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p53 and p73 in neurogenesis of the adult zebrafish *Danio rerio*

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Summary

Compared to the adult mammalian brain, the brain of the adult zebrafish *Danio rerio* exhibits a very high proliferative and regenerative potential. The adult mammalian brain in contrast has a very limited neurogenic capacity mainly restricted to two zones, the subventricular zone of the lateral telencephalic ventricles and the subgranular zone of the dentate gyrus of the hippocampus. In contrast, the zebrafish brain harbours 16 proliferation zones distributed all over the brain. The zebrafish has thus become a model for the study of adult neurogenesis and regeneration of nervous tissue. I characterized the expression of the two transcription factors p53 and p73 in the adult zebrafish brain. Both p53 and p73 were shown to play crucial roles in mammalian adult neurogenesis: p53 suppresses the self-renewal of adult neural stem cells and is involved in apoptotic death of neurons following damage. p73 is relevant for the survival of neurons, self-renewal and maintenance of neural stem cells as well as differentiation of precursor cells. It was thus of interest whether these genes have similar roles in the adult zebrafish brain.

I established a detailed map of the expression pattern of *p53* and *p73* mRNA and p53 protein in the adult zebrafish brain. *p53* and *p73* mRNA expression overlaps in many regions including neurogenic zones. The p53 protein is expressed in most of these regions indicating that the mRNA expression reflects the protein expression. The p53 protein is expressed in mature neurons, Type I cells (non-dividing radial glial cells) and Type IIIa and Type IIIb cells (neuroblasts) in the adult zebrafish telencephalon. In cells of the oligodendrocyte lineage and in Type II cells (dividing radial glial cells) an expression of the p53 protein is not detectable. After stab injury of the adult zebrafish telencephalon both *p53* and *p73* genes are up-regulated. p53 is up-regulated in Type I cells. In contrast to the uninjured brain, p53 is expressed in cells of the oligodendrocyte lineage following injury. Furthermore, target genes of p53 are up-regulated and apoptosis is induced after stab injury. These results suggest a role for p53 in constitutive and regenerative neurogenesis. However, $tp53^{M214K}$ mutant zebrafish do not show any phenotype. The structurally related p73 is expressed in a very similar pattern as p53 in the uninjured and injured zebrafish brain. Therefore, redundancy between p53 and p73 may occlude the manifestation of a phenotype in the p53 mutant. Taken together, the analysis of expression of both p53 and p73 in the adult zebrafish brain suggests a role of these genes during constitutive and regenerative neurogenesis. The future elucidation of the precise function of the two genes in these processes requires, however, double mutant analysis.

Zusammenfassung

Im Gegensatz zum Gehirn von adulten Säugetieren weißt das Gehirn des adulten Zebrabärblings *Danio rerio* eine sehr hohe Möglichkeit zur Proliferation und Regeneration auf. Das Gehirn von adulten Säugetieren hat im Gegensatz dazu eine sehr eingeschränkte Fähigkeit zur Neurogenese, welche hauptsächlich auf zwei Zonen beschränkt ist, die subventrikuläre Zone der lateralen Ventrikel des Telencephalons und die subgranuläre Zone des Gyrus dentatus des Hippocampus. Das Gehirn des Zebrabärblings besitzt indessen 16 proliferative Zonen, welche über das gesamte Gehirn verteilt sind. Der Zebrabärbling wurde deshalb zu einem Modell für die Untersuchung der adulten Neurogenese und Regeneration von Nervengewebe. Ich habe die Expression der beiden Transkriptionsfaktoren p53 und p73 im Gehirn von adulten Zebrabärblingen charakterisiert. Es wurde gezeigt, dass sowohl p53 als auch p73 wichtige Rollen in der adulten Neurogenese von Säugetieren spielen: p53 hemmt die Selbsterneuerung von adulten neuralen Stammzellen und ist am apoptotischen Zelltod von Neuronen nach einer Schädigung beteiligt. p73 ist wichtig für das Überleben von Neuronen, Selbsterneuerung und Erhaltung von neuralen Stammzellen und ebenso für die Differenzierung von Vorläuferzellen. Deshalb war es von Interesse, ob diese Gene ähnliche Rollen im Gehirn von adulten Zebrabärblingen besitzen.

Ich entwickelte eine detaillierte Karte von den Expressionsmustern von *p53* und *p73* mRNA und p53 Protein im adulten Gehirn von Zebrabärblingen. Die Expression von *p53* und *p73* mRNA überschneidet sich in vielen Bereichen inklusive neurogener Zonen. Das p53 Protein ist in den meisten dieser Bereiche exprimiert, was darauf hindeutet, dass die mRNA Expression die Expression des Proteins widerspiegelt. Das p53 Protein wird im Telencephalon von adulten Zebrabärblingen in reifen Neuronen, Typ I Zellen (sich nicht teilende radiale Gliazellen) und Typ IIIa und Typ IIIb Zellen (Neuroblasten) exprimiert. Eine Expression des p53 Proteins ist nicht erkennbar in Zellen die von Oligodendrozyten abstammen und in Typ II Zellen (sich teilende radiale Gliazellen). Nach einer Stichverletzung des Telencephalons von adulten Zebrabärblingen ist sowohl das *p53* als auch das *p73* Gen hochreguliert. p53 ist in Typ I Zellen hochreguliert. Im Gegensatz zu einem unverletzten Gehirn ist p53 nach einer Stichverletzung in Zellen die von Oligodendrozyten abstammen exprimiert. Des Weiteren sind nach einer Stichverletzung Zielgene von p53 hochreguliert und Apoptose wird induziert. Diese Ergebnisse weißen darauf hin, dass p53 eine Rolle in der konstitutiven und regenerativen Neurogenese spielen könnte. Allerdings weißen Zebrabärblinge mit einer *tp53*^{M214K} Mutation keinen Phänotyp auf. Das strukturell verwandte p73 zeigt ein ähnliches Expressionsmuster als p53 im unverletzten und verletzten Gehirn von Zebrabärblingen. Daher könnte eine Redundanz zwischen p53 und p73 eine Manifestation des Phänotyps in der p53 Mutante verdecken. Zusammengefasst, die Analyse der Expression von sowohl p53 als auch p73 im

Gehirn des adulten Zebrabärblings deutet auf eine Rolle dieser Gene während der konstitutiven und regenerativen Neurogenese hin. Die künftige Aufklärung der genauen Funktion dieser beiden Gene bei diesen Vorgängen benötigt jedoch die Analyse von doppelten Mutanten.

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Abbreviations

AIF	apoptosis inducing factor
aPKC	atypical protein kinase C
Ascl1a	achaete-scute complex-like 1a
ASPP	Ankirin repeats, SH3 domain, proline-rich protein
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
Bax	Bcl-2-associated X protein
bHLH	basic helix-loop-helix
BLBP	brain lipid binding protein
BMP	bone morphogenetic protein
BrdU	5-Bromo-2`deoxyuridine
CDK	cyclin-dependent kinase
Chk2	checkpoint kinase 2
CMZ	ciliary marginal zone
CNS	central nervous system
COP1	constitutively photomorphogenic 1
CSF	cerebrospinal fluid
DBD	DNA-binding domain
Dcx	doublecortin
def	digestive-organ expansion factor
DEL	dorsal ependymal lining
DG	dentate gyrus
DIG	digoxigenin
Dlx2	distal less homeobox gene 2
dpf	days post fertilization
dpl	days post lesion
EGFP	enhanced green fluorescent protein
Egfr	epidermal growth factor receptor
Fgf	fibroblast growth factor
FISH	fluorescent *in situ* hybridization
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GLAST	astrocytic-specific glutamate transporter
GST	glutathione-S-transferase
hpf	hours post fertilization

hpl	hours post lesion
HRP	horseradish peroxidase
Id1	inhibitor of DNA binding 1
INL	inner nuclear layer
ISH	*in situ* hybridization
MAP	mitogen-activated protein
Mash1	mammalian achaete-scute homolog 1
Mdm2	mouse double minute-2
Meis2	Meis homeobox 2
MGC	Müller glia cells
MPNST	malignant peripheral nerve sheath tumor
Msi1	Musashi homolog 1 (*Drosophila*)
NES	nuclear export signal
NGF	nerve growth factor
NLS	nuclear localization signal
NPC	neural precursor cell
NSC	neural stem cell
OB	olfactory bulb
OD	oligomerization domain
OPC	oligodendrocyte progenitor cell
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
Pgr	progesterone receptor
PIR2	p73-induced Ring finger protein 2
PR	proline-rich domain
Prok2	Prokineticin 2
PSA-NCAM	polysialylated neural cell adhesion molecule
Puma	p53-up-regulated modulator of apoptosis
REG	regulatory domain
RGC	radial glial cell
RMS	rostral migratory stream
ROS	reactive oxygen species
S100β	S100 calcium binding protein β
SAM	sterile alpha motive
SGZ	subgranular zone
Shh	sonic hedgehog
Sox2	SRY-box containing gene 2

