior to the top in all the images.

These constructs were micro-injected into one cell stage zebrafish embryos. At 24 hpf, the embryos were sorted for GFP expression in the anterior floorplate, mediated by the *shh* promoter (*-2.4shh:gfp*). This served as an internal control for all injections performed in this entire study. Transient analysis displays a mosaic pattern of expression and not all embryos injected show a similar expression profile, making it important to validate these results by proper quantification and repetition of injections.

Expression analysis revealed that the 350 bp *ar-A* (+2021 to +2381) alone was not able to drive *shh:gfp* expression in the retina (Fig. 17F). The other 1.5 kb construct (+549 to +2021) with the intronic sequences mediated the expression in both the GCL and INL of the retina (Fig. 17E) identical to that of the embryos injected with the full length *ar-A* construct (+549 to +2381) (Fig. 17D). The injections were repeated thrice and results are presented in Table 2. Taken together, these results led to the identification of an enhancer responsible for driving *shh:gfp* expression in the retina located between +549 and +2021 of the *shh* locus and was designated as *RetE* (Retinal Enhancer). In order to better understand the regulation of *shh:gfp* expression by *RetE* and for further studies, transient assay was not ideal. Therefore the injected embryos were raised to adulthood

and screened for stable transgenics and 4 independent lines were obtained for -*2.4shh:gfpRetE* that recapitulated transient results. These results confirm that *RetE* is enhancer mediating *shh:gfp* expression in the retina.

CONSTRUCT	RETINA EXPRESSION	NUMBER OF RETINAL EXPRESSING EMBRYOS/TOTAL NUMBER OF INJECTED EMBRYOS
1.9 kb *ar-A* (+549 to +2381)	+++	65/108
1.5 kb *RetE* (+549 to +2021)	++	75/123
350 bp *ar-A* (+2021 to +2381)	-	0/50

Table 2: Results of injections for each deletion construct are performed thrice and are tabulated. +/- indicates the presence or absence of retina expression. *RetE* enhancer were scored ++ as there were fewer ganglion cells that were GFP positive when compared to the 1.9 kb construct (+549 to +2381).

3. A 300 bp region is sufficient and necessary to drive *shh:gfp* expression in the retina

To further characterise the minimal region of the *RetE* required for retina expression, a deletion scan of 200 bp steps from the 5' and 3' of *RetE* (Fig. 18A) was performed. These constructs were cloned into the *-2.4shh:gfpSceI,* injected into zebrafish embryos and monitored for the transient expression. The results from the injections are presented in Table 2. From the 5' deletion series, only the construct that had a deletion from +549 to +729 was able to drive GFP expression in the retina (Fig. 18B). All the other deletion constructs failed to show any GFP expression in the retina (Table 3).

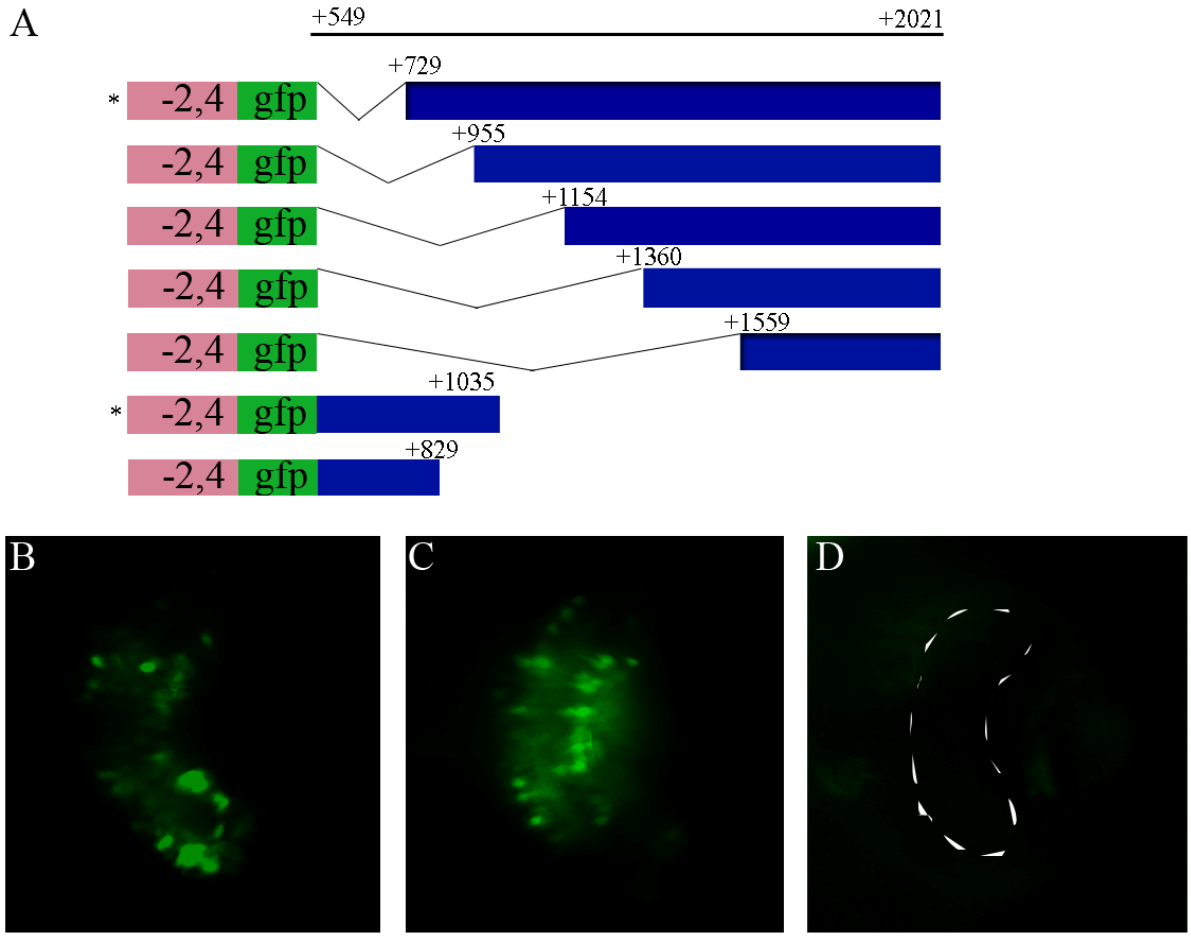


Figure 18: A series of deletion constructs were generated (A) to identify the minimal region required for *shh:gfp* expression in the retina. Two constructs carrying *shh* sequence from +729 to +2021 and +549 to + 1035 shown in asterisks were able to drive expression in the retina (B, C). Construct carrying *shh* sequences from +549 to +829 was not able to mediate retina expression (D). Anterior to the top in all images and imaged at 72 hpf.

The results presented above suggests that a region between +729 to +955 carried the regulatory information needed for *shh:gfp* expression in the retina. To further confirm this analysis, two corresponding 3' deletion constructs with the sequence + 549 to +1035 and +549 to + 829 were investigated. According to their expression pattern (Fig. 18C, D) it was evident that only the construct carrying the sequence +549 to +1035 was able to mediate *shh:gfp* expression in the retina. This deletion scan narrowed down the functional enhancer *RetE* between +829 to +955 of the *shh* locus.

CONSTRUCT	RETINA EXPRESSION	NUMBER OF RETINAL EXPRESSING EMBRYOS/TOTAL NUMBER OF INJECTED EMBRYOS	PERCENTAGE
+ 729 to +2021	++	78/128	59.3
+ 955 to +2021	-	3/109	2.7
+1154 to +2021	-	0/108	0.0
+1360 to +2021	-	0/107	0.0
+1559 to +2021	-	0/102	0.0
+ 549 to +1035	+	27/56	48.2
+ 549 to +829	-	0/98	0.0

Table 3: Results of injections for each deletion construct are performed thrice and are tabulated. +/- indicates the presence or absence of retina expression.

With the rough deletion scan, the activity of flanking sequences could have been missed so a larger fragment from +729 to +1035 was tested for retina expression (Fig. 19A). This was cloned downstream of *-2.4shh:gfpSceI,* injected and monitored for their expression. Expression analysis, indicate that the 300 bp region alone (+729 to +1035) was able to drive retina expression (Fig. 19B, 72% n=40). To evaluate the functional significance of this 300 bp region, another construct with an internal deletion of this 300 bp was generated. This was cloned as above and tested for retina expression. The construct that carried the 300 bp deletion failed to drive any GFP expression in the retina (Fig. 19C, 0% n=85). Taken together these data indicate that the 300 bp (+729 to +1035) *RetE* enhancer region is sufficient and necessary for *shh:gfp* expression in the zebrafish retina.

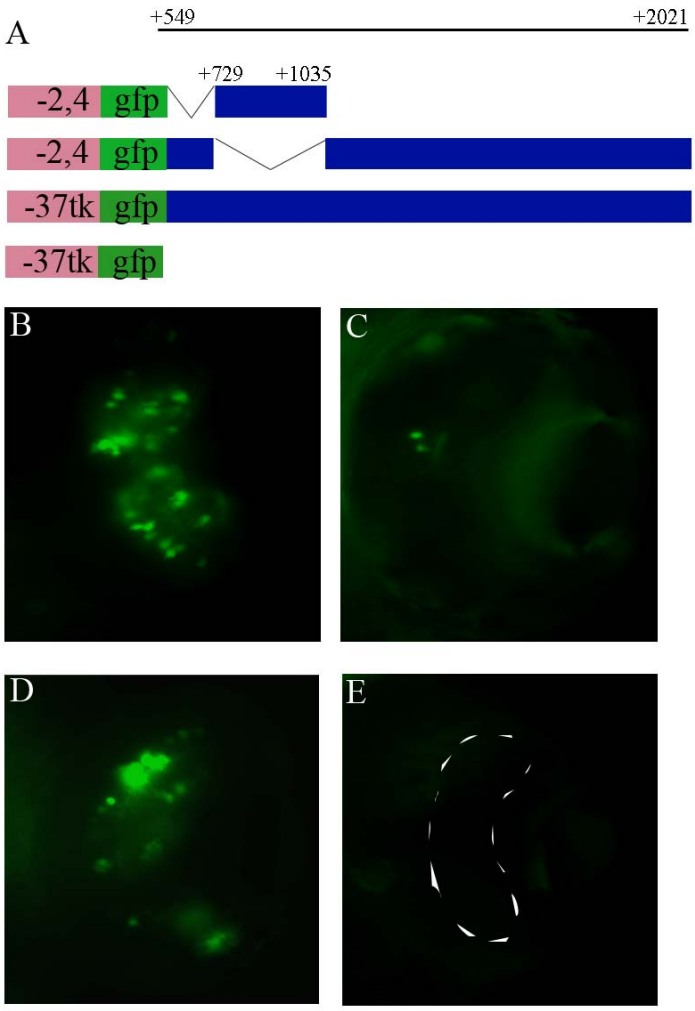


Figure 19: A 300 bp region is sufficient and necessary to drive expression in the retina. An outline of constructs generated are shown in the scheme A. Embryos injected with the *-2.4shh:gfp* plasmid carrying the minimal region +729 to +1035 drives expression in the retina (B). Embryos injected with the *-2.4shh:gfpRetE* plasmid with an internal deletion from +729 to +1035 failed to drive GFP expression in the retina (C). Embryos injected with the heterologous promoter *-37tk:gfpRetE* is also able to drive GFP expression in two layers (D) but the control embryos injected with *-37tk:gfp* does not show any GFP expression in the retina (E). Anterior to the top in all images and pictures taken at 72 hpf.

To test, if the regulatory activity of *RetE* is dependent on the -2.4*shh* promoter, the *RetE* enhancer was cloned downstream of a TATA box containing a minimal thymidine kinase promoter with *gfp* as reporter gene (Rastegar et al., 2002). The construct was injected and expression monitored at 72 hpf. The *-37tk:gfpRetE* was able to drive expression in both layers of the retina (Fig. 19D, 44% n=47). The control embryos injected with *-37tk:gfp* showed no GFP expression in the retina (Fig. 19E, 0% n=42 embryos). These results indicate that the enhancer activity is independent of the *shh* promoter.

4. A 40 bp core region of the *RetE* enhancer is required for *shh:gfp* expression

To define precisely the key regulatory sequence within the 300 bp region required for enhancer activity, a mutational screen over the entire region was performed. Non-overlapping mutations of 20 bp constructs termed M1 to M15 (Fig. 20A) were generated via a PCR based approach. The 15 constructs were cloned downstream of *-2.4shh:gfp*, injected, and the transient expression was monitored in the retina at 72 hpf. The *-2.4shh:gfp* alone drove expression in the anterior floor plate and this served as an internal control. Injections were repeated thrice and the statistics are shown in Table 4.

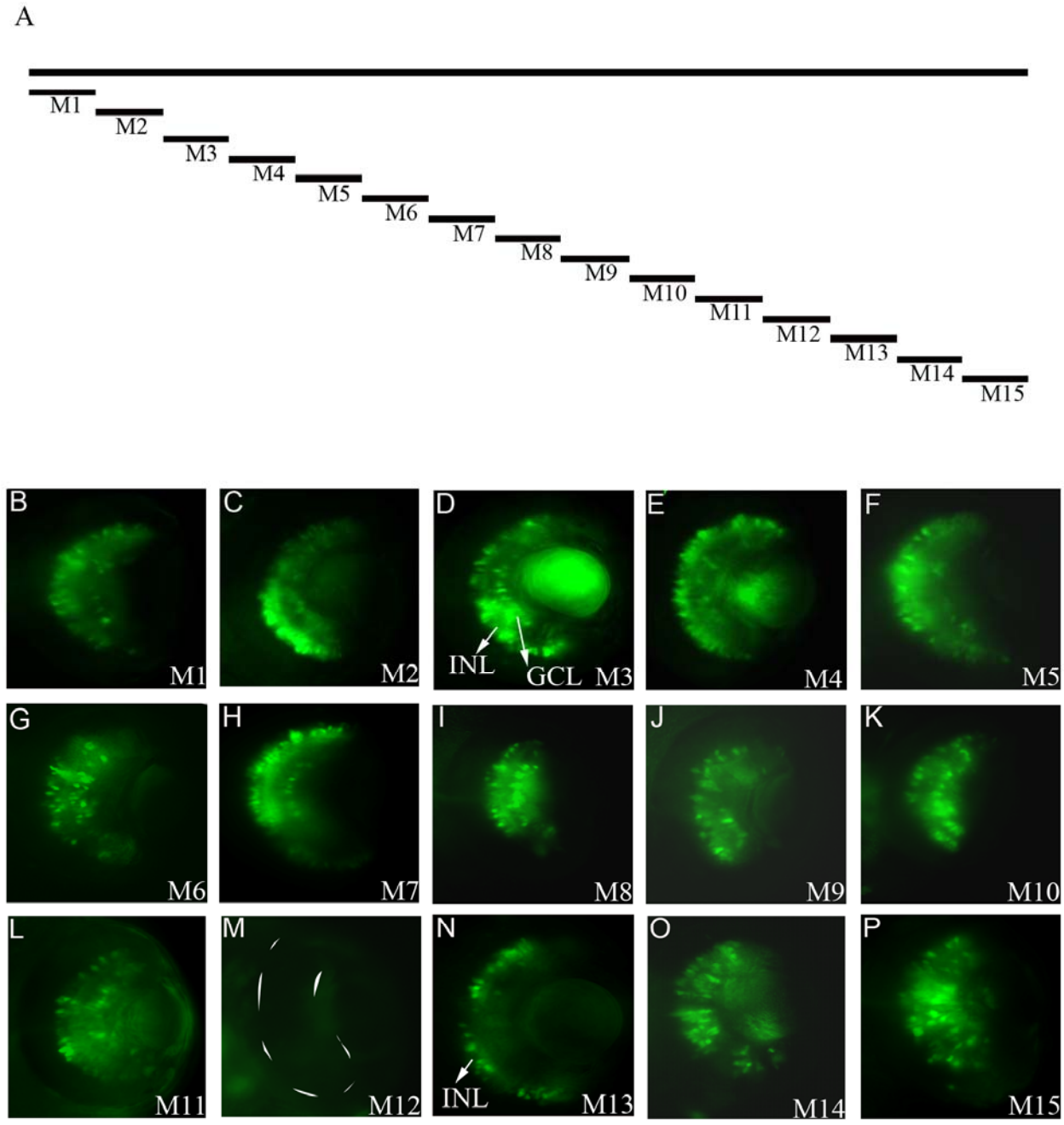


Figure 20: Fine mapping of region +729 to +1035. A mutational scan of 20 bp(A). Mutation M12 led to the complete loss of *shh:gfp* expression (M) whereas mutation M13 abolished *shh:gfp* expression in the GCL but not in the INL (N). Anterior to the top in all images and pictures taken at 72 hpf.

