The role of Sonic Hedgehog in regulating proliferation, cell survival, and cell-cycle exit in the zebrafish fin buds and neural-plate derived tissues

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Sergey Prykhozhij,
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Presented by
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The role of Sonic Hedgehog in regulating proliferation, cell survival, and cell-cycle exit in the zebrafish fin buds and neural-plate derived tissues

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ZUSAMMENFASSUNG


Effekt auf den Fgfsignalweg vermittelt wird. Im Kontrast dazu wirkt der Fgfsignalweg auf die Zellteilung direkt und unabhängig von seinem Effekt auf Shh.


Insgesamt zeigen meine Ergebnisse, dass Shh eine wichtige Rolle in der Regulation von Zellteilung und Zelltod während der Wirbeltierentwicklung spielt, und es beeinflusst diese Prozesse unterschiedlich in verschiedenen Geweben. Im Kontext der gepaarten Brustflossenanlagen reguliert Shh die Musterbildung und fördert die Zellproliferation durch
SUMMARY

The development of multicellular organisms depends on the integration between pattern formation and the regulation of cell number. The secreted signaling protein Sonic Hedgehog (Shh) has been implicated in directing both of these processes, suggesting it may participate in achieving this integration. The role of Shh in directing pattern formation in vertebrate model systems, including the zebrafish, has been well characterized during the last decades. Among the organs in which the patterning function of Shh has been best studied are the limb buds, the retina, and the neural tube. I therefore chose to study the role of Shh in regulating cell proliferation, cell death, and cell survival in these organs of the zebrafish. In addition, I also examined the interaction between Shh and several other factors directing cell proliferation and cell death, including the secreted signaling protein Fgf, and the transcription factor p53.

In the context of the zebrafish paired fin buds, I focused on the interplay between Shh and the Fgf signaling pathways. Shh directs pattern formation along the anterior/posterior axis of the vertebrate limb, whereas several Fgfs in combination direct pattern formation along the proximal/distal axis of the limb. In addition, Shh and Fgf signaling pathways in the limb bud are mutually interdependent. Therefore, I aimed to determine the relative importance of each pathway for proliferation in this organ. In zebrafish shh mutants, both proliferation and Fgf signaling in the pectoral fin buds are initially normal, but later are strongly reduced. Furthermore, pharmacological inhibition of Hh signaling for short periods has little effect on either Fgf signaling, or on cell-cycle gene expression, whereas long periods of inhibition lead to the downregulation of both. By contrast, even short periods of pharmacological inhibition of Fgf signaling lead to strong disruption of proliferation in the fin buds, without affecting Shh signaling. Activation of Fgf signaling by implantation of FGF4-soaked beads into shh mutant pectoral fin buds leads to the rescue of cell-cycle gene expression and proliferation in these organs. These results show that the role of Shh in this process is indirect, and is mediated by its effect on Fgf signaling. By contrast, the activity of the Fgf pathway affects proliferation directly and independently of its effect on Shh.
In neural-plate derived tissues such as the retina and the neural tube, Shh is essential for survival of cells during development. Here I identify p53 as the mediator of cell death in shh mutant since in the absence of Shh activity, p53 target genes are induced, and p53 loss leads to suppression of apoptosis in shh mutants. p53 induces apoptosis in the absence of Shh signaling by activating expression of the pro-apoptotic target genes puma and bax1, which induce the intrinsic pathway of apoptosis and whose level of expression correlates with the severity of apoptotic phenotypes. In support of the hypothesis that p53 activation results from loss of Shh signaling, p53 target gene expression and apoptosis are both suppressed by over-expression of dominant-negative PKA in shh mutants. To monitor p53 activation in living zebrafish embryos, I constructed a transgenic line expressing fluorescent protein under the control of the p53-driven promoter. Indeed, p53 reporter expression correlates very well with apoptosis levels in vivo, except in the early retina. Furthermore, p53 reporter can be induced by genotoxic drugs and colocalises with active-Caspase3. p53 reporter-positive cells were also found defective in their cell cycle progression at 48 hpf. Consistent with this result, proliferation assays on the double shh p53 mutant revealed that loss of p53 rescues normal cell-cycle exit and increases the rate of mitosis in the shh mutant retina. Moreover, differentiation of amacrine cells and photoreceptors was rescued in the double shh p53 mutant retina. These results show that in the absence of shh, p53 is required for the induction of apoptosis, and also regulates proliferation, cell-cycle exit and differentiation in the retina.

Taken together, my results show that Shh plays an important role in regulating both proliferation and cell survival during vertebrate development, and that it affects these processes distinctly in different tissues. In the context of the paired fin buds Shh directs pattern formation and in addition promotes cell proliferation, via activation of the Fgf signaling pathway. In neural-plate derived tissues Shh not only plays a patterning role, but also promotes cell survival and proliferation by antagonizing activation of the transcription factor p53.
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1 INTRODUCTION
**1.1 Signaling pathways in regulation of proliferation and cell death**

During development, organ growth is tightly regulated by controlling cell numbers. While cell number regulation remains one of the most complex problems in developmental and cell biology, work from many labs has shown that this is achieved by balancing proliferation and cell death within tissues. Localised regulation of cell numbers also contributes to organ shapes. Division and survival decisions inside the cell often require specific external signals. Several families of diffusible signaling molecules have been shown to affect both proliferation and cell death. In this thesis, I will focus on the Hedgehog signalling pathway and in particular on its role in regulating proliferation and cell death. In recent years, it has become clear that a true picture of signalling pathway function can be obtained from considering them a part of a network. This way of thinking influenced my work on Shh functions in proliferation and cell survival regulation by leading me to focus on interactions of Fgf signalling and p53 pathways with Shh signalling.

**1.2 Hedgehog signaling**

**1.2.1 Hedgehog family of proteins**

The curious name “Hedgehog” for this class of signaling molecules comes from the spiky phenotype of the Hedgehog (Hh) mutant Drosophila larvae identified in the forward genetic screen finished in 1980 (Nüsslein-Volhard and Wieschaus, 1980). Subsequently, similar genes were found in many other animals and in recent years our understanding of this class of signaling molecules has progressed dramatically. Undoubtedly, the Hh family of proteins is a key regulator of animal development, as it is involved in patterning and differentiation, regulation of proliferation and survival, may act as chemotactic cues and regulate morphogenesis via a combination of these functions.

Structural phylogenetics studies show that Hedgehog proteins contain two domains: Hedge and Hog domains, which have signaling function and protein splicing functions, respectively, and can be found independently in other proteins (Bürglin, 2008). Hh proteins appeared in early metazoan evolution, since sponges have some proteins containing the Hedge domain, and Cnidaria such as Nematostella vectensis have full Hh proteins. Most other Eumetazoa carry Hh genes except for some nematode species that lost these genes secondarily.
Regarding the main model systems, *Drosophila* carries a single *Hh* gene and vertebrates have three *Hh* genes: *Sonic Hedgehog* (*Shh*), *Indian Hedgehog* (*Ihh*) and *Desert Hedgehog* (*Dhh*). In zebrafish (*Danio rerio*), which underwent additional genome duplication, there are two *Shh* homologs (*Shha* and *tiggy-winkle hedgehog*), two *Ihh* homologs (*Ihha* and *Echidna Hedgehog*) and one *Dhh* homolog (Varjosaalo and Taipale, 2008).

### 1.2.2 Hedgehog biosynthesis, secretion and spreading in tissues

Hedgehog is initially produced as a 45 kDa precursor protein, which is then processed to a 19 kDa active signaling peptide HhNp (Fig. 1). First, the signal peptide is cleaved during precursor transport into the endoplasmic reticulum, then the C-terminal domain catalyzes intramolecular protein cleavage of N-terminal domain via a thioester intermediate. This intermediate then reacts with cholesterol through the hydroxyl group to form HhN molecule. The final HhNp molecule is formed when Skinny Hedgehog enzyme (or HHAT in vertebrates) attaches palmitate to HhN (Chamoun et al., 2001; Lee et al., 2001; Buglino and Resh, 2008) (Fig. 1). This lipid-modified form of Hedgehog can tightly attach to cell membranes of the producing cells. In both *Drosophila* and vertebrates, the secretion of Hh depends on Dispatched, a 12-span transmembrane protein homologous to Hh receptor Patched (Burke et al., 1999; Ma et al., 2002; Kawakami et al., 2002). Loss of *Dispatched* in all cases produces a phenotype similar to a complete loss of *Hh* signaling and results in accumulation of *Hh* proteins inside the secreting cells (Burke et al., 1999; Ma et al., 2002; Kawakami et al., 2002).
Figure 1. Hedgehog protein cleavage and lipid modification pathway. After its synthesis, the signal sequence of Hedgehog precursor protein is removed by the signal peptidase in the endoplasmic reticulum (1). The C-terminal part of Hedgehog then catalyzes coordinated intramolecular proteolytic cleavage and cholesterol moiety addition (2). The next step is addition of palmitic acid by Skinny Hedgehog (Ski) acyl-transferase (3). N-terminal part of Hedgehog (HhN) (24-197 aa) modified with cholesteroyl- and palmitate moieties (HhNp) is highly lipophilic and is the final active signaling molecule. The figure was redrawn with modifications from Varjosalo and Taipale, 2008.
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Whereas Hh processing and secretion are well understood, it is not clear, how Hh proteins spread in gradients across 50-300 µm depending on the organism and tissue (Zhu and Scott, 2004). Several studies propose that Hh spreads over long distances in the form of multimere complexes containing lipids (Zeng et al., 2001) or lipoproteins (Panakova et al., 2005; Calejo et al., 2006). However, a recent study in *Drosophila* (Katanaev et al., 2008) proposed two secretion pathways for Hh protein: a short range and a long-range pathway, whose function depends on a membrane microdomain protein Reggie-1, which suggests that additional mechanisms may be involved in Hh spreading. Since Hh spreads in tissues in lipoprotein complexes, what is the role of Hh lipid modifications in this process? Indeed, lipid modifications are required for Hh protein distribution *in vivo* (Guerrero and Chiang, 2007; Varjosalo and Taipale, 2008). Palmitoylation is necessary for Hh activity and long-range signaling in both *Drosophila* and vertebrates (Chamoun et al., 2001; Chen et al., 2004), and the effect of palmitoylation loss on long-range Hh signaling in vertebrates is due to defects in Hh lipoprotein complex formation. It is unclear whether cholesterol enhances or hinders Hh transport *in vivo* since different studies produced conflicting results, and the associated caveats include unequal protein expression, Dispatched-independent secretion and potential lack of palmitoylation (Varjosalo and Taipale, 2008).

Hh tissue spreading is controlled not only by lipid modifications but also by cell-surface Hh-binding molecules and proteoglycans in the extracellular space. Genetic studies on exostosin (EXT)/*tout velu* family of heparan sulfate proteoglycan (HSPG)-synthesizing enzymes in *Drosophila* have first suggested that HSPGs are involved in Hh spreading (Lin, 2004) by synthesizing Dally and Dally-like (Dlp) glypicans, glycosylphosphatidylinositol (GPI) – linked heparan sulfate proteoglycans, which are attached to the cell surface and whose polysaccharide chains protrude into the extracellular space. Overall, glypicans have two main functions in *Drosophila*: contributing to Hh spreading and cell-autonomously enhancing signaling pathway activation (Jiang and Hui, 2008). Loss of these proteins inhibits Hh signaling in *Drosophila* similarly to the loss of HSPG-synthesizing enzymes. In particular, in wing discs Hh fails to move into cell clones mutant for HSPG synthesizing enzymes leading to abnormal accumulation of the protein in more posterior regions. This requirement of HSPGs for Hh spreading is limited to lipidated HhNp, present in lipoprotein particles. By contrast, the non-lipidated form of Hh can move farther than HhNp and does not require HSPGs for its
movement. In addition, it has been shown, that HSPGs are involved in maintaining Hh protein stability. How Hh interacts with glypicans is not completely understood, but there is evidence in *Drosophila* that Shifted is crucial for this interaction (Glise et al., 2005; Gorfinkiel et al., 2005). Shifted is a secreted protein necessary for Hh spreading and signaling activation in developing wing blade, notum and eye tissues in the fly. In these tissues Shifted can bind both Hh and glypicans thus mediating their interactions. However, Shifted-mediated binding is not the only mechanism of Hh interaction with proteoglycans, which may also be direct. Moreover, Hh-glypican interaction may not even depend completely on polysaccharide chains of glypicans (Filmus et al., 2008). In contrast to *Drosophila*, where glypicans activate Hh signaling, mouse Glypican3 (GPC3) is a negative regulator of the Hh pathway (Capurro et al., 2008). Mice and humans lacking GPC3 exhibit an overgrowth phenotype. Mechanistically, GPC3 binds Hh proteins and the resulting complex becomes endocytosed and degraded, thereby reducing Hedgehog level in the tissues. Consistently, in the absence of GPC3, the level of Shh protein and expression of Hh target genes are significantly higher. Similarly to GPC3, *Drosophila* glypicans also induce endocytosis of Hh in a complex with Ptc releasing inhibition of Smo thus activating Hh signaling (Beckett et al., 2008).

There are two additional ways to regulate the Hh gradient (Varjosalo and Taipale, 2008). On the one hand, high levels of Hh signaling can induce secondary production of Hh proteins in cells responding to it (Tabata et al., 1992; Roelink et al., 1995). On the other hand, Hh signaling leads to its own down-regulation by inducing expression of Patched (Ptc), Hh receptor, and the complex is degraded in the lysosomes (Chen and Struhl, 1996; Incardona et al. 2000). Similarly, in vertebrates Hh induces expression of Hip1 binding Hh to the cell surface and thus preventing its spreading (Chuang and McMahon, 1999).

### 1.2.3 Hedgehog signaling pathway

Hedgehog signalling is a signal transduction pathway regulating proteolytic processing of its downstream effector transcription factors Cubitus interruptus (Ci) in *Drosophila* and Gli in vertebrates (Fig. 2; Lum and Beachy, 2004; Jiang and Hui, 2008; Varjosal et an and Taipale, 2008). In the absence of Hh signals, proteolysis leads to formation of repressive forms of Ci
and Gli factors, whereas when Hh ligands are present this proteolysis is inhibited and the full-length Ci and Gli can activate their target genes.

The most upstream signal transducer Smoothened (Smo) is inhibited by Hh receptor Ptc in the absence of Hh signal via a catalytic mechanism possibly involving oxysterols or vitamin D3 metabolites. Hh ligand binding to Ptc relieves Smo inhibition that Ptc mediates thus activating the signal transduction pathway. Hh ligands are aided in their binding to Ptc by proteoglycans, as well as membrane-spanning co-receptors Ihog and Boi (Cdo and Boc in vertebrates, respectively), stimulating Hh signaling (Allen et al., 2007; Tenzen et al., 2006; Yao et al., 2006; Zhang et al., 2006). Negative regulation of expression of these co-receptors by Hh signaling, transcriptional activation of negative Hh signaling regulators patched, hip1 and Gli degradation-promoting HIB/SPOP genes, negative regulators of Hh signaling, ensures robust negative feedback regulation of Hh signaling (Varjosalo and Taipale, 2008).

Upon Hh stimulation, Drosophila Smo (dSmo) accumulates at the membrane, is phosphorylated by Protein Kinase A (PKA), Casein Kinase I (CKI) and changes its conformation (Zhao et al., 2007). In vertebrates, GRK2 induces similar activatory phosphorylation of Smo thereby activating downstream signaling (Chen et al., 2004b; Meloni et al., 2006). The C-terminus of dSmo is a binding platform for multiple factors: Costal2 (Cos2), Ci, Fused kinase (Fu), PKA and several other kinases. Cos2 belongs to the kinesin superfamily and serves as a molecular scaffold protein for Ci phosphorylation by PKA, CKI and GSK3β. Without Hh, Cos2-mediated phosphorylation of Ci leads to Slimb-dependent ubiquitination and proteolytic processing of Ci into a repressive form CiR, while upon Hh stimulation, Cos2 is recruited by dSmo and cannot promote degradation of Ci. Inhibition of Ci degradation leads to accumulation of full-length activatory form CiA, for whose complete activation Suppressor-of-Fused (Sufu) must be removed from CiA by Fu-mediated phosphorylation. This phosphorylation occurs upon Cos2 binding to dSmo since Cos2 brings Fu close to Sufu bound to Ci. The Fu requirement for Sufu inactivation was confirmed by dispensability of Fu for Hh signaling upon Sufu loss (Ohlmeyer and Kalderon, 1998; Lefers et al., 2001).
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Figure 2. The Hedgehog signaling pathway in *Drosophila* and vertebrates.

Hh reception is enhanced by its binding to coreceptors Ihog/Boi in *Drosophila* and Cdo/Boc/Gas1 in mammals. *Drosophila* Dally and Dlp1 HSPGs bind and concentrate Hh molecules on the surface of the cell. Patched controls Hh signaling by inhibition of Smo. Lack of Smo activity allows phosphorylation of Ci/Gli (Gli2 and Gli3) by PKA, CKI, GSK3 and possibly other kinases, which leads to ubiquitination by Slimb/β-TrCP SCF subunits and proteasome-mediated proteolysis. This proteolysis generates truncated repressor forms CiR/GliR of full-length Ci/Gli. In *Drosophila*, Cos2 promotes phosphorylation of Ci acting as a molecular scaffold to bring Ci and its kinases in close proximity. Hh binding to Ptc blocks its inhibition of Smo. dSmo is then phosphorylated on the C-terminal domain by PKA and CKI, which leads to its activation and cell surface accumulation. In vertebrates, GRK2 can activate Smo by phosphorylation. Recruitment of Cos2-Fu by Smo leads to dissociation of Cos2-Ci complexes and reduction in Ci phosphorylation and abundance of repressor form of Ci. Hedgehog signaling also stimulates CiA activity by promoting Fu-mediated inhibition of Sufu. Hh-promoted expression of HIB targeting CiA for degradation is another negative-feedback loop in Hh signaling. In vertebrates, Sufu is the main Gli inhibitor and is inhibited by Cdc2l1 kinase. DYRK2 kinase targets Gli2 for degradation, while MAP3K10 activates Gli2 by inhibiting DYRK2. Additionally there is evidence for Gai (Gai) function in signaling downstream of Smo in both *Drosophila* and vertebrates. In brown are indicated *Drosophila* proteins playing both positive and negative roles in Hedgehog signaling regulation, negative regulators are in red and positive regulators are in green. The figure was modified from Jiang and Hui (2007).
The mechanism of Hh signaling in vertebrates differs from the one in *Drosophila* (Fig. 2; Varjosalo and Taipale, 2008). For its signal transduction the vertebrate Hh pathway unlike the one in *Drosophila* requires the primary cilium, a small microtubule-based structure with a sensory function in most vertebrate cells (Wong and Reiter, 2008). Mutations in intra-flagellar transport (IFT) components affecting primary cilium structure and function lead to Hh signaling inhibition in the neural tube, but to its apparent activation in the limbs. Consistent with the role of IFT in Hh signaling, Smo, Ptc, Sufu and Gli factors localize to the primary cilium, and there is evidence that the main role of the primary cilia is to promote processing of Gli proteins to both activatory and repressive forms. Hh signal transduction prior to Gli processing also occurs in the primary cilia, where Hh signaling is initiated when Smo is released from its Ptc-mediated inhibition. Ptc inhibition of Smo ciliary localization is removed by Shh stimulation leading to Smo recruitment to the primary cilium, where it co-localises with other Hh signaling components (Rohatgi and Scott, 2007). In contrast to Smo, Ptc leaves the primary cilium after binding Shh and cannot inhibit ciliary recruitment of Smo.

Functions of vertebrate homologs of Hh signaling mediators downstream of Smo are still unclear. The zebrafish Cos2 homolog has been suggested to negatively regulate Hh signaling and interact with Gli1 (Tay et al., 2004). However, mouse Cos2 homologs, Kif7 and Kif27, cannot bind either Smo or Gli factors and are functional kinesin motors in contrast to Cos2 (Varjosalo et al., 2006). Like in the case of Cos2, the zebrafish Fused homolog has been implicated in Hh signaling in the context of muscle pioneer generation (Wolff et al., 2003), whereas mouse Fused homologue is highly divergent and non-essential for Hh signaling (Jiang and Hui, 2008). Another difference of Hh signaling in vertebrates and in *Drosophila* is signal transduction downstream of Smo, and only recently kinases involved in this process were identified. Evangelista and colleagues (2008) identified Cdc2l1 kinase as necessary and sufficient for Hh signaling activation. Mechanistically, Cdc2l1 binds and inactivates Sufu, a potent Gli inhibitor. Another systematic kinase screen identified DYRK2 and MAP3K10 kinases involved in Hh signal transduction (Varjosalo et al., 2008). DYRK2 negatively regulates Hh signaling by phosphorylating and promoting degradation of Gli2, whereas MAP3K10 is a positive regulator of Hh signaling inhibiting DYRK2 and possibly other kinases. Identification of these kinases is important for understanding Hh signaling, because, like Ci, GLI2 and GLI3 can be phosphorylated and targeted to degradation in the absence of
Hh signals. When Hh signal is present, both proteins remain full-length and can activate target genes. Several kinases and protein degradation machinery are crucial for controlling Gli activity. PKA phosphorylation of Gli3 primes it for further phosphorylation by CKI, GSK3β and other kinases and subsequent ubiquitination by SCF ubiquitin ligase complex (Wang et al., 2000; Jiang, 2006). Moreover, Gli2 processing is also regulated by PKA, and Gli2 without PKA sites is much more active (Pan et al., 2009). How the newly identified kinases fit into the current models of Gli regulation is currently unclear.

1.3 The role of Sonic Hedgehog in regulating proliferation in development and tumourigenesis

There is now abundant evidence that Hh signaling is involved in regulation of proliferation. In most instances, Hh ligands stimulate proliferation by direct regulation of cell-cycle promoting genes, through activation of secondary signaling pathways inducing cell cycle progression or via other mechanisms that are still unknown. Several early studies found clear evidence that Hh signaling regulates proliferation. Fan and Khavari (1999) artificially expressed Shh in the mouse skin and showed that it can increase cell numbers and override p21 CDK inhibitor-mediated cell cycle arrest. This result suggested a potent proliferative activity of Shh and could potentially explain the origin of basal cell carcinoma. Similarly, Shh produced by Purkinje cells has been shown to promote granular cell precursor (GCP) proliferation in the developing cerebellum (Wechsler-Reya and Scott, 1999; Dahmane and Ruiz i Altaba, 1999). Increase in GCP proliferation by Shh was abolished by Hh signaling inhibition, and this effect could be observed both in cultured cell lines and in vivo. Tectum and neocortex are two other dorsal brain parts which require Shh for cell proliferation (Dahmane et al., 2001). These observations provided important clues to why Hh signaling is activated in tumours of the neural origin. Later studies also showed that Hh signaling is required for cell proliferation in several stem cell niches in the postnatal and adult brain (Machold et al., 2003; Palma et al., 2005). Cayuso and colleagues (2006) showed that Shh requirement for proliferation in the developing spinal cord is mediated by Gli factors. Subsequent studies on the mechanisms of Hh signaling-mediated proliferation found that Hh signaling is required for expression of cell cycle regulators. Direct effects of Hh signaling on expression of cell cycle regulators were identified in both *Drosophila* and vertebrates in several different tissues.
During *Drosophila* eye development Hh signaling is required for normal expression of cyclins and proliferation, and its activation suppresses the phenotype due to negative cell cycle regulator RBF (Retinoblastoma) overexpression. In this case, Hh signaling promoted cell cycle progression by transcriptional upregulation of D-type and E-type cyclins in target cells (Duman-Scheel et al., 2002). This transcriptional up-regulation of cell-cycle genes has been shown to occur as a direct response to promoter binding of Gli factors (Duman-Scheel et al., 2002; Yoon et al., 2002). In the mouse cerebellum, Kenney and Rowitch (2000) found that Shh signaling maintains GCP proliferation by regulating *cyclin D* gene expression. Expression profiling of genes in the GCP cells showed that Shh regulates cell cycle progression by inducing N-Myc, which activates its downstream transcription program (Oliver et al., 2003).