Sox10	SRY-box containing gene 10
SVZ	subventricular zone
TAD	transactivation domain
Tbr2	T-box brain 2
TET	tetramerization domain
TID	transcription inhibition domain
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UV	ultraviolet
ZO-1	zona occludens protein 1

Chapter 1

Introduction

1.1 Adult neurogenesis

For a long time it was thought that no new neurons can be generated in the adult mammalian brain. Now it is known that adult neurogenesis still takes place in the mammalian brain, including humans. However, compared to zebrafish, the neurogenic and regenerative capacities of mammals are very limited. It is important to investigate why adult zebrafish are endowed with such an enormous potential to generate new neurons and to repair lesions of the brain. This knowledge may guide efforts to develop therapies for human neurodegenerative diseases or brain injuries in the future.

1.1.1 Adult neurogenesis in mammals

To date the mouse brain is the best-studied model used for adult neurogenesis research. Two regions where adult neural stem cells are located and neurogenesis takes place predominantly are present in the adult mouse brain, namely the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch et al., 1999a; Garcia et al., 2004; Seri et al., 2001; Taupin and Gage, 2002). Both the SVZ and the SGZ are part of the telencephalon and contain neural stem cells characterized by a slow proliferation rate, the potential for self-renewal and generation of progeny of all neural lineages, notably neurons, astrocytes and oligodendrocytes.

1.1.1.1 Adult neurogenesis in the subventricular zone (SVZ)

The SVZ, which lies in the lateral walls of the lateral ventricles, is the most prominent site of adult neurogenesis in mammals. It contains four different cell types, ependymal cells, neural stem cells (NSCs), transit amplifying cells and neuroblasts, that form the neurogenic niche (Figure 1 A and B) (Doetsch et al., 1997).

Ependymal cells (E-cells) are multi-ciliated cells that build up an epithelium directly lining the wall of the ventricle. The E-cells are in direct contact with the cerebrospinal fluid (CSF) (Figure 1 B) and control the transport of factors from the CSF into the parenchyma. The beating of their cilia is essential for a normal CSF flow and concentration gradient formation of factors (Del Bigio, 1995; Riquelme et al., 2008). It was demonstrated that ependymal cells secrete noggin, which inhibits bone morphogenetic proteins (BMPs) thereby stimulating neurogenesis in the SVZ (Lim et al., 2000). Initially E-cells were thought to function as neural stem cells (Johansson et al., 1999) since they originate from embryonic radial glial cells which act as neural stem cells during development (Spassky et al., 2005). However, it was shown by several studies that ependymal cells do not seem to be the actual neural stem cells (Capela and Temple, 2002; Doetsch et al., 1999a; Spassky et al., 2005). Though, after injury E-cells are able to transform into radial glial-like cells and function as adult neural stem cells (Coskun et al., 2008; Johansson et al., 1999; Zhang et al., 2007). Since ependymal cells show no self-renewal and thus lack an important characteristic of stem cells, these cells may rather constitute a reservoir that can be activated upon injury (Carlen et al., 2009).

Directly underneath the ependymal cell layer the B-cells, which are the stem cells of the SVZ, are located. They exhibit electrophysiological and ultrastructural features of astrocytes. In addition B-cells express astrocytic markers as glial fibrillary acidic protein (GFAP), astrocytic-specific glutamate transporter (GLAST) and others (Doetsch, 2003; Doetsch et al., 1999a; Doetsch et al., 1997; Garcia et al., 2004; Seri et al., 2001). B-cells stem from embryonic radial glial cells and possess properties of these embryonic neural stem cells. For example, B-cells also contact the ventricle with short processes that extend through the ependymal layer. Additionally, they express the same markers such as Nestin and SRY-box containing gene 2 (Sox2). Moreover, they contact blood vessels as well with a long basal process (Figure 1 B). Therefore, like radial glia during development, B-cells retain an apical-basal polarity and are part of the ventricular epithelium (Mirzadeh et al., 2008; Shen et al., 2008). It was suggested that B-cells do not form a homogeneous population of neural stem cells in the SVZ (Merkle et al., 2007).

It has been shown in several studies that B-cells act as neural stem cells in the SVZ (Doetsch et al., 1999a; Doetsch et al., 1999b; Imura et al., 2003). B-cells give rise to C-cells, which are rapidly dividing transit amplifying progenitors. Due to this proliferation mode, relatively quiescent stem cells are able to divide infrequently, which protects them from accumulating mutations during each division. Type C-cells are characterized by the expression of markers like distal less homeobox gene 2 (Dlx2), mammalian achaete-scute homolog 1 (Mash1) or epidermal growth factor receptor (Egfr). Many signals like sonic hedgehog (Shh), nitric oxide or dopamine converge on C-cells. Thus they are emerging as important factors of the stem cell lineage (Ahn and Joyner, 2005; Hoglinger et al., 2004;

Romero-Grimaldi et al., 2008). Moreover, C-cells can give rise both to neuroblasts and to oligodendrocyte progenitor cells (OPCs) that give rise to mature oligodendrocytes (Kim et al., 2007; Menn et al., 2006).

As already mentioned above, C-cells give rise to neuroblasts, which are also called A-cells. Neuroblasts migrate as chains ensheated by astrocytes along the rostral migratory stream (RMS) into the olfactory bulbs (OBs) (Figure 1 C).There they differentiate into periglomerular interneurons and GABAergic granule neurons and integrate into the existing circuitry. A-cells show an expression of markers like polysialylated neural cell adhesion molecule (PSA-NCAM) or doublecortin (Dcx) (Doetsch and Alvarez-Buylla, 1996; Doetsch et al., 1997; Lois and Alvarez-Buylla, 1994; Lois et al., 1996; Luskin, 1993; Peretto et al., 1997). Moreover, it was shown that T-box brain 2 (Tbr2)-expressing A-cells are able to generate glutamatergic neurons as well in the OBs (Brill et al., 2009). Neuroblasts can be recruited to sites of neuronal death or injury in the cortex (Brill et al., 2009; Chen et al., 2004; Magavi et al., 2000). Furthermore, following demyelinating lesions the neuronal fate of neuroblasts is changed to a glial fate that leads to the generation of oligodendrocytes (Jablonska et al., 2010). In addition, it was demonstrated that cortical lesion induces enhanced proliferation and neurogenesis in the SVZ. Some of the newly generated neural progenitors migrate into the injured area where they differentiate into glial cells and to a lesser extent into neurons (Saha et al., 2013).

Until recently it was not clear whether adult neurogenesis and migration of neuroblasts do also exist in the adult human brain (Curtis et al., 2007; Sanai et al., 2004). However, it was shown that SVZ astrocytes exist in the walls of the human anterior lateral ventricles and some of them function as stem cells *in vitro* (Quinones-Hinojosa et al., 2006; Sanai et al., 2004). Additionally it was demonstrated that neuroblasts are continuously present in the anterior ventral SVZ and the RMS of the adult human brain (Wang et al., 2011).