Two regions important for *shh:gfp* expression in the retina were identified from the mutation scan. The mutation termed M12 (+957 to +976) (Fig. 20M) lead to complete loss of expression in the GCL and INL of the retina. These embryos still show expression in the anterior floorplate which serves as a positive control for the injections

(Table 4). Another adjacent 20 bp mutation M13 (+977 to +996) (Fig. 20N) retained only the INL expression, with the GCL expression lost (Table 4). All other mutant constructs showed expression in both GCL and INL of the retina (Fig. 20 B-L, O, P). The numbers of the injections are presented in Table 4. To validate the data, the embryos injected with M12 and M13 were raised to adulthood and then screened for stable transgenics. Two independent lines for each of the constructs were identified and they recapitulated an expression pattern (Fig. 21A, B) similar to that seen in transient expression studies. The data presented in this section suggest that a 40 bp core region (+957 to +996) is crucial for enhancer activity in the GCL and INL of the zebrafish retina.

Mutant construct	Floorplate	GCL and INL	Only INL	Total number of GFP embryos	Percentage
M1	84	84	-	109	77
M2	55	55	-	87	63.2
M3	56	56	-	70	80
M4	73	73	-	119	61
M5	56	56	-	78	71.3
M6	50	50	-	68	73.5
M7	33	33	-	50	66
M8	44	44	-	69	63.7
M9	46	46	-	88	52.2
M10	47	47	-	91	51.6
M11	47	47	-	80	58.7
M12	105	0	-	105	0
M13	47	0	47	80	58.7
M14	45	45	-	96	46.8
M15	43	43	-	102	42.1

Table 4: Injections of mutant constructs are performed thrice and the embryos expressing GFP in specific layers are tabulated. Expression in the floorplate served as the positive control for the micro-injections.

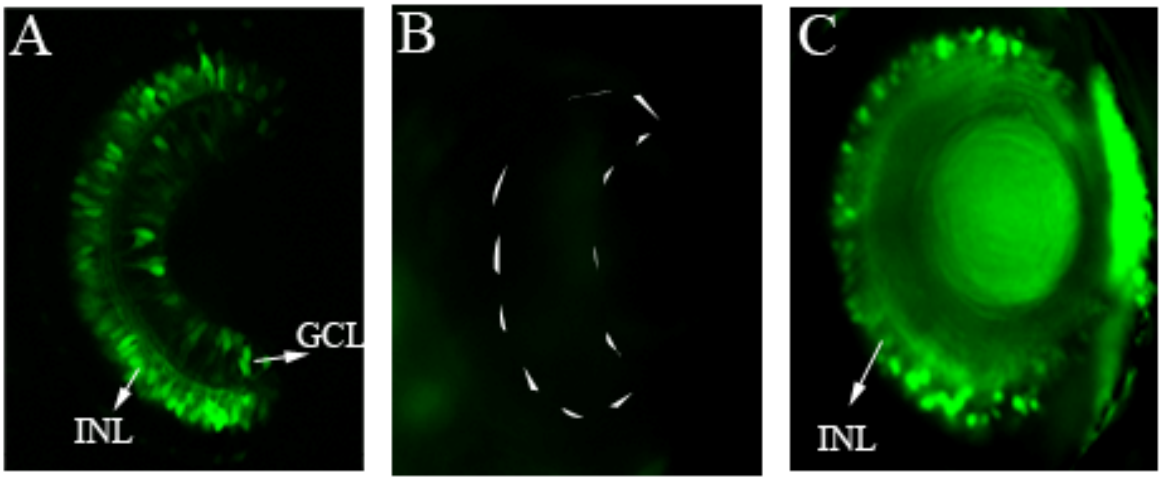


Figure 21: *Shh* expression pattern in the stable M12 and M13 transgenes. Transgenes carrying the M12 mutation failed to drive expression in the retina (B) while the transgene carrying the M13 mutation drive expression only in the INL (C) when compared to *-2.4shh:gfpRetE* (A). The M13 stable line had an overall weak GFP expression and had background expression in the lens.

5. Critical region of the *RetE* enhancer is conserved but not sufficient for *shh:gfp* expression

The mutational scan identified a 40 bp core region of the *RetE* enhancer required for *shh:gfp* expression in the retina. The 40 bp region was subjected to a comparative approach. Comparative genomics could provide insights into the evolutionary conserved nucleotides which may be more likely to be functionally relevant. Conserved blocks of non-coding sequences often represent functionally important domains that share function (Dickmeis and Muller, 2005). Shh intron 1 retinal enhancer sequence of sequence of chick, human and mouse corresponding to the zebrafish 40 bp (M12 and M13) were retrieved from ENSEMBL. Using the T-Coffee program, the sequences were aligned as in Fig. 22. The alignment revealed a block of conservation across the 40 bp with 24 out of the 40 nucleotides identical between zebrafish, mouse, human and chick (Fig. 22).

Figure 22: Multiple sequence alignment (T-Coffee software) of the 40 bp region. Astericks indicate the 100% conservation in M12 and M13 region between zebrafish and the other species.

To investigate whether the 40 bp region (+957 to +996) alone can recapitulate the *RetE* function in the retina, this fragment was cloned downstream of *-2.4shh:gfp*, injected into zebrafish embryos and the expression monitored at 72 hpf. Expression analysis revealed no detectable GFP expression in the retina (Fig. 23B, 0% n=76 embryos) suggesting that the highly conserved region alone is insufficient for retina expression.

A 300 bp (+729 to +1035) region of the *RetE* enhancer was sufficient for driving expression in the retina (Fig. 19B). Availability of significant conservation with human and mouse sequences (Fig. 22) led to the speculation that evolution would have allowed retina function to be retained in these species too. To test this, mouse and human *shh* sequence corresponding to the zebrafish 300 bp region (+729 to +1035) were amplified from their genomic DNA respectively. These were then cloned downstream of *-2.4shh:gfp*, injected and the retina expression monitored at 72 hpf. Expression analysis revealed that both mouse and human *shh* constructs failed to drive any GFP expression in the retina (Fig. 23D, 0% n=45 and 23E, 0% n=36). Control embryos were injected with *-2.4shh:gfpRetE729/1035* zebrafish sequence (Fig. 23B, 70% n=38). Taken together these data suggest a different mechanism for *shh* regulation in mouse and human or that sequences in the fish 300 bp could have been shuffled to other regions in other species.

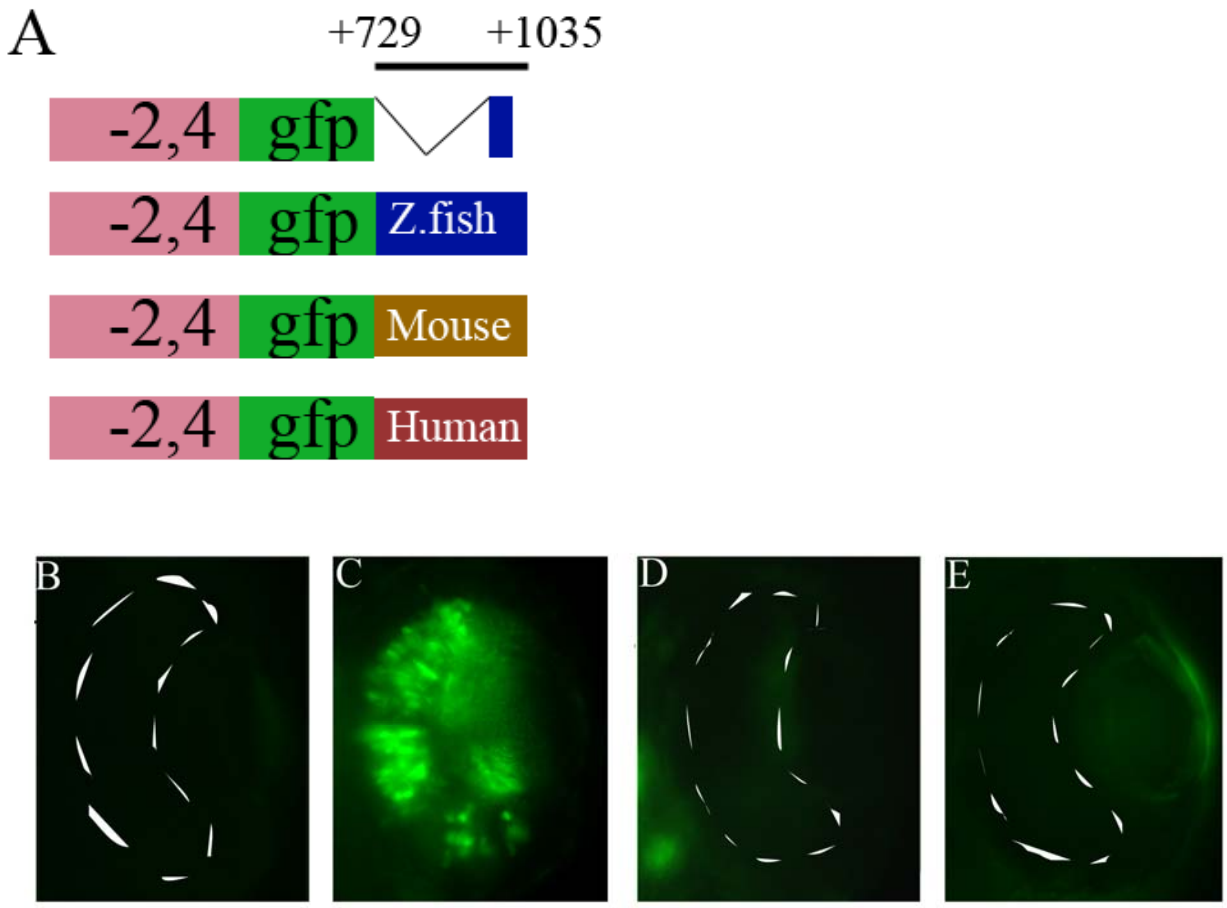


Figure 23: Outline of constructs depicted in A. Embryos injected with the *-2.4shh:gfpRetE40 bp* failed to drive *shh:gfp* expression in the retina (B). Embryos injected with *-2.4shh:gfpRetE*300 bp drive *shh:gfp* expression in the GCL and INL of the retina (C) but embryos injected with the corresponding mouse and human 300 bp region show no detectable GFP in the retina (D, E). All images at 72 hpf.

6. The conserved 40 bp region plus additional 5′ sequences are sufficient to drive expression

The conserved 40 bp region (+957 to +996) alone was insufficient for mediating *shh:gfp* expression. Earlier 5′ deletion series revealed that sequences 3′ to the 40 bp region are not capable of driving *shh:gfp* expression in the retina (Fig. 18A). To determine if other non -conserved 5′ sequences of the 300 bp *RetE* are required for expression, two constructs with roughly an addition of 5′ 85 and 5′ 170 bp to the core 40bp were generated (Fig. 24A) The 85 bp+40bp and 170 bp +40bp constructs were cloned downstream of *-2.4shh:gfp* and injected into zebrafish embryos and transient expression studied at 72 hpf.

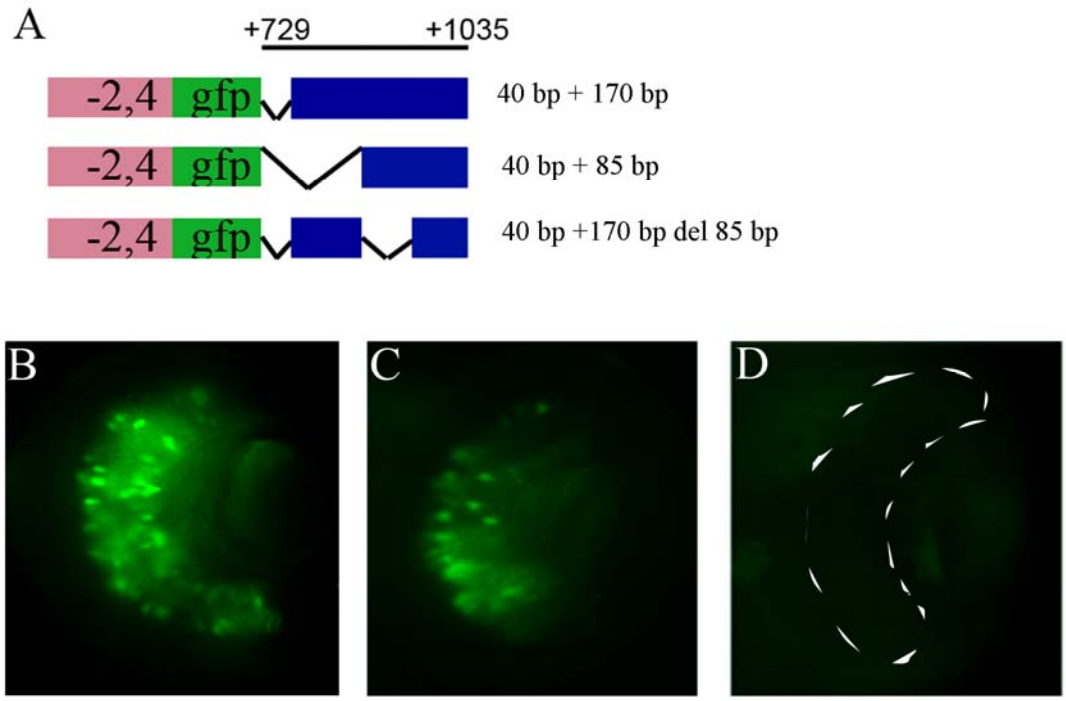


Figure 24: Overview of deletion constructs in A. Embryos injected with the 40 bp +85 bp and 40 bp+ 170 bp are able to drive GFP expression in both GCL and INL of the retina (B, C). Embryos injected with the 40 bp+ 170 bp with 85 bp internal deletion failed to drive any expression in the retina (D). Anterior to the top in B, C and D at 72 hpf.

CONSTRUCT	RETINA EXPRESSION	NUMBER OF RETINAL EXPRESSING EMBRYOS/TOTAL NUMBER OF INJECTED EMBRYOS
85 bp + 40 bp	+	51/91
170 bp + 40 bp	+	43/91
170 bp del 85 bp + 40 bp	-	0/71

Table 5: Results of the injections performed thrice are tabulated. +/- indicate the presence or absence of retina expression.

Expression analysis revealed that both the constructs were able to drive GFP expression in the GCL and INL of the retina (Fig. 24B, C) as construct -*2.4shh:gfpRetE729/1035* (Fig. 19). Addition of 85 bp could confer retina expression, to assess its functional importance it was deleted in the context of the +170 construct (Fig. 24A). This was then cloned downstream of -*2.4shh:gfp*, injected and the retina expression monitored at 72 hpf. This construct failed to drive retina expression (Fig. 24D). All the injections were performed thrice and the results are presented in Table 5. Altogether these data demonstrate that the conserved 40 bp region (+957 to +996) plus 85 bp additional 5′ sequences are sufficient for enhancer activity both in the GCL and INL of the retina.

7. The Ets factors Erm/ Pea3 were predicted to bind to the *RetE*

Regulatory regions are usually a composite of multiple transcription factor binding sites. To determine the transcription factors involved in the regulation of *RetE*, the 40 bp core region was analysed for binding site motifs using the PATCH program (TRANSFAC 6.0). Potential binding sites were identified by searching the genome with consensus sequences or using weight matrices. The PATCH program revealed binding sites for the ETS transcription factors Erm and Pea3. Erm and Pea3 possess a similar core consensus sequence "GGAA/T" (Brown et al., 1998). Three binding sites (BS1, BS2 and BS3) for Erm and Pea3 were identified both in M12 and M13 region. The BS3 exhibited a 100% matrix core which is well conserved among many species while BS1 and BS2 showed 75% and 83% identity respectively (Fig. 25). Erm and Pea3 belong to the ETS family of transcription factors and are reported to be targets of Fgf signalling in the zebrafish (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). They possess a 85-aminoacid ETS domain (Karim et al., 1990) that binds to DNA and regulates transcription (Laudet et al., 1999). Earlier findings have not illustrated a clear role of Erm and Pea3 in the retina and are to be addressed in detail.