Also during retinal development, Shh plays a major role in regulating proliferation. Early studies showed that Shh stimulates proliferation of retinal progenitors in tissue culture (Jensen and Wallace, 1997; Levine et al., 1997) and in the ciliary marginal zone (CMZ) of newly born mice and chickens (Moshiri and Reh, 2004; Moshiri et al., 2005). During mouse retina development, Shh positively regulates cell cycle progression probably by regulating *cyclin D1* expression, and without Shh cells cannot continue proliferating and exit the cell cycle becoming retinal ganglion cells (Wang et al., 2005). Consistently, in the zebrafish *shh* mutant retina, proliferation was reduced during neurogenesis (Stenkamp et al., 2002). Explaining positive effects of Shh on retinal proliferation, Locker and co-workers (2007) found that in *Xenopus* Shh signaling promotes retinal progenitor proliferation by shortening G1- and G2-phases of the cell cycle. This effect may be caused by Shh-mediated positive regulation of *cyclin D1*, *cyclin A2*, *cyclin B1* and *cdc25C* gene expression, which would lead retinal cells to faster cell cycle progression towards the final mitosis and cell-cycle exit.

Despite its widespread role in promoting proliferation, Shh does not function as the classical mitogen since it cannot make terminally differentiated cells enter the cell cycle from a quiescent phase and does not induce mitogen-activated protein kinase (MAPK) pathway (Roy and Ingham, 2002).

On the other hand, Hh signaling has also been shown to have a negative role on cell cycle progression. In the mouse gut, Ihh is required for cell cycle exit and differentiation of colonocytes both *in vivo* and in a colonocyte cell line through activation of p21 CDK inhibitor expression (van den Brink et al., 2004). Although there is strong evidence for a positive role of
Shh in retinal proliferation, studies in the zebrafish retina show that this signaling pathway is required for retinal cell-cycle exit. In zebrafish shh mutant retina and under conditions of Hh signaling inhibition by forskolin, most retinal cells fail to express p57Kip2 CDK inhibitor and do not exit the cell cycle (Shkumatava and Neumann, 2005; Masai et al., 2005). Consistent with these studies in vertebrates, in Drosophila retina development, Hh signaling is involved in promoting cell-cycle exit but only in a strict combination with Dpp signaling (Firth and Baker, 2005).

Regulation of other signaling pathways which in turn promote cell cycle progression may be another strategy for Hh signaling to regulate proliferation during development. One clear case of such a mechanism is regulation of proliferation during Drosophila wing disc development. In this context, Hh promotes proliferation by regulating Dpp expression, a morphogen of the TGF-β family. Wing disc cells lacking receptors for Dpp have proliferation defects suggesting a cell-autonomous role for Dpp signaling in regulating proliferation (Burke and Basler, 1996). Furthermore, Martín-Castellanos and Edgar (2002) provided complementary evidence showing that activated Dpp receptor activates cell cycle progression in wing disc cells.

Consistent with its role in regulating proliferation, Hh signaling activation was implicated in brain cancers such as glioma and medulloblastoma, skin cancer basal cell carcinoma (BCC), adenosarcomas of oesophagus, stomach, liver, colon and pancreas, rhabdomyosarcoma in the muscle, small-cell lung carcinoma, some cancers of breast, prostate (Rubin and Sauvage, 2006; Beachy et al., 2004). In addition to mentioned solid tumours, stroma-derived Hh ligands facilitate growth of B-cell lymphomas by providing survival signals (Lindemann, 2008). Inactivation of PATCHED and SUFU releases negative regulation of Hh signaling and is a frequent way to initiate tumour formation. Alternatively overexpression of Gli factors and activating mutations in SMO gene represent Hh signaling activation mechanisms leading to tumour formation. Therefore, cancers resulting from Hh signaling activation do not depend on Hh ligand stimulation but can be inhibited with Smo antagonists such as cyclopamine (Jiang and Hui, 2008). Hh signaling alterations and some signaling inputs ultimately result in a certain activity level of Gli factors, a Gli code. Gli codes change during tumourigenesis progression translating into different cancer cell behaviours, such as enhanced proliferation and eventually metastasis (Ruiz i Altaba et al., 2007). There are several potential
mechanisms by which Hh signaling can promote tumourigenesis: activation of proliferation, suppression of apoptosis, induction of epithelial-to-mesenchymal transition and promoting angiogenesis. These possibilities should be important considerations guiding the search for cancer therapies (Rubin and Sauvage, 2006).

1.4 Sonic Hedgehog and Fibroblast Growth Factors in vertebrate limb bud patterning and as potential limb bud mitogens

Limb development is highly amenable to experimental and genetic manipulation in several model organisms, and the main signaling pathways that direct limb development are well characterized (Fig. 3; reviewed in Capdevila and Izpisúa Belmonte, 2001; Niswander 2002; Tickle 2002). The zone of polarizing activity (ZPA), a small group of cells in the posterior mesenchyme, controls polarity along the anterior/posterior axis (Saunders and Gasseling, 1963). The secreted signaling protein Shh is expressed in the ZPA, and has been shown to mediate the effect of the ZPA during limb development (Fig. 3; Riddle et al., 1993; Chang et al., 1994; López-Martínez et al., 1995; Neumann et al., 1999). The apical ectodermal ridge (AER) is another major signaling center of the limb bud. It runs along the distal margin of the limb bud, and is the site of expression of several Fgf genes (Fig. 3; reviewed in Martin 1998). The AER is required for outgrowth and patterning of the limb along its proximal/distal axis, and can be functionally replaced by FGF-soaked beads in chicken embryos, indicating that Fgf signaling can mediate AER function (Fallon et al., 1994; Niswander et al., 1993). Furthermore, conditional inactivation of both Fgf4 and Fgf8 in the mouse AER leads to failure of proximal/distal outgrowth (Sun et al., 2002). Factors from the AER and ZPA form a mutual feedback loop that allows growth and patterning of the different axes to be coordinated. Thus fgf-4, which is expressed in the posterior AER, can be induced in the anterior AER of the chicken limb bud by ectopic Shh protein (Niswander et al., 1994; Laufer et al., 1994). Furthermore, removal of Shh activity from the zebrafish fin buds leads to loss of fgf4 and fgf8 expression in the AER (Neumann et al., 1999), and, conversely, removal of Fgf4 and Fgf8 activity from the mouse AER leads to loss of shh expression in the ZPA (Sun et al., 2002), indicating that each signaling pathway is required for the maintenance of the other pathway.
Figure 3. Shh and Fgf signaling pathways in limb patterning and outgrowth. Shh and Fgf signaling pathways are interdependent due to mutual regulation of expression and both pathways affect limb bud mesenchyme. Shh signaling can affect both anterior-posterior patterning and limb bud outgrowth. Fgf is mainly required for outgrowth and proximo-distal patterning.

Despite all the advances in defining how limb patterning occurs, our understanding of how proliferation during limb development is regulated is still fragmentary. More specifically, since cells in many different contexts proliferate in response to mitogens, it has been important to define the proliferation-stimulating molecules in the context of the vertebrate limb bud. The two best mitogen candidates in limb buds are Shh and Fgf proteins. Similar to Hh signaling, Fgf signaling positively regulates proliferation in a number of contexts. Indeed, Fgf1 and Fgf2 were initially identified as mitogenic factors in fibroblast tissue culture, and subsequently, other members of the FGF protein family were found to have a similar activity (Powers et al., 2000). Furthermore, Fgf signaling has also been shown to have a mitogenic activity in vivo during embryonic development. Thus FGF-4 is necessary for proliferation of the inner cell mass during early post-implantation development in the mouse (Feldman et al., 1995), and FGF-8 and FGF-17 are required for proliferation in the mouse dorsal midbrain (Partanen,
2007). In addition, Fgf signaling promotes proliferation of osteoblasts (Ohbayashi et al., 2002), of lens cells (Robinson, 2006), and during hematopoiesis (Moroni et al., 2006).

In zebrafish there is genetic evidence that Shh is required for fin bud outgrowth and cell proliferation (Neumann et al., 1999) and defects in digit formation in mice lacking shh gene may be due to the role of Shh in proliferation (Towers et al., 2008; Zhu et al., 2008). As the manuscript containing some of the data in this thesis was under review (Prykozhij and Neumann, 2008), a paper was published proposing a direct role of Shh in regulating proliferation in developing chick limb buds (Towers et al., 2008). Following observations that inhibition of Hh signaling for long periods leads to loss of posterior digits, the authors investigated whether this result is due to the morphogen or proliferation-promoting property of Shh. Although cyclopamine inhibition only led to a reduction of cyclinD2 without affecting cyclinD1 and N-Myc, implantation of Shh-soaked beads led to an increase of cyclinD1 and N-Myc expression. This induction of G1- and S-phase genes by Shh-soaked bead implantation was apparently independent of the AER region. Moreover, derepression of Hh signaling by loss of Gli3 led to a strong induction of cyclinD1, cyclinD2 and N-Myc.

In contrast to direct proliferation regulation by Shh during limb bud development, several studies suggest that Fgf signaling may be fulfilling this function. Dudley et al. (2002) surgically removed AER on the chick limb buds and analysed proliferation several hours after the cut. They found a clear decrease in proliferation in the underlying mesenchyme suggesting that proliferation during limb bud development is regulated by Fgf molecules released by AER. However, another interpretation of such experiments is that loss of AER also leads to defects of Shh signaling. In contrast to the AER removal studies, genetic inactivation Fgf4 and Fgf8 genes in the mouse limb buds leads to reductions in limb bud size most likely due to an increase in cell death since the proliferation rate is normal (Sun et al., 2002). Still, there may be other Fgf ligands besides Fgf4 and Fgf8, which may be sufficient to maintain proliferation (Niswander 2003). Thus, to date the relative contributions of Shh and Fgf signaling pathways in maintaining limb bud proliferation remain uncleal.

1.5 Sonic Hedgehog in zebrafish retinal development

From a developmental and functional point of view, the retina is a part of the nervous system. Its function is to perceive light and transduce the resulting information to the brain.
The retina has a highly ordered structure consisting of three cell layers interconnected by synapses localized in two layers (Fig. 4B; Bilotta and Saszik, 2001). There are seven main cell types in the retina: retinal ganglion cells (RGCs), amacrine cells, bipolar cells, horizontal cells, cone photoreceptors, rod photoreceptors and Müller glia (Fig. 4A). A recent study of *Xenopus* retina found that retinal cell types are born in the following order: RGCs, horizontal cells, cone photoreceptors, rod photoreceptors, amacrine cells, bipolar cells and Müller glia (Wong and Rapaport, 2009). RGCs are all located in a single layer and their axons transmit visual information to the optic tectum. Inner plexiform layer (IPL) is located between the RGC layer and the inner nuclear layer (INL) and contains synapses of RGCs with amacrine and bipolar cells. The INL follows the RGC layer and contains amacrine, bipolar, horizontal and Müller glia cells. Finally, the outer-most part of the retina is outer nuclear layer (ONL) containing cone and rod photoreceptors. Synaptic connections between INL and ONL are located in the outer plexiform layer, where bipolar and horizontal cells make synapses with photoreceptors. Outside of the neural retina lies retinal pigmented epithelium absorbing stray light and thereby protecting retinal cells from light-induced damage.

Conversion of multipotent retinal progenitors to differentiated retinal cell types occurs during the complex process of differentiation, which is influenced both by intrinsic and extrinsic factors with unclear relative contributions. In particular, the competence model suggests that retinal progenitors go through a number of intrinsically defined states and have different propensities to become specific cell types (Livesey and Cepko, 2001). Intrinsic factors are mainly transcription factors belonging to basic helix-loop-helix or homeobox families (Stenkamp, 2007), which alone or in combinations determine the fates of the progenitor cells. According to the competence model, extrinsic factors can influence the ratios of different cell types, but they cannot induce cell types of a different progenitor competence state. Despite some progress, it is still unclear to what extent the extrinsic factors control retinal cell fates.

The role of Shh during retina development is especially well-understood in zebrafish. Shh influences zebrafish retina throughout its development and its known functions consist of early proximo-distal patterning of the optic vesicle, promoting retinal neurogenesis and cell type differentiation and retinotectal projection of RGC axons (Stadler et al., 2004). After optic vesicle formation, Shh and Twhh are required for patterning this structure into optic stalk and
neural retina. In this context, Hh signaling is promoting optic stalk cell fate by activating Pax2 (Ekker et al., 1995; Macdonald et al., 1995) and Vax1 and Vax2 homeobox genes (Stadler et al., 2004). Indeed, the activation of Hh signaling leads to expansion of optic stalk tissue at the expense of neural retina (Macdonald et al., 1995; Masai et al., 2000), as in the neural retina, Hh signaling leads to inhibition of Pax6 expression and activation of Pax2 thereby converting the neural retina into the optic stalk. By contrast, inhibition of Hh signaling leads to expansion of the neural retina and reduction of the optic stalk.

During retinal neurogenesis, progenitors undergo final mitoses and differentiate into retinal cell types. Initiation of zebrafish retinal neurogenesis is induced by Fgf signaling from the optic stalk (Martinez-Morales et al., 2005). Neurogenesis is first induced in the ventro-nasal patch of the zebrafish eye at 28 hpf, and after RGCs are born, neurons are born in the INL and ONL in distinct waves (Hu and Easter, 1999). Shh expression in the retina is first initiated in newly-born RGCs and then spreads in a wave as new RGCs are born (Neumann and Nüsslein-Volhard, 2000). The requirement of Hh signaling for RGC development is mediated through positive regulation of the \textit{ath5} gene (Stenkamp and Frey, 2003; Neumann and Nüsslein-Volhard, 2000). Subsequent spreading of Shh expression in amacrine cells also occurs in a wave independent of Shh expression in RGCs (Shkumatava et al., 2004). In that study it has also been shown that Shh is required for differentiation of all retinal cell types except for some RGCs. Although it was previously proposed that Shh expressed by pigmented retinal epithelium is required for photoreceptor differentiation (Stenkamp et al., 2000), Shkumatava and colleagues (2004) conclusively show that short-range Shh signaling from neural retina is necessary and sufficient to induce photoreceptor and Müller glia differentiation. Another possibility suggested by Stenkamp (2007) is that Shh is required for amacrine and photoreceptor survival rather than differentiation. The role of Shh in Müller glia differentiation seems to be conserved since in the mouse retina Müller glia express Hh signaling target genes and require Shh for their normal organization (Wang et al., 2002). In the context of retinal differentiation, Shh also promotes cell-cycle exit of differentiating cells (Masai et al., 2005; Shkumatava and Neumann, 2005). Indeed, \textit{shh} mutant embryos had a pronounced delay in \textit{p57kip2} expression and blocking \textit{p57kip2} function with morpholino oligonucleotides leads to a \textit{shh} mutant phenocopy, that is lack of differentiation in the retina, cell-cycle exit defects and a high level of apoptosis in the retina.
Up to date, it is unclear how to reconcile the results concerning the functions of Shh in the retina. Since Shh signaling in the retina regulates neurogenesis, proliferation, cell-cycle exit and cell survival, an obvious question is how a single signaling pathway can control these distinct cell behaviors.
Figure 4. Retinal cell types and layer structure.
A. Illustration of the spatial relationships between different cell types in the neural retina.
Reproduced with modifications from http://webvision.med.utah.edu/
1.6 The survival function of Sonic Hedgehog signaling

Hh signaling pathway regulates cell survival during development in addition to its pattern formation, proliferation and differentiation functions in a variety of contexts (reviewed in Ingham and McMahon, 2001; Ulloa and Briscoe, 2007; Ruiz i Altaba et al., 2003). There are several examples for the survival role of Shh. In the mouse \( shh^{-/-} \) mutant, neural tube and ventral sclerotome showed high level of apoptotic cells (Borycki et al., 1999; Litingtung and Chiang, 2000). Similarly, surgical manipulations to prevent formation of the notochord in chick embryos lead to extensive apoptosis in the neural tube implicating Shh as a survival factor in the neural tube (Charrier et al., 2001). These findings are confirmed by the fact that restoration of Hh signaling by provision of Shh sources or de-repression of Hh signaling by deletion of \( gli3 \) gene, repressor of Hedgehog target genes, could suppress apoptosis (Charrier et al., 2001; Litingtung and Chiang, 2000). However, it is possible that cell survival depends on the patterning role of Shh in the neural tube (Briscoe and Ericson, 1999; Patten and Placzek, 2000). This seems not to be the case, as, for example, Cayuso and colleagues (2006) showed that the effect of Hh signaling on cell survival occurs much earlier than patterning or differentiation. Also, lack of Shh in chick embryos leads to striking apoptosis in the neural crest (Ahlgren and Bronner-Fraser, 1999), which is similar to the phenotype the mouse \( Smo \) gene knockout embryos with decreased proliferation and increased apoptosis in the branchial arches (Jeong et al., 2004). By contrast, studies of Shh in neural crest development in zebrafish confirmed an important role of Shh in cartilage differentiation, but did not identify the cell survival function of Shh signaling likely due to using cyclopamine treatments and a hypomorphic \( shh \) mutant (Wada et al., 2005; Eberhart et al., 2006). These results also suggest that the levels of Hh signaling required for cell survival may be lower than those required for differentiation. Zebrafish retina is another tissue requiring Shh for survival of most cell types as shown by \( shh^{-/-} \) mutant analysis (Shkumatava and Neumann, 2005).

However, there is still some controversy about the underlying mechanism of the survival role of Shh. Activation of pro-survival genes by Shh signaling represents the simplest scenario, and indeed, Gli1 can activate BCL2 gene in human cell lines by binding to several near-consensus sites in the BCL2 promoter, one of which plays a crucial role in Gli1-mediated induction. Likewise, Gli1 over-expression in the mouse keratinocytes (Bigelow et al., 2004)
and over-expression of the Gli3 dominant-active form in developing chick embryos leads to induction of \textit{Bcl2} (Cayuso et al., 2006). In contrast to this transcriptional mechanism, another study proposed that in the absence of Shh Patched1 mediates neural tube apoptosis, in a way that is independent of the canonical Shh signaling pathway and to function through more direct caspase activation (Thibert et al., 2003). Patched1 could function in this context as a dependency receptor, i.e. a receptor inducing cell death in the absence of cognate ligand binding (Chao, 2003; Guerrero and Ruiz i Altaba, 2003). However, another study (Cayuso et al., 2006) concluded that Hh survival function is mediated entirely by Gli transcription factors. Consistent with and complementary to the results in this thesis, Abe et al (2008) showed that activation of Hh signaling can suppress p53 pathway in tissue culture systems. To activate Hh signaling, the authors employed dominant-active Smo mutants and Gli transcription factor overexpression. They found that Hh signaling activation leads to phosphorylation of Hdm2 (human Mdm2) on serines 166 and 186. The phosphorylated Hdm2 becomes activated and catalyses ubiquitination of p53, which leads to suppression of p53-mediated tumour suppression in conditions of DNA damage and oncogenic stress. The authors concluded that Hh signaling induces expression of an unknown factor activating Mdm2. The results by Abe et al. (2008) raise the question whether p53 induces apoptosis \textit{in vivo} in the absence of Shh signaling. Taken together, the studies suggest that we still know very little about the role of Shh in cell survival and the signal transduction mechanisms activating cell death machinery in the absence of Shh. Another important topic is the fate of surviving cells when apoptosis due to \textit{shh} loss is suppressed.

1.7 \textbf{p53 pathway in apoptosis induction and cell cycle regulation}

Because of its tumour-suppressive role, p53 is one of the best-investigated proteins. The most fundamental function of p53 is to allow cells to respond to a variety of stresses as DNA damage, hypoxia, nucleotide depletion, and aberrant growth signals by oncogenes. Activation of p53 by DNA damage and oncogenes are considered of highest relevance for tumour suppression. The signal transduction from DNA damage to p53 is accomplished by ATM/ATR, Chk1 and Chk2 kinases (Fig. 5). Oncogenic signaling in mammalian systems leads to elevation of \textit{p14ARF (ARF)} gene expression, which activates p53 through Mdm2 inhibition (Fig. 5; Efeyan and Serrano, 2007). Both oncogenic signaling to ARF (Efeyan and
Serrano, 2007) and DNA damage response signaling (Vousden and Lane, 2007) may play important roles in p53-mediated tumour suppression. The best investigated functions of p53 are apoptosis induction, cell cycle arrest and senescence. However, recent studies (reviewed in Vousden and Lane, 2007) show that p53 regulates glycolysis and oxidative stress regulation, cell death by autophagy, repair of genotoxic damage and cell differentiation. p53 activation by AMP kinase due to a low glucose level helps cell survive starvation conditions (Jones et al., 2005). p53 also regulates metabolism under normal conditions by activating expression of SCO2, cytochrome c oxidase assembly factor, and thus promotes oxidative phosphorylation in mitochondria (Matoba et al., 2006) and this could be the reason why p53 mutant mice have low endurance. Additionally, p53 regulates expression of many proteins with anti-oxidant functions such as sestrins, ALDH4 and TIGAR (Vousden and Lane, 2007; Bensaad et al., 2007). Another novel function of p53 is to induce autophagy, digestion of cellular contents by vesicular organelles autophagosomes, by *DRAM* gene activation (Crighton et al., 2006).

Regulation of p53 activity and abundance depends on two factors: Mdm2 and Mdm4. While Mdm2 is required for degradation of p53, Mdm4 inhibits its activity (Marine and Jochemsen, 2004). For p53 to become activated and more abundant, the negative regulation by Mdm2 must be prevented. The precise mechanism is still unknown, however, the currently favored model is that in unstressed cells, Mdm2 controls p53 stability by ubiquitination, which targets p53 to proteasomal degradation, and Mdm4 blocks the transcription activation domain of p53 (Toledo and Wahl, 2006). Upon DNA damage and other stresses, Mdm2 becomes modified and promotes degradation of itself and Mdm4 (Stommel and Wahl, 2004). This eventually leads to higher p53 activity, resulting in an increase in Mdm2 expression further increasing p53 activity. Upon stress relief, Mdm2 starts targeting p53 and stops promoting Mdm4 degradation, which brings the system back to the unstressed condition. Due to importance of Mdm2 and Mdm4, factors regulating these proteins may be involved in p53 activation.