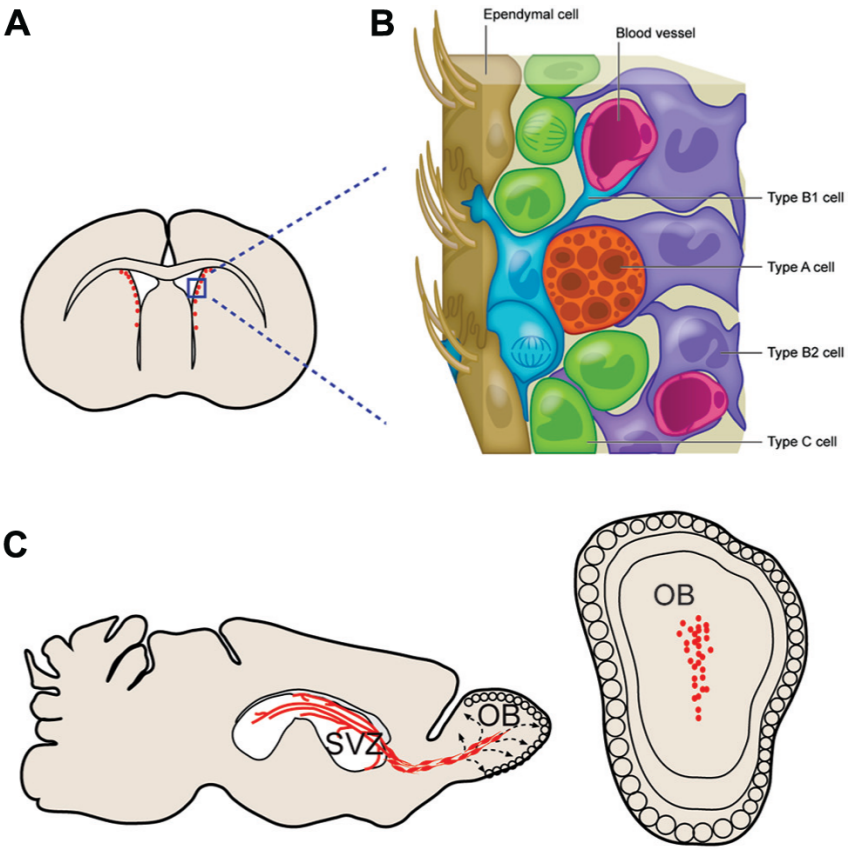


Figure 1: Adult neurogenesis in the SVZ of the mouse brain.

(A) Coronal view of the SVZ. (B) Enlarged view of the boxed area in (A) which shows the neurogenic niche of the SVZ comprised of E-, B-, C- and A-cells. Multiciliated ependymal cells (E-cells, brown) line the wall of the lateral ventricle. Neural stem cells (B-cells, blue) are located underneath the epidermal layer and are in contact with the ventricle and blood vessels (red). B-cells give rise to transit amplifying cells (C-cells, green) which in turn generate neuroblasts (A-cells, orange). A-cells migrate into the OBs via the RMS where they differentiate into interneurons. (C) Sagittal view of the mouse brain showing neuroblasts (red) migrating from the SVZ to the OB. Modified from (Gonzales-Roybal and Lim, 2013).

1.1.1.2 Adult neurogenesis in the subgranular zone (SGZ) of the hippocampus

Besides the SVZ, the SGZ of the DG in the hippocampus is the second site of adult neurogenesis in the mammalian brain (Figure 2 A) (Gage, 2000; Kempermann et al., 2004a). Neurogenesis in this region plays a role in memory and learning. Furthermore, abnormal hippocampal neurogenesis could be linked to epilepsy and depression for example (Dranovsky and Hen, 2006; Parent et al., 2006; Zhao et al., 2008). Gao and Chen showed, that traumatic brain injury promotes proliferation of NSCs in the hippocampus but neurogenesis is not increased since most of the newborn cells are glia.

There are distinct types of progenitors present in the SGZ that express different markers and show different morphological features. Radial glia like type 1 progenitors express progenitor markers as Nestin and Sox2 and, like B-cells in the SVZ, astrocytic markers as GFAP (Figure 2 B) (Encinas et al., 2006; Garcia et al., 2004; Kempermann et al., 2004b; Steiner et al., 2006; Suh et al., 2007). Contrary to B-cells in the SVZ, type 1 progenitors exhibit a radial morphology with a long apical process similar to embryonic radial glia (Figure 2 B) and they express radial glia markers like Vimentin or brain lipid binding protein (BLBP) (Encinas et al., 2006). Type 1 progenitors are thought to give rise to transit amplifying progenitors, which have only short processes and do not show a radial morphology.

The several types of transit amplifying progenitors can be distinguished by marker expression and morphology (Encinas et al., 2006; Lugert et al., 2010; Suh et al., 2007). Type 2A cells still express Sox2 and give rise to Type 2B (late stage transit amplifying progenitors) or Type 3 (neuroblasts) cells. Type 2B cells are characterized by the expression of both Nestin and Dcx, an immature neuronal marker. Whereas Type 3 cells are Nestin-negative and express only Dcx (Figure 2 B) (Dhaliwal and Lagace, 2011). When immature neurons differentiate finally into mature glutamatergic granule neurons there is down-regulation of Dcx and up-regulation of NeuN and calretinin (Figure 2 B). Newly generated neurons in the SGZ mature functionally and structurally within six to eight weeks (Jessberger and Kempermann, 2003; van Praag et al., 2002; Zhao et al., 2006).

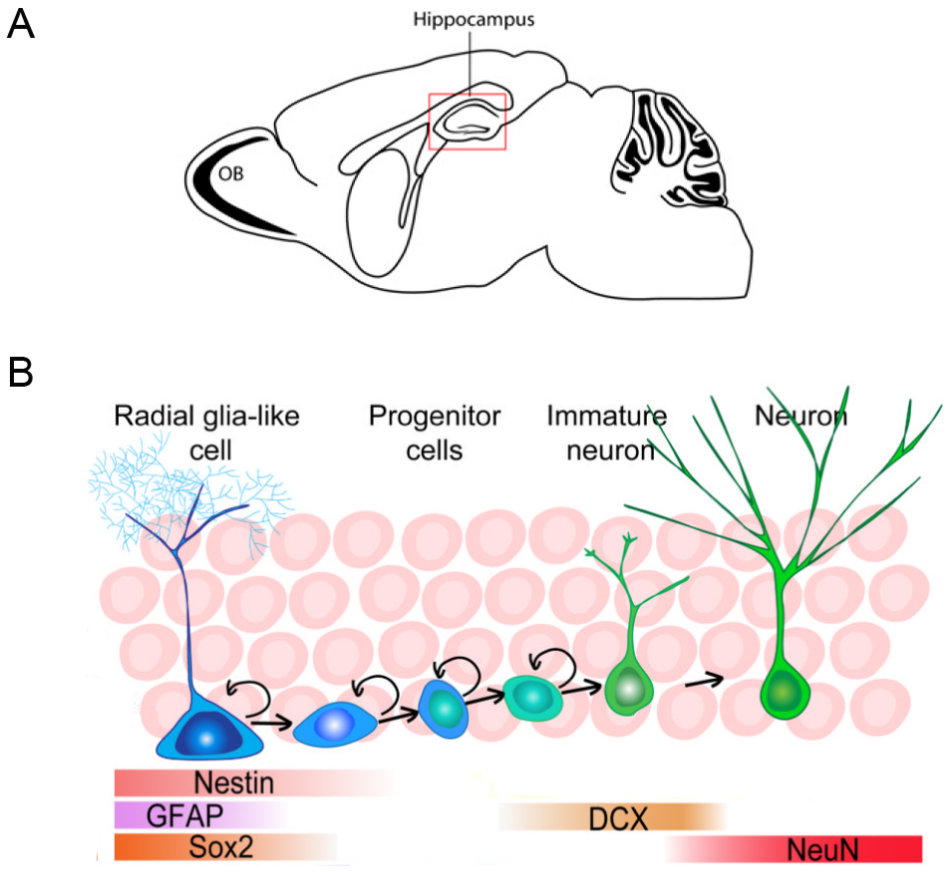


Figure 2: Adult neurogenesis in the SGZ of the mouse brain.
(A) Sagittal view of the mouse brain. The boxed area shows the hippocampus. (B) In the SGZ of the DG in the hippocampus radial glia-like cells give rise to transit amplifying progenitor cells, immature and mature granule neurons. The different cell types during the different stages of the differentiation process are characterized by the expression of distinct markers. Modified from (A) (Hsieh, 2012) and (B) (Bonaguidi et al., 2011).