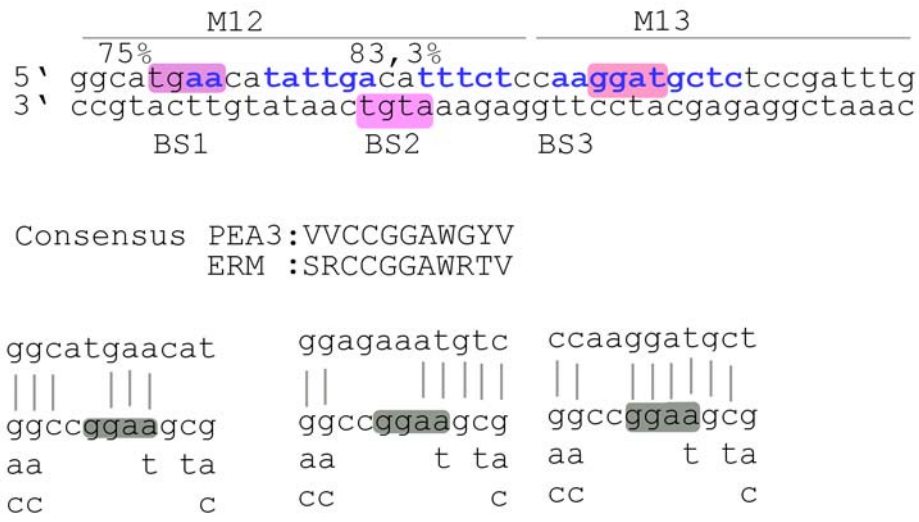


Figure 25: Predicted binding motifs for Erm and Pea3 in M12 and M13 region of the *RetE* enhancer. Three motifs identified are represented as BS1, BS2 and BS3 respectively and are indicated as pink boxes. The blue letters indicate the conserved nucleotides. Erm and Pea3 share a common core motif GGAA/T in their consensus binding sequence.(V = G/ A / C, R = A /G, S= G /C, W= T/A, Y= C/T).

8. Pea3/ Erm intact binding site is required for *shh:gfp* expression in the GCL

The search for binding site motifs indicated that M13 possessed a strong core sequence for Erm/Pea3 transcription factors. To elucidate the functional significance, mutations targeting the core sequence in M13 (GGAT) were designed using a PCR based strategy. The mutations were introduced into the *RetE* (1.5 kb). This was then cloned downstream of *-2.4shh:gfp*, injected and the transient expression monitored at 72 hpf. The embryos displayed a complete loss of *shh:gfp* expression in the GCL while expression in the INL was intact (Fig. 26B, 62% n=81). This result is consistent with the earlier findings where mutations across the entire M13 also led to loss of GFP expression in the GCL (Fig. 20N). Control embryos injected with *-2.4shh:gfpRetE* showed expression in both layers of the retina (Fig. 26A, 63% n=39). Taken together, these findings suggest that *shh:gfp* expression in the GCL is independently regulated from that of INL involving Pea3 and Erm factors.

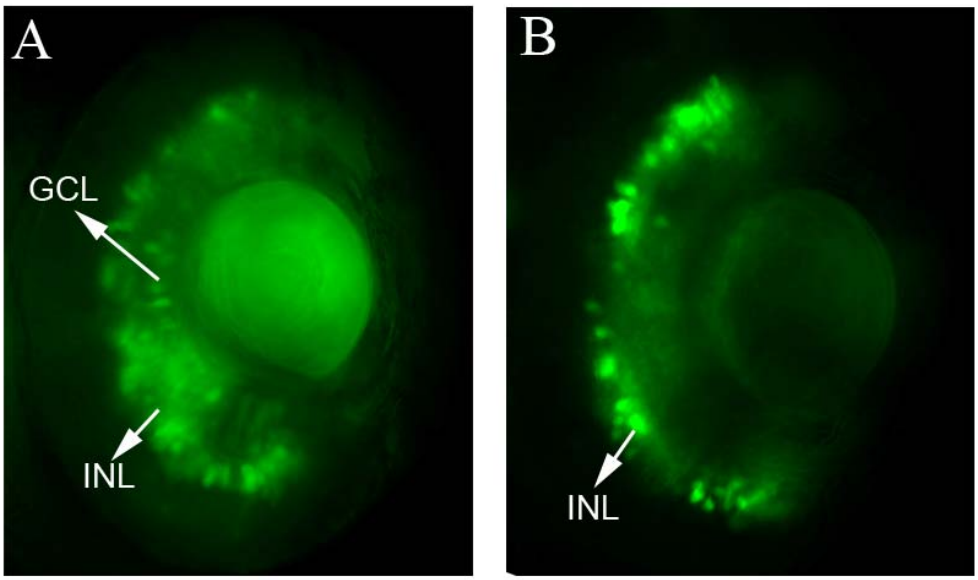


Figure 26: *Shh:gfp* expression in the GCL requires an intact Erm/Pea3 binding site. Embryos injected with the plasmid *-2.4shh:gfpRetE* carrying mutations in the Erm/Pea3 core motif of M13 show GFP expression only in the INL while expression in the GCL is lost (B) . Control embryos injected with *-2.4shh:gfpRetE* show GFP expression in both GCL and INL of the retina (A). Images taken at 72 hpf.

9. Pea3 and Erm are expressed in the zebrafish retina

Mutational studies suggest the importance of the binding motifs of Erm/Pea3 for driving expression in the GCL. To understand whether the expression of these factors coincides with that of the retinal enhancer, Pea3 and Erm mRNA expression in the zebrafish retina was studied using whole mount *in-situ* hybridisation with digoxigenin labelled antisense Pea3 and Erm probes. The *in-situs* were performed on two stages of embryos one at 34 hpf when neurogenesis has already initiated and another at 72 hpf when retinal neurogenesis is complete. The expression of *erm* was ubiquitous in the retina at 34 hpf (Fig. 27A), at 72 hpf the expression was just limited to a single layer of lens epithelial cells surrounding the lens (Fig. 27B). The expression of Pea3 differed at 34 hpf (Fig. 27C) with a faint staining in the GCL and central retina and in a layer of the lens epithelium, and at 72 hpf the expression was retained only in the lens epithelium (Fig. 27D). Taken together, *in -situ* results indicate that both Erm and Pea3 are expressed in the zebrafish when retinal neurogenesis is active.

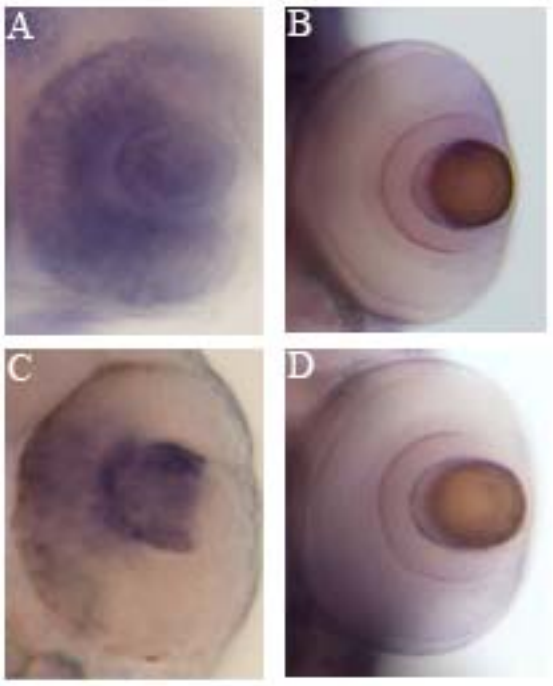


Figure 27: Expression pattern of Erm and Pea3. Erm is expressed ubiquitously in the retina and lens at 34 hpf (A) and later only in the lens epithelium at 72 hpf (B). Pea3 is expressed faintly in the GCL and central retina and in the lens epithelium at 34 hpf (C) and later at 72 hpf (D) seen only in the lens epithelium.

10. Pea3 and Erm act redundantly in promoting *shh:gfp* expression in the retina

Pea3 and Erm are expressed in the zebrafish retina and mutation of their binding sites indicates that they are important for *RetE* activity in the retina. In order to elucidate their role in regulating *shh* expression in the retina, a knock-down approach using morpholinos (MOs) was performed. Morpholinos designed against the translation initiation site or splice site of the gene (Draper et al., 2001) were ordered from Genetools and prepared for microinjection as described (Nasevicius and Ekker, 2000). Morpholinos were injected into *-2.4shh:gfpRetE* transgenic embryos and the effect on the retina expression monitored at 48 hpf. Embryos injected with either Pea3 MO or Erm MO (1 µg/µl) showed normal GFP expression in GCL and INL of the retina (Fig. 28B n=30, Fig. 28C n=27).

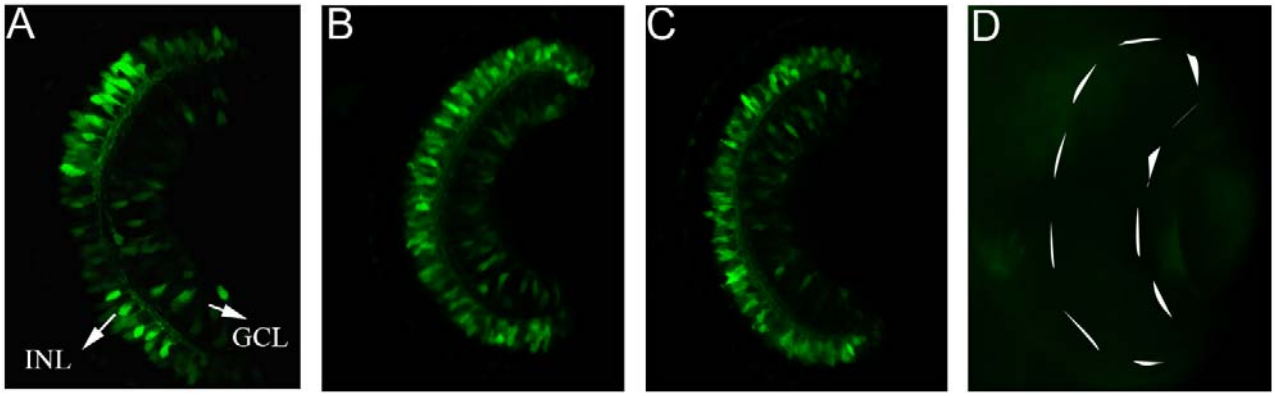


Figure 28: Morpholino based downregulation of Pea3 and Erm. Control embryos injected with mismatch Erm MO + Pea3 MO show normal GFP expression in the GCL and INL (A). Transgenic embryos injected with either Pea3Mo or Erm Mo (1,0 µg/µl) had no effect on the expression pattern (B, C) while those injected with a mixture of both the morpholinos at the same concentration (1,0 µg/µl) showed a complete loss of GFP expression in the retina (D). Confocal pictures at 48 hpf and anterior to the top in all images.

To test whether Pea3 and Erm act redundantly in controlling *shh:gfp* expression, a cocktail of both Pea3 MO and Erm MO (1 µg/µl) were injected into *-2.4shh:gfpRetE* transgenic embryos. Expression analysis on the retina at 48 hpf revealed a complete loss of GFP expression in both GCL and INL of most of the injected embryos (Fig 28D n=51). Frequently only 1-2 dots of expression were seen at the initiation site. Embryos injected with a mixture of Pea3 and Erm control morpholinos (1 µg/µl) show normal GFP expression in the retina (Fig. 28A, n=28). These findings provide evidence that both transcription factors act redundantly for regulating *shh:gfp* expression in the retina.

11. PEA3 and ERM bind *in-vitro* to the binding motif in M13 region

To investigate whether, the identified motifs in M13 bind ERM or PEA3, ElectroMobility Shift Assays (EMSA) using GST fusion proteins were performed. Only the ETS domain which mediates the transcriptional activity of Erm and Pea3 (Brown et al., 1998) was cloned downstream of into the GST expression plasmid. The GST proteins were produced in E. coli, then purified using a Gluthathione Sepharose 4B resin and analysed on a SDS polyacrylamide gel. As shown in the Fig. 29, a 36 kDa and 32 kDa GST fusion protein of ERM and PEA3 were isolated, respectively. There were also several minor bands present in the elute which indicates partial degradation during

purification.

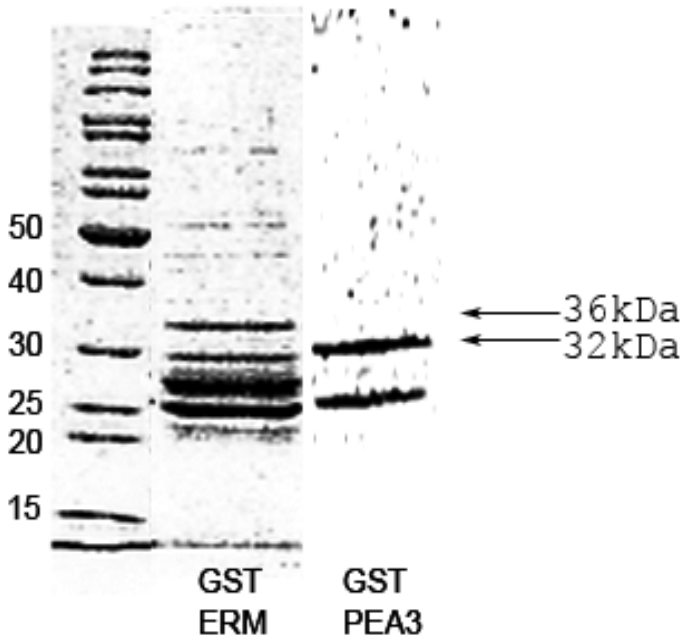
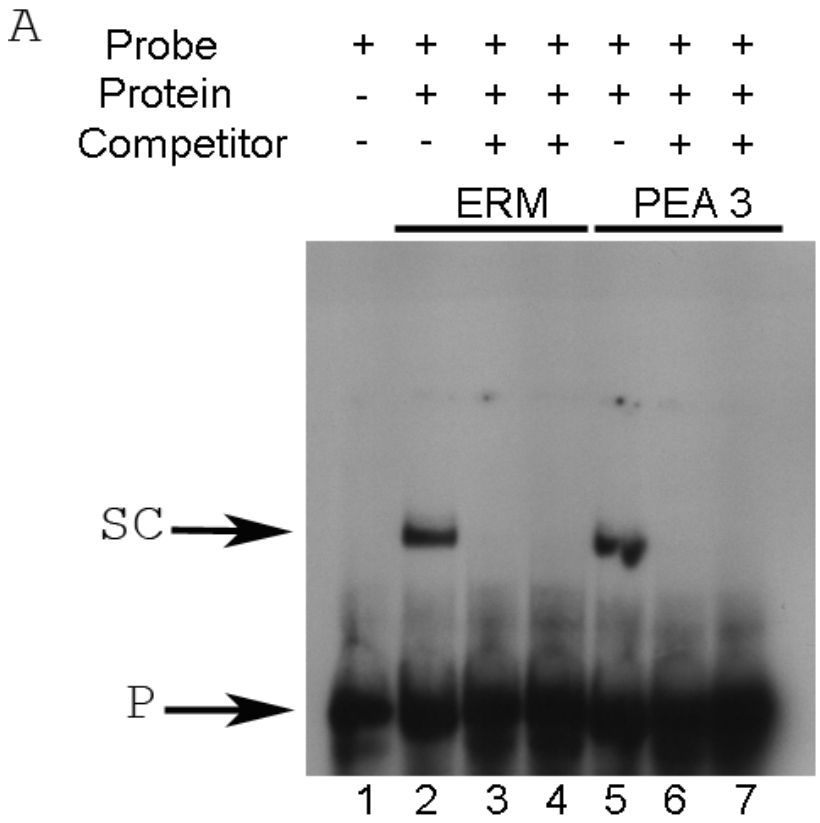


Figure 29: Purification of GST-ERM and GST-PEA3 fusion proteins. The purified GST-ERM is 36 kDa and purified GST-PEA3 is around 32 kDa indicated by arrows. The presence of multiple bands indicate the partial degradation during purification and this does not affect the DNA binding activity in latter experiments.