Cell cycle regulation is another important function of p53. Cell cycle consists of several phases dividing the time from one cell division to the next. G1-phase is the period when cells growth and preparation to DNA replication occur. During S-phase DNA is replicated and in the second growth G2-phase, cells prepare to divide through mitosis (M-phase). To prevent inappropriate cell cycle progression upon damage, cells employ cell-cycle checkpoints, in
which p53 plays a key role. One of the p53-mediated cell responses is cell-cycle arrest, a transient block of cells in G1 or G2/M phase (reviewed Brown et al., 2007). Induction of CDKN1A gene encoding p21 cyclin-dependent kinase (CDK) inhibitor by p53 leads to cell cycle arrest before G1/S transition. p21 inhibits Cdk4/6-mediated phosphorylation and inactivation of Retinoblastoma (Rb) protein. Indeed, loss of CDKN1A strongly impairs p53-mediated cell cycle arrest in fibroblasts after irradiation (Brugarolas et al., 1995). Unphosphorylated Rb represses E2F-regulated cell cycle genes involved in S-phase progression. Expression of p21 leads to accumulation of unphosphorylated Rb and maintenance of S-phase cell cycle gene repression. Thus, a model emerged that p53 regulates cell cycle directly through p21-mediated CDK inhibition and indirectly, through Rb, inhibits expression of S-phase cell cycle genes (Liebermann et al., 2007). More recently, several studies established that members of the miR-34 family are p53 targets with important roles in p53-mediated G1/S transition regulation (reviewed in He et al., 2007). More specifically, miR-34 can inhibit translation of CDK4, CDK6, cyclinE2 and E2F3 thereby blocking G1- to S-phase cell cycle progression. Another mechanism of cell cycle regulation by p53 is repression of C-MYC, a gene involved in G1/S cell cycle progression. Induction of G2/M arrest was found to be a much more complex process with many potential effectors induced by p53 (Brown et al., 2007). p53 regulates CyclinB-Cdk1 complex expression by inhibiting NF-Y transcription factor which regulates expression of CyclinB and Cdk1. Moreover, expression of p53 targets p21 and GADD45α inhibits Cdk1 kinase activity. Cdc25C is required for activatory dephosphorylation of Cdk1, and p53 prevents this Cdk1 dephosphorylation by repressing expression of Cdc25C gene. p53 also influences localisation of CyclinB-Cdk1 complexes by activating 14-3-3σ gene. 14-3-3σ prevents nuclear localization of CyclinB-Cdk1 complex and Cdc25C. GADD45α is another p53 target involved in G2/M cell-cycle arrest, which disrupts CyclinB-Cdk1 complex. The list of other p53-regulated genes with less well-characterized functions in G2/M cell-cycle arrest includes Reprimo, MCG10 and Gtse-1.

The essential tumour-suppressive role of p53 is induction of apoptosis. Most studies suggest that p53 mediates apoptosis via the intrinsic mitochondrial apoptotic pathway. Transcriptional and transcription-independent activities (TIA) of p53 were proposed to be involved in the process. As mutations in p53 affect both its transcription activity and its localization and activity in mitochondria, it has not been possible to unambiguously separate
the two activities (Schuler and Green, 2005). It has been shown that p53 rapidly localizes to mitochondria after stress induction. Interestingly, proline-rich domain of p53 is not required for p53-mediated transactivation but is necessary for induction of apoptosis. P72R mutation in this domain enhanced p53 apoptotic activity through more efficient mitochondrial localization due to enhanced nuclear export. In addition, p53 physically interacts with Bcl-2 and Bcl-xL anti-apoptotic proteins suggesting a functional similarity of p53 to BH3-only pro-apoptotic Bcl-2 proteins (Yee and Vousden, 2003). \textit{In vitro} models of p53 TIA showed that purified p53 from DNA-damaged cells combined with Bax could permeabilise mitochondrial and liposomal membranes.

The transactivation function of p53 is clearly required for apoptosis induction by p53. Some of p53 targets regulate generation of reactive oxygen species (ROS) with a strong damaging potential for DNA, membrane lipids and proteins. ROS excess may lead to apoptosis probably through disruption of mitochondrial membranes. Importantly, ROS generation can be both suppressed and induced by p53 (Michalak et al., 2005). Suppression of ROS generation can be achieved by p53-responsive sestrin genes PA26 and Hi95. Sestrin proteins PA26 and Hi95 are enzymes producing peroxiredoxins maintaining cellular redox balance. p53 also induces expression of ALDH4 proline degradation enzyme protecting cells from increased levels of ROS. TIGAR is a p53 target which negatively regulates glycolisis by enzymatically reducing the level of Fructose-2,6-bisphosphate (Bensaad et al., 2007). Thus, glucose becomes diverted into the pentose pathway generating NADPH, a potent anti-oxidant, which neutralizes reactive oxygenespecies (ROS) and contributes to cell survival. By contrast, induction of ROS may also be induced by p53. For instance, PIG8 and PIG3 p53 target genes were implicated into generation of ROS and induction of apoptosis.

Intrinsic pathway of apoptosis is activated by disruption of the outer mitochondrial membrane and subsequent release of cytochrome C. Cytochrome C binds Apaf1 to form the apoptosome complex which serves to activate Caspase9 and downstream effector caspases (Fig. 6). Apaf-1, the integral component of the apoptosome (Fig. 6), was shown to be a transcriptional p53 target (Fortin et al., 2001). However, loss of APAF-1 does not completely protect cells from death and in p53-deficient mice its expression is not affected. Intrinsic apoptotic pathway is activated by BH3-only Bcl2 family proteins, which interact with downstream pro-apoptotic Bcl2 family proteins Bax and Bak. Pro-apoptotic Bcl2 family
proteins are antagonized by anti-apoptotic Bcl2 family members. There is now abundant evidence that p53-mediated apoptosis can be inhibited by Bcl2 overexpression, removal of BAX and BAK genes and by loss of BH3-only Bcl2 family member PUMA (Michalak et al., 2005). Consistent with these findings, p53 regulates expression of Bcl2 family genes. BAX was the first identified p53 target involved in intrinsic pathway of apoptosis (Selvakumaran et al., 1994). Although an increased Bax level is not sufficient for cell death, it enhances induction of apoptosis. Another p53 target is BID is a BH3-only Bcl2 family member (Sax et al., 2003), whose uncleaved form has a low pro-apoptotic activity but can be cleaved by Caspase-8 and other caspases generating tBid form capable of disrupting mitochondrial membrane (Fig. 6). This cleavage reaction serves as a connection of intrinsic and extrinsic pathways. However, bid mutant mice had normal apoptosis after γ-irradiation suggesting a contributory role for Bid in p53-mediated apoptosis. NOXA is another BH3-only Bcl-2 family p53 target involved in apoptosis and tumour suppression. NOXA is induced by p53 after γ-irradiation and under conditions of oncogenic stress, and it loss leads to a significant reduction of apoptotic levels after genotoxic stress (Oda et al., 2000). The most essential BH3-only Bcl-2 family gene for p53-dependent apoptosis is PUMA, which is induced by p53 under a variety of stress conditions (Nakano and Vousden, 2001). Apoptosis induction by Puma requires both Bax and Bak, suggesting that Puma acts upstream of these factors, by neutralizing anti-apoptotic Bcl-2 proteins, and thus helps Bax and Bak multimerise and permeabilize the outer mitochondrial membrane leading to activation of apoptosis (Fig. 6; Chipuk and Green, 2008). Furthermore, loss of PUMA protects cells from p53-mediated apoptosis in mouse and zebrafish (Kratz et al., 2006; Nakano and Vousden, 2001).

**Figure 5.** An overview of the p53 pathway inputs, signal transduction and outputs. DNA damage, oncogenes, hypoxia, nutrient depletion and other factors are the stresses activating p53 response. Signal transducers such as ATM, ATR, Chk2 and p14ARF are involved in control of p53 activity by phosphorylation or direct binding. Activity of both p53 and Mdm2 can be regulated by ATM and perhaps other kinases. Regulation of p53-Mdm2 negative feedback loop and of Mdm4, p53 inhibitor, leads to p53 activation. Additional post-translational modifications, histone acetyl transferases (HAT) and cofactor binding regulate the specificity of p53 promoter binding. Subsequent expression of p53 target genes involved in DNA repair (DDB2, DDIT4, GADD45a, TRIM22), cell-cycle arrest (CDKN1A, SFN, TP53I3, CDC25) or apoptosis (PUMA, NOXA, BAX, BID, PIG3) leads to depicted outcomes. The figure was adopted with modifications from Riley et al. (2008).
INTRODUCTION

The diagram illustrates the p53-inducing stresses and their signaling pathways. Stress signals are transmitted through various transducers such as ATM, ATR, Chk2, and p14ARF. These transducers then phosphorylate p53, which leads to its transcription and dimerization with Mdm2. Additional modifications by CBP, p300, PCAF, etc., can further influence p53 activity. p53 binds to p53RE (response element) and recruits cofactors for its target gene expression. The effector proteins include DDB2, DDIT4, GADD45a, TRIM22, CDKN1A, SFN, TP53I3, CDC25, PUMA, NOXA, BAX, BID, and PIG3. The outcomes include DNA repair, cell-cycle arrest, and apoptosis.
Cells with active p53 typically face a choice between p53-mediated apoptosis and cell cycle arrest. However, the choices of cells with active p53 are often much more complex than this simple binary choice. In different tissues, p53 induction in response to irradiation may occur or not, and even when p53 protein level increases no apoptosis may be induced. The mechanism(s) for these differences is still unknown, although special post-translational modifications of p53 and its co-factor interactions may play a role (Murray-Zmijewski et al., 2008). Several post-translational modifications influence the choice between p53-mediated apoptosis and cell cycle arrest (Das et al., 2008). Ser46-phosphorylated p53 has a higher propensity to activate pro-apoptotic genes than \( p21 \). After severe DNA damage, p53 becomes acetylated at Lys120 and preferentially activates \textit{Bax} and \textit{Puma}. By contrast, ubiquitination of Lys320 of p53 leads to preferential activation of cell-cycle inhibitory genes by p53. Other known mechanisms for regulating the outcomes of p53 activation are gene-specific chromatin modification and protein cofactor binding to p53.
Intrinsic pathway of apoptosis is initiated at the outer mitochondrial membrane by Puma, Noxa, Bax, tBid and p53 which induce pore formation in this membrane. These pores allow release of cytochrome C from mitochondria, which can complex with Apaf-1 protein and Caspase9 to form the apoptosome complex. Caspase9 in the apoptosome complex proteolytically activates Caspase3, which in turn activates Caspases6 and 7. Active caspases cleave many proteins to eventually induce apoptosis. p53 regulates the intrinsic pathway of apoptosis by either itself affecting outer mitochondrial membrane permeability or inducing expression of genes which do this (shown in mars red color in this figure). Extrinsic pathway of apoptosis is activated by ligands (such as TRAIL or FASL) binding to their receptors, death receptors. Ligand-bound death receptors become activated, complexes of adaptor molecules assembled on them activate Caspase8, an initiator caspase activating Caspase3. Caspase 8 also cleaves Bid protein transforming it into a highly active BH3-only mitochondria-permeabilizing factor. For clarity only the best-investigated factors are shown and their interactions among each other and with anti-apoptotic Bcl-2 proteins are not depicted.
1.8 Evidence for interaction of Hedgehog and p53 pathways

Activation of Hh signaling has oncogenic properties in a number of contexts. To understand how Hh signaling is linked to tumorigenesis, it is necessary to discover the connections between the Hh pathway and other oncogenes and tumour suppressors. p53 is considered the most important tumour suppressor and interacts with many proteins and pathways. Interestingly, Hh signaling is one of p53-interacting signaling pathways. Wetmore and colleagues (2001) have provided the first evidence for a genetic interaction of p53 and Shh signaling pathway in the context of medulloblastoma development. Mice or humans heterozygous for patched mutation (patched+/–) show a strongly increased incidence of medulloblastoma most likely due to an increased level of Hh signaling. The authors also found that p53 loss in patched+/– background leads 95% of mice to develop medulloblastoma compared to only 14% of patched+/-p53+/- mice. p53 also interacts genetically with Sufu, a negative regulator of Shh signaling (Lee et al., 2007). While Sufu+/- heterozygote mice did not develop medulloblastoma with higher frequency, Sufu+/- p53+/- heterozygotes had medulloblastoma incidence of 58%, suggesting that activated Hh signaling can cause medulloblastoma development if not controlled by p53. In basal cell carcinoma (BCC) mutations in patched and p53 are found with a higher incidence (Kim et al., 2002; Reifenberger et al., 2005). Moreover, in gliomas requiring Hh signaling for development and maintenance (Clement et al., 2007) p53 mutations are the most frequent genetic alterations (Ohgaki and Kleihues, 2007). Cancer stem cells in glioma tumours present activated Hh signaling due to higher expression of Gli1, which is required for cancer stem cell self-renewal and overall tumour proliferation and survival. Studies of genetic interactions give important clues for further research but do not provide mechanistic explanations of the observed phenomena. A recent study by Stecca and Ruiz i Altaba (2009) described a negative feedback loop between p53 and Gli1 in the mouse, which helps explain the genetic interaction of p53 and Shh signaling. The authors observed that p53 can inhibit Gli1-mediated proliferation, neurosphere formation and positive transcriptional regulation in luciferase assays. p53 also promoted cytoplasmic localization of Gli1, which interferes with its normal nuclear localization and may direct Gli1 to proteosomal degradation. Closing the loop, Gli1 overexpression led to a striking increase of Mdm2 expression and down-regulation of p53 in the mouse brain and several cell lines (Stecca and Ruiz iAltaba, 2009). These results are
consistent with the previously reported finding that Hh signaling can negatively regulate p53 pathway (Abe et al., 2008).

1.9 Zebrfish as a model system

Different organisms have been used as model systems for understanding fundamental biological problems and pathologies. More precisely, a model system can be defined as an organism with useful features together with information resources and powerful experimental tools, which enable convenient and productive research. The zebrafish (Danio rerio) as a model system was established in early 1980s by George Streisinger and co-workers. In the last 20-30 years zebrafish has become very popular due to the fact that it is a vertebrate of small size with relatively short life cycle and rapid external development, high number of progeny and can be easily maintained in the lab. Moreover, the embryo is transparent, a property important for in vivo microscopy, and transgenic technologies enable imaging of a variety of cells and processes in the zebrafish embryos. Finally, forward and reverse genetic approaches are available to uncover and characterize gene functions.

1.10 Aims of this thesis

The work presented here aimed to investigate the role of Shh in controlling proliferation and cell survival. Shh and Fgf signalling pathways are good candidate mechanisms how to control proliferation during limb development, since they are both known to directly regulate proliferation in other contexts. However, it has been an open question whether and how they work together to regulate proliferation of limb bud cells. I set out to investigate this question using shh–/– mutant analysis, pharmacological inhibition of Hh and Fgf signaling and activation of Fgf signaling using recombinant FGF protein.

Shh is known to provide survival signals in a way that is yet unknown. My aim was to investigate the existence of a common mechanism underlying the survival function of Shh in different tissues. I have identified p53 pathway as the apoptosis-inducing mechanism in shh–/– mutant and created a reporter to study p53 activation. Another aim was to confirm that normal Hh signaling pathway is required to control p53 activity. Since p53 also affects cell cycle progression, I aimed to understand how p53 affects proliferation and differentiation of retinal progenitor cells in the absence of Shh.
2 Results
2.1 Shh and Fgf signaling pathways in regulating fin bud cell proliferation

2.1.1 Loss of Shh signaling leads to reduction of cell cycle gene expression correlating with Fgf signaling status

In order to investigate the role of Shh in regulating cell-cycle progression in the pectoral fin buds, I analyzed the expression of G1- and S-phase cell-cycle genes in the pectoral fin buds of zebrafish shh−/− mutants. Analysis was focused on cyclinD1, pcna and mcm5, which are generally used as markers of proliferating cells in zebrafish (Shepard et al., 2004; Ryu et al., 2006). Expression of cyclinD1 is necessary for G1-progression and S-phase entry, while pcna and mcm5 are necessary for DNA replication during S-phase (Ohtani, 1999). As a control, I analyzed expression of replication protein A1 gene (ra1), which is expressed constitutively in all cells of the fin bud. At 32 hpf, cyclinD1, pcna, mcm5 and ra1 are expressed at very similar levels in wild-type and in shh−/− mutant fin buds (Fig. 7C-F, I-L). Since expression of the Shh target patched1 (ptc1) (Concordet et al., 1996) is absent from shh−/− mutant fin buds at all stages (Fig. 7A, M), these results indicate that expression of G1- and S-phase cell-cycle genes is independent of Shh at 32hpf. Examination of these cell-cycle genes at 38hpf, however, reveals that cyclinD1, pcna, and mcm5 expression are lost in shh−/− mutant fin buds, while ra1 remains unaltered (Fig. 7O-R, V-X), suggesting that cell-cycle progression becomes dependent on Shh signaling at later stages. Since the expression of Fgf ligands in the AER depends on Shh activity (Capdevila and Izpisúa Belmonte, 2001; Neumann et al., 1999), I also tested whether the activity of the Fgf signaling pathway in shh−/− mutant fin buds correlates with the observed reduction in cell-cycle gene expression. Using the Fgf-target pea3 as a marker for Fgf signaling (Roehl and Nüsslein-Volhard, 2001), I find that pea3 expression in shh−/− mutant pectoral fin buds is identical to wild-type fin buds at 32hpf, but is strongly reduced at 38hpf (Fig. 7B, H, N, U). This result is consistent with the observation that Shh is necessary for maintenance of Fgf expression in the AER, and suggests a correlation between the activity of Fgf signaling and the expression of cell-cycle genes in shh−/− mutant fin buds. Taken together, these results show that expression of G1- and S-phase cell-cycle genes is initially normal in shh−/− mutant pectoral fin buds, but is later lost, and that this shift correlates with a similar loss of Fgf signaling activity at later stages.
Figure 7. G1- and S-phase cell-cycle gene expression in fin buds of \( shh^{-/-} \) mutant correlates with the Fgf signaling status.

Wild-type embryos and \( shh^{-/-} \) mutant embryos at 32 hpf (A-L) and 38 hpf (M-Y) were analysed for expression of the Shh target patched1 (ptc1) (A, G, M; T), the Fgf target pea3 (B, H, N, U), the cell-cycle genes cyclinD1, pcna, and mcm5 (C-E, I-K, O-R, V-X), and replication protein A1 (ra1) (F, L, S, Y). The Shh target ptc1 was expressed in the posterior part of wild-type fin buds at 32 and 38 hpf stages (A, M), but its expression was absent in \( shh^{-/-} \) mutant fin buds (G, T). The Fgf signaling target pea3 was expressed at comparable levels in wild-type and \( shh^{-/-} \) mutant fin buds at 32 hpf stage (B, H). At 38 hpf pea3 was still strongly expressed in the wild-type fin buds (N), but almost completely absent in the \( shh^{-/-} \) mutant fin buds (U). cyclinD1, pcna and mcm5 were expressed strongly in both wild-type and \( shh^{-/-} \) mutant fin buds at 32 hpf stage (C-E, I-K). At 38 hpf these genes were still strongly expressed in the wild-type fin buds (O-R), but downregulated in the \( shh^{-/-} \) mutant fin buds (V-X). Expression of ra1 was similar in both wild-type and \( shh^{-/-} \) mutant fin buds at 32 and 38 hpf stages.

Since the loss of G1- and S-phase cell-cycle genes in \( shh^{-/-} \) mutant fin buds occurs relatively late, and only after Fgf signaling is lost, selective inhibition of Hh signaling using the plant alcaloid cyclopamine (Incardona et al., 1998) was employed to determine the time period of inhibition necessary to affect cell-cycle progression. Cyclopamine inhibits the action of Smoothened protein, which transduces the Hh signal after it becomes released from...
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Patched1-mediated inhibition (Chen et al., 2002). The use of cyclopamine allows inhibition of Hh signaling for varying periods of time, and thereby temporal control over the signaling inhibition. My aim was to find duration of cyclopamine treatment sufficient to inhibit Hh signaling, but leaving Fgf signaling largely unaffected, thereby uncoupling the two pathways from each other. I find that a 6-hour treatment from 34 to 40 hpf with 100 μM cyclopamine is sufficient to inhibit ptc1 expression almost completely (Fig. 8A, D), but has little effect on expression of the Fgf-target pea3 (Fig. 8B, E). Likewise, this treatment has little or no effect on cyclinD1, pcna, mcm5 and ra1 expression (Fig. 8C, G-I, F, J-L). By contrast, however, a 13-hour cyclopopamine treatment from 34 to 47 hpf leads to loss of both ptc1 and pea3 expression (Fig. 9A, B, D, E), and also leads to strong reduction of cyclinD1, pcna, and mcm5 expression, but without affecting ra1 (Fig. 9C, G-I, F, J-L). These results show that loss of Shh signaling leads to loss of cell-cycle gene expression only after a 13-hour delay, indicating that this is likely to be an indirect effect. Since after this delay period cell-cycle gene expression loss correlates closely with reduction of Fgf signaling in response to Shh inhibition, Fgf5s are very good candidates for mediating the effect of Shh on cell-cycle progression in the fin bud.
Figure 8. Cyclopamine inhibition for 6 hours does not strongly affect Fgf signaling and cell-cycle gene expression.