Sox2-positive type 2 progenitors with a non-radial morphology are able to self-renew. Moreover, a single Sox2-positive type 2 progenitor cell can give rise to an astrocyte and a neuron. In addition, non-radial type 2 progenitors can give rise to radial type 1 progenitors, which suggests a reciprocal relationship between these progenitor states. It was hypothesized that the non-radial Sox2-positive Type 2 cells are the self-renewing, multipotent neural stem cells in the hippocampus. These NSCs can give rise to quiescent Type 1 radial progenitors, which could function as a reservoir of rarely dividing cells (Suh et al., 2007). These findings indicate that the lineage relationship between progenitor cells in the SGZ is still controversial. Thus there is need of further lineage tracing studies to identify the relationships between the distinct progenitors in the SGZ.

In contrast to the SVZ (see chapter 1.1.1.1), adult neurogenesis in the human DG has been consistently reported and 700 new neurons are added in an adult human hippocampus each day (Beckervordersandforth et al., 2014; Eriksson et al., 1998; Spalding et al., 2013).

1.1.2 Adult neurogenesis in zebrafish

In comparison to mammals (Figure 3 A), zebrafish show a much higher proliferative and regenerative potential. In the adult zebrafish brain 16 regions could be identified where adult neurogenesis takes place (Figure 3 B) (Adolf et al., 2006; Grandel et al., 2006; Kaslin et al., 2008; Zupanc et al., 2005). In these regions homologs of the two mammalian stem cell niches in the SVZ and the hippocampus are included, as well as additional stem cell niches in the telencephalon and all other subdivisions of the brain.

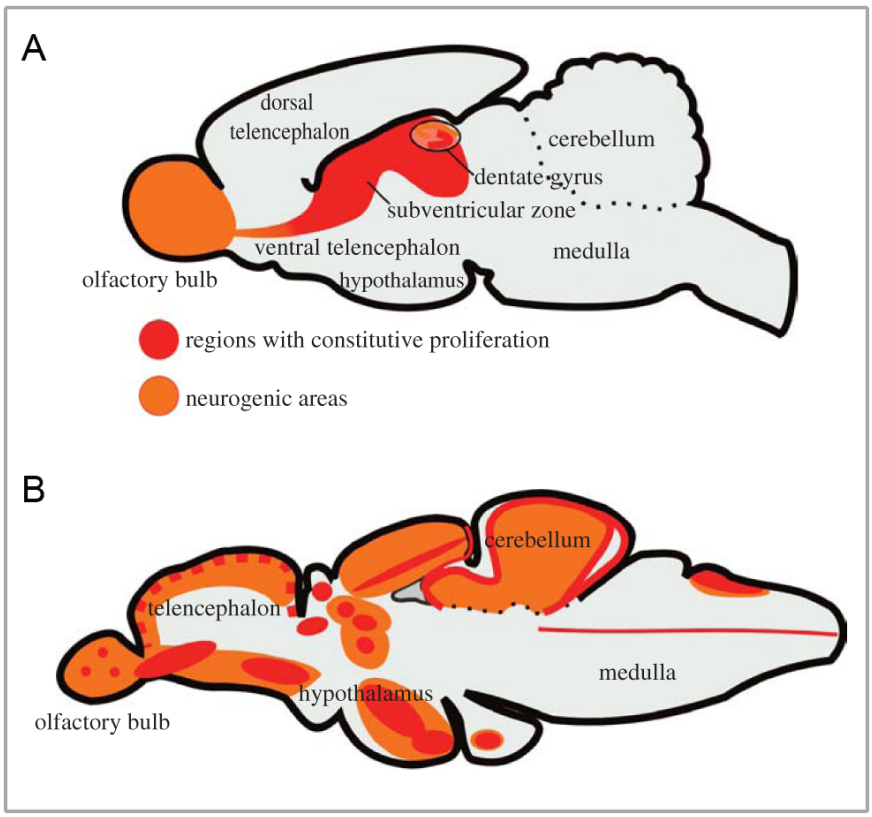


Figure 3: Adult neurogenesis in the mouse and zebrafish brain.

Parasagittal schematic overview of the adult mouse (A) and zebrafish (B) brain showing the proliferation zones. In the adult mouse brain proliferation (red) and neurogenesis (orange) take place

in only two regions of the telencephalon, the SVZ of the lateral ventricles and the SGZ of the hippocampal DG. Whereas the adult zebrafish brain shows proliferation and neurogenesis in all subdivisions of the brain. Modified from (Kaslin et al., 2008).

Due to these characteristics of the adult zebrafish telencephalon, it is an interesting model for studying adult neurogenesis from a comparative point of view. The olfactory bulbs and the dorsolateral telencephalon of the adult zebrafish brain which was suggested to be homologous to the mammalian hippocampus (Broglio et al., 2005) can be used to compare the homologous proliferation zones of mammals and zebrafish.

However, the development of the teleostean telencephalon differs from the development of the mammalian telencephalon. The telencephalon of teleosts is formed by eversion which leads to the formation of an external ventricle. Therefore, the proliferative zones of the adult zebrafish telencephalon are located both internally and at the outer surface of the telencephalon. In contrast, the mammalian telencephalon is generated by evagination of the neural tube that leads to an internal ventricular zone (Figure 4) (Broglio et al., 2005; Folgueira et al., 2012; Mueller and Wullimann, 2009; Wullimann and Mueller, 2004).

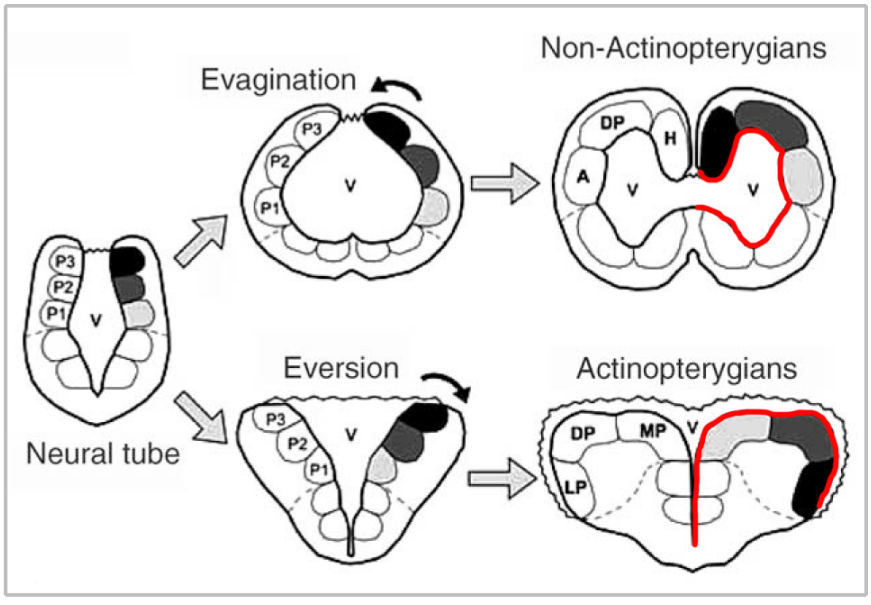


Figure 4: Development of the telencephalon.

Schematic representation of the process of eversion and evagination of the neural tube during the development of the telencephalon of actinopterygians (ray-finned fish) and non-actinopterygians, respectively. Eversion (outward folding) of the actinopterygian telencephalon leads to a ventricular zone that is present internally but also covers the outer surface (red). Evagination (inward folding) of the non-actinopterygian telencephalon results in a ventricular zone that is only located internally (red).

A: amygdale; DP: dorsal pallium; H: hippocampus; LP: lateral pallium; MP: medial pallium; P1, P2, P3: the three main subdivisions of the pallium; V: ventricle. Modified from (Broglio et al., 2005).