Synthetic oligonucleotides harbouring the M12+M13 region (*RetE* probe) were annealed and then 5′ end labelled with ^{32}P using T4 kinase. ERM or PEA3 were incubated with the probe (Fig. 30B) and EMSA was performed. No specific complexes for Erm and Pea3 were observed when the reaction mixture contained only labelled probe (Fig. 30A, lane 1). However, the GST-ERM or GST-PEA3 protein formed a specific complex with the labelled probe in lane 2 and 5 respectively (Fig. 30A). In order to test the specificity of the binding, 50-100 fold molar excess of cold probe (*RetE*) were used for competition (Fig. 30B). The formation of the complex was completely hindered upon competition with the excess of cold *RetE* oligos in lane 3, 4, 6 and 7 respectively (Fig. 30A).



B

RetE probe 5′ GGGCATGAACATATTGACATTTCTCCAAGGATGCTCTCCGATTTGTTT 3′
3′ CCCGTACTTGTATAACTGTAAAGAGGTTCCTACGAGAGGCTAAACAAA 5′

RetE competitor 5′ GGGCATGAACATATTGACATTTCTCCAAGGATGCTCTCCGATTTGTTT 3′
3′ CCCGTACTTGTATAACTGTAAAGAGGTTCCTACGAGAGGCTAAACAAA 5′

Figure 30: *In vitro* binding of ERM and PEA3 to the M12+M13 motif (A). Lane 1 indicates the 40 bp probe alone, Lanes 2 and 5 shows the DNA- Protein complexes that are retarded in the gel and Lanes 3,4,6,7 shows the successive competition through cold probes in a range of 50-100 fold. Sequence of probe and competitor used are provided (B). P= free probe and SC= shifted complex.

The binding specificity was also further subjected to competition using M13 motif oligos, wildtype and mutant (Fig. 31C) in the range of 50-100 fold excess. Lane 2 displays the shifted complex produced on binding of ERM and PEA3 (Fig. 31A, B). The binding specificity was reflected by the reduced amount of complex formed when M13 wildtype cold oligos were added in lanes 3 and 4 (Fig 31A, B). However, complex formation was not inhibited by adding excess mutated M13 oligo in lane 5 and 6

respectively (Fig. 31A, B). Altogether these data reveal that ERM and PEA3 can bind to motifs in the *RetE* region and these can be competed either with *RetE* or M13 wildtype oligos but not with the M13 mutant oligos.

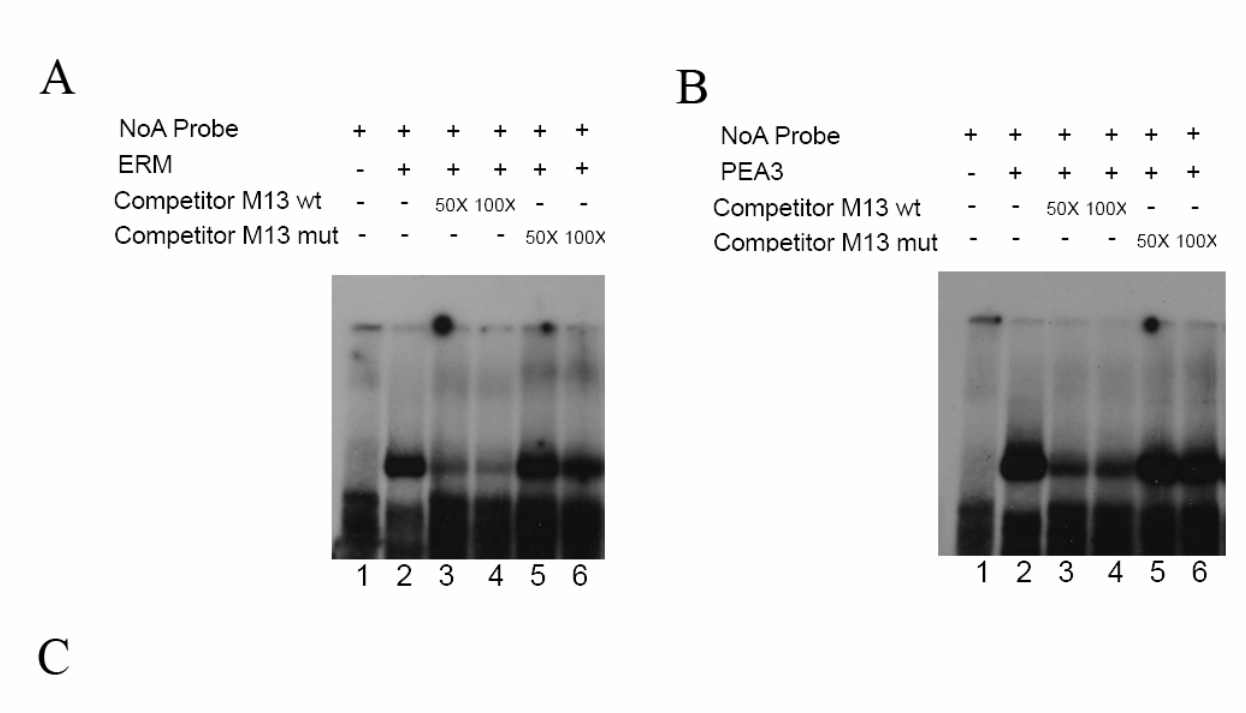


Figure 31: In vitro binding of ERM and PEA3 to RetE probe and specific cold competition using M13 wildtype and M13 mutant oligos, mutated nucleotides in violet (A, B). In both the gels, lane 1 carries only the probe, lane 2 shows the binding of ERM and PEA3 with a shifted complex produced. Lane 3 and 4 shows the cold competition (50-100 fold) using M13 wildtype oligos with a partial loss in the complex and lanes 5 and 6 shows the cold competition (50-100 fold) using M13 mutant oligos where the competing potential is lost. Sequence of probe and competitor used are provided (C). P= free probe and SC= shifted complex.

12. PEA3 and ERM can also bind to weak motifs in M12

In order to determine if ERM and PEA3 bound specifically only to the M13 motif of the *RetE* probe, EMSA was carried out using *RetE* probe carrying mutation in the M13 core motif (Fig. 32B). The *RetE* probe with M13 mutation was incubated with ERM or PEA3 and EMSA were performed. Lane 2 and 3 (Fig. 32 A) display the shift produced upon binding of ERM and PEA3 to the mutant probe. This suggests that ERM and PEA3 can also recognise and bind the two other motifs in M12 region.

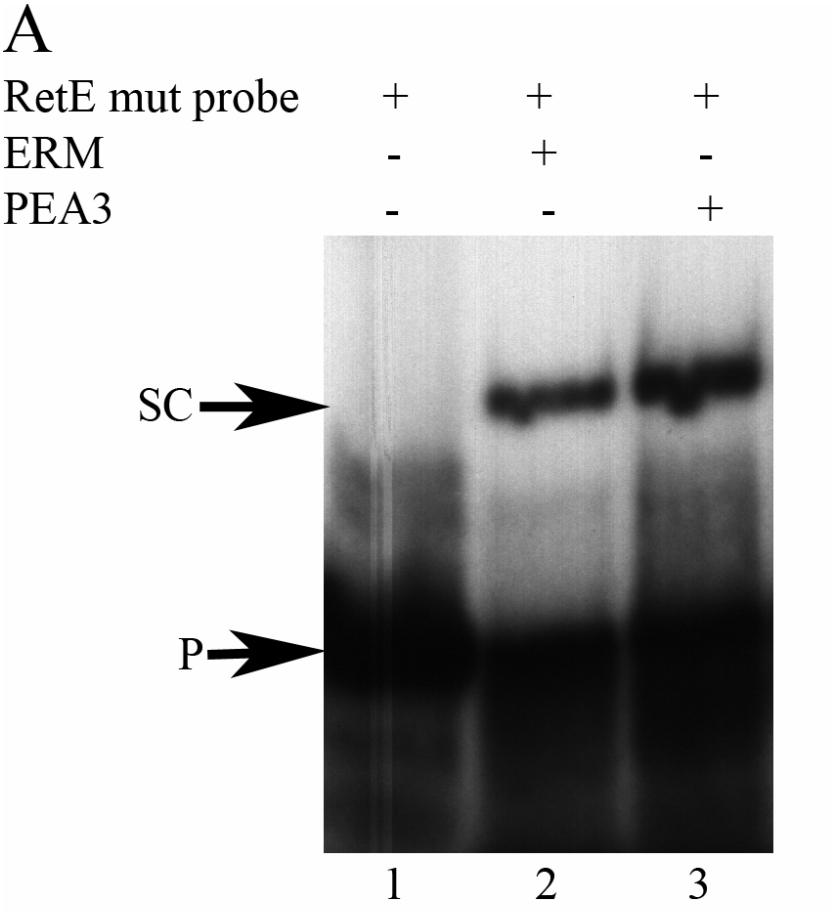
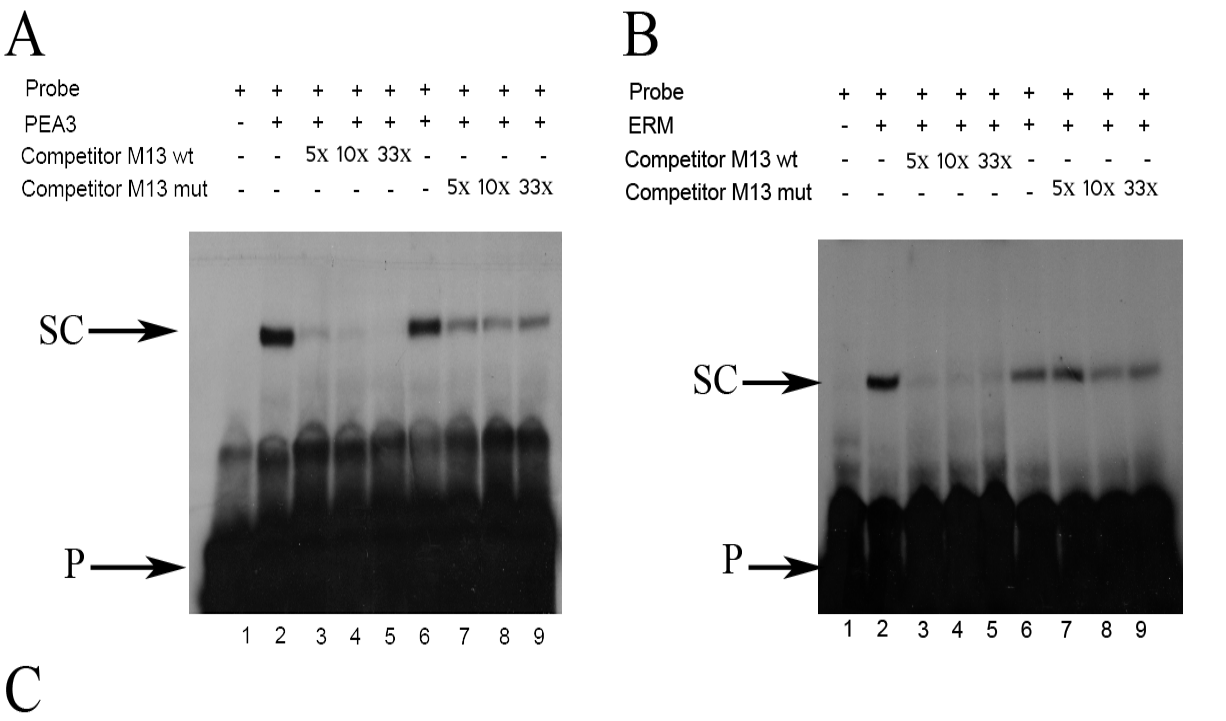


Figure 32: In-vitro binding of ERM and PEA3 with mutant RetE probe, mutated nucleotides in violet (B). Lane 1 shows the mutant probe alone, while lane 2 and 3 shows the mutant probe binding to ERM and PEA3 and thereby producing a shifted complex that is retarded in the gel. P= free probe and SC= shifted complex.

In order to verify this, EMSA was conducted using an oligo containing the M12 motif (Fig. 33C) as the probe. The M12 probe labelled with ^{32}P was incubated with the proteins and subjected to EMSA. Both ERM and PEA3 can recognise the motifs in the M12 region (Fig. 33A, B- lane 2). To check the specificity of this binding, the shifted bands were competed using cold oligos of M13 wildtype and M13 mutant (Fig. 33C) in 5-33 fold excess. The M13 wildtype oligo competed very efficiently at even 5 fold excess in lane 3 (Fig. 33A, B). The mutant M13 oligo also displayed competition at the range of 10 to 33 fold excess in lane 8 and 9, respectively (Fig. 33A, B). Thus some unspecific competition was noted. This suggests that an excess of cold M13 wildtype in the reaction can readily compete with the weaker motifs in the M12 region and could displace the binding thus proving that M13 interaction with the proteins are much stronger than M12 in accordance with the PATCH results (Fig. 25).



C

M12 probe	5′ GGCATGAACATATTGACATTTCTC 3′
	3′ CCGTACTTGTATAACTGTAAAGAG 5′
M13 wt competitor	5′ TTTCTCCAAGGATGCTCTCCGATTTGTTTCT 3′
	3′ AAAGAGGTTCCTACGAGAGGCTAAACAAAGA 5′
M13 mut competitor	5′ TTTCTCCAAAAGCGCTCTCCGATTTGTTTCT 3′
	3′ AAAGAGGTTTTCGCGAGAGGCTAAACAAAGA 5′

Figure 33: *In vitro* binding of ERM and PEA3 to the M12 probe and competition using M13 wildtype and M13 mutant oligos (A, B). In both the gels, lane 1 carries only the probe, lane 2 and 6 shows the binding of protein with a shifted complex produced. Lane 3, 4 and 5 show the cold competition (5-33 fold) using M13 wildtype oligos with a complete loss in the formation of the complex and lanes 7, 8 and 9 shows the cold competition (5-33) using M13 mutant oligos where there is partial competition seen. Sequence of probe and competitor used are provided (C). P= free probe and SC= shifted complex.

Taken together, the EMSA studies reveal that both ERM and PEA3 can bind to both M12 and M13region of *RetE*, and competition results indicate that the M13 protein interaction is more efficient than the M12 protein interaction.

13. Fgfs are required for the initiation and propagation of the *shh:gfp* wave in zebrafish

Erm and Pea3 act downstream of FGF signalling (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001) and earlier studies have clearly indicated that Fgf from the optic stalk is required for the initiation of retinal neurogenesis (Martinez-Morales et al., 2005). To decipher the influence of FGF signalling on the *shh* wave in the retina, transgenic embryos were exposed to SU5402 treatment, a potent inhibitor of Fgf signalling at the level of the receptor tyrosine kinases (Mohammadi et al., 1997). Transgenic embryos *-2.4shh:gfpRetE* were exposed to 16 µM SU5402 at two different time points. In the first treatment, embryos were exposed from 24 hpf before *shh:gfp* expression is initiated in the retina whereas in the second treatment, embryos were exposed from 34 hpf when 2-3 dots of *shh:gfp* expression are already visible at the ventro-nasal region (Fig. 34A). Failure to express in the retina may be due to the loss of initiation of expression in Fgf blocked embryos. The effect of Fgf inhibition on the *shh:gfp* expression in the retina was monitored at 48 hpf. Control embryos were treated with DMSO in a similar way (Fig. 34B, D).