Wild-type embryos were treated with 100 μM cyclopamine (cyA) (D-F, J-L) or with the carrier 0.5 % ethanol (EtOH) (A-C, G-I) for 6 hours from 34 to 40 hpf (A-L) and analysed for the expression of ptc1, pea3, cyclinD1, pcna, mcm5 and ra1. Expression of ptc1 was nearly completely lost after the treatment (A, D). Hh signaling inhibition for 6 hours led to a small change in pea3 expression in fin buds (B, E). Comparably small changes in expression after the treatment were observed for cyclinD1, pcna, mcm5 and ra1 (C,F; G, J; H, K; I, L).
Figure 9. Cyclopamine inhibition for 13 hours is sufficient to inhibit Fgf signaling and cell cycle gene expression.
Wild-type embryos were treated with 100 μM cyclopamine (cyA) (D-F, J-L) or with the carrier 0.5 % ethanol (EtOH) for 13 hours from 34 to 47 hpf (A-C, G-I) and analysed for the expression of ptc1, pea3, cyclinD1, pcna, mcm5 and ra1. Expression of ptc1 was lost after the 13-hour (A, D) inhibition period. After 13-hour Hh signaling inhibition, fin bud pea3 expression was strongly decreased (B, E). Likewise, expression of cyclinD1, pcna and mcm5 in fin buds was strongly downregulated (C, F; G, J; H, K). Expression of ra1 gene was only mildly affected by 13-hour cyclopamine treatment (I, L).
2.1.2 Fgf signaling inhibition leads to rapid loss of cell-cycle gene expression and proliferation decrease in the fin buds

Following the observation that Hh signaling inhibition strongly affects expression of cell-cycle genes after a 13-hour cyclopamine treatment, I decided to investigate how rapid the response of the same genes is to inhibition of Fgf signaling. For this purpose, I used the chemical inhibitor SU5402, which inhibits signaling by Fgf receptor in mouse cell culture lines (Mohammadi et al., 1997) and in zebrafish embryos (Roehl and Nüsslein-Volhard, 2001). I find that treatment with 10 μM SU5402 for 3 hours between 36 and 39 hpf leads to nearly complete loss of the Fgf-target *pea3* (Fig. 10A, D), while expression of the Shh target *ptc1* is hardly affected (Fig. 10B, E), indicating that, under these conditions, Fgf signaling is blocked whereas Shh signaling is still intact. This 3-hour inhibition of Fgf signaling is sufficient to cause a nearly complete loss of expression of *cyclinD1* (Fig. 10C, F), *pcna* (Fig. 10G, J) and *mcm5* (Fig. 10H, K) in fin buds, while *ra1* (Fig. 10I, L) is unaffected. Interestingly, *cyclinD1*, *pcna*, and *mcm5* expression are also lost from the branchial arch primordia following this treatment (Fig. 10C, F, G, J, H, K). Consistent with the loss of expression of G1- and S-phase genes after 3 hours of SU5402 treatment, the number of cells labelled with 5-bromodeoxyuridine (BrdU) is also strongly reduced in the fin buds under these conditions (Fig. 11A, B). These results show that the effect of Fgf signaling on cell-cycle progression in the fin buds is much more rapid than the effect of Shh signaling, since there is a severe down-regulation of cell-cycle genes already after 3 hours of Fgf signaling inhibition.
Figure 10. Fgf signaling inhibition leads to loss of G1- and S-phase cell cycle gene expression. Wild-type embryos were treated with 10 μM SU5402 (A-C, G-I) or the carrier 0.125 % DMSO (D-F, J-L) for 3 hours from 36 to 39 hpf. SU5402 treatment strongly downregulated the expression of *pea3* FGF signaling target (A, D), but had only a small effect on *ptc1* expression in fin buds (B, E). SU5402 treatment also caused strong down-regulation of *cyclinD1* (C, F), *pcna* (G, J) and *mcm5* (H, K). Expression of *ra1* was not affected by SU5402 treatment (I, L).
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Figure 11. Fgf signaling inhibition reduces proliferation in zebrafish fin buds. Wild-type embryos were treated with 10 μM SU5402 (A) or 0.125 % DMSO (B) for 3 hours from 36 to 39 hpf. After 3-hour treatment with either SU5402 or DMSO, embryos were injected with 10 mM BrdU solution into the yolk and incubated for 1 hour in the same solutions before fixation. S-phase progression, as revealed by BrdU labeling, was strongly inhibited after SU5402 treatment, in comparison to control embryos (n = 10, at least 2 fin sections per embryo were analysed) (A, B).

Since blockage of Fgf signaling with SU5402 leads to rapid loss of G1- and S-phase gene expression both in the pectoral fin buds and in the branchial arches, I also checked whether Fgf signaling is required for proliferation in other tissues. Therefore, an inhibitor treatment at 20 hpf was performed, at a stage at which many embryonic cells are still proliferative. After 3 hours of treatment with 10 μM of SU5402, expression of *pea3* is almost completely lost in these embryos, but *cyclinD1*, *pcna*, *mcm5* and *ral* expression is unaltered (Fig. 12A-C, G, H; D-F, I, J). Furthermore, while SU5402 treatment at 36 hpf leads to loss of cell-cycle genes from both the pectoral fin buds and the branchial arches, it has no effect on the same genes expressed in the retina and the optic tectum. These results indicate that Fgf signaling is not generally required for proliferation in the whole embryo, but that it instead directs expression of cell-cycle genes specifically in the pectoral fin buds and in the branchial arches.
Figure 12. Fgf signaling inhibition at 20 hpf does not lead to global reduction of cell-cycle gene expression. Wild-type embryos were treated with 10 μM SU5402 (A-C, G,H) or 0.125 % DMSO (D-F, I, J) for 3 hours from 20 to 23 hpf. SU5402 treatment caused a strong downregulation of \textit{pea3} gene expression (A, D), but expression of \textit{cyclinD1}, \textit{pena}, \textit{mcm5} and \textit{ra1} genes was not changed in SU5402-treated embryos compared to control ones (B, E; C, F; G, I; H, J).

### 2.1.1 Activation of Fgf signaling in \textit{shh}^{-/-} mutant induces cell-cycle gene expression and proliferation

The results presented so far strongly suggest that Fgf signaling is directly required for cell-cycle progression in zebrafish fin buds, while Shh plays an indirect role via its regulation of Fgf expression. However, since the Shh and Fgf signaling pathways in the limb bud are coupled by a feedback loop mechanism, it is difficult to change the activity of one pathway without affecting the other. Therefore, I decided to use a gain-of-function experiment to uncouple the Fgf pathway completely from the Shh pathway, by providing an ectopic source of Fgf protein in \textit{shh}^{-/-} mutant fin buds, and asking if this ectopic source of Fgf signaling is able
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to rescue cell-cycle progression in the absence of Shh. For this purpose, heparin gel beads were soaked with recombinant human FGF4 protein and implanted into fin buds on the right hand side of shh⁻/⁻ mutant embryos at 29-32 hpf. The fin buds on the left hand side were not implanted and served as an internal control in these experiments. Implanted embryos were then grown to 50 hpf and gene expression was analysed by *in situ* hybridisation. I find that FGF4-soaked beads induce *pea3* expression in shh⁻/⁻ mutant fin buds (Fig. 13A). Furthermore, *cyclinD1* (Fig. 13B), *pcna* (Fig. 13C) and *mcm5* (Fig. 13D, D’, D’’) transcripts are also induced in the fin buds implanted with FGF4-soaked beads. Consistent with these results, I also detect increased incorporation of BrdU in bead-implanted shh⁻/⁻ mutant fin buds, compared to unimplanted fin buds (Fig. 14A, B). Finally, there is increased growth of shh⁻/⁻ mutant fin buds with implanted FGF4-soaked beads (Fig. 13D’, D’’, 14C, D), further supporting the conclusion that Fgf signaling is able to restore outgrowth in the absence of Shh. This increase in fin size after bead-implantation is somewhat variable and depends on bead position relative to the fin bud, with the largest outgrowth observed when beads are located right inside the bud (Fig 14C, D). Taken together, these results indicate that Fgf signaling is sufficient to direct proliferation in zebrafish fin buds in the absence of Shh.
Figure 13. Human FGF4 bead implantation leads to induction of Fgf signaling and cell cycle gene expression in \( shh^{-/-} \) mutant.

Fin buds on the right hand side of \( shh^{-/-} \) mutant embryos were implanted with FGF4-soaked heparin beads at 29-32 hpf, grown until 50 hpf and fixed (A-D). \( shh^{-/-} \) mutant embryos show upregulation of \( pea3 \) (A), \( cyclinD1 \) (B), \( pcna \) (C) and \( mcm5 \) (D) expression in response to the FGF4-soaked beads on the implanted side. Fin buds are outlined by dotted lines in panels A to D. A non-implanted fin bud on the left hand side shows no \( mcm5 \) expression (D’), while an implanted fin bud on the right-hand side of the same embryo (D”) shows restored \( mcm5 \) expression. Fin buds implanted with FGF4 beads show increased outgrowth (D”), compared to non-implanted control fin buds (D’).
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Figure 14. Human FGF4 bead implantation induces fin bud proliferation and outgrowth in shh<sup>-/-</sup> mutant embryos.

Fin buds on the right hand side of shh<sup>-/-</sup> mutant embryos were implanted with FGF4-soaked heparin beads at 29-32 hpf, grown until 50 hpf and fixed (A-D). For anti-BrdU staining, embryos were first implanted and then injected with 10 mM BrdU solution at 38 hpf before fixation at 50 hpf. The non-implanted fin bud shows few BrdU-labeled nuclei (C), while an FGF4 bead-implanted fin bud (D) has extensive BrdU labeling (sections of both sides of 10 bead-implanted embryos were analysed). Fin buds implanted with FGF4 beads show increased outgrowth (D), compared to non-implanted control fin buds (C).
2.2. The mechanism of the survival function of Shh

2.2.1 Apoptotic phenotype of zebrafish shh<sup>−/−</sup> mutant

Previous work on the survival function of Shh leads to the question whether different instances of apoptosis due to loss of Shh signaling share the same mechanism or may have different mechanisms or be secondary consequences of the lack of differentiation signals provided by Shh. To approach this question systematically, I analysed *shh<sup>−/−</sup>* mutant and wild-type embryos at several stages for apoptosis patterns and levels using whole-mount ApopTag staining, and using fluorescent TdT-mediated dUTP-TMRRed nick end labeling (TUNEL) staining on retinal sections. Representing most of the early zebrafish development, embryos were analysed at 24, 30, 36, 48 and 60 hpf. Mutants in *shh* gene cannot be clearly recognized until around 24 hpf, so 24 hpf was the earliest stage analysed. Apoptosis levels in *shh<sup>−/−</sup>* mutant were much higher than in wild-type at all stages. Wild-type embryos showed a low-level stereotypic pattern of apoptosis (Fig. 15D-F, I, J) previously described in zebrafish (Cole and Ross, 2001). At 24 hpf, most of the neural tube of *shh<sup>−/−</sup>* mutant except for its anterior part contained high numbers of apoptotic cells (Fig. 15A). At 30 and 36 hpf, neural tube apoptosis persisted, but apoptotic cells became localized more dorsally (Fig. 15B, C). Since neural crest cells also undergo apoptosis in the absence of Shh, dorsal apoptotic cells in zebrafish *shh<sup>−/−</sup>* mutant at 24 and 30 hpf are very likely neural crest cells (Fig. 16A, B). At 48 and 60 hpf, neural tube apoptosis became even more dorsally restricted, its level decreased, and apoptotic cells appeared in the retina (Fig. 15G, H).
Figure 15. Apoptosis levels in shh<sup>−/−</sup> mutant and wild-type embryos by whole-mount ApopTag staining.
Wild-type (D-F, I, J) and shh<sup>−/−</sup> mutant (A-C, G, H) embryos were stained for apoptotic cells using the ApopTag protocol. At all five stages analysed (24 hpf (A, D), 30 hpf (B, E), 36 hpf (C, F), 48 hpf (G, I), 60 hpf (H, J)) shh<sup>−/−</sup> mutant embryos had dramatically increased levels of apoptosis in the neural tube decreasing over the course of development compared to wild-type. At 48 and 60 hpf stages, high level of retinal apoptosis was also observed in shh<sup>−/−</sup> mutant embryos.

Retinal apoptosis was analysed in shh<sup>−/−</sup> mutant and wild-type embryos at 24, 36, 48 and 60 hpf using TUNEL staining. At 24 hpf, both wild-type and shh<sup>−/−</sup> mutant retinas typically contained very few TUNEL-positive cells (Fig. 16A, E). By contrast, midbrain region of 24 hpf shh<sup>−/−</sup> mutants contained a high number of apoptotic cells (Figure 16A) showing a different timing of apoptosis in the shh<sup>−/−</sup> mutant retina and midbrain. Wild-type and shh<sup>−/−</sup> mutant retinas at 36 hpf were very similar in their low numbers of apoptotic cells and had significant apoptosis in their lenses (Fig. 16B, F). At 48 hpf, apoptosis level in shh<sup>−/−</sup> mutant retinas increased in comparison to that in the wild-type retinas (Fig. 16C, G). Finally, at 60 hpf there is a dramatic difference in apoptotic levels in wild-type and shh<sup>−/−</sup> mutant retinas, with
the mutant retina containing many apoptotic cells (Fig. 16D, H). These results are consistent with the result reported by Neumann and Nüsslein-Volhard (2000) that apoptosis in the shh\(^{-/-}\) mutant retina is not increased before 48 hpf. The distinct timing of apoptosis in different regions of shh\(^{-/-}\) mutant embryos may suggest different apoptotic mechanisms or reflect differences in Shh expression requirements for cell survival in different tissues, but the same mechanism of apoptosis induction.

**Figure 16.** Retinal apoptosis in zebrafish shh\(^{-/-}\) mutant and wild-type embryos during development.

Wild-type (A-D) and shh\(^{-/-}\) mutant (E-H) retinal sections were TUNEL stained. The following stages were analysed: 24 hpf (A, E), 36 hpf (B, F), 48 hpf (C, G) and 60 hpf (D, H). There is an increase in retinal apoptosis level in shh\(^{-/-}\) mutant over the course of development, whereas in wild-type there is no such increase.

### 2.2.2 Anti-apoptotic factor expression in shh\(^{-/-}\) mutant

Apoptosis is generally induced when pro-apoptotic factors outweigh anti-apoptotic factors in a cell. One model to understand apoptosis induction is that of the death threshold, which suggests that cumulative influence of both pro- and anti-apoptotic factors matters and the final outcome may not be attributed to a single factor (Lowe et al., 2004). Bcl2 family consists of both pro- and anti-apoptotic factors, and their balance is essential for cell survival. Zebrafish contains 12 pro-apoptotic and 5 anti-apoptotic bcl-2 family genes, which were functionally characterized by Kratz and colleagues (2006). Bcl2, Bcl2l, Mcl1a and Mcl1b were
confirmed as pro-survival factors, and Mcl1 proteins were found to be essential for cell survival during early embryonic development. As a first step toward understanding apoptosis in shh\(^{--}\) mutant, I analysed expression of these anti-apoptotic Bcl2 family genes in wild-type and shh\(^{--}\) mutant embryos. Bcl2, bcl2l, mcl1a and mcl1b all have a broad expression pattern in both wild-type and shh\(^{--}\) mutant embryos consistent with their basic function in regulating apoptosis at the level of mitochondria (Fig. 17). All of these genes are also expressed at very similar levels in wild-type and shh\(^{--}\) mutant embryos suggesting that there is no dramatic change in anti-apoptotic Bcl2 family gene expression in the absence of Shh (Fig. 17). However, it is not possible to exclude some tissue-specific differences in expression of these genes.

**Figure 17.** Expression of anti-apoptotic Bcl2 family genes in shh\(^{--}\) mutant versus wild-type zebrafish embryos at 24 hpf.

In situ whole-mount analysis of bcl2, bcl2l, mcl1a and mcl1b expression in shh\(^{--}\) mutant and wild-type embryos at 24 hpf.
2.2.3 The involvement of p53 pathway in apoptosis in $shh^{-/-}$ mutant

Given the lack of clear differences in anti-apoptotic factor expression between $shh^{-/-}$ mutant and wild-type embryos, I asked if induction of pro-apoptotic factors may explain increased apoptosis in the developing $shh^{-/-}$ mutant embryos. Therefore, I checked if p53 pathway mediates apoptosis in $shh^{-/-}$ mutant embryos. Analysis of p53 target gene expression showed that $p53$, $cyclinG1$, $bax1$, $puma$ and $p21$ are specifically induced in $shh^{-/-}$ mutant embryos at 24 hpf (Fig. 18A, D; B, E; C, F; G, I; H, K). Similarly, at 56 hpf, $p53$ was up-regulated in the $shh^{-/-}$ mutants in the brain, retina and branchial arches (18L, M) relative to wild-type embryos (18N, O). To confirm the results of in situ hybridizations, I performed quantitative PCR (qPCR) analyses of p53 target genes on $shh^{-/-}$ mutant versus wild-type embryos at 24 hpf and 56 hpf. qPCR clearly showed the induction of $p53$, $mdm2$, $cyclinG1$, $bax1$ and $puma$ at both stages (Fig. 19). CDK inhibitor gene $p21$ had the highest fold induction of expression. Of the pro-apoptotic genes, $bax1$ and $puma$, but not $noxa$, were clearly induced in $shh^{-/-}$ mutants at both 24 hpf (Fig. 19A) and 56 hpf (Fig. 19B). Puma is one of the most potent inducers of mitochondrial apoptotic pathway and the main pro-apoptotic p53 target in zebrafish and mammals (Kratz et al., 2006; Nakano and Vousden, 2001). Induction of $puma$ relative to $bax1$ was higher in $shh^{-/-}$ mutant embryos at 24 hpf (Fig. 19A) than at 56 hpf (Fig. 19B) correlating with a higher level of apoptosis at 24 hpf than at later stages (Fig. 15). Higher in situ and qPCR signals for p53 may indicate a higher level of p53 thereby creating a positive feedback loop. However, it was reported that p53 activates an internal promoter of its own gene leading to production of a short p53 isoform $\Delta 113p53$ (Robu et al., 2007). Strongly elevated expression of $\Delta 113p53$ could indeed be detected in $shh^{-/-}$ mutant, whereas the level of full-length p53 cDNA was very similar in both wild-type and $shh^{-/-}$ mutant (Fig. 19C). This result rules out the positive feedback loop of full-length p53 expression and suggests that expression of $\Delta 113p53$ occurs similarly in different cases of p53 activation.
RESULTS

Figure 18. Expression of p53 target genes in 24 and 56 hpf shh\(^{-/-}\) mutant versus wild-type. 24 hpf wild-type and shh\(^{-/-}\) mutant embryos were \textit{in situ} hybridised with probes against p53 (A, D), cyclinG1 (B, E), bax1 (C, F), puma (G, I) and p21 (H, K). These p53 target genes are expressed at a higher level in shh\(^{-/-}\) mutant, and higher expression occurs in neural tube, retina and somites. 56 hpf wild-type and shh\(^{-/-}\) mutant embryos were also hybridized with the probe against p53 gene (L, M, N, O). The hybridizations show increased expression of p53 in shh\(^{-/-}\) mutant embryos (L, M) compared to wild-type ones in some brain regions, retina and branchial arches.

Timing of ectopic apoptosis due to genetic defects can help understand the reasons for its induction. High level of apoptosis in the shh\(^{-/-}\) mutant occurs at 24 hpf, when morphological defects of the mutant are striking. In order to understand apoptosis and p53 activation timing better, I performed \textit{in situ} hybridisation against p53 and ApopTag staining on embryo clutches from heterozygous shh\(^{+/+}\) parents at 1 somite, 6 somite, 8 somite, 10 somite, 12 and 14 somite stages (Fig. 20). Increased apoptosis and induced p53 expression could be first identified in shh\(^{-/-}\) mutant embryos at the 10 somite stage (Fig. 20M-P) and then progressed as development proceeded. Consistent with the requirement of Shh for survival in many different tissues, p53 expression and apoptosis occurred in a broad pattern (Fig. 20). Another notable observation is that the onset of p53 activation and apoptosis in shh\(^{-/-}\) mutant occur rapidly and at a defined developmental time point since 8 somite (Fig. 20I-L) and 10 somite (Fig. 20M-P) stages are separated by only around 1 hour. These results show that
control of p53 activity by Shh signaling starts early in development before most cells exited the cell cycle and differentiated.

**Figure 19.** Quantitative PCR analysis of p53 target activation and p53 isoform expression in shh\(^{+/--}\) mutant embryos.

qPCR analyses were performed at 24 and 56 hpf (A, B). qPCR assays for p53, p21, mdm2, cyclinG1, bax1, puma, noxa and gapdh were performed on both wild-type and shh\(^{+/--}\) mutant embryos, and expression of p53 targets was normalized using gapdh expression as a reference gene. The panels show p53 target gene expression in mutant embryos relative to that in wild-type siblings (A, B). All p53 targets except noxa show up-regulation. qPCR assays were performed on three different clutches of embryos and standard deviations are shown.

C. Semiquantitative PCR for full length p53, Δ113p53 and β-actin expression in wild-type and shh\(^{+/--}\) mutant at 24 hpf and 56 hpf. p53 is expressed at similar levels in both wild-type and shh\(^{+/--}\) mutant, whereas Δ113p53 is expressed at a much higher level in shh\(^{+/--}\) mutants. PCR was normalized using β-actin expression.
**Figure 20.** Onset of increased p53 expression and apoptosis in shh^{--} mutant occurs during somitogenesis. 

*p53 in situ* was used to identify time points with activated p53 protein (A, B, E, F, I, J, M, N, R, S, V, W). ApopTag staining was used as an assay for apoptosis (C, D, G, H, K, L, P, Q, T, U, X, Y). The following stages were analysed 1so (A-D), 6so (E-H), 8so (I-L), 10so (M-P), 12so (Q-T), 14so (U-X). At early stages it was not possible to identify the genotype of the embryos. From 10somite stage on, shh^{--} mutant embryos could be identified based on higher p53 expression and ApopTag staining because they made one quarter of the batch. The stainings were repeated two times and 40 embryos were analysed for each staining.
Expression analysis clearly showed activation of p53 pathway in $shh^{-/-}$ mutant. To confirm p53 pathway’s importance for apoptosis in $shh^{-/-}$ mutant, I performed p53 morpholino oligonucleotide (MO) knock-down (Nasevicius and Ekker, 2000). MO against p53 gene were used previously to knock down p53 expression during early zebrafish development (Langheinrich et al., 2002). Injection of p53 morpholino but not of control morpholino could indeed suppress elevated apoptosis in $shh^{-/-}$ mutant at 24 hpf without affecting levels of normal developmental apoptosis (Fig. 21A). At 56 hpf in $shh^{-/-}$ mutant exhibits an increased level of apoptosis in the retina, which could be suppressed by MO knock-down of p53 but not by control MO (Fig. 21B). p53 generally activates intrinsic apoptotic pathway, which is inhibited by Bcl2 family proteins. To confirm the importance of the intrinsic apoptotic pathway, I expressed EGFP-bcl2 or EGFP mRNA in $shh^{-/-}$ mutant and wild-type embryos. Indeed, EGFP-bcl2 successfully suppressed elevated apoptosis in $shh^{-/-}$ mutant at 24 hpf, whereas EGFP did not have any effect (Fig. 21C). This result suggests that p53-mediated apoptosis in $shh^{-/-}$ mutant occurs via the intrinsic pathway. However, a number of studies in tissue-culture systems showed that Bcl2 overexpression may lead to inhibition of p53 transcriptional activity (Froesch et al., 1999; Beham et al., 1997; Ryan et al., 1994). Such a situation would obscure our results by suppressing p53 pathway upstream of its effects on mitochondria. Therefore, I also analysed expression of p53 targets $p53$ and $cyclinG1$ after injection of EGFP-bcl2 or EGFP mRNA and failed to find any differences in expression of the p53 targets in these experimental conditions (Fig. 21D). Therefore, it is unlikely that in this system overexpression of EGFP-bcl2 can block p53 transcriptional activity.
Figure 21. p53 mediates apoptosis in shh<sup>−/−</sup> mutant by intrinsic apoptosis pathway.  
A. Elevated apoptosis observed in in shh<sup>−/−</sup> mutant at 24 hpf could be suppressed by injection of p53 morpholino but not of control morpholino. 
B. Retinal apoptosis observed in in shh<sup>−/−</sup> mutant at 56 hpf could be suppressed by injection of p53 morpholino but not by injection of control morpholino (n = 10 and 40 sections for shh<sup>−/−</sup> mutant injected with p53 morpholino; n = 10 and 34 sections for in shh<sup>−/−</sup> mutant injected with control morpholino).  
C. Apoptosis in in shh<sup>−/−</sup> mutant could be suppressed by injection of EGFP-bcl2 mRNA but not by injection of EGFP RNA. Injection of EGFP-bcl2 also reduces the level of normal developmental apoptosis.  
D. The level of expression of p53 and cyclinG1 in both in shh<sup>−/−</sup> mutant and wild-type embryos was not affected by injection of EGFP-bcl2 mRNA relative to the control injection of EGFP mRNA.