The progenitor cells located in the adult zebrafish telencephalon are heterogeneous concerning their proliferation rate. The proliferation intensity varies along the dorsoventral and rostrocaudal axis of the telencephalon. In the anterior telencephalon proliferation can be detected along the complete ventricular zone. However, in more posterior parts of the telencephalon proliferation is restricted to different subdomains (Lindsey et al., 2012). The most proliferative region is present in the ventral subpallium near the OBs (Vv) (Figure 5 A) (Adolf et al., 2006; Ganz et al., 2010; März et al., 2010a). In this region almost exclusively 5-Bromo-2`deoxyuridine (BrdU)-positive and S100 calcium binding protein β (S100β)-negative cells are present and any quiescent cells are located there (Lindsey et al., 2012; März et al., 2010a). This region in the medial subpallium is homologous to the mammalian SVZ and mainly generates neurons that migrate to the OBs via the RMS as well (Adolf et al., 2006; Byrd and Brunjes, 1998, 2001; Grandel et al., 2006; Kishimoto et al., 2011). Additionally, this region is able to generate neurons that migrate to the adjacent parenchyma, which is in contrast to mammals (Adolf et al., 2006; Kishimoto et al., 2011). In the posterolateral zone of the dorsal telencephalic area at the border between Dl and Dp (Figure 5 A and B), which is homologous to the hippocampus of mammals, and also in other ventricular regions the proliferation rate is lower (Grandel et al., 2006; März et al., 2010a; Zupanc et al., 2005). Though, proliferating cells are not only located at the ventricular zone. In the parenchyma proliferating cells could be detected as well. These cells are OPCs that give rise to mature oligodendrocytes and self-propagate (Adolf et al., 2006; März et al., 2010b).
Furthermore, zebrafish do not possess an ependyma, a multi-ciliated ependymal layer covering the whole ventricular surface, like mammals (Duan et al., 2008; Johansson et al., 1999). In zebrafish only the roof of the telencephalic ventricle and the dorsomedial regions of the proliferative zones are covered by an ependymal layer (DEL, dorsal ependymal lining) (D and Dm; Figure 5 A and B) (Lindsey et al., 2012). Thus, the stem cell niche of zebrafish bears resemblance to the fragmented ependymal lining of the stem cell niches of birds and reptiles (Garcia-Verdugo et al., 2002).

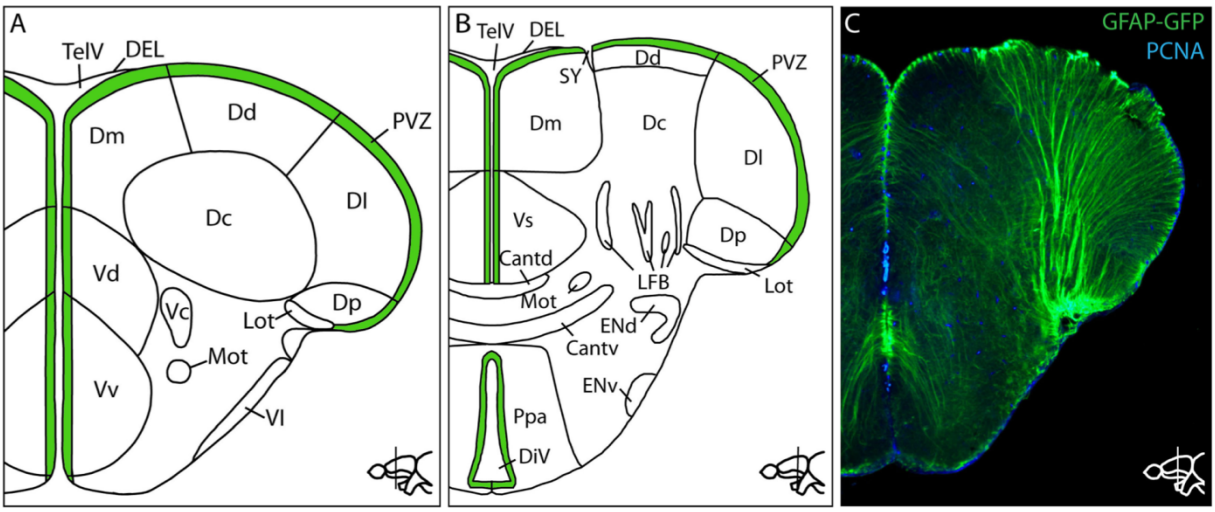


Figure 5: Anatomy of the adult zebrafish telencephalon.

Schematic of transverse sections through the anterior (A) and posterior (B) zebrafish telencephalon. The different anatomical subdomains are indicated. Proliferative periventricular zones are shown in green. (C) Radial glial cells possess long processes that end at the pial surface. The radial glial cells in the adult zebrafish telencephalon are marked by immunostaining against GFP in the *Tg(GFAP-GFP)* line. Co-staining with the proliferation marker proliferating cell nuclear antigen (PCNA) allows to distinguish between PCNA-negative Type I and PCNA-positive Type II cells (see (März et al., 2010a)). Cantd: commissural anterior, pars dorsalis; Cantv: commissural anterior, pars ventralis; Dc: central zone of the dorsal telencephalic area (D); Dd: dorsal zone of D; DEL: dorsal ependymal lining; DiV: diencephalic ventricle; Dl: lateral zone of D; Dm: medial zone of D; Dp: posterior zone of D; Lot: lateral olfactory tract; ENd: entopeduncular nucleus, dorsal part; ENv: entopeduncular nucleus, ventral part; LFB: lateral forebrain bundle; Mot: medial olfactory tract; PPa: parvocellular preoptic nucleus, anterior part; PVZ: periventricular zone; SY: sulcus ypsiloniformis; TelV: telencephalic ventricle; Vc: ventral nucleus of the ventral telencephalic area (V); Vd: dorsal nucleus of V; Vl: lateral nucleus of V; Vp: postcommisural nucleus of V; Vs: supracommisural nucleus of V; Vv: ventral nucleus of V. From (Schmidt et al., 2013).

1.1.2.1 Constitutive adult neurogenesis in zebrafish

1.1.2.1.1 Neural progenitors in the telencephalon of adult zebrafish

During mammalian development radial glial cells act as embryonic neural stem cells. At the end of development most of the radial glial cells convert into astrocytes and ependymal cells. Some of these astrocytes retain stem cell potential in the SGZ and SVZ (Alvarez-Buylla and Garcia-Verdugo, 2002; Alvarez-Buylla et al., 2001; Alvarez-Buylla et al., 2002; Doetsch,

2003; Kriegstein and Alvarez-Buylla, 2009). The adult neural stem cells in the zebrafish telencephalon are so-called radial glial cells (RGCs). The cell bodies of these RGCs are located at the ventricular zone. They are able to self-renew and to generate new neurons. Furthermore, the RGCs exhibit a radial morphology which is characterized by a large triangular soma and a long radial process that reaches the pial surface of the telencephalon (Figure 5 C). There, the RGCs terminate with their endfeet either at the pial surface itself or on blood vessels (Lam et al., 2009; Lindsey et al., 2012; Rothenaigner et al., 2011). Moreover, the RGCs in the adult zebrafish telencephalon express the same markers as embryonic radial glia, like S100β, Nestin, GFAP and BLBP (Adolf et al., 2006; Lam et al., 2009; Pellegrini et al., 2007). In contrast to the mammalian brain, no bona fide astrocytes have been identified in the adult zebrafish brain until now (Adolf et al., 2006; Grandel et al., 2006; März et al., 2010a; März et al., 2011). Since the adult zebrafish brain seems to retain embryonic features it might thus be more capable of regeneration and adult neurogenesis.

The two different types of RGCs in the adult zebrafish telencephalon can be distinguished by the expression of the proliferation marker proliferating cell nuclear antigen (PCNA) (Figure 5 C), which is highly expressed in the G1-phase of the cell cycle and maintained throughout division (Bravo et al., 1987), or incorporation of BrdU, which labels the S-phase of the cell cycle (Gratzner, 1982). Both cell types express the markers mentioned above. Quiescent Type I cells are characterized by the expression of S100β, GFAP and BLBP but lack PCNA expression and are negative for BrdU ($S100\beta^+$, $GFAP^+$, $BLBP^+$, $PCNA^-$, $BrdU^-$). Whereas slowly cycling Type II cells express S100β, GFAP and BLBP and additionally PCNA and they incorporate BrdU ($S100\beta^+$, $GFAP^+$, $BLBP^+$, $PCNA^+$, $BrdU^+$) (Figure 6) (März et al., 2010a; Schmidt et al., 2013). Most of the RGCs are Type I cells and therefore quiescent. Activated Type II RGCs are both able to self-renew by symmetric division and to give rise to neuroblasts (Type III cells) by asymmetric division (Figure 6) (Rothenaigner et al., 2011). The newly generated neuroblasts carry on to proliferate and start to express markers for a neuronal differentiation state like PSA-NCAM and proneural markers like achaete-scute complex-like 1a (Ascl1a) (Figure 6). Subsequently they either enter the RMS to reach the OBs or they leave the periventricular zone and migrate deeper into the parenchyma (Schmidt et al., 2013). The two different types of neuroblasts can be characterized by the expression of distinct markers. Both Type IIIa and Type IIIb cells express PCNA and PSA-NCAM but only Type IIIa cells express Nestin and glial markers. Furthermore, all the different progenitor cell types express the progenitor and stem cell marker Sox2 (März et al., 2010a). There exists heterogeneity in marker expression and cellular composition of stem cell niches in the adult zebrafish telencephalon. Type II cells are not evident in the RMS where Type III cells are predominantly located and Type I cells are clustered in the ventral ventricular zone of the subpallium for example. In the rest of the telencephalic ventricular zone these distinct cell

types are present in a scattered fashion (Ganz et al., 2010; März et al., 2010a). It has been hypothesized that there are additional cell types present in the stem cell niches that are either additional types of progenitors or cells that contribute to the architecture of the stem cell niche (Lindsey et al., 2012).