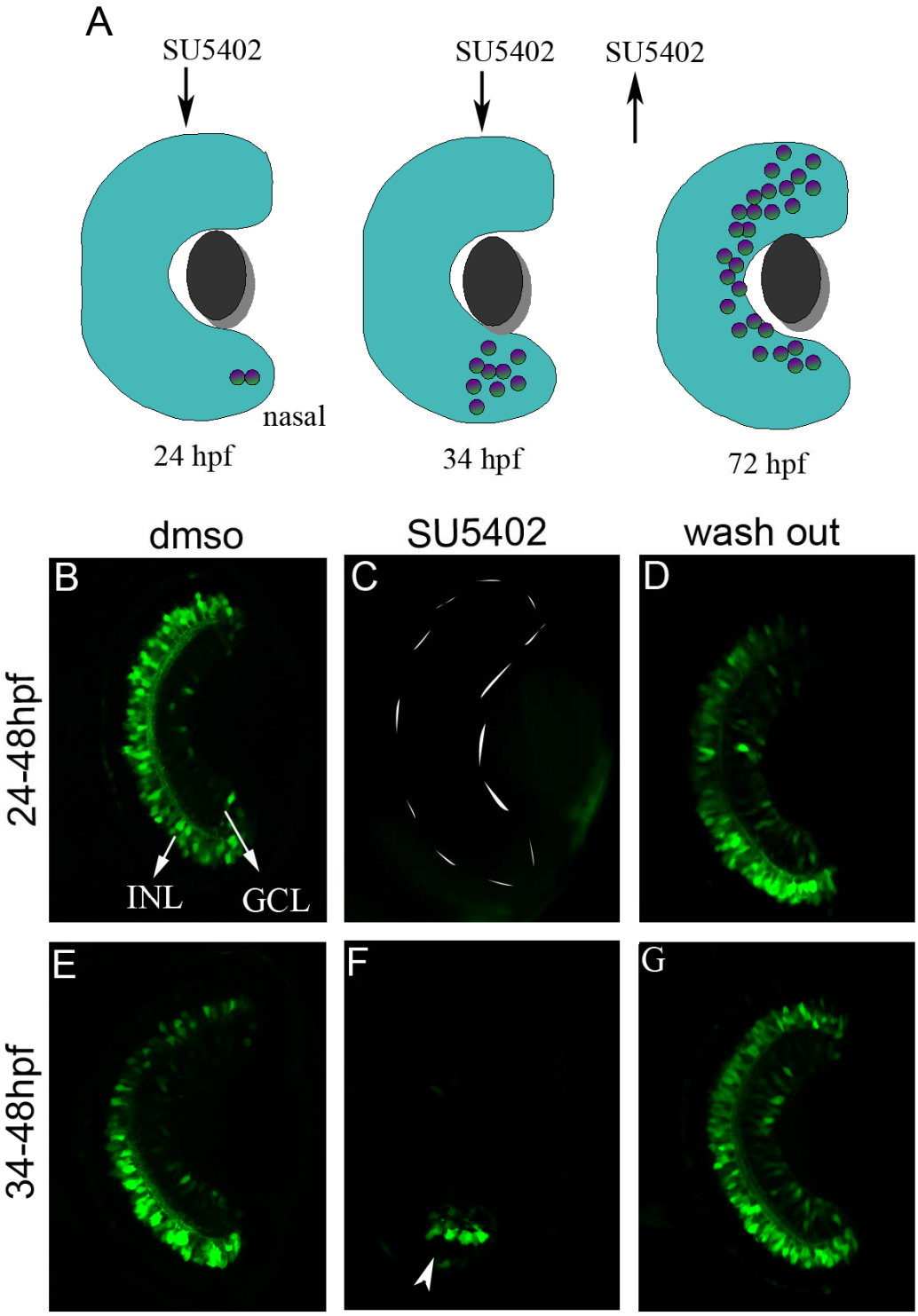


Figure 34: The SU5402 inhibition assay shows that Fgfs are required for initiation and propagation of the *shh:gfp* wave. An outline of the treatment is shown in scheme A. Treatment of embryos with DMSO at any time frame does not show any alteration in *shh:gfp* transgene expression (B, E). Embryos treated with SU5402 from 24-48 hpf show a complete loss of GFP expression in the retina (C) while those treated from 34-48 hpf shows few GFP (arrowhead) cells at the initiation point (F). Expression is recovered when the embryos are removed from SU5402 and after several washes in fish water allowed to grow until 72 hpf. Confocal pictures at 48 hpf (B, C, E, F) and 72 hpf (D, G) with anterior to the top in all images.

Embryos exposed to SU5402 from 24-48 hpf blocked the initiation of *shh:gfp* and its subsequent spread in the retina (Fig. 34C, n=58). On the other hand, embryos exposed to SU5402 from 34 hpf allowed *shh:gfp* expression to be initiated at the ventronasal position but the propagation of the wave was completely blocked in these embryos (Fig. 34F, n=35). After 48 hpf, both the experimental batch of embryos were removed from the inhibitor, followed by several washes in fish medium and allowed to grow in the same until 72 hpf. Expression analysis of these embryos at 72 hpf showed a complete recovery of *shh:gfp* expression in the GCL and INL of the retina (Fig. 34D, G). Control embryos treated with DMSO showed normal *shh* expression pattern (Fig. 34B, E, n=38). Thus this experiment demonstrates that Fgf signalling is required for both the initiation and propagation of the *shh:gfp* wave in the zebrafish retina.

14. Several Fgfs participate in the regulation of *shh:gfp* expression in the retina

Fgfs are known to be expressed in the zebrafish eye. Fgf3 and Fgf8 are expressed in the optic stalk (Herzog et al., 2004; Reifers et al., 2000; Tsang et al., 2002) and are important for initiation of retinal neurogenesis (Martinez-Morales et al., 2005). To determine which of these Fgfs influence the *shh:gfp* expression in the retina, a knock-down approach using morpholinos (MO) were performed. Fgf3 and Fgf8 morpholinos designed against the translation initiation site of the gene were injected at a concentration of 0.5 -2.0 µg/µl into *-2.4shh:gfpRetE* transgenic embryos at the one cell stage and expression in the retina was monitored at 48 hpf.

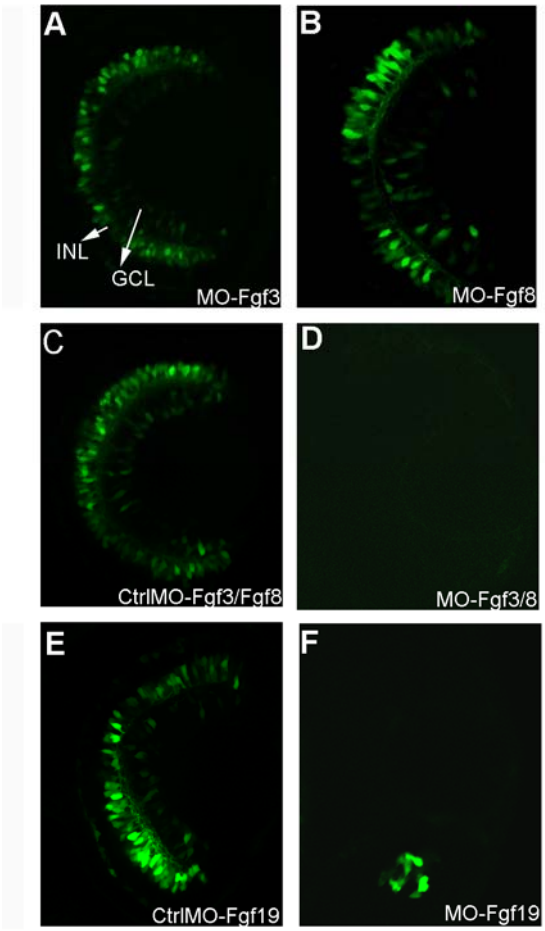


Figure 35: Several Fgfs are required for *shh* transgene expression in the retina. Embryos injected with either Fgf3 or Fgf8 MO show no effect on the transgene expression in the retina (A, B) while those injected with a mixture of Fgf8MO and Fgf3MO block the transgene expression completely (D). Control embryos injected with mismatch Fgf3 and mismatch Fgf8 MOs have a normal GFP expression pattern (C). Embryos injected with Fgf19MO show only initiation at the ventral nasal patch and subsequent spread is blocked (F). Control embryos injected with mismatch Fgf19 MO had normal GFP expression pattern (E). Confocal pictures at 48 hpf with anterior to the top in all images.

The injection of Fgf3 (0.8 µg/µl) and Fgf8 MOs (1.6 µg/µl) alone led to no loss of *shh:gfp* expression in the retina (Fig. 35A, n=31, Fig. 35B, n=39). However a mixture of both Fgf3 and Fgf8 MOs showed a complete loss of GFP expression in transgenic embryos (Fig. 35D, n=41). Those injected with a mixture of mismatch MOs of Fgf3 and Fgf8 show normal transgene expression pattern (Fig. 35C, n=45). This suggests that both signalling molecules are required for initiation and maintainance of *shh:gfp* expression in the transgene. Another Fgf molecule, Fgf19 is expressed in the neural retina (Miyake et al., 2005), to check if it could also be a candidate regulating *shh:gfp* expression, a similar knock down approach was performed. Fgf19 MO (1 µg/µl) was injected and the downregulation gave a significant result with only initiation of *shh:gfp* expression at the

ventronasal patch noticed in more than 50% of the injected embryos (Fig. 35F, n=58) when compared to those injected with mismatch Fgf19 MO (Fig. 35E, n=39). The subsequent spread of expression was blocked in the embryos thus demonstrating that Fgf19 is a key molecule required for the propagation and not for the initiation of the *shh:gfp* wave in the zebrafish retina. Fgf1 in chick have been reported to be required for progression of neurogenesis (McCabe et al., 1999), but Fgf1 MO had no effect on the transgene expression pattern (data not shown).

Thus inhibitor and morpholino studies clearly indicate that multiple Fgfs play a crucial role in establishment and propagation of the *shh:gfp* wave in the zebrafish eye.

15. *Shh* acts in an auto-regulatory manner to drive expression in the retina

Previously it has been suggested that *Shh* is necessary for the propagation of the *shh* expression wave through the retina of the zebrafish embryo (Neumann and Nuesslein-Volhard, 2000). The results from the earlier section of the thesis suggest that Fgfs play crucial roles in the initiation and propagation of *RetE* driven expression in the retina. To re-examine the role of Hh signalling in this process, inhibitor and mutant studies were performed. Cyclopamine is an inhibitor of *shh* signalling that acts at the level of the smoothened receptor and has been shown to block *shh* expression in the zebrafish retina (Neumann and Nuesslein-Volhard, 2000). Transgenic embryos *-2.4shh:gfpABC#28* were treated with 100 µM cyclopamine from 24 hpf to 48 hpf. When analysed for expression in the retina, however all the treated embryos showed normal GFP expression (discussed later).

Recent studies (Masai et al., 2005) have also reported that cyclopamine is inefficient to block *shh:gfp* transgene expression in the retina and have utilised another pharmacological compound Forskolin. This compound acts efficiently in blocking *shh* expression. Forskolin (Sigma) an activator of adenylcyclase activates PKA and thus negatively blocks *shh* signalling. Transgenic embryos (*-2.4shh:gfpABC#28*) were treated with Forskolin (0.3 µM) from 24-48 hpf. Forskolin treatment produced the characteristic phenotype of U-shaped somites and bending of the body axis (Barresi et al., 2000)(Fig. 36B) and transgene expression in the retina at 48 hpf was restricted only to the initiation site at the ventro-nasal patch (Fig. 36D, n=24) and propagation of the wave was completely blocked in these embryos. Control embryos in DMSO show normal

expression with GFP in both GCL and INL of the retina (Fig. 36C, n=18).

Figure 36: Inhibition of *shh* signalling using Forskolin from 24 hpf to 48 hpf. Treated embryos at 48 hpf exhibit U-shaped somites and bend axis (B) and in the retina, transgene expression is limited to the initiation site at the ventronasal position with propagation blocked (D). Control embryos in DMSO show normal phenotype and GFP expression in the retina (A, C).

To further confirm the role of *shh* signalling, two of the *shh* signalling mutants were analysed for retina expression. *Slow-muscle omitted (smu)* has a mutation in the *shh* signal transduction receptor smoothened (Varga et al., 2001) and *sonic-you (syu)* has a deletion of the *shh* gene (Schauerte et al., 1998). The *smu*^{b577} and *syu*^{t4} fish were crossed into *-2.4shh:gfpABC#15* transgenic line, allowed to grow and the F1 progeny screened to identify ABC15*Smu*$^{-/-}$ and ABC15*Syu*$^{-/-}$ fish respectively. The expression of *shh:gfp* transgene in these mutants was compared with that of *-2.4shh:gfpABC#15* (Fig. 37A). The ABC15 *Smu*$^{-/-}$ showed no GFP expression in the retina (Fig. 37B) and this is consistent with earlier published results where it is reported that *Smu*$^{-/-}$ embryos do not possess a normal optic stalk (Varga et al., 2001). The ABC15 Syu$^{-/-}$ showed very weak GFP expression at the ventronasal position (Fig. 37C) in only 25% of the homozygous mutants while the other 75% had no GFP expression in the retina. This could potentially be due to the penetrance of the mutation in these embryos.

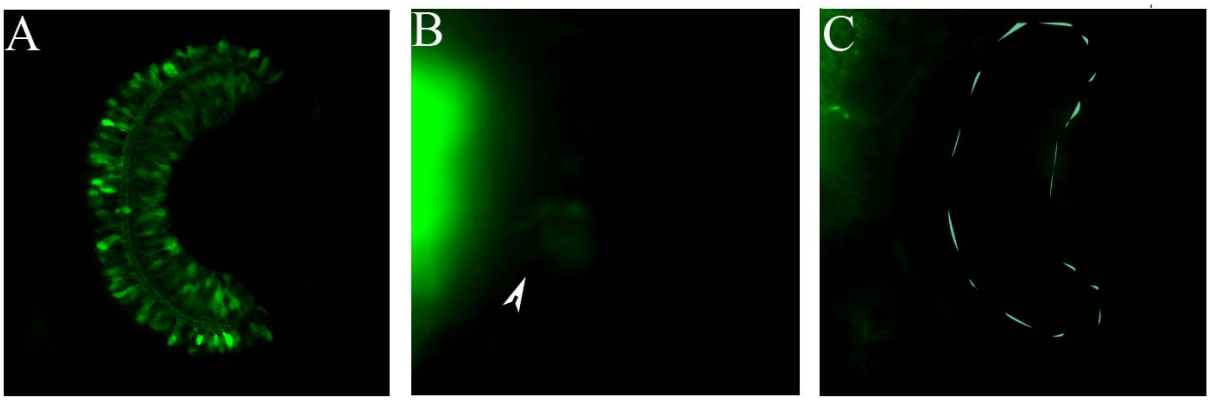


Figure 37: Role of *shh* signalling in retinal neurogenesis. *shh* signalling mutants *sonic-you* show a very faint expression at the initiation site (arrowhead) (B) when compared to wildtypeABC (A) while *slow muscle omitted* show complete loss of retina expression (C). Confocal images at 72 hpf with anterior to the top in all images.

Taken together forskolin and mutant studies support the auto-regulatory function of *shh* during its spread in the zebrafish retina.

I have identified a cis-regulatory enhancer *RetE* regulating *shh:gfp* expression in the zebrafish retina. This enhancer is located in the first intron of the *shh* locus and mediates expression in the GCL and INL of the retina. Deletion and mutation studies identified a 40 bp region of the *RetE* important for retina expression. The 40 bp region by itself is insufficient and requires a neighbouring 85 bp sequence to confer retina expresssion. Search for transcription factors in this region identified three binding sites for Erm and Pea3 factors of the ETS family. Biochemical and morpholino studies suggest that both Erm and Pea3 regulate *shh* transgene expression in the eye. Erm and Pea3 are downstream of Fgf signalling and it was reported that Fgfs secreted from the optic stalk are responsible for the initiation of neurogenesis. Inhibitor and morpholino assays performed in this study indicate a co-operative role between several Fgfs for the initiation and propagation of the *shh:gfp* wave. Also s*hh* was shown to be required for the spread of the wave in an auto-regulatory manner. The pharmacological inhibition and mutant studies carried out suggest that several distinct Fgfs and *shh* act sequentially and that the propagation of the *shh:gfp* wave depends on a co-operation between the Fgf and *shh* signalling pathways.