2.2.4 shh<sup>−/−</sup> p53<sup>−/−</sup> double mutant phenotypes

p53 requirement for apoptosis occurring due to shh loss could be shown using morpholino knock-down. However, MO have limited perdurance and mostly exert their effects during the first 2 days of development. Thus, it is difficult to study late effects of gene loss
using morpholino knock-downs. Furthermore, genetic null mutations are generally perceived superior to morpholino knock-down for loss-of-function experiments. Therefore, to confirm the role of p53 in apoptosis in shh\(^{-/-}\) mutant and to study the late effects of p53 loss on the phenotype of shh\(^{-/-}\) mutant, p53\(^{dfl}\) mutation (Berghmanns et al., 2005), a null mutation of p53, was genetically combined with shh\(^{ld}\) null mutation. The double mutant shh\(^{-/-}\)p53\(^{-/-}\) was protected from ectopic apoptosis in shh\(^{-/-}\) mutant at 24 hpf, but normal apoptosis was not affected (Fig. 22A). I also checked the levels of apoptosis in the retina of wt, shh\(^{-/-}\) and shh\(^{-/-}\)p53\(^{-/-}\) embryos at 72 hpf and found that the high level of apoptosis observed in shh\(^{-/-}\) mutant at 72 hpf was successfully suppressed by p53 loss (Fig. 22B).

**Figure 22.** Rescue of apoptosis in shh\(^{-/-}\) mutant by loss of p53.
Apoptosis staining at 24 hpf and at 72 hpf in wt, shh\(^{-/-}\) and shh\(^{-/-}\)p53\(^{-/-}\) is shown in this figure. Apoptosis was assayed in whole-mount in 24 hpf embryos using ApopTag staining (A) and on retinal sections in 72 hpf embryos using TUNEL staining (B). A. Elevated apoptosis in shh\(^{-/-}\) relative to wild-type is rescued by p53 loss in shh\(^{-/-}\)p53\(^{-/-}\) B. Highly elevated retinal apoptosis in shh\(^{-/-}\) relative to wild-type is likewise rescued by p53 loss in shh\(^{-/-}\)p53\(^{-/-}\). The figure shows representative pictures. All stainings were performed on at least 6 embryos for each genotype.

When cells survive due to suppression of apoptosis, the most interesting question is what fate they acquire. Acquisition of particular fates during development is tightly linked to
the cell-cycle exit of differentiating cell precursors. Therefore, another interesting question is whether p53 affects failure of cell-cycle exit in the retina of \textit{shh}^{+/−} mutants. In zebrafish retina, Shh signaling was proposed to serve as a differentiation and cell-cycle exit signal in addition to being a survival factor (Masai et al., 2005; Shkumatava et al., 2004; Shkumatava and Neumann, 2005). Therefore, I wanted to suppress retinal apoptosis and examine whether p53 contributes to the differentiation phenotype of \textit{shh}^{+/−} mutant zebrafish embryos. Another important goal is to identify p53-independent effects of Shh on retinal differentiation and retinal progenitor cell-cycle exit. To achieve these goals, retinal sections of wt, \textit{shh}^{−/−} and \textit{shh}^{−/−}p53^{−/−} mutant embryos were stained with antibodies against retinal differentiation markers. Retinal ganglion cells (RGCs) identified by anti-zn5 staining were present in retinas of embryos of all three genotypes, but they appeared more abundant in \textit{shh}^{+/−}p53^{+/−} retinas (Fig. 23A-C). A subset of amacrine cells identified by anti-parvalbumin antibodies were found in large numbers in the inner plexiform layer of the wild-type retina (Fig. 23D), whereas \textit{shh}^{−/−} mutant retina has none of these cells (Fig. 23E). Strikingly, \textit{shh}^{−/−}p53^{+/−} embryos show a partial rescue of amacrine cell differentiation (Fig. 23F). Another more widely expressed marker of differentiated amacrine cells is GAD67, whose staining revealed a high number of amacrine cell in the wild-type retina (Fig. 23M) and their virtual absence in the \textit{shh}^{−/−} retina (Fig. 23N). In the \textit{shh}^{−/−}p53^{+/−} retina the number of GAD67-positive amacrine cells strongly increased compared to the \textit{shh}^{−/−} retina (Fig. 23O). Differentiation of red-green cone and rod photoreceptors is normal in wild-type embryos (Fig. 23G, J), whereas in \textit{shh}^{−/−} cells of these types are almost completely absent (Fig. 23H, K). As in the case of amacrine cells, differentiation of photoreceptors is rescued in \textit{shh}^{−/−}p53^{+/−} mutant (Fig. 23I, L). These results imply that p53 prevents differentiation of all cell types in the \textit{shh}^{−/−} mutant retina. However, differentiation of two other cell types was not rescued in the \textit{shh}^{−/−}p53^{+/−} mutant retina. Müller glia is an abundant late-differentiating cell type which is required to maintain the environment inside the retina. A high number of these cells were present in the wild-type retina at 72 hpf (Fig. 24A) in contrast to the \textit{shh}^{−/−} mutant retina where Müller glia were absent (Fig. 24B). This differentiation defect could not be rescued by p53 loss (Fig. 24C). Bipolar cells are a retinal cell type involved in signal transduction during light perception, which was identified by antibodies against PKC\textalpha in the wild-type retina at 72 hpf (Fig. 24D). The \textit{shh}^{+/−} mutant retina lacked bipolar cells (Fig. 24E) and this bipolar cell differentiation defect could not be
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rescued in $shh^{-/-}p53^{+/+}$ mutant retina (Fig. 24F). Taken together, these data suggest that previously described retinal differentiation defects of $shh^{-/-}$ mutants (Shkumatava et al., 2004) in the cases of amacrine cells and photoreceptors are mediated by p53.

Figure 23. Rescue of photoreceptor and amacrine cell differentiation in $shh^{-/-}$ mutant by $p53$ loss.

The figure shows photos of stained retinal sections from 72 hpf wild-type (wt) (A, D, G, J, M), $shh^{-/-}$ (B, E, H, K, N) and $shh^{-/-}p53^{+/+}$ mutants (C, F, I, L, O). The sections are oriented with their anterior side to the top. All sections are stained with phalloidin-Alexa568 to visualize the overall lamination of the retina. Antibody against zn5 antigen (zn5) labels retinal ganglion cells in the retina. Wild-type embryos (A) have both ganglion cell layer and the optical nerve labeled. Ganglion cell layer is also relatively normal in $shh^{-/-}$ mutant (B), and loss of $p53$ in $shh^{-/-}p53^{+/+}$ mutant leads to a significant increase in the intensity of zn5 staining (C). Amacrine cells are successfully labeled in wild-type retina with an antibody against parvalbumine (D), but are normally absent in $shh^{-/-}$ mutant (E). In $shh^{-/-}p53^{+/+}$ mutant a significant number of them appears (F) due to rescue of differentiation. Antibody staining against Zpr1 protein expressed in red-green double cones identifies the photoreceptors in wild-type embryos (G), their lack in $shh^{-/-}$ mutant (H) and rescue of their differentiation in $shh^{-/-}p53^{+/+}$ mutant (I). Antibody staining against Zpr3 protein expressed in rod photoreceptors identifies them in wild-type embryos (J), their lack in $shh^{-/-}$ mutant (K) and rescue of their differentiation in $shh^{-/-}p53^{+/+}$ mutant (L).
## RESULTS

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RESULTS

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**Figure 24.** Loss of p53 does not rescue differentiation of Müller glia cells and bipolar cells in the retina of shh–/– mutants. Staining against glutamine synthetase, a marker for Müller glia, reveals a high number of these cells in wild-type embryos (A). In both shh–/– and shh–/–p53–/– mutants (B, C) there are very few of Müller glia. Staining against PKCα, a marker of bipolar cells in the retina, identifies these cells in the wild-type embryos (D), but no bipolar cells are present in appropriate retina regions in either shh–/– and shh–/–p53–/– mutants (E, F). All stainings were performed on at least 6 embryos for each condition and representative pictures are shown.

Since loss of p53 rescued differentiation in the shh–/– mutant retina, a question arises whether p53 affects cell-cycle exit in the retina. A likely possibility is that p53 loss induced cell-cycle exit of the precursor cells which later differentiated. According to this view, p53 would be responsible for failure of cell-cycle exit in the shh–/– mutant retina. An alternative view that failure of cell-cycle exit can induce p53 activation in the shh–/– mutant retina is attractive but is not consistent with the available data since p53 activation is a much earlier event than cell-cycle exit in the retina. To confirm the first possibility, I decided to directly analyze S-phase progression using the BrdU incorporation assay in the wt, shh–/– and shh–/–
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p53\textsuperscript{–/–} mutant retinas. BrdU incorporation assays were performed at 48 and 56 hpf, since at these stages cell-cycle exit in the retina is being completed. Indeed, at 48 hpf wild-type retina contains BrdU-positive cells not only at the retinal margin but also at the basal surface of the retina (Fig. 26A) and by 56 hpf all cells except cells in the marginal zone exit the cell cycle (Fig. 26B). In the \textit{shh}\textsuperscript{–/–} mutant retina, high numbers of cells remained in S-phase at both stages (Fig. 26A, B). Interestingly, in the \textit{shh}\textsuperscript{–/–}p53\textsuperscript{–/–} mutant retina the pattern and extent of cell-cycle exit are very similar to the wild-type situation (Fig. 26A, B). Since cell-cycle exit in the zebrafish retina is induced by \textit{p57kip2} expression, I checked if this gene is expressed in \textit{shh}\textsuperscript{–/–} mutant upon loss of p53. In fact, at 38 hpf, strong \textit{p57kip2} expression was present in large areas of wild-type and \textit{shh}\textsuperscript{–/–}p53\textsuperscript{–/–} mutant retinas, but was absent from the \textit{shh}\textsuperscript{–/–} mutant retina (Fig. 25C). By contrast, \textit{p53} was highly expressed only in the \textit{shh}\textsuperscript{–/–} mutant retina and was nearly absent in wild-type and \textit{shh}\textsuperscript{–/–}p53\textsuperscript{–/–} mutant retinas (Fig. 25C). These striking results suggest that the cell-cycle exit defect in the \textit{shh}\textsuperscript{–/–} mutant retina is clearly dependent on the p53 function. Since p53 is well known to inhibit cell cycle progression, it is possible to predict that the cell-cycle exit defect in the \textit{shh}\textsuperscript{–/–} mutant retina may not be a case of ectopic uncontrolled proliferation but rather a cell cycle block in S-phase. Supporting this prediction requires a way to label cells with activated p53 to better understand their cell cycle properties and eventual progression to apoptosis.

\textbf{Figure 25.} Loss of \textit{p53} rescues cell cycle exit defect in \textit{shh}\textsuperscript{–/–} mutant.

S-phase progression in wild-type, \textit{shh}\textsuperscript{–/–} and \textit{shh}\textsuperscript{–/–}p53\textsuperscript{–/–} mutants at 48 and 56 hpf was studied by BrdU labeling. Confocal sections are shown with the anterior side to the top.
A. At 48 hpf most cells in the wild-type retina have already exited the cell cycle. In the \textit{shh}\textsuperscript{–/–} mutant retina, however, cell cycle exit is almost completely absent. By contrast, cell-cycle exit in the \textit{shh}\textsuperscript{–/–}p53\textsuperscript{–/–} mutant retina is much more advanced and quite similar to the situation in the wild-type retina.
B. At 56 hpf in the wild-type retina BrdU-positive cells are found only in the ciliary marginal zone (CMZ) and in the \textit{shh}\textsuperscript{–/–} mutant retina cells still failed to exit the cell cycle. Like in the wild-type retina, cells of \textit{shh}\textsuperscript{–/–}p53\textsuperscript{–/–} mutant have mostly exited the cell cycle and proliferative cells are located only in the CMZ.
C. \textit{In situ} hybridizations against \textit{p57kip2} and \textit{p53} in wild-type, \textit{shh}\textsuperscript{–/–} mutant and \textit{shh}\textsuperscript{–/–}p53\textsuperscript{–/–} mutant retinas.
## RESULTS

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2.2.5 Hedgehog pathway activation rescues apoptosis in shh−/− mutants

The results so far demonstrate that p53 is essential for elevated apoptosis in the shh−/− mutant. However, it is not yet clear how the absence of Shh signaling activates p53 pathway. Abe et al. (2008) suggested the existence of an unknown factor inhibiting p53 pathway, which can be induced by Gli-mediated Hh signaling activation. Since their work was performed on human and mouse cell lines, I wanted to confirm if this conclusion would also apply to the situation of shh genetic loss in zebrafish. Protein kinase A (PKA) is a negative regulator of Hh signal transduction from Smo to Gli transcription factors in vertebrates. To better understand the connection between shh loss and p53 activation in vivo, I activated canonical Hh/Gli signaling pathway by injection of dominant-negative PKA GFP fusion (dnPKA-GFP) mRNA (Hammerschmidt et al., 1996; Ungar and Moon, 1996) into progeny of fish heterozygous for shh deletion. Given the limited stability of this mRNA, I chose to analyse the phenotypes due to dnPKA-GFP over-expression at 12 somites stage, when shh−/− mutant embryos already show elevated expression of p53 target genes and a high level of apoptosis. Indeed, overexpression of dnPKA-GFP led to a high level of ptc1 expression in 52 out of 61 (85 %) embryos (Fig. 26A, N), whereas 9 out of 61 (15 %) which probably received little mRNA showed normal uninduced expression of ptc1 for wild-type and shh−/− mutant, respectively (Fig. 26B, C, N). All of EGFP-injected embryos showed an uninduced level of expression, of which 16 out of 61 (26 %) embryos showed shh−/− mutant level of patched1 expression (Fig. 26E) and 45 out of 61 (74 %) had wild-type level of expression (Fig. 26D, N). p53 pathway activity after either dnPKA-GFP or EGFP over-expression was assessed by an in situ staining for cyclinG1, a well-characterized p53 target gene. Only 5 out of 45 (11 %) dnPKA-GFP injected embryos expressed cyclinG1 at a perceptibly higher level than the rest 40 embryos (Fig. 26F, G, N), and the difference between these two categories of embryos was slight. By contrast, injection of EGFP mRNA didn’t lead to a decrease in proportion of shh−/− mutant embryos with high expression of cyclinG1 (15 out of 57 (26 %)) (Fig. 26I, N). In the case of EGFP-injected embryos, the difference of cyclinG1 expression between wild-type and shh−/− mutant embryos was very pronounced (Fig. 26H, I). Consistent with its effect on cyclinG1 expression, dnPKA-GFP also led to a reduction in the number of embryos with elevated apoptosis levels (5 out of 65 (7.7 %)) (Fig. 26K, N) compared to 17 out of 66 (26 %) in the EGFP-injected sample (Fig. 26M, N) and the number of apoptotic cells in dnPKA-GFP-injected embryos was smaller than
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in EGFP-injected ones. The number of embryos with a low level of apoptosis correspondingly increased to 60 out of 65 (92%) in the dnPKA-GFP injected sample (Fig. 26J, N) compared to 49 out of 66 (74%) in the EGFP-injected sample (Fig. 26L, N).
Figure 26. Hh signaling activation by dnPKA-GFP decreases p53 target cyclinG1 expression and suppresses apoptosis in shh^{+/−} mutant embryos.

Embryos at the 12 somite stage from shh^{+/−} parent fish were injected with either dnPKA-GFP (A-C, F, G, J, K, N) or EGFP mRNA (D, E, H, I, L, M, N) and in situ stained for patched1 (A-E), target gene of Hh signaling, and for cyclinG1 (F-I), p53 target gene, and ApopTag staining was performed to characterize the level of apoptosis (J-M). Injection of dnPKA-GFP mRNA led to expression of patched1 at a high level in most embryos (A) compared to a few embryos which received little dnPKA-GFP mRNA (B, C) and EGFP-injected embryos (D, E). Few of the embryos injected with dnPKA-GFP showed a higher level of cyclinG1 expression (G) and this level was not very different from that of the majority of embryos (F). The difference of cyclinG1 expression after EGFP mRNA injection in higher-expressing (I) and lower-expressing (H) embryos was very large. ApopTag staining of EGFP-injected shows that around one quarter of embryos have a much higher level of apoptosis (M) than most of the embryos with a low level of apoptosis (L). Injection of dnPKA-GFP mRNA leads to a strong decrease in the number of embryos with increased apoptosis and its level (K) and a decrease in low-apoptosis proportion of embryos (J). Summary of the results of this experiment (N) shows two categories for each staining: high and low, which in the case of patched1 staining indicate ectopic activation of Hh signaling or lack thereof and in the cases of cyclinG1 and ApopTag indicate relative levels of gene expression and apoptotic cell numbers, respectively. Proportions of embryos with the presented phenotypes are shown in the lower right corner of each image. The results presented are representative of three independent experiments.
2.2.6 Creating and characterizing zebrafish p53 reporter line

Our ability to study gene expression using \textit{in situ} hybridization (ISH) is important to study gene transcripts in developing embryos. However, ISH does not allow real-time analysis of cells expressing particular transcripts and is difficult to combine with many antibody stainings and other histochemical methods. Advent of fluorescent proteins has overcome this problem by enabling researchers to create reporter systems based on particular promoters driving expression of genes encoding fluorescent proteins. My aim was to develop a transgenic p53 reporter to study the behavior and characteristics of cells with active p53. I implemented a strategy similar to the one previously employed by Zhang et al. (2001). Human 2.4 kb CDKN1A promoter encoding p21 was used as the basis for the p53 reporter, 13 copies of the optimal p53 binding sites (PG13) (Kern et al., 1992) were inserted in front of this promoter as an enhancer. Tol2kit transgenic system (Kwan et al., 2007) was then used to assemble the final construct. In short, PG13p21 promoter, nlsEGFP and polyA DNA parts were assembled by recombination inside the destination vector used for transposon-based transgenesis (Fig. 27). The destination vector additionally provided EGFP transgene specifically expressed in the heart, which facilitates transgenic embryo screening and identification of carrier fish.
RESULTS

Figure 27. The Tol2kit-based strategy to make PG13::nlsEGFP-polyA p53 reporter. 5’ entry vector containing human p21 promoter with PG13 enhancer, middle entry vector encoding nlsEGFP and 3’ entry vector containing polyA signal were LR recombined with pDestCG2 vector (not shown), which serves as a carrier of the construct for transgenesis. LR recombination resulted in PG13p21::nlsEGFP-polyA construct inside the pDestCG2, which provides attachment sites for the transposase and a transgenic marker for positive embryo screening.

Figure 28. Activation of PG13p21::nlsEGFP p53 reporter by treatment with roscovitine. Wild-type embryos carrying PG13p21::nlsEGFP transgene were incubated for 6 hours at 24 hpf with either 50 µM roscovitine or 0.4 % DMSO in E3 medium. DMSO-treated embryos did not show any significant expression of the reporter (the upper panel), but Roscovitine was quite effective in inducing p53 reporter expression (the lower panel). All embryos show green-heart transgenesis marker. The experiment was repeated two times and 20 embryos were used for each treatment.
The p53 reporter zebrafish transgenic line represents a potentially general tool to study p53 activation and regulation. Studies of p53 in zebrafish employed camptothecin, a genotoxic drug, or roscovitine, an Mdm2 inhibitor, to activate p53 (Langheinrich et al., 2005; Lee et al., 2007). I chose treatment with roscovitine to verify activation of the p53 reporter. Treatment with roscovitine for 6 hours at 24 hpf could efficiently induce p53-mediated apoptosis, which was prevented by p53 knockdown (Langheinrich et al., 2005). Wild-type embryos carrying the p53 reporter transgene were incubated either with 50 µM roscovitine or 0.4 % DMSO as a control for 6 hours starting at 24 hpf. As a result, p53 reporter expression was strongly up-regulated in the brain and retina of the roscovitine-treated but not of control DMSO-treated embryos (Fig. 28). This result confirms the utility of the p53 reporter transgenic line in the context of drug-induced p53 activation and suggests that this line is a valuable tool for other investigations of p53 function.

2.2.7 p53 reporter activation in \textit{shh}^{−/−} mutant

The next step to characterize the utility of the newly-generated p53 reporter was to introduce it into \textit{shh}^{−/−} mutant. After obtaining PG13p21::nlsEGFP \textit{shh}^{+/−} fish, expression of the p53 reporter was photographed in \textit{shh}^{−/−} mutant and wild-type embryos at 24, 30, 36 and 48 hpf (Fig. 29). At all stages analysed wild-type embryos had a low background level of the p53 reporter expression consistent with previous observations that p53 activation is normally under tight control. By contrast, at all analysed stages the level of the p53 reporter expression in \textit{shh}^{−/−} mutants was high. At 24 hpf, \textit{shh}^{−/−} mutants had a very broad expression of the p53 reporter in the retina, neural tube and somites. Over the next 24 hours of development, expression of the p53 reporter in \textit{shh}^{−/−} mutants becomes restricted to midbrain and retina regions (Fig. 29). These changes in p53 reporter expression are most certainly caused by the clearance of cells by p53-mediated apoptosis. To confirm the specificity of the p53 reporter induction in \textit{shh}^{−/−} mutant embryos, I knocked down p53 expression using morpholino oligonucleotides. High level of p53 reporter expression in \textit{shh}^{−/−} mutant at 24 hpf was readily inhibited by p53 MO but not by control MO injection (Fig. 30). This result confirms that p53 is essential to mediate the expression of its transcriptional activity reporter transgene.
RESULTS

Figure 29. p53 reporter PG13p21::nlsEGFP expression in wild-type and shh<sup>−/−</sup> mutant embryos during development.
Wild-type and shh<sup>−/−</sup> mutant embryos carrying PG13p21::nlsEGFP p53 reporter were photographed at 24, 30, 36 and 48 hpf. Expression of the p53 reporter is highly elevated in mutant embryos in the retina, nervous system and somites in comparison to the p53 reporter expression in wild-type at all stages.