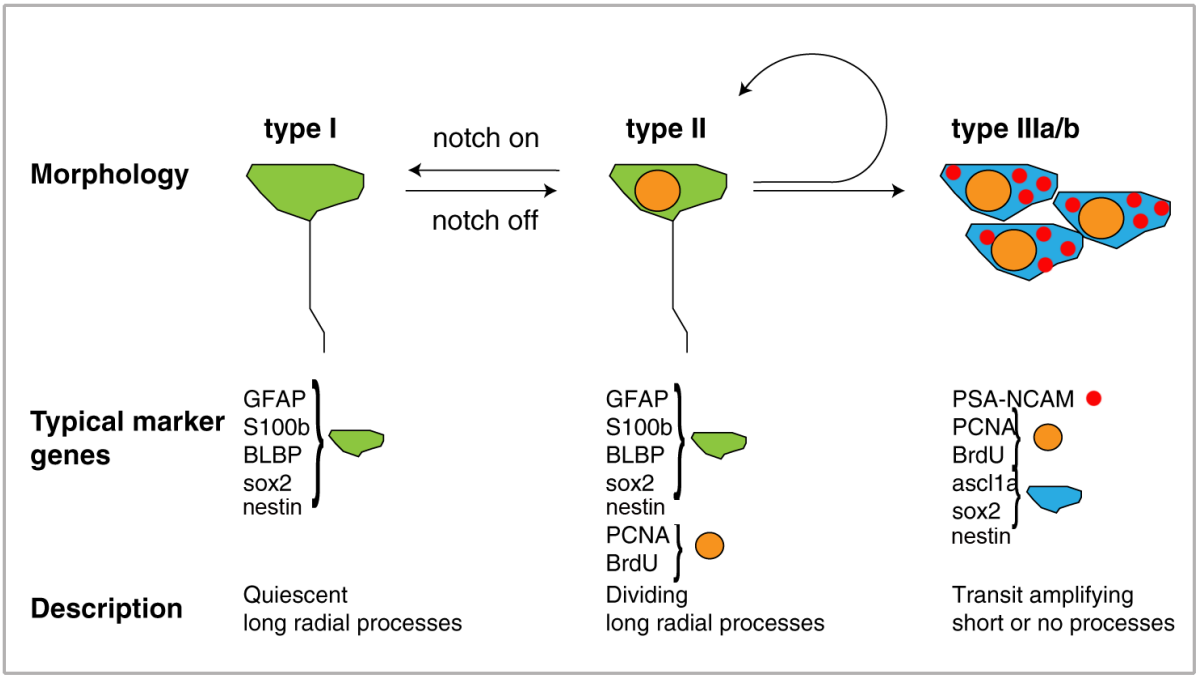


Figure 6: The different types of progenitor cells in the adult zebrafish telencephalon.
The markers expressed by Type I, II and III cells are indicated. Type II cells give rise to Type III cells and are able to self-renew. Activation of notch pushes Typ2 II radial glial cells into quiescence whereas blocking of notch leads to the transformation of quiescent Type I into proliferating Type II cells (Chapouton et al., 2010). Modified from (Schmidt et al., 2013).

In the embryonic mouse brain (Nadarajah et al., 2001) but also in the embryonic fish brain (Peukert et al., 2011) the long processes of RGCs provide a scaffold for new-born neurons that migrate out of the ventricular zone. It was suggested that this is also the case in the adult zebrafish brain (Lam et al., 2009; Pellegrini et al., 2007) but evidence for this hypothesis is still missing. Furthermore it was shown that in anterior regions of the RMS neuroblasts are associated with blood vessels during migration towards the OBs (Kishimoto et al., 2011). Therefore, it could be that blood vessels are more important guidance cues than the processes of RGCs. However this has to be further investigated. In addition, it has been demonstrated that most of the new-born neurons do not migrate to the pial surface but settle in periventricular areas (Kroehne et al., 2011).

Furthermore, adult neurogenesis in the zebrafish brain is linked to circadian clock activity. The core clock feedback loop was shown to be active in all known progenitor cell types of the telencephalon (Weger et al., 2013). Moreover, the progesterone receptor (pgr) is widely expressed in the adult zebrafish brain showing the highest expression in RGCs. The expression of pgr is up-regulated by estrogens (Diotel et al., 2011). Estrogen treatment leads to a reduced cell proliferation in several brain regions and most likely affects survival and cell migration without affecting differentiation (Diotel et al., 2011; Makantasi and Dermon, 2014).

1.1.2.1.2 Additional neurogenic sites in the adult zebrafish brain

Not only the adult zebrafish telencephalon but also other regions of the brain possess proliferative potential. However, other neurogenic sites than the telencephalon are less well investigated. Nevertheless it was suggested that the different stem cell niches in the adult zebrafish brain differ in terms of their architecture. These differences may lead to different neurogenic potentials of the distinct niches.

Especially the cerebellum exhibits substantial neurogenesis (Grandel et al., 2006). Proliferating neural progenitors in the cerebellum express the progenitor markers Meis homeobox 2 (Meis2), Musashi homolog 1 (*Drosophila*) (Msi1), Sox2 and Nestin. Additionally, these cells express cytoskeletal elements such as atypical protein kinase C (aPKC), zona occludens protein 1 (ZO-1), γ-tubulin and β-catenin and thus show neuroepithelial characteristics (Kaslin et al., 2009). The proliferating cells in the cerebellum do not express radial glial markers but there are some radial glial-like cells present in the neurogenic niche that express Vimentin, BLBP and GFAP. These cells seem not to be stem cells but rather function as a scaffold for migrating neuroblasts. Since blocking of fibroblast growth factor (Fgf) leads to a decreased proliferation, Fgf may be crucial for the activity of stem cells in the cerebellum (Kaslin et al., 2009).

A second site of adult neurogenesis in the zebrafish brain is present in the optic tectum. The caudal, medial and lateral margins of the periventricular grey zone (PGZ) exhibit proliferating cells (Grandel et al., 2006; Ito et al., 2010; Kaslin et al., 2008; Marcus et al., 1999; Zupanc et al., 2005). Dividing cells in these regions do not express glial markers but the neural progenitor markers Sox2 and Msi1 and PCNA (Ferri et al., 2004; Kaneko et al., 2000). As in the cerebellum the proliferating cells exhibit neuroepithelial properties and express aPKC, ZO-1 and γ-tubulin (Del Bene et al., 2008; Oteiza et al., 2008). Furthermore non-proliferating radial glial-like cells can be found next to dividing neuroepithelial-like progenitors and they can be detected in the deeper layer of the PGZ (Ito et al., 2010). Moreover, the progenitor cells build up a multipotent progenitor pool since they can differentiate into oligodendrocytes

and radial glia as well as in GABAergic and glutamatergic neurons (Higashijima et al., 2004; Ito et al., 2010; Marusich et al., 1994; Mueller and Wullimann, 2002).
The boundary between the midbrain and the hindbrain harbours another neurogenic site (Chapouton et al., 2006). In this region of the brain, cells expressing *her5:gfp* form a cluster at the ventricle of the midbrain/hindbrain boundary. These *her5:gfp*-positive cells exhibit features of stem cells. Some of these cells divide slowly, express stem cell markers as BLBP, GFAP, Msi1 or Sox2 and are able to differentiate into glia and neurons (Chapouton et al., 2006).