Discussion

1. Cis-regulatory enhancers mediate *shh:gfp* expression in the retina

In the zebrafish retina, *shh* spreads from the initiation site at the ventronasal patch in a wave-like manner (Neumann and Nuesslein-Volhard, 2000). *Shh* is expressed in the GCL and amacrine cells of the INL in the retina (Shkumatava et al., 2004). A novel cis-regulatory enhancer (*RetE*) located in the first intron of the *shh* gene responsible for driving its expression in the retina was identified. Systematic deletion analysis of the enhancer led to the identification of a 300 bp region that is both sufficient and necessary for driving *shh* expression. By a second series of mutations across this region using a linker scanning approach, a minimal 40 bp enhancer region required for *shh:gfp* expression in specific layers was identified. Sequence alignment among several species showed considerable homology across the 40 bp core region but is not sufficient to for the enhancer function in the retina. The core 40 bp region requires an additional 85 bp of 5' sequences for enhancer activity. This observation is similar to recent reports

(Poulin et al., 2005) for the Dc2 enhancer (424 bp) of the dachshund gene in mice driving expression in the hindbrain, forebrain and retina. *In vivo* analysis showed that a 100% conserved 144 bp minimal region of the enhancer is necessary but insufficient to recapitulate the expression.

The additional 85 bp sequence included in the construct is conserved with 39 out of 85 nucleotides identical between zebrafish and human. However, no candidate transcription factor sites are apparent. In a similar investigation, Hutcheson et al have shown that the bHLH transcription factor *ath5* is regulated by an enhancer harbouring a pair of highly conserved E-boxes in the Xenopus retina (Hutcheson et al., 2005). These highly conserved E-boxes are necessary but insufficient for transgene expression and require an additional 48 bp to confer tissue specific expression (Hutcheson et al., 2005). But why should gene regulatory sequences require hundreds of base pair long sequences when most transcription factors (TFs) are capable of recognising short 6-12 bp degenerate sites? It may be due to clustering of multiple transcription factor binding sites since enhancers are modular. TFs have a combinatorial role that leads to the tissue specificity of the enhancer. Thus, it is intriguing to speculate that multiple factors interact with *RetE* and participate in *shh* gene regulation.

RetE requires a minimal 40 bp core and additional 5' 85bp for retina expression. Clearly not all of this sequence is conserved. TF-binding modules are rather flexible in their orientation. It may be that they got rearranged so they would not be detected by pairwise comparisons. Why are some stretches such as that including the Erm and Pea3 binding sites strongly conserved and the others not? Perhaps strict spatial requirements for protein protein interaction exist between neighbouring TFs in these cases, maintaining a particular sequence through evolution.

2. Pea3 and Erm regulate *RetE* activity in the retina

Mutational and protein binding assays in this study have identified Pea3/Erm binding sites in the *RetE* enhancer, necessary for its activity in the retina. Erm and Pea3 activate *RetE* activity in the retina through the binding sites located in both the M12+M13 region of the *RetE* enhancer.

Erm, Er81 and Pea3 are members of the PEA3 subfamily of ETS transcription factors (Chotteau-Lelievre et al., 2001; Chotteau-Lelievre et al., 2003). They are co-expressed in

several tissues and organs and are generally described as transactivators (de Launoit et al., 1997; de Launoit et al., 2000). PEA3 subfamily members have been shown to be involved in a number of processes including neuronal path finding (Arber et al., 2000; Livet et al., 2002) and to play an important role in HER2/Neu- mediated mammary oncogenesis (Shepherd et al., 2001). Their role and function are not precisely known, but deregulation of their expression is often associated with carcinogenesis (Oikawa, 2004; Oikawa and Yamada, 2003). Erm and Pea3 are important recipients of Fgf signalling (Firnberg and Neubuser, 2002; Munchberg et al., 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001) but Er81 is not dependent on Fgf signalling (Roussigne and Blader, 2006). Fgf signalling activates the ERK/MAP kinase pathway and leads to the upregulation of the expression of PEA3 subfamily members. Erm and Pea3 both possess ETS domain that are 95% conserved and share a similar consensus sequence (Brown et al., 1998). Their combined activity could regulate the enhancer activity.

In this study, several lines of evidence favour the notion that both Erm and Pea3 are required for regulating *RetE* activity in the retina. First the expression of Erm and Pea3 precedes that of *shh* in the zebrafish retina (Neumann and Nuesslein-Volhard, 2000). Erm is expressed ubiquitously while Pea3 is expressed in the GCL and in the central region of the retina at 34 hpf. In contrast, Pea3 was reported to be exclusively expressed in the GCL of the chick retina (McCabe et al., 2006). Second, knockdown using Erm and Pea3 completely abolished the *RetE* activity in the retina. Third, *in-vitro* binding assays demonstrate that both Erm and Pea3 could bind to the motifs in the *RetE* sequence. Taken together all these suggests that the retinal enhancer requires Erm and Pea3 in controlling *RetE* mediated *shh:gfp* expression in the retina.

Point mutations in the M13 binding site for Erm and Pea3 lead specifically to the loss of *shh:gfp* expression in the GCL. Thus, the Erm and Pea3 site in the M13 sequence is specifically required for GCL expression. This is in agreement with the different mechanism of regulation in the GCL and INL suggested by the analysis of the *lakritz* mutant. *lakritz* mutants (lak) carry mutations in the *ath5* gene and lack RGCs (Kay et al., 2001). It was also shown that in *lak* mutants, the S*hh* wave in the INL is independent on the *shh* wave in the GCL (Shkumatava et al., 2004). It is tempting to speculate that Erm and Pea3 are not sufficient to drive expression in GCL but require GCL specific cofactors to cooperate with. Mutation of the Erm and Pea3 binding site in the M13

region would abolish this interaction with this unknown GCL specific cofactor, thereby leading to loss of *shh:gfp* expression specifically in the GCL.

In contrast, the interaction of Erm and Pea3 with the weaker less conserved M12 binding motifs are required for expression in both layers of the retina. Mutation in the M12 region led to the complete loss of *shh:gfp* expression in both GCL and INL while that in M13 retained only expression in the INL. Thus *shh:gfp* expression in the GCL and INL can be seperated. The mutational data indirectly provide evidence that in addition to Erm and Pea3 several other cofactors have to interact with the retinal enhancer to render it functional in the retina. No candidate transcription factor binding sites were identified in the M12 region. The complexity of this enhancer is further underscored by the fact that the 40 bp marked by the M12 and M13 core is not sufficient to drive expression and requires in addition 85 bp upstream of this core region.

Comparison of the expression profile of zebrafish Pea3 with known embryonic genes, suggests potential partner proteins and/or target genes for PEA3. For example, pax-2.1 appears to be coexpressed with PEA3 in neural cells and at the mid-hindbrain border (Krauss et al., 1991). A direct interaction between Pax and ETS-domain proteins has previously been demonstrated on the murine MB-1 promoter (Fitzsimmons et al., 1996). Thus, Pax 2 or Pax6 are candidates that could interact with Erm and Pea3 as both factors are key players for retina patterning (Ekker et al., 1995; Macdonald et al., 1995; Take-uchi et al., 2003). However, motif search identified no binding sites for these factors in the *RetE* sequence and therefore a role for them is unlikely in regulating the retinal enhancer.

Very few PEA3 group member target genes have been currently reported. Most of them encode proteinases required for extracellular matrix degradation, such as MMP-1, MMP-9 and MMP-3, MT1-MMP and MMP-7 (Bosc et al., 2001; Crawford et al., 2001; Habelhah et al., 1999; Lynch et al., 2004) or adhesion molecules such as Icam-1 (de Launoit Y, 1998), Osteopontin (El-Tanani et al., 2004) or Cyclooxgenase-2 (Funaoka et al., 1997). Neu and glutathione peroxidase (Drevet et al., 1998; Xing et al., 2000) are also targets of the PEA3 group members. AP-1 is a transcription factor consisting of Jun/Fos family proteins interacting with adjacent Ets domain factors in a large number of promoter/enhancer elements (Wasylyk et al., 1993; Westermarck and Kahari, 1999). Two novel interacting partners for Pea3 and Erm were identified using a yeast one-hybrid screen (Greenall et al., 2001; Guo et al., 2006). USF1 interacts with the Pea3 via its

HLH domain and enhances binding of Ets proteins (Greenall et al., 2001); the other factor LPP is a coregulatory protein that affects PEA3 function (Guo et al., 2006). However, except for AP1, there is no evidence for these factors being expressed in the zebrafish retina, and binding motifs for others were not identified in the *RetE* sequence. Mutation of a weak binding motif for AP1 did not lead to loss of the expression in the retina.

PEA3 interacting factors could be identified by yeast one-hybrid or two hybrid screens. One could potentially also use CHIP or mass spectrometry techniques but there is a limitation that they require a relatively large amount of retinal cells. Another possibility is to identify TFs from the large transcription factor screen going on in our lab. Candidates expressed in the retina at the right time could be selected and later examined in detail for possible interactions.

3. Forskolin is more efficient than cyclopamine in blocking *shh:gfp* wave

Shh plays an important role in specification of the optic stalk (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003). *Shh* has been implicated in the propagation of retinal differentiation while initiation of this process is independent of Hh signalling in vertebrates (Neumann, 2001; Neumann and Nuesslein-Volhard, 2000). In zebrafish, *shh* is initiated at the ventral nasal position and then spreads subsequently in a wave like manner (Neumann and Nuesslein-Volhard, 2000). When Hh signalling was inhibited at 26 hpf using the teratogenic compound cyclopamine, RGC differentiation is still initiated close to the optic stalk but further differentiation is blocked (Neumann and Nuesslein-Volhard, 2000). In contrast, Kay and his colleagues have reported that cyclopamine was inefficient in blocking shh expression when treated from 24 hpf (Kay et al., 2005). In order to understand the role of *shh*, cyclopamine treatment was also performed on *-2.4shh:gfpABC15* and *-2.4shh:gfpRetE* transgenic embryos from 24 hpf to 48 hpf. When analysed for GFP expression, all embryos displayed normal GFP expression in both the GCL and INL of the retina. I used Forskolin as it was reported by Masai et al (2005) that Forskolin treatment blocks neurogenesis more effectively than cyclopamine. Forskolin increases the activity of PKA thereby negatively regulating Hh. Embryos *(-2.4shh:gfpRetE)* treated with Forskolin from 24 hpf showed only initiation of *shh:gfp* and a block in the spread of the wave. This result is in agreement with earlier

reports suggesting an autoregulatory function for *shh* in the retina.

Recently, it was reported that cyclopamine has a relatively low affinity for Smo (Romer et al., 2004; Williams et al., 2003) and furthermore, cyclopamine is a hydrophobic chemical and white precipitates form immediately after diluting and applying into embryo medium and this could also explain the incomplete inhibition of Hh signalling in the eye. Cyclopamine purchased from two different companies (BIOMOL and TRC) were ineffective in blocking Hh activity and these may not be as potent as the earlier source of cyclopamine (W. Gaffield) that are no longer available (Neumann et al., 1999).

Masai et al (2005) have reported that the level of PKA activity is much higher in forskolin-treated embryos using an antibody against phosphorylated CREB. Although it still remains to be elucidated how exactly PKA inhibits the Hh signalling pathway in vertebrates, it is generally accepted that PKA negatively regulates Hh-dependent Gli activation by promoting the cytoplasmic sequestration of Gli1 and generating the repressor forms of Gli2 and Gli3 (Ingham and McMahon, 2001). It is possible that the ratio of the repressor form to the activator form of Gli proteins is higher following forskolin treatment than it is after cyclopamine treatment. High predominance of repressor activity of Gli proteins over activator activity may be necessary to inhibit progression of neuronal production. Progression of neurogenesis is more severely affected in Gli-MO-injected retinas (Masai et al., 2005). Another possibility is that the activation of CREB contributes to the forskolin-induced defects in neurogenesis. Because phosphorylation of CREB by PKA activates the transcription of cyclin D1 (Lonze and Ginty, 2002), CREB-mediated activation of cyclin D1 expression may inhibit the cell-cycle exit of retinoblasts in concert with the blockade of Hh-mediated Gli activation.

4. Fgfs cooperate for the progression of the *shh:gfp* wave in the retina

My results show that several Fgfs are required for the initiation and propagation of the *shh:gfp* wave in the retina. The impact of Fgf signalling on *shh* transgenes has not been reported in detail. The data from my work indicate that Fgf8 and Fgf3 are required for initiation and propagation of *shh* transgenes expression in the retina. This is consistent with the data shown earlier that Fgf signalling emanating from the optic stalk in

zebrafish to be sufficient and necessary to initiate retinal differentiation (Martinez-Morales et al., 2005). The onset and spreading of differentiation seems, however, to have species-specific spatial organization. In zebrafish, the first RGCs are generated in the ventral retina close to the optic stalk and differentiation spreads in the naso-dorsal direction, unfolding in a fan like manner (Hu and Easter, 1999) whereas in chick initiation is seen in central retina which then spreads peripherally (Martinez-Morales et al., 2005) .

My data also indicate a role for Fgf19 in the propagation of the *shh:gfp* wave in cooperation with that of Fgf3 and Fgf8 signalling. The cooperative activity of Fgf family members with overlapping expression domains seems to be a common theme for patterning in different regions of the nervous system. The sequential action of Fgf8 and Fgf17 during the development of the midbrain-hindbrain organiser illustrates this concept (Reifers et al., 2000). Fgf8/17/18 functions together with Fgf9/16/20 during notochord formation in Ciona embryos (Yasuo and Hudson, 2007). Using morpholino knockdown they have shown that Fgf signalling acts in two phases of notochord formation. The early induction step involves a choice between notochord and neural fates and this is mediated by Fgf9/16/20 alone while the second phase is required for maintainance of the notochord fate and this is mediated by the combined effect of Fgf9/16/20 and Fgf8/17/18 (Yasuo and Hudson, 2007).

A combinatorial activity of Fgf24 and Fgf8 is responsible for much of the Fgf signalling controlling posterior mesoderm development in zebrafish (Draper et al., 2003). A morpholino knockdown approach had indicated that Fgf8 and Fgf3 cooperate in the patterning and neurogenesis of the hindbrain (Maves et al., 2002; Walshe et al., 2002; Walshe and Mason, 2003), otic placode (Leger and Brand, 2002; Maroon et al., 2002); forebrain and eye patterning (Picker and Brand, 2005).

Morpholino knockdown and inhibitor assays performed in this work support the notion that several Fgfs act together in promoting transgene expression in the retina. The results presented here show that the knockdown with a cocktail of Fgf3 and Fgf8 MOs show a complete loss of *shh:gfp* expression in the retina. Zebrafish Fgf3 mutant (*lia*) and Fgf8 mutant (*ace*) have a normal optic stalk and retinal neurogenesis is normal in these embryos (Herzog et al., 2004; Shanmugalingam et al., 2000) as reported by the expression pattern of the early RGC marker *ath5*. Ath5 expression is similar in both wildtype and mutant embryos until 32 hpf thereafter showing a slight decrease in the

mutants (Martinez-Morales et al., 2005). Furthermore, no *ath5* expression is detected in the retina of the zebrafish Fgf3 and Fgf8 double mutants which have an otherwise normal optic stalk (Martinez-Morales et al., 2005). Fgf3/Fgf8 morphants have no *shh:gfp* expression in the retina and this would support a reduntant role of Fgf8 and Fgf3 for the initiation of the transgene expression. There could exist a cooperative role with Fgf3 and Fgf8 having an early function during initiation and Fgf19 required later for propagation of the *shh:gfp* wave in the zebrafish eye.

5. *Shh* and Fgf act in concert for propagation of the *shh:gfp* wave in the retina

In zebrafish, postmitotic cells are initially generated in the ventronasal retina adjacent to the optic stalk, and neuronal differentiation progresses to the entire neural retina (Hu and Easter, 1999). The progression of differentiation of retinal ganglion cells (RGCs) in the zebrafish retina requires the signalling molecule Hedgehog (Hh) (Neumann and Nuesslein-Volhard, 2000). The results from this study suggest an autoregulatory function for *shh* in the zebrafish retina. Initial studies in the zebrafish retina reported that initiation is independent of *shh* signalling (Neumann and Nuesslein-Volhard, 2000). This in agreement with the data presented in this work, where transgenic embryos (-*2.4shh:gfpABC15*) treated with an inhibitor of Hh signalling (Forskolin) show only 1-2 dots of GFP expression at the ventral nasal position with a block in the spread. -*2.4shh:gfpABC15Syu* mutant embryos display a similar pattern with only transgene initiation in the retina. Mutant and inhibitor studies clearly show a requirement for *shh* during its propagation arguing for an interactive function between *shh* and Fgf in the zebrafish retina.