Figure 30. p53 knock-down inhibits PG13p21::nlsEGFP reporter expression in shh<sup>−/−</sup> mutant embryos at 24 hpf.
Progeny of heterozygous shh<sup>−/−</sup> embryos carrying PG13p21::nlsEGFP was injected with either p53 morpholino or control morpholino and the reporter was imaged at 24 hpf.

Shh exerts its effects on cell survival in specific regions of the developing embryo, whereas expression of the p53 reporter seems ubiquitous. To verify whether different cells
RESULTS

express p53 reporter at different levels, I injected H2BmRFP1 mRNA into oocytes carrying p53 reporter and analysed hindbrain and midbrain regions of wild-type and $shh^{+/−}$ at 24 hpf. Wild-type embryos expressed almost no p53 reporter in either hindbrain or midbrain (Fig. 31). By contrast, many cells expressed p53 reporter in $shh^{+/−}$ mutants in hindbrain and midbrain regions (Fig. 31A, B). p53 reporter nlsEGFP is expressed at very different levels in different cells in $shh^{+/−}$ mutant embryos as is clearly seen in a hindbrain section by very different relative levels of nlsEGFP and H2BmRFP1 (Fig. 31A). To understand whether p53 reporter expression was consistent with the embryo regions requiring Shh for cell survival, I analysed p53 reporter expression and apoptosis by TUNEL staining in different embryo regions at 24 hpf on embryo cryosections (Fig. 32). Especially striking was expression of the p53 reporter in the neural tube. Midbrain, hindbrain and spinal cord expressed nlsEGFP in $shh^{+/−}$ mutant p53 reporter fish and had a high number of TUNEL-positive cells (Fig. 33B, C, D). By contrast, in the $shh^{+/−}$ retina despite the pronounced expression of p53 reporter there was no elevated apoptosis at 24 hpf. This result is interesting and unexpected, because the spread of $shh$ expression in the retina starts at 28 hpf (Neumann and Nüsslein-Volhard, 2000). Activation of p53 in $shh^{+/−}$ mutant retinal cells before 28 hpf indicates a requirement of Shh perception at an earlier stage to control p53 activity. In the absence of Shh, neural crest cells undergo apoptosis (Ahlgren and Bronner-Fraser, 1999). Consistently, some p53 reporter-positive cells in the midbrain section (Fig. 32B) are located in the lateral regions characteristic of neural crest cells. In the spinal cord section (Fig. 32D) p53 reporter labels somite cells, which may be sclerotome or muscle cells requiring Shh for their survival (Borycki et al., 1999). I then analysed apoptosis wild-type and $shh^{+/−}$ mutant carrying p53 reporter at 56 hpf by staining with TUNEL. Sections of wild-type embryos contained very little signal for p53 reporter and TUNEL staining and most signal was due to auto-fluorescence (Fig. 33). By contrast, all the sections from $shh^{+/−}$ mutant had a strongly increased level of apoptosis and p53 reporter expression. Especially strong apoptosis and p53 reporter expression were detected in the $shh^{+/−}$ mutant retina (Fig. 33A). This observation is different from the result at 24 hpf, when $shh^{+/−}$ mutant retina had very little apoptosis, and suggests that accumulation of pro-apoptotic factors eventually induces cell death in the $shh^{+/−}$ mutant retina. Midbrain and hindbrain regions of $shh^{+/−}$ mutants at 56 hpf also contained many apoptotic and p53 reporter-positive cells, which is similar to the situation in the $shh^{+/−}$ mutant at 24 hpf (Fig. 33B, C). Taken together, the results at 24 and 56
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hpf in shh<sup>−/−</sup> mutant suggest that p53 reporter-positive cells persist during development and gradually become cleared by apoptosis rather than die simultaneously at a defined time point.

**Figure 31.** Cell-specific levels of PG13p21::nlsEGFP reporter expression in brain regions of wild-type and shh<sup>−/−</sup> mutant embryos at 24 hpf.
A. Hindbrain regions of the wild-type and shh<sup>−/−</sup> embryos containing PG13p21::nlsEGFP p53 reporter and injected H2BmRFP1. For the shh<sup>−/−</sup> mutant embryo, both image stack projection and single section images are shown.
B. Midbrain regions of the wild-type and shh<sup>−/−</sup> embryos containing PG13p21::nlsEGFP p53 reporter and injected H2BmRFP1.
Figure 32. p53 reporter expression and apoptosis in \textit{shh}\textsuperscript{−/−} mutant and wild-type at 24 hpf. 24 hpf wild-type and \textit{shh}\textsuperscript{−/−} embryos carrying p53 reporter transgene (PG13p21::nlsEGFP) were transversally sectioned and stained with TUNEL and DAPI, nlsEGFP signal is endogeneous (A-D). A. Sections of \textit{shh}\textsuperscript{−/−} and wild-type retinas show that p53 reporter is expressed at a high level in \textit{shh}\textsuperscript{−/−} retina, but there is no increase in apoptosis. B. Transverse sections of midbrain regions of \textit{shh}\textsuperscript{−/−} and wt. C. Transverse sections of hindbrain regions of \textit{shh}\textsuperscript{−/−} and wt. D. Transverse sections of hindbrain regions of \textit{shh}\textsuperscript{−/−} and wt. In midbrain (B), hindbrain (C) and spinal cord (D) p53 reporter expression highly correlates with increased apoptosis and a proportion of p53 reporter positive cells are also TUNEL-positive.
Figure 33. p53 reporter expression and apoptosis in $shh^{-/-}$ mutant and wild-type at 56 hpf. 56 hpf wild-type and $shh^{-/-}$ embryos carrying p53 reporter transgene (PG13p21::nlsEGFP) were transversally sectioned and stained with TUNEL and DAPI, nlsEGFP signal is endogeneous (A-C). A. Retinal section. B. Hindbrain section. C. Midbrain section.
2.2.8 p53 reporter-positive cells in shh−/− mutant are positive for apoptotic markers

Since p53 is known to induce apoptosis, it is very important to characterize apoptotic phenotype in p53 reporter-positive cells. p53 reporter is usually expressed in the embryo regions with elevated levels of apoptosis as revealed by TUNEL staining (Figs 32, 33). However, since TUNEL is quite a late marker of apoptosis many TUNEL-positive cells in shh−/− mutants lack detectable p53 reporter expression. This is most likely due to protein degradation in the dying cells and longer persistence of apoptotic cell death. An alternative possibility of p53-independent apoptotic cell death can be excluded because p53 loss efficiently suppresses apoptosis in the shh−/− mutant. To characterize the apoptotic phenotype of p53 reporter-positive cells, I stained 24 hpf shh−/− mutant embryos with TUNEL or anti-active Caspase3. Wild-type embryos were not used for these stainings because they contain extremely few p53 reporter positive cells. Although in the shh−/− mutant there were many TUNEL-positive cells, few of them were also positive for p53 reporter nlsEGFP (Fig. 34A). In double-positive cells, TUNEL signal occupied the center of the cell and p53 reporter nlsEGFP was located at the cell periphery. This result contrasts with the normally nuclear localization of nlsEGFP, but the cytoplasmic localization of p53 reporter nlsEGFP is consistent with nuclear breakdown in the apoptotic cells (Kramer et al., 2008). Next I wanted to see whether p53 reporter co-localizes with active Caspase3. The number of active Caspase3-positive cells in the hindbrain of shh−/− mutants was much lower than that of TUNEL-positive cells (Fig. 34B) suggesting a much shorter persistence of Caspase3 activity in apoptotic cells. Strikingly, in most examined cells cytoplasmic active Caspase3 was clearly co-localised with p53 reporter nlsEGFP (Fig. 34B). This result confirms that cell death program is activated in cells with active p53.
Figure 34. Co-localisation of apoptotic markers with p53 reporter expression.  
A. Labeling p53 reporter nlsEGFP-positive cells with TUNEL. In some cells, TUNEL and nlsEGFP are present. These cells are labeled with arrows.  
B. Anti-activeCaspase3 labeling of p53 reporter nlsEGFP-positive cells. Cells undergoing apoptosis but not small apoptotic corpses have both nlsEGFP and activeCaspase3 in the cytoplasm (labeled with arrows). DAPI staining shows that activeCaspase3-positive cells have condensed nuclei.
2.2.9 Cell cycle behavior of p53 reporter-positive cells in shh$^{-/-}$ mutant

Development of the PG13p21::nlsEGFP p53 reporter zebrafish transgenic line allows a better understanding of how cells with activated p53 behave in vivo. In particular, this line enabled a better, easier and more versatile visualization of cells with active p53 as described in previous sections. Additionally, cell-cycle behavior of p53 reporter-positive cells can be studied (Zhang et al., 2001). Observations of cell-cycle exit rescue in shh$^{-/-}$ retina by p53 loss raise a question how cell cycle is regulated by p53 in the absence of Shh. Since p53 can induce cell-cycle arrest, one can explain the cell-cycle exit experiments (Fig. 26) by proposing that p53 inhibits cell cycle progression of retinal progenitors in the absence of Shh. To address this hypothesis, I used Fluorescence-activated cell sorting (FACS) method to analyse cell cycle properties of p53 reporter-positive cells. For FACS analysis, cell cycle distribution was determined using Propidium Iodide (PI) DNA staining since cells have different DNA contents in different cell cycle stages. Natural EGFP fluorescence was used to sort p53 reporter-positive cells. Thus, it is possible to compare cell cycle distributions of different cell populations directly. I applied the PI/EGFP-based FACS analysis to wild-type and shh$^{-/-}$ mutant embryos at 48 and 56 hpf, the stages used for the cell cycle exit experiment, expecting this analysis to help me understand the influence of p53 on retinal progenitor cell cycle progression. For this experiment the best samples would be dissected eyes. However, since it is technically challenging to obtain sufficient numbers of dissected eyes from zebrafish embryos, I used embryo heads for FACS-based experiments. In the case of shh$^{-/-}$ mutants, eyes contain a high proportion of all p53 reporter-positive cells in the head, which makes FACS likely to provide information on p53 reporter activation in the retina. FACS data show light scattering properties of the cells allowing correct cell populations to be selected for analysis as shown in Fig. 35A for 48 hps shh$^{-/-}$ mutants. PI and EGFP staining signals enable determination of cell cycle distributions of all cells (All cells), GFP-positive (GFP+) and GFP-negative (GFP-) cells for both wild-type and shh$^{-/-}$ mutant heads (Fig. 36). At 48 hpf in shh$^{-/-}$ mutant p53 reporter-positive cells have a very low G2/M cell percentage at 3 %, whereas 21.7 % of shh$^{-/-}$ mutant GFP- cells, 13.5 % of all shh$^{-/-}$ mutant cells and 11.5 % of wild-type cells were mitotic (Fig. 36B). This very low G2/M cell percentage in shh$^{-/-}$ mutant heads was accompanied by a higher S-phase proportion and a slightly higher G1-phase percentage relative to other samples (Fig. 36B). At 56 hpf, cell cycle distribution of p53 reporter-positive shh$^{-/-}$ mutant cell remained
similar to that at 48 hpf with G2/M proportion of 4.5%. However, at 56 hpf there was a dramatic reduction in G2/M proportions most probably due to cell-cycle exit in wild-type and \( shh^{-/-} \) p53 reporter-negative cell populations, making all populations similar in their mitotic indices. Moreover, the S-phase percentages of p53 reporter-positive and negative cells in \( shh^{-/-} \) mutants at 56 hpf became similar. Nevertheless, the FACS data clearly indicate that p53 reporter-positive cells in \( shh^{-/-} \) mutants are defective in cell-cycle progression and completing mitoses required for birth of differentiated cells at 48 hpf.

**Figure 35.** FACS analysis of cell cycle distribution of cells with active p53 reporter in wild-type and \( shh^{-/-} \) mutant heads. Heads of wild-type and \( shh^{-/-} \) embryos carrying PG13p21::nlsEGFP reporter were dissociated to single cells to analysed using flow cytometry. DNA was stained using propidium iodide and EGFP fluorescence was detected directly.

A. Example of FACS data for 48 hpf \( shh^{-/-} \) PG13p21::nlsEGFP. In the upper row, the left-most panel shows the distribution of cells according to their scatter properties. The middle panel shows the PI staining distribution (FL3A vs FL3W). The right panel shows the distribution of EGFP signal (FL1H) relative to the DNA PI staining signal (FL3W). The lower row of panels shows cell cycle distributions of cells with different levels of p53 reporter expression. The left panel shows cell cycle distribution of all cells in the sample, where peaks in different shadings correspond to cells with different DNA content. The middle panel shows cell cycle distribution of p53 reporter-positive cells, where notable shortage of G2-phase/mitotic cells is evident. The left panel shows the cell cycle distribution of p53 reporter-negative cells.

B. Cell cycle phase quantification of wild-type and \( shh^{-/-} \) head samples at 48 and 56 hpf. Mutant samples are classified into All cells, GFP+ and GFP-, and the data for wild-type samples is presented as All Cells. G1, S and G2/M cell cycle phases and sub-G1 are the categories distinguished on the basis of PI DNA staining. Left panel shows samples at 48 hpf and the right panel shows 56 hpf samples. FACS experiments were repeated 3 times at both 48 and 56 hpf and representative data are shown.
RESULTS

A

48 hpf shh/- PG13p21::nlsEGFP

B

<table>
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<th>48 hpf PG13p21::nlsEGFP</th>
<th>56 hpf PG13p21::nlsEGFP</th>
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<td>shh +/- GFP -</td>
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- G1
- S
- G2/M
- sub-G1
2.2.10  p53 regulates proliferation in the $shh^{-/-}$ mutant retina

Information about cell cycle behaviour of p53 reporter-positive cells in $shh^{-/-}$ mutant obtained using FACS is important, but it doesn’t provide direct information on how these cells behave in the retina. Therefore, a direct analysis of the cell cycle progression of retinal cells had to be done. As a first step, BrdU labeling of S-phase cells was performed on wild-type and $shh^{-/-}$ mutant retinas at 48 hpf. In the wild-type retina, there are very few p53 reporter-positive cells and they are BrdU-negative (Fig. 36). By contrast, $shh^{-/-}$ mutant retina contains many p53 reporter-positive cells and nearly all of them are BrdU-positive, whereas some p53 reporter-positive cells present in the brain are BrdU-negative (Fig. 36). This result contrasts with the FACS data in whole-head cell populations since in the retina p53 reporter-positive cells have a low G1-phase proportion. It also suggests that in other head regions of $shh^{-/-}$ mutants, p53 reporter-positive cells may be predominantly in G1-phase. Therefore, cell-cycle regulation by p53 in the absence of Shh may be tissue-specific.

![Figure 36](image)

**Figure 36.** BrdU labeling of 48 hpf wild-type and $shh^{-/-}$ mutant retinas carrying p53 reporter. Retinal cryosections of wild-type and $shh^{-/-}$ mutant embryos labeled with 1-hour BrdU pulse were stained with anti-BrdU and anti-GFP antibodies. Image stacks were obtained using confocal microscopy and projected.
RESULTS

Since FACS-based experiments showed that p53 reporter-positive cells have defects in their progression into G2-phase and mitosis, I next analysed this aspect of cell cycle progression in the shh<sup>−/−</sup> mutant retina. Staining cells for Ser10-phosphorylated form Histone3 (pH3) enables studies of mitotic cell populations, since pH3 is a reliable marker for cells in late G2-phase and mitosis (Hendzel et al., 1997). pH3 also lends itself well to quantification of proliferation due to relatively low numbers of positive cells. In addition pH3 can be co-localised with the EGFP signal from the p53 reporter transgene. Using these tools, I analysed cell cycle progression of retinal cells during neurogenesis at 34 and 48 hpf. At 34 hpf, in the wild-type retina there is ongoing neurogenesis and the level of proliferation is still high, whereas in shh<sup>−/−</sup> mutants neurogenesis is defective and the rate of retinal mitosis is lower than in their wild-type siblings (Stenkamp et al., 2002). Wild-type retina at 48 hpf is completing neurogenesis, and a high number of cells are going through their final mitoses. To investigate the influence of p53 on retinal proliferation in the shh<sup>−/−</sup> mutant, I first studied co-localisation of pH3 with p53 reporter nlsEGFP in wild-type and shh<sup>−/−</sup> mutant retinas at 34 and 48 hpf (Fig. 37A). At both stages, wild-type embryos had very few cells expressing p53 reporter. By contrast, in the shh<sup>−/−</sup> mutant retina at both 34 and 48 hpf most cells were p53 reporter-positive (Fig. 37A). The number of pH3-positive retinal cells was clearly smaller in the shh<sup>−/−</sup> mutant than in the wild-type at both 34 and 48 hpf (Fig. 37A). Moreover, some mitotic cells in the shh<sup>−/−</sup> mutant retina were p53 reporter-negative suggesting that not all mitotic cells experienced p53 influence (Fig. 37A). However, these co-localisation data are not very useful to quantify the influence of p53 on cell cycle progression in the shh<sup>−/−</sup> mutant retina because p53 reporter nlsEGFP nuclear signal becomes cytoplasmic in mitosis and may not co-localise with pH3 chromatin signal. In addition, wild-type and shh<sup>−/−</sup> mutant p53 reporter-negative cell populations are different and there is some variation in p53 reporter expression in different cells and embryos. Therefore, as a more direct approach I used genetic inactivation of p53 to understand its role in cell cycle regulation in the shh<sup>−/−</sup> mutant retina. At 34 and 48 hpf stages, I obtained samples of wild-type, the shh<sup>−/−</sup> mutant and the shh<sup>−/−</sup> p53<sup>−/−</sup> embryos and stained retinal sections with anti-pH3 antibody to identify mitotic cells and with DAPI to visualize cell DNA. At both 34 and 48 hpf there were fewer mitotic cells in the shh<sup>−/−</sup> mutant than in the wild-type retina with the difference less striking at 48 hpf than at 34 hpf (Fig. 37B). Strikingly, in the double shh<sup>−/−</sup> p53<sup>−/−</sup> mutant there was a clear increase in mitotic cell numbers and
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retina size at both 34 and 48 hpf (Fig. 37B). To normalize mitotic cell numbers with respect to the total cells in the retina, mitotic and total cells were counted for each retinal section image and mitotic cell percentages were calculated. In the first experiment at 34 hpf, wild-type retina contained 13.9 % (Standard Deviation(SD) = 1.53 %) pH3-positive cells, shh\(^{-/-}\) mutant retina had 7.13 % (SD=1.48%), which is almost 2 times fewer (Fig. 37C). By contrast, 34 hpf shh\(^{-/-}\) p53\(^{-/-}\) retinas had 11.5 % (SD=1.79%) pH3-positive cells, which is statistically different from the shh\(^{-/-}\) mutant retinal mitotic rate (t-test P<0.001) and almost reached the wild-type mitotic rate (Fig. 37C). This result suggests that in the absence of Shh decreased proliferation in the retina is due to p53 activity. Another possibility is that p53 loss by itself is sufficient to increase retinal proliferation. Most evidence in the literature suggests that p53 regulates proliferation only under conditions of stress or upon its ectopic activation. However, to address this possibility, I repeated analysis of mitotic rates at 34 hpf in wild-type, p53\(^{-/-}\), shh\(^{-/-}\) and the shh\(^{-/-}\) p53\(^{-/-}\) retinas (Fig. 37D). Clearly, p53 loss on its own is not sufficient to increase the mitotic rate, since mitotic rates of the wild-type retina (11.8 %, SD=1.73 %) and p53\(^{-/-}\) mutant retina (12.02 %, SD=1.46 %) are not statistically different (t-test, P=0.67). In the second experiment at 34 hpf, the mitotic rate in the shh\(^{-/-}\) mutant retina (7 %, SD=1.38 %) was also significantly lower than in the wild-type retina (t-test, P<0.001). Showing the rescue of retinal proliferation, the mitotic rate of the shh\(^{-/-}\) p53\(^{+/+}\) retina (12.1 %, SD=1.9 %) was even slightly higher than the wild-type retinal mitotic rate, but the difference was not significant (t-test, P=0.55). I then checked proliferation at 48 hpf in wild-type, shh\(^{-/-}\) and the shh\(^{-/-}\) p53\(^{-/-}\) retinas (Fig. 37E). Mitotic rates in wild-type and shh\(^{-/-}\) mutant retinas were 11.1 % (SD = 2 %) and 7.16 % (SD = 1.25 %), respectively, and significantly different from each other (t-test, P<0.001) (Fig. 37E). Similar to the results at 34 hpf, loss of p53 in the shh\(^{-/-}\) mutant (shh\(^{-/-}\) p53\(^{-/-}\)) retina led to a striking increase in the mitotic rate to 11.6 % (SD = 1.54 %), which is a complete rescue of mitosis since the difference between wild-type and shh\(^{-/-}\) p53\(^{-/-}\) mitotic rates is not significant (t-test, P= 0.34). The results at both 34 and 48 hpf conclusively demonstrate that p53 is responsible for decreased mitotic rates in the shh\(^{-/-}\) mutant retina during neurogenesis.
**RESULTS**

**Figure 37.** p53 regulates mitotic rate in \( shh^{-/-} \) mutant during neurogenesis.

A. pH3 staining of PG13p21::nlsEGFP p53 reporter retinas. Wild-type and \( shh^{-/-} \) mutant at 34 and 48 hpf were analysed. At both stages, wild-type retinas had a high level of mitotic cells and no expression of p53 reporter. By contrast, at the same stages mutant retinas had a lower number of mitotic cells and most retinal cells were p53 reporter-positive. Not all mitotic cells in the \( shh^{-/-} \) mutant retinas were clearly p53 reporter-positive.

B. pH3 / DAPI staining of wild-type, \( shh^{-/-} \) and \( shh^{-/-} p53^{-/-} \) retinas at 34 and 48 hpf. At both stages, mitotic rate in the \( shh^{-/-} \) retina was lower than in the wild-type retina. In the \( shh^{-/-} p53^{-/-} \) retina the mitotic rate almost reached that of wild-type retina.

C. Statistical analysis of pH3/DAPI stainings in wild-type, \( shh^{-/-} \) and \( shh^{-/-} p53^{-/-} \) retinas at 34 hpf, which are shown in B. 10 embryos and 20 retinal sections were analysed for each genotype.

D. Statistical analysis of the second pH3/DAPI staining experiment in wild-type, \( p53^{-/-} \), \( shh^{-/-} \) and \( shh^{-/-} p53^{-/-} \) retinas at 34 hpf. 10 embryos and 20 retinal sections were analysed for each genotype.