1.1.2.1.3 Oligodendrocytes in the adult zebrafish telencephalon

In the adult mammalian brain slowly cycling OPCs, which express the transcription factors SRY-box containing gene 10 (Sox10) and Olig2, are the major proliferating cell population (Dawson et al., 2003; Geha et al., 2010). They are distributed throughout the brain and produce oligodendrocytes that can take part in myelin repair (Gensert and Goldman, 1997; Nait-Oumesmar et al., 1999; Polito and Reynolds, 2005). It was shown that OPCs act as multipotent stem cells in vivo (Rivers et al., 2008; Zawadzka et al., 2010). In the parenchyma of the adult zebrafish telencephalon mature oligodendrocytes, proliferating OPCs, slowly proliferating OPCs and quiescent OPCs are located. These cells belonging to the oligodendrocyte lineage also express Sox10 and Olig2 like in mammals. Furthermore, quiescent OPCs seem to be the major cell type present in the parenchyma of the adult zebrafish telencephalon. In contrast to the mammalian CNS, where more oligodendrocytes than OPCs are present, in the zebrafish telencephalon much more OPCs than mature oligodendrocytes exist. They probably form a pool of resting OPCs that can be activated to proliferate when there is need of. Since more neurons, which have to be myelinated, are produced in the zebrafish than in the mammalian brain, more OPCs may be required to produce enough mature oligodendrocytes (März et al., 2010b).

1.1.2.2 Regenerative adult neurogenesis in zebrafish

The zebrafish central nervous system (CNS) exhibits enormous regenerative properties. In contrast, in the mammalian brain regenerative adult neurogenesis is very limited. It is likely that the local environment in most areas of the mammalian brain is not suited for a long-term survival of newly generated neurons and that the zebrafish brain environment could be more permissive to regeneration. Furthermore, the number of newly generated neurons in the mammalian brain is far too low to replace the lost neurons (Arvidsson et al., 2002; Chen et al., 2004; Kroehne et al., 2011; Zupanc, 2008). After stroke for example, reactive neurogenesis is induced in the mammalian brain but the majority of newborn neurons is not maintained for a long time due to lack of functional integration into the circuitry and a permissive environment (Arvidsson et al., 2002; Nakatomi et al., 2002; Yamashita et al., 2006). In addition, after traumatic brain injury a proliferative response of progenitor cells takes place, which leads only to the production of new astrocytes but any neurons are generated (Buffo et al., 2008). Therefore the question arises why zebrafish show this exceptional ability for regeneration of not only axons but also whole neurons. Since zebrafish show a life-long body growth, that also includes the formation and incorporation of new neurons, this continuous growth could lead to a suitable environment for regeneration of injured neural tissue.

1.1.2.2.1 Regeneration of the retina

Contrary to mammals, zebrafish have a huge ability to regenerate injured organs including kidney, heart, fins as well as the CNS (Becker and Becker, 2008; Diep et al., 2011; Kuscha et al., 2012; Lien et al., 2012; Singh et al., 2012). The adult zebrafish retina is able to regenerate completely after distinct types of lesion, notably light-induced damage of photoreceptors (Vihtelic and Hyde, 2000), stab lesions (Fausett and Goldman, 2006; Senut et al., 2004), heat damage (Raymond et al., 2006), toxic lesion of inner retinal neurons (Fimbel et al., 2007) and surgical excision of retinal tissue (Cameron, 2000). The regeneration process is dependent on Fgf signaling (Hochmann et al., 2012). In the zebrafish retina two different sources are present, that can generate new cells during neurogenesis and regeneration, the Müller glia cells (MGC) and the ciliary marginal zone (CMZ). The MGC are located in the inner nuclear layer (INL) of the retina. They are able to generate photoreceptor progenitors of the rod lineage. The CMZ is located at the interface between ciliary epithelium and neural retina. Cells in the CMZ allow for a life-long growth of the retina and neurogenesis since they give rise to all types of neurons. Furthermore the CMZ is

associated with blood vessels. The MGC play a role after injury since they show an up-regulation of GFAP and re-enter the cell cycle. In addition they de-differentiate and acquire characteristics of multipotent retinal progenitors in the CMZ (Raymond et al., 2006). MGC give rise to proliferating multipotent progenitor cells, which generate all types of neurons to repair the damaged tissue (Bernardos et al., 2007; Fausett and Goldman, 2006; Fimbel et al., 2007; Goldman, 2014; Lenkowski and Raymond, 2014).

1.1.2.2.2 Regeneration of the optic nerve

In addition to the retina, zebrafish are also capable of regenerating the optic nerve. They are able to recover visual function within 20-25 days (Kaneda et al., 2008), which is very fast in comparison to goldfish (30-50 days) (Kato et al., 1999), the major model organism used to investigate optic nerve regeneration in fish. In mammals such as rat and mouse, optic nerve injury leads to massive cell death and irreversible impairment of the visual system (Agudo et al., 2008; Berkelaar et al., 1994; Bonfanti et al., 1996). Contrary, lower vertebrates like frogs (Humphrey and Beazley, 1985; Scalia et al., 1985) or zebrafish can restore visual function owing to survival of retinal ganglion cells.
Thus, the reason for optic nerve regeneration is rather the survival and regeneration of original retinal ganglion cells than proliferation of these cells. This is in contrast to regeneration of the retina (see chapter 1.1.2.2.1) and the spinal cord (see chapter 1.1.2.2.3) where proliferation of progenitor cells is crucial for proper regeneration. It was demonstrated that after optic nerve crush or transsection almost all retinal ganglion cells survive. More than half of the surviving cells regrew their axons to the target area within one week after injury. In addition, multipotent retinal stem cells are able to generate new cells to replace lost ones after damage (Zou et al., 2013).

1.1.2.2.3 Regeneration of the spinal cord

After spinal cord transsection spinal cord axons are regenerated (Becker et al., 1997) and whole neuronal assemblies are rebuilt with complete restoration of function after injury (Ayari et al., 2010; Cameron, 2000; Clint and Zupanc, 2001; Ito et al., 2010; Kirsche, 1965; Reimer et al., 2008; Zupanc, 2008; Zupanc and Ott, 1999; Zupanc and Zupanc, 2006). Fish show an almost normal swimming behavior 6 weeks after injury as demonstrated by behavioral tests (Becker et al., 2004). However, when challenged by swimming against a flow they show a

worse performance than uninjured fish. Thus, the recovery process is limited in some aspects (van Raamsdonk et al., 1998). The regenerating spinal cord motor neurons originate from *olig2:EGFP*-positive ependymoradial glial cells. This process is regulated by Shh signaling (Reimer et al., 2009; Reimer et al., 2008).

1.1.2.2.4 Regeneration of the brain

The reactions of the mammalian brain following stab injuries have intensively been investigated (for example (Buffo et al., 2005; Dimou et al., 2008; Hampton et al., 2004; Magnus et al., 2007; Robel et al., 2011)). Immediately after injury recruitment and proliferation of microglia takes place. Microglia remove the cell debris and via their signaling activity they participate in a process called reactive gliosis, which is the major hallmark of the reaction to mammalian brain injury (Hampton et al., 2004; Koshinaga et al., 2000). Reactive gliosis is characterized by hypertrophy of astrocytes and in severe lesions proliferation of astrocytes as well. Moreover, GFAP and other astrocytic markers and immature glial markers as nestin and BLBP are up-regulated in astrocytes (Buffo et al., 2008; Robel et al., 2011; Sofroniew, 2009). Additionally, the transcription factor Olig2 is highly up-regulated at the site of the lesion and OPCs start to proliferate near the lesion. These events result in impaired neuronal regeneration (Buffo et al., 2005; Dimou et al., 2008; Hampton et al., 2004; Magnus et al., 2007; Robel et al., 2011; Sofroniew, 2009). Altogether, these different reactions can finally lead to the formation of a glial scar. On the one hand, this scar seals the injured tissue off but on the other hand it also prevents regeneration of axons and neurons (Robel et al., 2011; Sofroniew, 2009).