Hhs and Fgfs are closely expressed in many tissues of the developing embryo, including the telencephalon, optic vesicles and retina (Crossley et al., 2001). It has also been shown, specifically in the frontonasal process, that local retinoid signalling maintains local *shh* and Fgf8 expression, thereby coordinating forebrain and facial morphogenesis. When retinoid signalling is transiently disrupted, forebrain tissue is absent and the eyes are fused (Schneider et al., 2001). Retinoic acid also controls expression of *shh* and Fgf8 in the limb bud (Helms et al., 1996; Stratford et al., 1999).

A few examples of Hh and Fgf interactions are known, in the eye. In the Medaka eye and mid-hindbrain boundary, injected *shh* induces *spalt* gene expression in the proximal

optic vesicle, and this requires Fgf signalling, because dominant negative XFD co-injections block *spalt* induction (Carl and Wittbrodt, 1999). It is thought that Fgf may specify a competence domain and Hh specifies the dorsoventral extent of *spalt* expression. In a similar manner, co-expression of Fgf8 and *shh* in the vertebrate mid-hindbrain boundary and rostral forebrain induces dopaminergic neurons. When Fgf4 is also present, hindbrain 5HT neurons are induced (Ye et al., 1998). On the other hand, in the vertebrate limb, Fgf8 is required for the induction and maintenance of *shh* expression, via protein kinase C, which then leads to the upregulation of Fgf4 (Johnson and Tabin, 1997; Lu et al., 2001).

Fgf and Hh signalling have an intimate relationship and a positive feedback loop exists in forebrain patterning in zebrafish. In particular, they are implicated in ventral telencephalon and diencephalon patterning where *shh* expression depends on signalling by Fgf3 and Fgf8 in the hypothalamus, ventral thalamus and zona limitans intrathalamica (Walshe and Mason, 2003). Moreover, the expression of Fgf3 and Fgf8 is at least in part under the control of Hh signalling in the forebrain (Miyake et al., 2005). Recently, the zebrafish Fgf19 has been identified to function downstream of the Hh pathway in the forebrain patterning similarly to Fgf3 and Fgf8 (Miyake et al., 2005). It could be that both *shh* and Fgf19 act in parallel during the propagation of the *shh:gfp* wave in the retina. The *shh* signalling mutant *smoothened* (Varga et al., 2001) shows complete loss of *shh* transgene expression suggesting that Fgf3 and Fgf8 require midline *shh* for initiation of retinal neurogenesis. *Smu* mutants lack the optic stalk (Varga et al., 2001). On the other hand inhibitor and morpholino studies show that *shh:gfp* expression is dependent on Fgf3, Fgf8 and Fgf19 for its initiation and propagation in the zebrafish retina. Thus an interplay between Fgf and *shh* signalling exists during the *shh:gfp* wave in the retina (Fig. 37).

6. The mechanism controlling *Shh* expression in the zebrafish retina

Shh plays an important role as a morphogen in vertebrate eye development (Esteve and Bovolenta, 2006; Russell, 2003). *Shh* secreted from the midline is required for patterning of the retina (Ekker et al., 1995; Take-uchi et al., 2003; Varga et al., 2001). Blocking *shh* with cyclopamine from 26 hpf leads to initiation of *shh* transgene expression while the propagation of the wave is affected (Neumann and Nuesslein-Volhard, 2000),

indicating that *shh* is required for its own regulation in the zebrafish eye. However, recent studies argue against this role and have reported that only blocking *shh* early in zebrafish embryos around 13 hpf has an effect on retinal neurogenesis (Kay et al., 2005).

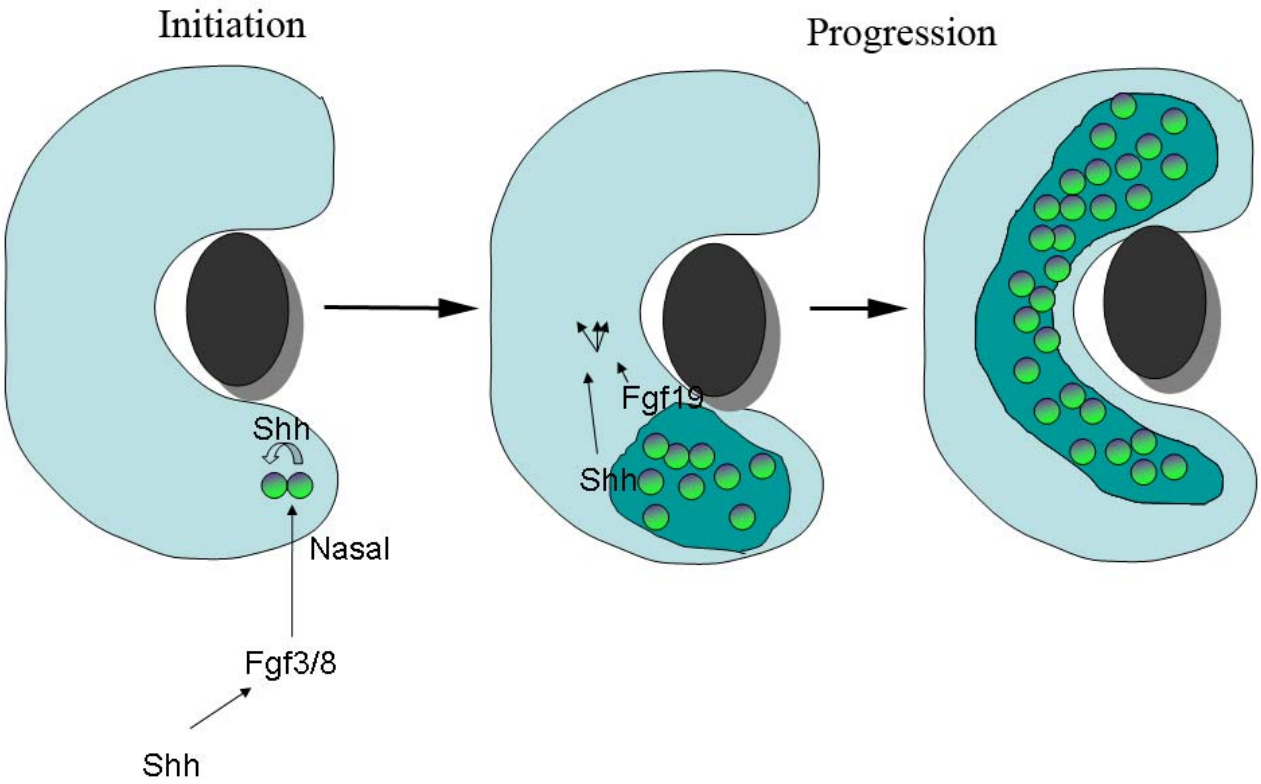


Figure 38: A model for shh wave in the zebrafish retina. There exists a weak gradient of *shh* in the retina during the early stages of zebrafish development. Midline shh is required for Fgf3 and Fgf8 to initiate neurogenesis in the retina. The newly formed RGCs secrete Hh which then acts parallely with Fgf19 and the wave spreads over the already established gradient.

Masai et al (2005) have documented a short range signalling activity of *shh* that is required for propagation of neurogenesis. Pharmacological inhibition and mutant studies from my thesis suggest an auto-regulatory function for *shh* during its expression in the eye. These results and the observations from others led to postulate the following model; early midline *Shh* signalling could create a gradient of *shh* in the retina. This midline source of *shh* is also required to induce Fgfs in the optic stalk to initiate neurogenesis in the retina. The Fgfs act as a gateway and once neurogenesis has been initiated the newly born RGCs secrete Hh creating a higher concentrated source of Hh similar to the observations seen in Drosophila and chick (Dominguez and Hafen, 1997; Zhang and Yang, 2001b). This Hh then acts in parallel with Fgf19 present in the neural retina to propagate the *shh:gfp* wave on the already established gradient leading to expression in specific layers of the retina.

Materials and methods

Fish stocks

Zebrafish (Danio rerio) were maintained at 28° C, referring to The Zebrafish Book (Westerfield, 1993). The developmental stages of the embryos were determined by the hours post fertilization (hpf) and by morphological features, as described by Kimmel et al (1995). Embryos analysed for retina were grown in fish water containing 0.003% Phenylthiourea (PTU) to prevent pigmentation.

Whole mount *in-situ* hybridisation

Dixoxigenin whole mount *in-situ* hybridisation was carried out as described. Zebrafish embryos with the chorion at the desired stage were fixed in BT fix (4% paraformaldeyde, 4% sucrose, 0.12mM $CaCl_2$, 0.1M NaPi pH 7.4) at 4°C overnight. After fixation, embryos were rinsed with PBS, 2x5 min and dechorinated with forceps. For storage, embryos were dehydrated stepwise through a series of methanol-PBS from 30%-70% and kept at -20°C. For further use, embryos were rehydrated through the methanol series and then washed 4x5 mins in PTW (1xPBS, 0.1% Tween 20). Embryos were treated with Proteinase K (10 µg/ml) in PTW for 2-6 mins depending on the stage of the embryos, followed by two washes in PTW, refixation in BT fix for 20 mins and washes in PTW 2x5 mins. After this treatment, embryos were transferred to hybridisation buffer (HYB: 50% Formamide, 5x SSC, 0.5 mg/ml yeast RNA, 50 µg/ml heparin, 0.1 % Tween 20.9 mM citric acid). Pre-hybridisation for 3-4 hrs at 65°C, then the buffer was replaced by fresh HYB containing 1/400 dilution of the dixoxigenin labelled antisense RNA probe and embryos were incubated overnight at 65°C. For embryos older than 48 hpf there are some additional steps before proteinase K treatment as follows; after rehydration and PTW washes the embryos are washed in H_2O for 1x5 min and then incubated at -20°C for 7 mins in acetone followed by a quick wash in H_2O for 5 mins which causes the embryos to swell. These additional steps are aimed at providing better penetration of the probe into the older embryos.

Embryos were washed serially 2x30 min in 50% formamide/50% 2xSSC, 0.1% Tween

20; 1x15 min in 2x SSC, 0.1% Tween 20 ; 2x30 min in 0.2x SSC, 0.1% Tween 20 and 1x 5 min blocking buffer(1x PBS, 0.1% Tween 20, 5% sheep serum, 0.2% BSA, 1% DMSO). The embryos were kept in blocking buffer at room temperature for 2 hours and then incubated in 1/4000 dilution of the anti- dixoxigenin alkaline phosphatase Fab fragments. The antibody incubation was done overnight at 4°C.

Embryos are then washed in PTW 6x20 mins followed by 2x5 mins rinsing in staining buffer (100 mM Tris-HCl pH 7.9, 100mM NaCl, 0.1% Tween 20, 50mM $MgCl_2$). The bound antibody was revealed by adding the substrates, NBT and BCIP (0.34 mg/ml and 0.175 mg/ml). Reaction was stopped by repeated rinses in PTW. The antisense probes of Erm and Pea3 were *in-vitro* transcribed from the pCSII plasmids (Munchberg et al., 1999) which were linearised with Not1 and transcribed with T7 polymerase along with DIG labelling mix.

Plasmid Constructions

All cloning was done following standard procedures (Sambrook, 2001). The *−2.4shh:gfp* plasmid was constructed by inserting the *−2.4shh* promoter (Chang et al., 1997; Muller et al., 1999) as a Sal1, Xho1 fragment in the pCS2:gfp vector (Blader and Strähle, unpublished data). Plasmids *−2.4shh:gfpA*, *−2.4shh:gfpB* and *−2.4shh:gfpC* were constructed by inserting PCR-amplified NotI/KpnI fragments corresponding to positions +549 to +2381, +2382 to +3592, +3593 to +5366, respectively. Amplification primers contained in addition Spe1 and Sfi1 restriction sites. *−2.4 shh:gfpAC* and *−2.4shh:gfpAB* plasmids were created by inserting ar-A as NotI/SpeI PCR fragment into *−2.4shh:gfpC* and *−2.4shh:gfpB*. The *−2.4shh:gfpABC* plasmid harbours the ar-C fragment in the Sfi1/Kpn1 sites of *−2.4shh:gfpAB* (Ertzer et al., 2007). During the course of the work the SceI meganuclease protocol became available (Thermes et al., 2002). The efficiency of obtaining stable transgenics is higher with this approach. Thus the I-Sce1 *−2.4shh:gfpABC* plasmid was constructed by inserting double-stranded oligonucleotides containing an I-Sce1 restriction site into the Sal1/Kpn1 restriction sites of *−2.4shh:gfpABC*. Plasmid *-2.4shh:gfp RetE*, were constructed by inserting PCR-amplified NotI/SpeI fragments corresponding to positions +549 to +2021 of *shh* gene into the *-2,4shh:gfp* Sce I plasmid (Ertzer et al., 2007). The *-37tk:gfpRetE* were generated by

inserting digested NotI/ KpnI fragments of RetE from *-2.4shh:gfp RetE* and then ligating into the NotI/ KpnI sites of the *-37tk:gfp* (Rastegar et al., 2002) plasmid.

Zebrafish *shh*, Mouse *Shh* and Human *SHH* corresponding to +729 to +1035 (of the zebrafish *shh* loci) were amplified by PCR from plasmid or genomic DNA and inserted into the Not1/ Spe 1 sites of the *-2,4shh:gfp* Sce I plasmid. The oligos used are as follows;

Z *shh* fwd	TTA GCT GCG GCC GCG TCC GCG CGT TGA GAC G
Z *shh* rev	AGT TAC GCA CTA GTA TTA AGT GTA ACC ATC
H *SHH* fwd	TAT AAT GCG GCC GCG CTC AGA GCC CCC ACG TTT C
H *SHH* rev	GCA TCC ACT AGT GAA AAC TAA AGT GAT GCA A
M *Shh* fwd	GTG CAT GCG GCC GCT CTA ACT ACC TGT ATT CT
M *Shh* rev	CAT CCA CTA GTC TCG ATT TGG CTG GGA GAT TG

The 40 bp enhancer was cloned by designing oligos over the entire sequence with digested 5' Not1 and 3' Spe1 ends. The oligos were then annealed at 100 °C for 5 mins and then cloned into the *-2,4shh:gfp* Sce I plasmid. The sequence of the oligos are as follows;

RetE Fwd	ggc cgc T GAA CAT ATT GAC ATT TCT CCA AGG ATG CTC TCC GAT TTG a
RetE Rev	ctagt CAA ATC GGA GAG CAT CCT TGG AGA AAT GTC AAT ATG TTC A gc

Deletion Constructs

The 5' deletion constructs were generated with different 5' primers with Not 1 sites and a common 3' primer with SpeI site. The oligos are as follows

+ 729 to +2021	TTA GCT GCG GCC GCG TCC GCG CGT TGA GAC G
+ 955 to +2021	TGA TGA GCG GCC GCC ATG AAC ATA TTG ACA TTT
+1154 to +2021	GAC TCA GCG GCC GCT TTA ATC TGA CTA ATA T
+1360 to +2021	CGA TCA GCG GCC GCA GAT TTG TGT TGC TTA A
+1559 to +2021	ATG TGA GCG GCC GCT TGA ACT TCT GAC CC
3' PRIMER	TCT TAC ACT AGT CTC CCT TTG AAA GAC TGA G

Similarly the 3' deletion constructs were created with a common 5' primer with Not 1

site and different 3' primers with Spe1 site and cloned into the Not 1/Spe1 site of the -2.4*shh:gfpSceI* plasmid (Ertzer et al., 2007). PCR were performed as follows: 30 cycles of 1min denaturation at 94°C, 1 min annealing at 58°C, 1 min elongation at 72°C and final elongation at 72°C for 10 mins. The constructs were all sequenced to avoid errors during amplification. The oligos used are as follows:

+549 to +1035 AGT TAC GCA CTA GTA TTA AGT GTA ACC ATC

+549 to +829 AGT TAC GCA CTA GTA CCA CCA CAG TCC C

5' PRIMER TAT AAG CTA TGC GGC CGC GTA ATT CTT TCG CCT TTC GAA ATC TG

The other deletion constructs were also cloned in a similar way. The oligos used are as follows:

+778 to +1035 fwd TTA GCT GCG GCC GCG GAT GTC CCG ACG GAT G

+778 to +1035 rev AGT TAC GCA CTA GTA TTA AGT GTA ACC ATC

+875 to +1035 fwd TTA GCT GCG GCC GCA CAT ACA TGT TCA TAT C

+875 to +1035 rev AGT TAC GCA CTA GTA TTA AGT GTA ACC ATC

For small internal deletion cloning, PCR amplication was carried out in two steps, the first PCR with the 5' outer primer and 3' deletion primer and 3' outer primer and 5' deletion primer. These initial PCRS were then mixed in equal amount and the final PCR carried out with the outer primers alone. The oligos used are as follows;

Del +729 to +1035

5' outer primer TTA GCT GCG GCC GCG TCC GCG CGT TGA GAC G

3 ' deletion primer GTG TCT GAT TCT AAT CTG TCA AAT TCA CAC ATA TGA CCA G

3' outer primer AGT TAC GCA CTA GTA TTA AGT GTA ACC ATC

5' deletion primer CTG GTC ATA TGT GTG AAT TTG ACA GAT TAG AAT CAG ACA C

Del +875 to +952

5' outer primer TTA GCT GCG GCC GCA CAT ACA TGT TCA TAT C

3 ' deletion primer CAT GCC CTA TAT TTC AAT CTA TAT TTA GC

3' outer primer AGT TAC GCA CTA GTA TTA AGT GTA ACC ATC

5' deletion primer GAA ATA TAG GGC ATG AAC ATA TTG

Mutational constructs

The mutations were generated through PCR where the primers were designed with nucleotide transitions. The mutation was performed every 20 bp non-overlapping over the +728 to +1035 bp region of the *shh* gene. The PCR were performed in steps, first PCR

was done using 5' Wt primer and 3' mutated primer (designated as B), 3′ wt primer and 5' mutated primer (designated as A). The PCR product from the above were then mixed in equal amounts and a final PCR was done using the 5' and 3' wt primers. The PCR were sequenced and then cloned into the *-2.4shh:gfpSceI* plasmid. The sequences of the oligos used are as follows with the mutations underlined;

5' wt primer TAT AAG CTA TGC GGC CGC GTA ATT CTT TCG CCT TTC GAA ATC TG

3' wt primer TCT TAC ACT AGT CTC CCT TTG AAA GAC TGA G

MUT1A

ACT TAT ATA CCA GAG TAT ACC AGG CGC GCG TTA TCT GTT T

MUT1B

AAA CAG ATA TCG CGC GCC TGG TAT ACT CTG GTA TAT AAG T

MUT2A

GGG TGA GCG GTA TAT ATT CAA CGC GTC TCA ACG CGC GGA C

MUT2B

TGA ATA TAT ACC GCT CAC CCA TAA AAC GTG ATG TCC CGA

MUT3A

CAG GCG CGC GTT ATC TGT TTG CGG GGT ACA AGC ACT TTA G

MUT3B

TCA GGT GCG GCA CTC ATC CGC TAA AGT GCT TGT ACC CCG C

MUT4A

ATA AAA CGT GGA TGT CCC GAT AAG CAG ACA TTA TGT TCA G

MUT4B

TAA GCA GAC ATT ATG TTC AGT CTG AAG GGA CTG TGG TGG TCA GAA

MUT5A

CTC AGA AAG TCA CAA CAA CTG AGG TAT GTC AGT CTC GTG AGA GC

MUT5B

CCT CAG TTG TTG TGA CTT TCC TGA GTC AGG TGC GGC ACT CAT CCG

MUT6A

TCT GAA GGG ACT GTG GTG GTC AGA ACG CAC TGA CTC TAC AGT GAT

MUT6B

TAT ATT TCA A TC TAT ATT TAA TCA CTG TAG AGT CAG TGC G

MUT7A

TAT GTC AGT CTC GTG ACA GCC GGG CGC GAG CCA GGG CGC G

MUT7B

AGC GAT ATG AAC ATG TAT GTC GCG CCC TGG CTC GCG CCC G

MUT8A

TAA ATA TAG ATT GAA ATA TAG TGC GTG CAC CTG CGC TAT C

MUT8B

GTG CGT GCA CGT GCG CTA TCT AGT CAG ACA GAC CGC AGC C

MUT9A

ACA TAC ATG TTC ATA TCG CTC GAC TGA GTG AGT TAT GAT T

MUT9B

CTC AGT CGA GCG ATA TGA ACA TGT ATG T

MUT10A

TAG TCA GAC AGA CCG CAG CCC GTT TAT CTA TCT ATC TAT G

MUT10B

TGC CCT GAT AAG CAG CCT GCC ATA GAT AGA TAG ATA AAC G

MUT11A

ATG AAT CAT CCG CTG AGG CAT GAA CAT ATT GAC ATT TCT C

MUT11B

GAG AAA TGT CAA TAT GTT CAC ATT TCA GCG GAT GAT TCA T

MUT12A

CAG GTG CGC CAG TGC CCT CTC AAG GAT GCT CTC CGA TTT G

MUT12B

CAA ATC GGA GAG CAT CCT TGA GAG GGC ACT GGC GCA CCT G

MUT13A

TGA ACA TAT TGA CAT TTC TCT GGA AGC ATC TCT TAG CCC A

MUT13B

TAG CCC ATT AAT AAG TAG AAA TGG GCT AAG AGA TGC TTC CA

MUT14A

CTC CAA GGA TGC TCT CCG ATT TGC CCT CGT CCG CCG GC

MUT14B

TCT GTC ATT AAG TGT AAC CAT CCC GAT TTG CCA GCG GAC GAG GG

MUT15A

AAG CAA CCG TGT CCG GCG ACA GAT TAG AAT CAG ACA C

MUT15B

GCC GGA CAC GGT TGC TTT AGC CCA TTA ATA AGT AG

The Pea3/Erm binding site mutation in M13 was performed similarly, the oligos used are as follows;

Mut A CCA AAA GCA CTC TCC GAT TTG T

Mut B GGA GAG TGC TTT TGG AGA AAT G

Construction of GST Pea3 and GST Erm plasmids

For GST constructs of Erm and Pea3, the ETS domain alone (Brown et al., 1998) was amplified using the following primers and cloned into the BamH1 –Xho1 site of the pGEX-4T3 expression vector (Promega).

Erm forward	ATT AAT GGA TCC ACG GCC CTC CAT ATC
Erm reverse	ATT AAT CTC GAG CAT CGG GAT CAC AGA C
Pea3 forward	ATT AAT GGA TCC TTC GTG AAG GTG CCC
Pea3 reverse	ATT AAT CTC GAG CTT CTG GCT CAC ACA C

Microinjection and expression analysis

Transgenes were excised from plasmids and separated by agarose gel electrophoresis followed by purification with the Qiaquick Kit (QIAGEN). Eggs were dechorionated using Pronase E as described (Westerfield, 1993). Dechorionated eggs were transferred to agar-coated plastic dishes containing 10% Hank's solution (Westerfield, 1993). Before injection, phenol red was added to 0.1% final concentration. DNA fragments were injected into the yolk of 1- to 2-cell stage zebrafish embryos at a concentration between 50 and 100 ng/µl. Injections of I-Sce1-modified plasmids were performed as described (Thermes et al., 2002), with some modifications. Embryos were placed in 10% Hank's solution and injected at room temperature. DNA was injected through the chorion into the cytoplasm of one cell stage embryos. The injection solution contained 10 ng/µl plasmid DNA, I-Sce1 meganuclease buffer 0.5× (New England Biolabs), 1 µg/µl I-Sce1 meganuclease (New England Biolabs) and 0.1% phenol red. GFP-expressing embryos

were raised to adulthood and transgenic carriers were identified by crossing with wild-type fish. GFP expression was analysed with a Leica DMIRBE inverted microscope and Leica confocal microscope.

Morpholino injections

Morpholino oligonucleotides (MOs) were synthesized by Gene-Tools, LLC (Corvallis, OR). The sequences of MOs used are as follows:

Pea3 MO 5′ AATCCATGCCTTAACCGTTTGTGGT 3′
Ctrl Pea3 MO 5′ AATCgATGCgTTAAgCcTTTcTGGT 3′
Erm MO 5′ GTTCCTGCATGTGAGACTTATTTGG 3′
Ctrl Erm MO 5′ GTTgCTGCATcTGAcAgTTATTTcG 3′
Fgf3 MO 5′ CATTGTGGCATGGCGGGATGTCGGC
Ctrl Fgf3 MO 5′ CATTcTGGCATcGCcGGATaTCaGC 3′
Fgf8 MO 5′ GAGTCTCATGTTTATAGCCTCAGTA 3′
Ctrl Fgf8 MO 5′ GACTCTGATCTTTATAGCgTCAcTA 3′
Fgf19 MO 5′ CAGTGACAAAGAGTAAGAGGAGCAT 3′
Ctrl Fgf19 MO 5′ AGTcACAAAcAGTAAcAGcAGgAT 3′

The MOs were injected at a concentration of 0,5-4 μg/μl at a volume of 0.15– 0.25 nl into one- to two-cell embryos. All morpholinos were prepared and injected as described (Nasevicius and Ekker, 2000).

SU5402 and Forskolin treatment

GFP transgenic embryos were dechorinated and were grown in PTU water. SU5402 (Calbiochem) was diluted to stock concentration of 3 mM in DMSO. Embryos were incubated in a 16 μM working concentration from 24 hpf to 48 hpf and 34 hpf to 48 hpf respectively and later washed several times in fish water and were mounted for pictures. Forskolin (Sigma) at a working concentration of 0.3 mM in DMSO was applied to dechorinated embryos from 24 hpf to 48 hpf and then analysed for expression.

Expression and purification of GST proteins

One colony of E.coli (BL 21) transformed with plasmid GST-Pea3 and GST-Erm was grown in 3ml LB containing amphicillin (100 µg/ml) overnight at 37°C to provide a starter culture. The overnight culture was then added to 300 ml LB with 100 µg/ml amphicillin and grown to 37°C with agitation to A 600 of O.D 0.6. The expression of fusion proteins was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.5 mM. The induction was done at 37°C for 3-4 hrs. The bacteria were then harvested by centrifugation at 4000 rpm for 20 mins. After freeze thawing in liquid nitrogen twice the pellet is suspended in 12,0 ml of extraction buffer (1X PBS, 0.1% Triton, 1 mM DTT, 0.3 mM PMSF, lysozyme (10 µg/ml) and sonicated on ice for 2 mins. The sonicate was then centrifuged at 4000rpm for 40 mins at 4°C.

For purification, the extract (6 ml) was incubated with 300 µl resin (Glutathione-Sepharose 4B twice prewashed with 1X PBS, 0.1 % Triton and twice with 1X PBS, 0.1 % Triton, 0.5 M NaCl) at room temperature for 10 or 30 minutes. After this incubation, the unbound proteins were removed by washing with TEN (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5% NP40) and PBS. The bound proteins were eluted twice by incubation with 300 µl elution buffer containing Glutathione (2.5 mg/mL Glutathione in 50 mM Tris-HCl pH 8, 20% Glycerol, 1 mM DTT, 0.5 mM PMSF) for 10 minutes at 4°C with mild shaking. The purity and protein contents were determined in Coomassie blue stained SDS-Polyacrylamide gel.

Gel Retardation Assay

For preparation of the probe, 1 µg synthetic oligonucleotides were annealed in the presence of NEB buffer 3 at 100°C for 10 minutes. The probes were then slowly cooled to room temperature. For labelling, 1 µg of annealed oligo was end labelled in the presence of γ ^{32}P ATP by T4 kinase. 100 to 200 ng of recombinant protein (GST-Pea3, GST-ERM) was incubated with binding buffer (20 mM HEPES pH 7.9, 40 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0. 2 mg/ml BSA, 5 % FICOLL) and 1µl of probe (40,000 cpm) for 45 minutes as described previously (Chang et al., 1997; Overdier et al., 1994). In competitor experiments, 50 to 100 fold excess of cold oligo nucleotide was added.

Electrophoresis was performed in 6 % polyacrylamide gel. The gel was pre-run for 2 hours at 100 voltage. Samples were loaded and the gel was run for 1 hour at 100 voltage. The gel was then subsequently exposed to the x-ray film (Kodak X-Omat) at -80°C overnight. The gel was then processed for autoradiography. The oligos used are as follows 5′ to 3′.

RetE WT FWD	GGG CAT GAA CAT ATT GAC ATT TCT CCA AGG ATG CTC TCC GAT TTG TTT
RetE WT REV	AAA CAA ATC GGA GAG CAT CCT TGG AGA AAT GTC AAT ATG TTC ATG CCC
RetE MUT FWD	GGG CAT GAA CAT ATT GAC ATT TCT CCA AAA GCG CTC TCC GAT TTG TTT
RetE MUT REV	AAA CAA ATC GGA GAG CGC TTT TGG AGA AAT GTC AAT ATG TTC ATG CCC
13 WT FWD	TTT CTC CAA GGA TGC TCT CCG ATT TGT TTC T
13 WT REV	AGA AAC AAA TCG GAG AGC ATC CTT GGA GAA A
13 MUT FWD	TTT CTC CAA AAA CGC TCT CCG ATT TGT TTC T
13 MUT REV	AGA AAC AAA TCG GAG AGC GCT TTT GGA GAA A
12 WT FWD	GGC ATG AAC ATA TTG ACA TTT CTC
12 WT REV	GAG AAA TGT CAA TAT GTT CAT GCC

Publications

Rathnam, S., Rastegar, S., Ertzer, R. and Strahle, U. (under prep)
Shh and Fgfs act sequentially for the propagation of the *shh:gfp* wave in the zebrafish retina.

I carried out all the experiment concerning cloning, microinjection, imaging, establishment of transgenic lines, protein expression, EMSA, knock down and inhibitor assays. Transgenic lines for midline expression studies were generated by Raymond Ertzer. I made the first draft of the manuscript that is under correction.

Ertzer, R., Muller, F., Hadzhiev, Y., Rathnam, S., Fischer, N., Rastegar, S. and Strahle, U.
(2007). Cooperation of sonic hedgehog enhancers in midline expression. *Dev Biol* **301**, 578-89.

I carried out the transient expression analysis for the ar-A and ar-C and AC fragments to confirm the synergistic effects of the enhancers.

Acknowledgements

First of all I would like to express my sincere thanks to my supervisor Prof. Dr. Uwe Strähle for giving me the opportunity to work under his guidance. He was always ready to answer my little doubts and I learned a lot from him.

I am grateful to Sepand for his constant support, encouragement and discussion. The thesis would not have come to its its final shape without the valuable inputs from Uwe, Thomas, Sepand and Masa.

I would like to thank Dr. Jochen Wittbrodt for being my second supervisor and adviser. I thank all the members of the jury for accepting to preside over my thesis defense.

I would also like to thank all my colleagues in the lab from Strasbourg to Karlsruhe for making my days in the lab so memorable. With an international atmosphere in the lab there was a great exchange between cultures and I always enjoyed it. I specially thank Christelle, Maryam and Isa for the friendly atmosphere and fun, Sandeep for all the coffee theories and nativity in the lab, Lixin for always ready to help me with my weekend crossings. I thank Nadine.F, Nadine.G, Nadine.B and Tina for their technical support and fish maintainance. People from the IGBMC imaging facility helped me a lot during my initial work in confocal microscopy and I thank them for their efforts.

I thank the funding agencies BIF (visiting fellowship), AFM and Forschungszentrum for their financial support during my thesis.

Finally I would like to give a big hug to my parents for giving me the freedom to pursue my career, to my husband Vino and my sister Rathi for all the love and support and my friends Leena and Chandu for the wonderful friendship we share.

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