E. Statistical analysis of pH3/DAPI stainings in wild-type, \( shh^{-/-} \) and \( shh^{-/-} p53^{-/-} \) retinas at 48 hpf, which are shown in B. 10 embryos and 20 retinal sections were analysed for each genotype.
3 Discussion
3.1 Direct and indirect regulation of proliferation by Shh in different contexts

The cell-cell signaling events that direct vertebrate limb development have been the subject of intense research for more than a hundred years. This provides an excellent foundation for investigating the mechanisms whereby pattern formation is integrated with proliferation. This integration means that signaling pathways involved in patterning also exert their effects on proliferation. In this project, I have focused on two of the main signals important for patterning and growth of the vertebrate limb: the Shh and Fgf signaling pathways. While both signals are crucial for outgrowth of the limb bud, it has been very challenging to uncouple these signals from each other, since expression of Shh depends on Fgf signaling, and vice versa. For example, while AER ablation experiments have been interpreted as causing failure of limb outgrowth because they lead to loss of Fgf gene expression in the limb bud (Fallon et al., 1994; Niswander et al., 1993; Dudley et al., 2002), AER ablation simultaneously leads to loss of Shh expression from the ZPA (Niswander et al., 1994; Laufer et al., 1994), and so cannot be used to separate the effect of Fgf signaling on proliferation from the effect of Shh signaling. In order to overcome this situation, I have used a combination of loss-of-function and gain-of-function experiments in the zebrafish model system to uncouple Shh from Fgf signaling in the pectoral fin bud, and have assessed the effect of each signal on fin bud cell cycle gene expression and proliferation independently of the other signal.

My results show that the effect of Shh on proliferation during limb development is indirect, and is mediated by its effect on Fgf expression in the AER. Inhibition of Shh signaling leads to loss of cell cycle progression only after a relatively long delay period of around 13 hours, which correlates with a concomitant loss of Fgf signaling. Inhibition of Fgf signaling, on the other hand, leads to disruption of cell cycle progression very rapidly, after only 3 hours of inhibitor treatment, and this occurs even though activity of the Shh pathway is still present. This rapid effect of Fgf on cell-cycle progression suggests a direct transcriptional response of cell-cycle genes to the Fgf pathway in the limb bud, which is consistent with the direct mitogenic effect of Fgf signaling shown for several cell types in tissue culture.

Since Hh signaling has also been shown to have a direct mitogenic effect on some cell types, it is perhaps surprising that Shh directs proliferation indirectly in the vertebrate limb.
bud. However, there is at least one previous example of such an indirect effect of Hh signaling on proliferation. During *Drosophila* wing development, Hh is necessary for growth of the wing imaginal disc, but this effect is mediated via the Hh-dependent expression of Decapentaplegic (Dpp), a member of the Tgf-β family of secreted signaling proteins (Roy and Ingham, 2002; Burke and Basler, 1996; Martín-Castellanos and Edgar, 2002). Furthermore, the proliferative response of different cell types to Hh is clearly context-dependent, and Hh signaling can even function as a negative regulator of the cell-cycle in some cell types (Neumann, 2005). Interestingly, this negative effect of Hh signaling on proliferation also appears to be indirect in some cases. In the rodent colonic epithelium, for example, Hh signaling stimulates cell-cycle exit by antagonizing the Wnt pathway (van den Brink et al., 2004). In the *Drosophila* retina, on the other hand, Hh signaling has both positive and negative effects on cell-cycle progression (Firth and Baker, 2005). Cell-cycle arrest of cells in front of the retinal differentiation wave depends on Hh signaling in combination with Dpp, while cell-cycle re-entry behind the wave front also depends on Hh signaling, but in this case mediated by the Notch signaling pathway (Firth and Baker, 2005; Baonza and Freeman, 2005).

My results also show a context-dependent effect of Fgf signaling on cell-cycle progression. Thus Fgf signaling is clearly essential for cell-cycle progression in the pectoral fin buds and in the branchial arches, since expression of G1- and S-phase cell-cycle genes in these tissues is lost after only 3 hours of inhibition of the Fgf pathway. Inhibition of Fgf signaling fails to affect cell cycle progression in other organs, however, such as the retina and the optic tectum, or at earlier stages of development. The Fgf signaling pathway is therefore not a global mitogenic signal in the zebrafish embryo, but instead directs proliferation in a highly tissue-specific manner. Altogether, the evidence thus indicates that both the Hh and Fgf pathway affect cell cycle progression in some cell types but not in others, and that this effect can be either direct or indirect. The control of cell-proliferation in multicellular organisms can therefore only be understood in a context-dependent manner, and my results help to shed light on this question in the context of the vertebrate limb bud. The molecular mechanisms by which different cell types respond distinctly to the same signal are still poorly understood, but will undoubtedly be unravelled by future research.
3.2 Interaction of p53 and Shh in regulation of cell survival, cell cycle exit, and differentiation

3.2.1 p53 mediates apoptosis in the absence of Shh in zebrafish

During development cell number is controlled not only by the rate of proliferation, but also by the rate of cell death. Since Shh has been implicated in the regulation of apoptotic cell death, I examined the role played by zebrafish Shh in directing apoptosis during organogenesis. In this project I focused on the elevated apoptosis in the neural tube and retina of the \( shh^{-/-} \) mutant. I asked if lower Bcl2 family gene expression in the \( shh^{-/-} \) mutant can be the reason for cell death. However, no striking differences in expression of Bcl2 family genes \( bcl2, bcl2l, mcl1a \) and \( mcl1b \) were found between wild-type and \( shh^{-/-} \) mutant embryos (Fig. 17). Although I cannot exclude some tissue-specific expression differences between wild-type and \( shh^{-/-} \) mutants, these results suggest that Shh signaling does not regulate expression of anti-apoptotic Bcl2 family genes globally. Another possibility is that Shh regulates survival through a different mechanism. However, activation of Hh signaling can induce expression of Bcl2 gene in mouse keratinocytes (Bigelow et al., 2004), chick spinal cord (Cayuso et al., 2006) and mouse lymphoma cells, where Hh signaling is required for survival through maintenance of Bcl2 expression (Dierks et al., 2007).

Further expression analyses showed that p53 target genes such as \( p53 \) itself, \( p21, mdm2, cyclinG1, bax1, puma \) were up-regulated in the \( shh^{-/-} \) mutant relative to wild-type through most of development as confirmed by in situ hybridization and qPCR assays (Figs. 18, 19). Up-regulation of \( p53 \) gene expression occurred by p53-mediated transcription from an internal promoter generating a short \( p53 \) transcript encoding an inactive p53 isoform (Chen et al., 2005; Robu et al., 2007). p53 is indeed required for elevated apoptosis in the absence of Shh, since morpholino knockdown of p53 rescued apoptosis in the neural tube and in the retina of the \( shh^{-/-} \) mutant (Fig. 21). Over-expression of EGFP-bcl2 (Langenau et al., 2005) also rescued elevated apoptosis in the \( shh^{-/-} \) mutant showing that intrinsic apoptotic pathway is activated. In contrast to the studies showing that Bcl2 can inhibit p53 activity (Froesch et al., 1999; Beham et al., 1997; Ryan et al., 1994), p53 target gene expression remained normal after EGFP-bcl2 over-expression (Fig. 21), which suggests that effects of Bcl2 on p53 activity may be species- or tissue-specific. This result is also consistent with my observation that Bcl2
family gene transcription is unaffected by loss of Shh. These results suggest that Shh is necessary to inhibit the activity of p53 but it is unclear how direct this inhibition is. To address this question, I then studied the onset of p53 pathway activation and elevated apoptosis in the \( shh^{-/-} \) mutant, which turned out to happen at the 10somite stage (Fig. 20). The broad pattern of p53 target gene expression and apoptosis in the \( shh^{-/-} \) mutant at the 10somite stage argues against the idea that cells die due to differentiation failure, since few cells in the wild-type express neuronal differentiation marker HuC at this stage (Kok et al., 2007). Early requirement of Shh for cell survival during development is consistent with the suggestion that Hh signaling affects cell survival independently from and earlier than affecting cell fate (Cayuso et al., 2006).

The results above raise a question whether p53 activity can be suppressed by Hh signaling activation. Indeed, activation of Hh signaling using \( \text{dnPKA-GFP} \) can effectively suppress p53 target gene expression and apoptosis in the \( shh^{-/-} \) mutant at the 12somite stage (Fig. 26). As this thesis project was in progress, Abe et al. (2008) provided evidence that Hh signaling regulates stability of p53 by activating Mdm2 in human cell lines probably through induction of an unknown Mdm2-activating factor. Stecca and Altaba (2009) characterized the interaction between p53 and Gli1, a downstream Hh signaling mediator, in wild-type chick neural stem cells. These authors found that Gli1 can suppress p53 activity by up-regulating Mdm2 level. They also showed that p53 can interfere with Gli1 functions, which creates a negative feedback loop. These studies are consistent with and support my observations that p53 mediates apoptosis in the absence of Shh, and that Shh is required to control p53 activity \textit{in vivo}. Due to lack of antibodies against zebrafish Mdm2, I could not determine if Hh signaling up-regulates Mdm2 level in zebrafish. Nevertheless, since p53 activity can be inhibited by Hh signaling activation in human cell lines, chick neural stem cells and zebrafish embryos, the same mechanism is likely involved.

Concerning p53 regulation, the question is whether Hh signaling is required for establishment or maintenance of p53 activity control. Due to the localized nature of Shh expression, it is unlikely that cells would require persistent Shh signaling to maintain p53 activity control. Another possibility is that Shh is required to maintain control of p53 activity only during a certain period of development. Shh is not involved in the earliest stages of p53 control since p53 can already be activated in late gastrulation (Kratz et al., 2006), which is
much earlier than 10 somite stage. However, p53 activity control appears to become dependent on Shh signaling after the onset of Shh expression.

### 3.2.2 p53 regulates proliferation and differentiation in the \textit{shh}^{{\text{−/−}}} mutant retina

The insight that p53 is essential for apoptosis due to \textit{shh} loss allows investigating how apoptosis suppression affects proliferation and differentiation. The retina was chosen as a system to examine these aspects of apoptosis suppression. To suppress apoptosis throughout development of \textit{shh}^{{\text{−/−}}} mutants, double \textit{shh}^{{\text{−/−}}} \textit{p53}^{{\text{−/−}}} mutants were generated. Elevated apoptosis was suppressed in these double mutants both in the neural tube at 24 hpf and in the retina at 72 hpf (Figs. 21, 22). I then studied how suppression of apoptosis affects differentiation in the \textit{shh}^{{\text{−/−}}} mutant retina. In fact, in the \textit{shh}^{{\text{−/−}}} \textit{p53}^{{\text{−/−}}} mutant retina, RGC staining is strongly increased, many amacrine cells, which are absent in the \textit{shh}^{{\text{−/−}}} mutant retina, appear and both cone and rod photoreceptors are nearly completely rescued (Fig. 23). However, differentiation of bipolar and Müller glia cells was not rescued in the \textit{shh}^{{\text{−/−}}} \textit{p53}^{{\text{−/−}}} mutant (Fig. 24). These results show that Shh is required not for differentiation of most retinal cell types, but simply to promote survival of retinal progenitor cells. In contrast, no rescue Müller glia differentiation in the \textit{shh}^{{\text{−/−}}} \textit{p53}^{{\text{−/−}}} mutant retina supports previous work in the mouse showing that Shh is required for Müller glia development (Wang et al., 2002). Several previous studies in zebrafish have addressed how suppression of p53-mediated apoptosis affects retinal differentiation. In zebrafish mutants of DNA polymerase delta 1 subunit (\textit{pold1}) many cells die of apoptosis, and suppression of apoptosis in the retina by \textit{p53} knockdown rescues eye size and morphology (Plaster et al., 2006). Yamaguchi and colleagues (2008) found that in the case of the zebrafish \textit{primase1} mutant \textit{p53} knockdown led to the rescue of amacrine cell and photoreceptor differentiation. By contrast, \textit{p53} knockdown in \textit{chromatin assembly factor 1b} (\textit{caf1b}) mutant did not lead to rescue of retinal differentiation (Fischer et al., 2007). Differentiation rescue by loss of \textit{p53} activity in the \textit{shh}^{{\text{−/−}}} mutant retina is similar to the \textit{p53} knockdown results in \textit{pold1} and \textit{primase1} mutants. However, Shh likely affects differentiation by regulating p53 activity and through other mechanisms, whereas mutations of \textit{pold1} or \textit{primase1} were proposed to induce genotoxic damage. By contrast, \textit{caf1b} is required for both cell survival and differentiation of most cell types. Although p53 inhibits retinal
differentiation in the $shh^{+/−}$ mutant, it promotes differentiation in mouse embryonic stem cells after DNA damage by decreasing Nanog expression (Lin et al., 2005), and reduces spontaneous differentiation of human stem cells (Qin et al., 2007). Thus, the effect of p53 on differentiation is strongly context-dependent.

$p53$ loss in the $shh^{+/−}$ mutant rescues two aspects of retinal proliferation: cell-cycle exit of retinal cell progenitors (Fig. 25) and mitoses during neurogenesis (Fig. 37). Moreover, cyclin-dependent kinase inhibitor $p57kip2$ expression is rescued in the $shh^{+/−} p53^{+/−}$ mutant retina (Fig. 25C), which correlates with normal cell-cycle exit. These findings may represent two sides of the same phenomenon, since active cell-cycle exit must result in more mitoses of differentiating cells. Therefore, rescue of cell-cycle exit may be necessary and sufficient to rescue the mitotic rate in the $shh^{+/−}$ mutant retina. However, rescue of the mitotic rate in the $shh^{+/−} p53^{+/−}$ mutant retina may also result from lack of p53-activated cell cycle inhibitory gene expression. $p57kip2$ loss in the $shh^{+/−} p53^{+/−}$ mutant is required to understand the relative contributions of the two mechanisms. However, since $p57kip2$ knockdown results in phenotypes too variable to make clear conclusions, this experiment was not performed. Previous studies of cell-cycle exit in the zebrafish retina (Masai et al., 2005; Shkumatava and Neumann, 2005) showed that $p57kip2$ expression is regulated by Hh signaling. My results support this conclusion and uncover that in the $shh^{+/−}$ mutant p53 mediates lack of $p57kip2$ expression. Thus, a model can be proposed that Hh signaling is required for $p57kip2$ retinal expression by controlling activity of p53. Concerning the mechanism of $p57kip2$ regulation by p53, there are several studies suggesting that p53-mediated $Notch1$ expression may be responsible for lack of $p57kip2$ retinal expression. $Notch1$ is a p53 target in human keratinocytes (Lefort et al., 2005) and myeloid and lymphoid cells (Secchiero et al., 2009). Moreover, Katoh and Katoh (2008) found that in human and mouse embryonic stem cells Gli2 prevents p53 from activating $Notch1$ gene expression, which establishes a connection between p53, Hh and Notch pathways. In zebrafish, $Notch1$ homologs are also likely to be p53 targets since they contain optimal p53 binding sites (personal observation). Notch signaling inhibits $p57kip2$ expression during zebrafish neural tube development (Park et al., 2005), in pancreas development (Georgia et al., 2006), in the adult intestinal crypts (Riccio et al., 2008) and during eye lens development (Jia et al., 2007). It will be important to check if p53 blocks cell-cycle exit by activating Notch signaling.
3.2.3 p53 reporter expression in tissues requiring Shh for survival

Another important aim in this project was development of a p53 reporter line in zebrafish to study p53 activation. In the \( shh^{-/-} \) mutant p53 reporter-positive cells were detected in the retina as early as 24 hpf leading to the conclusion that extraretinal Shh signaling is required for control of p53 activity during retinal development, since no Shh is produced in the retina at 24 hpf (Fig. 32). In addition, p53 reporter activation and apoptosis were detected in the midbrain, hindbrain and spinal cord regions of the neural tube (Fig. 32, 33), consistent with the previous studies on the Shh survival function in these tissues (Charrier et al., 2001; Britto et al., 2002). p53 reporter expression but no elevated apoptosis were detected in somites of the \( shh^{-/-} \) mutant (Fig. 29, 32). However, p53 may mediate apoptosis in the somites of the \( shh^{-/-} \) mutant at earlier stages, which would make the elevated p53 reporter expression fully consistent with the role of Shh in sclerotome cell survival and myogenic differentiation (Borycki et al., 1999). Locations of p53 reporter-positive cells in the lateral midbrain and of apoptotic cells in streams from the neural tube in whole-mount stainings at 24 hpf suggest their neural crest origin (Figs. 32, 15). These observations agree with the fact that Shh ensures survival of these cells (Ahlgren and Bronner-Fraser, 1999). Taken together, these results show that p53 reporter is detected in most regions, in which Shh plays the cell survival role. p53 reporter-positive cells could also be visualized in terms of their progression through S-phase, mitosis and apoptotic phenotypes (Figs. 34, 36,37). Moreover, FACS sorting of p53 reporter-positive cells enabled studying their cell cycle distribution and helped understand how p53 affects cell cycle progression in the \( shh^{-/-} \) mutant retina (Fig. 35). Finally, p53 reporter activation could also be induced by p53 activating drugs such as roscovitine (Fig. 28). These results clearly show that the transgenic p53 reporter line is more advantageous than \textit{in situ} hybridizations for detecting expression of p53 target genes, or even than antibodies against p53 (Lee et al., 2008), which are used for visualizing cells with active p53. The current form of the p53 reporter is based on nlsEGFP, but it is certainly possible to replace nlsEGFP with fluorescent proteins of other colours or with degradable fluorescent proteins to obtain a dynamic picture of p53 activity.
3.3 Conclusions and Outlook

Previous studies have shown that both Shh and Fgf signaling are crucial for outgrowth of the vertebrate limb. The results presented here show that the role of Shh in this process is indirect, and is mediated by its effect on Fgf signaling. By contrast, the activity of the Fgf pathway affects proliferation directly and independently of its effect on Shh. These results show that Fgf signaling is of primary importance in directing outgrowth of the limb bud, and clarify the role of the Shh-Fgf feedback loop in regulating proliferation.

The work presented here has also clarified several aspects of the role played by p53 in mediating Shh-directed control of cell survival, cell-cycle exit and differentiation. In the absence of Shh p53 mediates apoptosis in the neural tube and the retina. In the context of the retina, p53 is the mechanism downstream of Shh regulating proliferation, differentiation and survival. It is fascinating that a single mechanism can regulate such different biological processes, and previously Shh would be envisioned to control these processes through distinct mechanisms. On the other hand, there are several different mechanisms downstream of p53 as well, which means that the findings in this thesis point us in the promising direction to search for additional important mechanisms.

The role of Shh-regulated Bcl2 expression versus p53 pathway control in different cases of Shh-controlled cell survival should be uncovered by future studies. Another interesting question is how p53 pathway activation is controlled temporally and spatially during development in the absence of Shh. Moreover, future research will be necessary to unravel p53-mediated Hh signaling functions in the context of proliferation and differentiation in different model systems and tissues.
4 MATERIALS AND METHODS

4.1 Fish stocks, maintenance and identification

Wild-type Tupfel Long Fin (TLF), sonic-you\textsuperscript{14} (shh null mutation, Schauerte et al., 1996) and p53\textsuperscript{dcl} (Berghmanns et al. (2005); obtained from Schulte-Merker laboratory) heterozygous fish were used. Fish were maintained according to standard protocols. Embryos were grown in E3 embryo medium at 28 °C with or without the addition of 0.003% 1-phenyl-2-thiourea (PTU) (Sigma, cat# P7629) to inhibit pigmentation. Staging was performed according to hours post-fertilization (hpf) (Westerfield, 1995). Identification of \textit{shh}\textsuperscript{-/-}p53\textsuperscript{-/-} was performed by first selecting acridine orange-negative embryos and then performing PCR on embryo tails with pairs of the following primers to check the embryos for either wild-type or mutant alleles as described in Berghmanns et al. (2005): p53\text{ID\_for:} GATAGCCTAGTGCGAGCACACTCTT, p53\text{wt\_ID\_rev:} AGCTGCATGGGGGGAT and p53\text{mut\_ID\_rev:} AGCTGCATGGGGGGAA. Alternatively, only PCR genotyping was performed. The p53 genotype of adult fish was identified using the same PCR assay on fin clips. Fin clips were obtained by anesthetizing adult fish in 0.02 % Tricaine and then cutting small pieces of the caudal fin. All DNA samples for identification were prepared by boiling in 20-50 μl of 50 mM NaOH at 95 °C for 10 minutes and then neutralizing the resulting lysate with 1M Tris-HCl pH 8.2 as described previously (Meeker et al., 2007).

4.2 Chemicals and solutions

\textbf{60x stock solution E3 saline:}

\begin{itemize}
  \item 34.8g NaCl
  \item 1.6g KCl
  \item 5.8g CaCl\textsubscript{2} x 2H\textsubscript{2}O
  \item 9.78g MgSO\textsubscript{4} x 6H\textsubscript{2}O
  \item H\textsubscript{2}O ad 2l
  \item pH 7.2 with NaOH
  \item autoclave
  
\end{itemize}

for 1x use 16ml/l + 100μl methylene blue solution
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10x loading buffer:
- 50% glycerol
- 1x TE
- 0.25% bromophenol blue
- 0.25% xylene cyanol

TE:
- 10 mM Tris/HCl pH 7.4
- 1mM EDTA pH 8.0

50xTAE:
- 242 g Tris base
- 57.1 ml glacial acetic acid
- 100ml 0.5M EDTA
- Add ddH2O to 1 liter and adjust pH to 8.5.

10xPBS:
- 70g NaCl;
- 62.4g Na2HPO4 x2H2O;
- 3.4g KH2PO4
- pH7.4

DEPC H2O:
- Di-ethyl pyrocarbonate (DEPC) was dissolved in H2O at 0.1 %. The solution was incubated overnight at 37 °C and then autoclaved.

Acridine Orange
- Acridine Orange (Molecular Probes, Invitrogen, cat# A1301) was dissolved at 10 mg/ml in water and used at 1:5000 dilution for live apoptosis staining

4.3 Microinjection of mRNA and morpholino

p53 morpholino (GCGCCATTGCTTTGCAAGAATTG; Langheinrich et al., 2005) was purchased from Gene Tools and was injected into 1-cell stage at 0.5 mM. Five-mismatch control morpholino against her4 gene (GTACGACTCATTGGTGTCTGTGTTG) was used as a control morpholino. To make RNA for over-expression experiments, required pCS2+ vectors were linearised and transcribed using mMessage mMACHINE SP6 (Ambion, cat # AM1340).

The following plasmids were used to prepare RNA for microinjections:
- pCS2+dnPKA-GFP (Moon lab)
- pCS2+EGFP
- pCS2+EGFP-bcl2 (Look lab)
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pCS2+H2BmRFP1 (provided by Gilmour lab)
pCS2+FA Transposase (Tol2kit)

4.4 p53 reporter fish generation

4.4.1 p53 reporter construction

Human CDKN1A/p21 2.4 kb promoter was amplified from RPCI-11 Human BAC clone (Imagenes, RPCIB753F15397Q) using the following primers:

HindIII_p21prom_for : AAGCTTGGGCACTCTTGTCCTGG
BamHI_p21prom_rev: GGATCCCTGACTCTTCGGCAG

The resulting PCR product was TOPO-cloned into pCR2.1 (Invitrogen) and then subcloned into p5E-MCS vector using HindIII and BamHI restriction sites. The resulting plasmid was named p5E-p21. PG13 enhancer (13 copies of an optimal p53 binding site CCTGACTTTGACTGCTGC) was synthesised by GeneArt (custom product). The synthetic PG13 fragment contained KpnI and HindIII restriction sites for directional cloning into p5E-p21. Insertion of PG13 into p5E-p21 resulted into the final 5’-entry vector p5E-PG13p21.

To generate the construct for injection into embryos, p5E-PG13p21 was recombined with pME-nlsEGFP, p3E-polyA and pDestCG2 vectors (Tol2kit, Kwan et al., 2007).

4.4.2 p53 reporter injection and carrier fish identification

pDestCG2-PG13p21::nlsEGFP-polyA plasmid was mixed with FA Transposase RNA to final concentrations 15 ng/μl and 20 ng/μl in DEPC H₂O, respectively. The mix was injected into wild-type oocytes, and injected embryos were grown to adulthood. Subsequently, F0 founder fish were outcrossed into wild-type and sonic-you backgrounds to generate F1 transgenic animals, which were used for the experiments and outcrossed once more to generate F2 generation.
4.5 General cloning procedures

4.5.1 RNA purification

For cloning:
To collect total embryo RNA, 30-50 embryos at the required stage were frozen in liquid nitrogen. RNA was then purified using RNeasy Mini kit (Qiagen, cat# 74104) according to the manufacturers’ protocol and eluted in DEPC H₂O.

For quantitative and semi-quantitative PCR:
- Transfer embryos (up to 100) to Eppendorf tube. Remove water with glass Pasteur pipette.
- Add 400 µl TRIzol Reagent and directly grind until embryos are dissolved.
- Then add 600 µl TRIzol reagent and mix.
- At this point leave 5 min at room temperature and continue, or freeze the lysate at –20°C.
- Add 200 µl chloroform for each ml reagent and shake the tube vigorously for 15 seconds.
- Leave 5-10 min at room temperature.
- Centrifuge at 12000g for 5 min
- From now on one should work RNase free!
- Transfer upper aqueous phase to new tube and precipitate with 0.5 ml isopropanol (for each ml reagent that was initially used)
- Leave 15 min or longer at room temperature.
- Centrifuge at 12000g for 10 min at 4°C.
- Remove supernatant, centrifuge short to collect and remove remaining supernatant.
- Add 1 ml 75% ethanol, vortex, and centrifuge 5 min.
- Remove 75% ethanol. Centrifuge short to collect and remove remaining ethanol. Dry pellet for 5 min at room temperature.
- Dissolve pellet in the desired amount of RNase free water (50µl).
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- Measure RNA concentration and run on a gel for short time to verify that degradation has not occurred (The upper 28S band should have twice the intensity of the lower 18S band).

4.5.2 cDNA synthesis

cDNA first-strand synthesis for cloning was performed using SuperScript III Reverse Transcriptase system (Invitrogen, cat# 18080-051) and to prepare cDNA for quantitative PCR Quantitect Reverse Transcription Kit (Qiagen, cat# 205311) was used according to manufacturers’ instructions.

4.5.3 Cloning probe template plasmids

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<th>Primer name</th>
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4.6 Quantitative Real-time PCR and semiquantitative PCR

cDNA samples prepared using Quantitect cDNA synthesis kit from whole embryos or embryo heads were used for quantitative real-time PCR after a 10-fold dilution. SYBR® Green PCR Master Mix (Applied Biosystems, cat# 4309155) was used to prepare qPCR reactions, which were run on the ABI7500 qPCR machine.

Table 2. Primers for PCR assays

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4.7 Sequencing

DNA Sequencing was performed by either EMBL Genomics Core Facility or by GATC Biotech.
4.8 Probe synthesis

4.8.1 Linearising DNA for *in vitro* transcription reactions

- Take 10 μg of a plasmid of interest and set up a digest reaction in 100 μl.
- Incubate for several hours or overnight at 37 C and run 5 μl on gel to check for linearity.
- Add 1 μl of Proteinase K (20 mg/ml) and 5 μl of 10 % SDS and incubate at 50 C for 1h. Add 100 μl water.
- Add an equal amount of 25:24:1 (v/v) phenol-chloroform-isoamyl alcohol and vortex.
- Separate the aqueous phase which contains the DNA from the organic phase by centrifugation at 8000 rpm for 1 min.
- In order to precipitate the DNA, add a 0.1 volume of 3 M sodium acetate, pH 5.5, to the aqueous phase and then 2 volumes of absolute ethanol. Incubate at –20 °C overnight or for shorter periods at –80 °C (20–30 min).
- Recover the precipitated DNA by centrifugation in the microfuge at 10 000 rpm for 15 min. Remove the ethanol with care and dry the pellet in a desiccator or 50 °C oven for 5 minutes.
- Wash with 1 ml of 70% (v/v) ethanol/DEPC. Remove the ethanol solution with care and dry the pellet in a desiccator or 50 °C oven for 5 minutes.
- Resuspend dried DNA in 20 μl of DEPC-treated water and store at –20 °C.

4.8.2 *In vitro* transcription reactions

*In vitro* transcription reactions for mMessage mMachine SP6 (Ambion, cat# AM1340) and DIG RNA Labeling Kit (Cat # 11175025910)) were performed according to manufacturers’ instructions.

4.8.3 Purification of RNA from *in vitro* reactions

After transcription, the DNA template is digested by the addition of 2 μl RNAse free DNase (Roche 776785) for 15 min at 37°C.

- Add 30 μl DEPC H2O and 30 μl LiCl precipitation solution (7.5 M)
- Chill for 2 hours at -20 °C
MATERIALS AND METHODS

- Centrifuge at 4°C for 30 min at 10,000 g
- Add 1 ml of 70% Ethanol/DEPC H₂O to the pellet
- Centrifuge at 4 °C for 5 minutes
- Remove the supernatant, shortly air dry and resuspend in 30 µl DEPC H₂O

4.9 Histochemical methods

4.9.1 In situ RNA hybridisation
RNA in situ hybridisations were performed according to Jowett (2001). Probes were made using Roche DIG RNA Labeling Kit (Cat # 11175025910). Antisense probes were made to detect expression of the following genes: *ptc1, pea3* (Norton et al., 2005), *cyclin D1* (Shkumatava and Neumann, 2005), *pcna, mcm5, ra1, bcl2, bcl2l, mcl1a, mcl1b, p53, mdm2, bax1, puma*. cyclinG1 probe was prepared from pCMV-SPORT6.1-cyclinG1 (RZPD Clone ID IMAGp998A0314862Q1) plasmid, which was digested with EcoRI and transcribed with T7 polymerase. Cloning of plasmids for making probes is described in the section on cloning. Templates for making anti-sense probes were prepared either by linearizing plasmids with restriction enzyme digests or by PCR with primers containing phage promoters.

4.9.2 Cryosectioning procedure
- Fix dechorionated embryos in 4% PFA/PBS for 1h at RT or O/N at 4°C
- Wash with PBS
- Incubate 2 hours at RT (O/N – a few days at 4°C) in 20% sucrose/PBS
- Remove embryos from sucrose and place in plastic moulds (Polysciences)
- Take off sucrose as well as possible
- Cover embryos with Tissue-Tek (Plano, cat# 4383)
- Align embryos in required position (ventral to the mold bottom) under the microscope
- Place molds on dry ice or into -80 °C freezer so that Tissue-tek hardens (about 20 min)
- Detach blocks by pressing hard with the thumb against the mold bottom
MATERIALS AND METHODS

- place blocks on holders
- place a holder in the cryostat and start to cut
- try to find the plane containing embryos by trimming 30-50μm sections
- cut embryos in 12μm sections
- transfer sections in a line to slide (super frost slides, Roth), 5-7 sections per line are optimal
- leave to dry at RT at least 1h

4.9.3 Antibody staining on cryosections

4.9.3.1 Standard antibody staining protocol

- Rehydrate cryosections in PBS with 0.1 % Tween-20 (PBST) for 1 hour
- Block for 1 hour with blocking solution (4 % normal goat serum in PBST).
- Incubate with desired primary antibody for several hours at RT or overnight at 4 °C
- Wash slides 3 times for 10 min in PBST
- Incubate for 2 hours with a fluorescently-labeled secondary antibody in blocking solution (1:400)
- Wash slides 3 times for 10 min in PBST
- Embed the slides in moviol and let dry before microscopy

The following antibodies were used in this thesis:

- Anti- zpr1 - Developmental Studies Hybridoma Bank, 1:100
- Anti-zpr3 - Developmental Studies Hybridoma Bank, 1:100
- Anti-zn5 - Developmental Studies Hybridoma Bank, 1:50
- Anti-parvalbumine – Chemicon (MAB1572), 1:200
- Anti-GAD67 – Chemicon (AB5862P), 1:100
- Anti-glutamine synthetase – BD Biosciences (cat# 610517), 1:500
- Anti-PKCα – Santa Cruz Biotechnology (sc-208), 1:100
- Anti-GFP (mouse) – Molecular Probes, Invitrogen (A11120), 1:200
- Anti-GFP (rabbit) – Molecular Probes, Invitrogen (A11122), 1:200
- Anti-BrdU (mouse) – Roche (cat# 1-299-964), 1:100
MATERIALS AND METHODS

Anti-pH3-S10 (rabbit) – Abcam (ab5176-100), 1:400
Anti-Digoxigenin-Alkaline Phosphatase – Roche (cat# 11093274910), 1:2000
Anti-activeCaspase3 – BD Pharmingen (cat# 557035), 1:200

4.9.3.2 BrdU staining protocol

- Rehydrate cryosections in PBST for 1 hour
- Treat with 4N HCl for 20 min
- Wash slides 3 times for 5 min in PBST
- Block for 1 hour with blocking solution
- Incubate with the mouse anti-BrdU antibody diluted in blocking solution (1:100) (Roche, cat# 1 170 376) overnight
- Wash slides 3 times for 10 min in PBST
- Incubate for 2 hours with the anti-mouse Alexa Fluor 488 antibody (Invitrogen, cat# A11001) diluted in blocking solution (1:400)
- Wash slides 3 times for 10 min in PBST
- Embed the slides in moviol and let dry before microscopy

4.9.4 Apoptosis stainings

4.9.4.1 TUNEL assay on whole-mount embryos

Whole-mount TUNEL staining was performed as described in Berghmanns et al., (2005).
- Fix embryos overnight in 4% paraformaldehyde and dehydrated in PBS/methanol series; 50%, 70%, 95%, and 100% (5 minutes each at room temperature (RT))
- Embryos were incubated in 100% acetone at –20°C (10 min) and rinsed three times in PBS containing 0.1% Tween-20 (PBST) (5 minutes each)
- Embryos were permeabilized by incubation in fresh 0.1% sodium citrate in PBST (15 minutes, RT), followed by three rinses in PBST (5 minutes each)
- Embryos were assayed by TUNEL by using the In Situ Cell Death Detection Kit, TMR Red (Roche).
4.9.4.2  TUNEL assay on cryosections

- Wash cryosections with PBST for 30 min
- Permeabilise by incubating slides for 2 min in ice-cold 0.1 % Sodium Citrate with 0.1 % Triton X-100
- Wash cryosections with PBST 2 times for 5 min
- Incubate with TUNEL reaction mix diluted twice with TUNEL dilution buffer (cat # 1966006001) for 1 hour
- Wash cryosections with PBST 2 times for 5 min

4.9.4.3  ApopTag In Situ Apoptosis Detection Kit

The protocol uses the ApopTag kit from Chemicon (cat# S7100).

- Fix embryos for 2 h at RT or overnight at 4 °C in 4 % PFA/PBST
- Wash in PBST and transfer to methanol for 30 min at -20 °C
- Rehydrate embryos in a graded methanol:PBST series (75 % MeOH, 50 % MeOH, 25 % MeOH) for 5 min each
- Wash once for 5 min in PBST (Washing volume is 0.5 ml)
- Digest embryos in proteinase K solution in PBST (10 /ul) at RT. 24 hpf embryos should be digested 2 minutes, embryos of all other stages should get 10 minutes for each additional day of development
- Rinse 2 times in PBST
- Postfix in PFA/PBST for 20 min at RT
- Wash 5 times for 5 min in PBST
- Postfix for 10 min at -20 °C with pre-chilled EtOH : acetic acid in ratio 2 : 1
- Wash 3 times for 5 min in PBST
- Remove very carefully all the liquid from embryos (use vacuum pump with 10 ul tip attached), add 37 ul of Equilibration buffer and incubate for 1 h at room temperature
- Remove very carefully the previous buffer. Add 25 ul of Reaction buffer and 8 ul of the TdT enzyme
MATERIALS AND METHODS

- Incubate overnight at 37 °C
- Wash with 250 ul of Stop/Wash Buffer for 3 hours at 37 °C
- Wash 3 times for 5 min with PBST
- Block with 2 mg/ml BSA, 5 % goat serum in PBST for 1 h at RT
- Remove the liquid as before and add 500 ul of anti-Dig-AP Fab 1:2000 in blocking solution (Roche)
- Wash 4 times for 30 min in PBST
- Wash 3 times for 5 min with 50 % Staining Buffer and develop staining with NBT-BCIP solution (1:100) in 50 % Staining Buffer
- Wash with PBST and store in 4 % PFA/PBST at 4 °C

4.9.4.3 Acridine Orange apoptosis staining

Dechorionated embryos were incubated with 2 µg/ml of Acridine Orage (AO) in E3 embryo medium for 30 minutes. The traces of AO were removed by 5 changes of the embryo medium. Embryos were then imaged using a fluorescent microscope.

4.10 Chemical inhibitors and treatment procedures

FGF signaling inhibitor SU5402 (Calbiochem, cat # 572630) was dissolved in DMSO at 8 mM. Treatment was performed with a 10 µM solution of SU5402 or a corresponding control DMSO solution in E3 embryo medium. Cyclopamine (Toronto Research Chemicals, cat# C988400) was dissolved in ethanol at 20 mM. Treatment was performed with 100 µM solution of cyclopamine or control ethanol solution in E3 embryo medium. Roscovitine was purchased from Sigma (cat # R7772), resuspended in DMSO at 12.5 mM and used for treatments at 1:250 dilution in E3 medium.

4.11 Bead implantation

Bead implantation was performed as described before (Norton et al., 2005). Recombinant human Fgf4 protein (R&D Systems, cat# 235-F4-025) was dissolved at a concentration 250 ng/µl in PBS with 0.1% bovine serum albumin and mixed in proportion 1:1 with the mix of heparin-acrylamide beads (cat# H5263) filtered through a 70 µm Cell strainer (BD Falcon, cat# 352350).
4.12 Fluorescence Activated Cell Sorting methods

4.10.1 EGFP/Propidium Iodide cell cycle analysis protocol

The following protocol is suitable for quantifying cell cycle distribution of EGFP-positive cells. It was provided by Marcel Souren and modified on the basis of a published flow cytometry protocol (Schmid and Sakamoto, 2001), which allows imaging of both Propidium Iodide and EGFP.

- Decapitate 40-50 embryos for each condition
- Transfer to a very small petri dish and remove as much liquid as possible
- Add 3 ml of Trypsin solution (Sigma, cat# T3924)
- Either triturate using an extended Pasteur pipette or 1 ml tip (for the latter it is important to press the tip down to the bottom of the dish)
- Check for complete dissociation visually or under a microscope
- Add 4 ml of TM-1% PEG with 10 % goat serum
- Transfer the solution to a 15 ml Falcon tube
- Centrifuge for 5 min at 1200 g at 4 °C
- Remove supernatant with the vacuum pump and add 4 ml of TM-1% PEG with 10 % goat serum and centrifuge for 5 min at 1200 g at RT
- Remove supernatant and resuspend cells in 2 ml of cold PBS
- Put a cell strainer on a FACS tube and pipet cell suspension through it
- Add 2 ml μl of 2 % Paraformaldehyde in PBS and incubate for 1 hour at 4 °C
- Centrifuge for 5 min at 1200 g at 4 °C and wash once in 5 ml of PBS
- Add 1 ml of 70 % ethanol at -20 °C drop-wise while mixing the cell suspension on slow vortex regime and incubate overnight
- Centrifuge for 5 min at 1200 g at 4 °C and wash twice in 3 ml of PBS
- Resuspend in 1 ml of PI buffer

Materials
- TM-1% PEG: 100 mM NaCl, 5 mM KCl, 5 mM Hepes, 1 % (w/v) PEG 20000 pH 7.0
- PBS
- Normal goat serum
MATERIALS AND METHODS

- PI buffer: 980 µl PBS + 10 µl propidium iodide + 10 µl RNase A

4.13 Confocal microscopy, image processing and analysis

Microscopy was performed using Leica SP2 confocal microscope, images were processed using ImageJ software. Additional image adjustments were made using Adobe Photoshop CS2 and panels of images assembled using Adobe illustrator CS2. For counting nuclei, Cell Counter plugin of ImageJ was employed.
5 PUBLICATIONS

Prykhozhij SV, Neumann CJ.  
Distinct roles of Shh and Fgf signaling in regulating cell proliferation during zebrafish pectoral fin development.  

Fischer S, Prykhozhij S, Rau MJ, Neumann CJ.  
Mutation of zebrafish caf-1b results in S phase arrest, defective differentiation, and p53-mediated apoptosis during organogenesis.  
Cell Cycle. 2007 Dec 1;6(23):2962-9.

Vanoosthuyse V, Prykhozhij S, Hardwick KG.  
Shugoshin 2 regulates localization of the chromosomal passenger proteins in fission yeast mitosis.  
6 REFERENCES


REFERENCES


REFERENCES

111. I Masai, Yamaguchi M, Tonou-Fujimori N, Komori A, Okamoto H: The hedgehog-PKA pathway regulates two distinct steps of the differentiation


170. P Secchiero, E Melloni, MG di Iasio, M Tiribelli, E Rimondi, F Corallini, V Gattei, G Zauli: Nutlin-3 upregulates the expression of Notch1 in both myeloid and lymphoid leukemic cells, as part of a negative feedback anti-apoptotic mechanism. Blood 2009.


207. M Yamaguchi, Fujimori-Tonou N, Yoshimura Y, Kishi T, Okamoto H, Masai I.: Mutation of DNA primase causes extensive apoptosis of retinal neurons


7 Appendix

7.1 Abbreviations

AER   apical ectodermal ridge
AP    Alkaline phosphatase
BAC   bacterial artificial chromosome
bHLH  basic helix-loop-helix
bp    base pairs
BSA   bovine serum albumin
BrdU  5-bromodeoxyuridine
°C    degree Celsius
CDK   Cyclin-dependent kinase
cDNA  complementary DNA
Ci    Cubitus interruptus
CiA   Cubitus interruptus activator form
CiR   Cubitus interruptus repressive form
CKI   Casein kinase I
CNS   Central nervous system
C-terminus Carboxy-terminus of a peptide
Da    Dalton
DAPI  4',6-Diamidin-2'-phenylindol-dihydrochlorid
DMSO Dimethylsulfoxide
DNA   Deoxyribonucleic acid
dNTP  deoxy-A/C/G/T-trisphosphate
Dpp   Decapentaplegic
EGFP  Enhanced green fluorescent protein
EtOH  Ethanol
FACS  Fluorescence-activated cell sorting
Fgf (FgfR) Fibroblast growth factor (receptor)
GCP   Granular cell precursor
GPC3  Glypican3
GS    Glutamine synthetase
h     hour(s)
H2BmRFP1 Histone2B red fluorescent protein 1
Hh    Hedgehog
hpf   hours post fertilization
IFT   Intraflagellar transport
IPL   Inner plexiform layer
ISH   In situ hybridisation
kB    kilo bases
M     mol/l
MO    Morpholino oligonucleotide
mRNA messenger RNA
nls nuclear localisation signal
N-terminus Amino-terminus (of a peptide)
O/N Overnight
ONL Puter nuclear layer
PBS Phosphate-buffered saline
PCR Polymerase chain reaction
PFA Paraformaldehyde
pH3 phospho-serine10 Histone3
PG13 13 copies of an optimal p53 binding site
PI Propidium Iodide
PKA Protein kinase A
PKC Protein kinase C
Ptc Patched
PTU 1-phenyl-2-thiourea
qPCR quantitative PCR
RBF Drosophila retinoblastoma
Rb Retinoblastoma
RGC Retinal ganglion cells
RNA Ribonucleic acid
RPE Retinal pigmented epithelium
RT Room temperature
Shh Sonic hedgehog
Smo Smoothened
TE Tris/EDTA
TGFβ Transforming growth factor β
TLF Tupfel long fin
TUNEL TdT-mediated dUTP-biotin nick end labeling
ISH In situ hybridization
ZPA Zone of polarising activity
Curriculum Vitae

Name
Sergey Prykhozhij

Born
16th March 1983 in Kishenev, Moldova

Nationality
Belarusian

A. University Education and Research

European Molecular Biology Laboratory and University of Heidelberg

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University of Edinburgh

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<td>2003 – 2005</td>
<td>University of Edinburgh</td>
<td>Edinburgh,</td>
<td>Bachelor of Science (Honours) in Molecular Biology (First Class Honours Degree, 76 %).</td>
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<td>Focus on genetics, genomics, molecular and structural biology with strong practical and research components.</td>
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<td>Summer research project on purification of kinetochore complexes from metaphase-arrested fission yeast Schizosaccharomyces pombe cells.</td>
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Belarusian State University

2000 – 2003

Minsk, Belarus

Belarusian State University, Faculty of Biology, Biotechnology group (marks: 4.99 out of 5).

General studies of biology, mathematics and natural sciences, humanitarian subjects with later focus on molecular biology, genetics and biochemistry.

Research work on the role of Interleukin-8 in inflammation and sepsis in the lab of Dr. Nikolai Voitenok

B. Conference attendance

- Poster presentation at Zebrafish Genetics and Development Conference “Identifying pathways involved in apoptosis in sonic-you mutant and p57Kip2 morphant” in Amsterdam, 2007
- A selected talk “Analysis of apoptotic pathways in zebrafish Sonic hedgehog mutant and p57Kip2 morphant” at the Horizons PhD symposium in Göttingen 2007

C. Awards

1997 - 5th place in Brest region Chemistry Olympiad
1998, 2000 - First diploma in Belarus Republican Biology Olympiad
2000 - Bronze medal at the International Biology Olympiad (IBO) held in Antalia, Turkey in July 9th –16th, 2000.
2003 - Darwin Trust Scholarship to study at the University of Edinburgh toward Honours BSc degree in Molecular Biology
2005 - Amersham Prize for the Top Molecular Biology student of 2005 at the University of Edinburgh
2005 – EMBL Pre-doctoral Fellowship

D. Publications