Initially, proliferative and cellular responses to injury of the brain of adult teleosts had been mainly studied in the cerebellum of *Apteronotus leptorhynchus*. It was shown that apoptosis reaches a maximum at 30 min after the damage and declines from 2 to 20 days post lesion (dpl) (Zupanc et al., 1998). Furthermore, proliferation peaks at 5 dpl (Zupanc and Ott, 1999) and microglia/macrophages exhibit increased levels from 3 dpl onwards with the highest level at 10 dpl (Zupanc et al., 2003). Moreover, GFAP-positive radial fibers start to increase from 8 dpl onward and their levels remain elevated until 100 dpl (Clint and Zupanc, 2001).

In mammalian neural tissue, necrotic cell death is predominant after injury (Liou et al., 2003; Vajda, 2002). However, apoptosis does also occur in regions surrounding the necrotic injury core (Liou et al., 2003). Necrosis leads to much stronger inflammatory responses than apoptosis, which results in a progressive loss of neural cells and scar formation (Zhang et al., 1997). In contrast, in lesions of the adult cerebellum of fish apoptosis seems to remove damaged cells (Zupanc et al., 1998). The different mechanisms to eliminate damaged cells

may be one of the factors that account for the enormous regenerative potential of injured neural tissue in fish.

In the injured adult zebrafish telencephalon microglia are rapidly recruited to the site of injury. Additionally, radial fibers get disorganized and over 90% of newborn cells express the neuronal marker Hu at 7 dpl. Prokineticin 2 (Prok2) is transiently up-regulated near the lesion site (Ayari et al., 2010).

März et al. showed that after stab injury of the adult zebrafish telencephalon first microglia accumulate at the site of the lesion and start to proliferate, which is similar to results from other teleosts and mouse. Another early response to injury in zebrafish is accumulation of OPCs at the lesion site. In contrast to the mammalian brain, this accumulation is only transient and the OPCs show only a moderate proliferation. Thus, there is no formation of a glial scar as in the mammalian brain. Subsequently, RGCs up-regulate expression of Nestin, S100β and GFAP and some cells show hypertrophic swellings, which is also observed in mammalian astrocytes (März et al., 2011). In agreement with data from mouse (Robel et al., 2011) and *Apteronotus leptorhynchus* (Clint and Zupanc, 2001), zebrafish show also a delayed increase of GFAP expression. Furthermore, Type II and Type III cells strongly proliferate at the ventricular zone and generate new neurons. However, proliferation is restricted to the injured hemisphere. In all, the lack of a strong response of OPCs and the massive increase in proliferation following stab injury of the adult zebrafish telencephalon may be the reason for the high regenerative ability of the zebrafish brain (März et al., 2011). In contrast to the observations made by März et al. another study did not observe an accumulation of OPCs at the site of the lesion. This may be due to the different injury techniques used in the two studies (Baumgart et al., 2012).

Moreover, it was demonstrated that after injury of the adult zebrafish telencephalon new neurons are born. *her4.1*-positive radial glial progenitor cells residing at the ventricular zone proliferate and give rise to neuroblasts. These neuroblasts migrate to the site of the lesion and differentiate into neurons which express mature neuronal markers and survive for more than 3 months (Kroehne et al., 2011). Accordingly, Baumgart et al. confirmed the generation and long-term survival of neurons following injury of the adult zebrafish telencephalon. Contrary to constitutive neurogenesis, where newborn neurons settle mainly at the subventricular zone, in regenerative neurogenesis newborn neurons migrate longer distances into the injured parenchyma (Baumgart et al., 2012; Kroehne et al., 2011). In a further study it was shown that the transcription factor Gata3 plays an important role in regenerative neurogenesis of the adult zebrafish telencephalon. It is only expressed in lesioned brains and knock-down of Gata3 decreases the proliferative response of RGCs after injury. Furthermore, the generation of new neurons as response to an injury is also reduced. The induction of Gata3 following injury may be dependent on Fgf. Thus, Gata3 seems to be

necessary for proliferation of progenitors and generation and migration of neurons following injury (Kizil et al., 2012b). In addition, *cxcr5*, a chemokine receptor, is involved in regenerative neurogenesis. *Cxcr5* is expressed both in neurons and at the ventricular zone of the adult zebrafish telencephalon and it is up-regulated upon injury. Knock-down of *cxcr5* leads to reduced production of new neurons but proliferation of RGCs is not changed. Therefore, *cxcr5* may play a role in the differentiation of RGCs into neurons but may not be required for their proliferation (Kizil et al., 2012a). Another factor that influences regenerative neurogenesis may be inflammation. Inflammation is necessary and sufficient to induce reactive proliferation and regenerative neurogenesis. In addition, inflammation is able to induce the expression of Gata3 (Kyritsis et al., 2012). Contrary, in mammals inflammation prevents regeneration of neural tissues (Hoehn et al., 2005; Iosif et al., 2006). Furthermore, it was shown that in aged zebrafish brains neurogenesis is decreased due to enhanced quiescence of RGCs. In addition, regenerative neurogenesis is much weaker than in younger zebrafish (Edelmann et al., 2013).

Moreover, it was demonstrated that excitotoxic brain injury by using quinolinic acid results in cell death, recruitment of microglia and enhanced proliferation and neurogenesis in the injured telencephalic hemisphere. RGCs generate neurons, which migrate to the site of the injury and survived for at least 8 weeks. In the mammalian brain neuroblasts migrate to the lesion site as well, but in contrast to the zebrafish brain, these neurons fail to survive for a long time (Skaggs et al., 2014). Thus, the reactions to excitotoxic brain injury in zebrafish are similar to the reactions following stab injury.

1.2 The p53 family of transcription factors

The p53 family of transcription factors consists of three members, namely p53, p63 and p73. p53, which was discovered first in 1979 (DeLeo et al., 1979; Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero et al., 1979), is the best-known and the most prominent member since it is an important tumor suppressor inducing cell cycle arrest and apoptosis. The two p53-related proteins p63 (Yang et al., 1998) and p73 (Kaghad et al., 1997) were discovered almost 20 years later in 1998 and 1997, respectively.

All three members of the p53 family of transcription factors show a high similarity of the domain structure, especially in the DNA-binding domain. However, p63 and p73 are much more closely related to each other than to p53 (Belyi et al., 2010; Kaelin, 1999). Due to their similarity, p63 and p73 are able to transactivate p53 target genes which causes apoptosis

and cell cycle arrest. However, it is assumed that several p53 target genes respond differently to p53, p63 and p73 (Levrero et al., 2000; Murray-Zmijewski et al., 2006). p53, p63 and p73 exhibit overlapping functions but they also have distinct physiological roles in cells (De Laurenzi and Melino, 2000; Levrero et al., 2000; Murray-Zmijewski et al., 2006; Pietsch et al., 2008).

The transcription factors p53, p63 and p73 oligomerize and bind to the DNA as tetramers, which is essential for their functions. It was shown that p53 forms only homotetramers, whereas p63 and p73 are also capable of forming heterotetramers *in vitro* (Davison et al., 1999; Joerger et al., 2009).

It is well known that p53 is a tumor suppressor protein and that the p53 gene is frequently mutated in human cancers (Hollstein et al., 1996; Hollstein et al., 1991). However, the situation for the two other members of the p53 family is contradictory. Since p63 and p73 map to regions in the human genome, that often show alterations in cancers, this suggests that both are tumor suppressors as well (Kaelin, 1999; Kaghad et al., 1997; Yang et al., 1998). Though, it was revealed, that p63 and p73, in contrast to p53, are only rarely mutated in human tumors (Deyoung and Ellisen, 2007; Irwin and Kaelin, 2001a; Melino et al., 2003). Furthermore, in most cases p53-deficient mice develop normally but they are prone to develop spontaneous tumors (Donehower et al., 1995; Donehower et al., 1992). However, few female p53-deficient embryos die because they exhibit a failure of neural tube closure, which leads to exencephaly and subsequent anencephaly. Additionally, these embryos often show craniofacial malformations (Armstrong et al., 1995; Sah et al., 1995). In contrast to p53-deficient mice, p63- and p73-deficient mice are less cancer prone but they all show severe developmental defects (Mills et al., 1999; Yang et al., 1999; Yang et al., 2000). However,(Flores et al.)found out that mice heterozygous for mutations both in p63 and p73 ($p63^{+/-};p73^{+/-}$) developed a variety of tumors. Moreover, they demonstrated that mice heterozygous for mutations both in p53 and p63 ($p53^{+/-};p63^{+/-}$) or p53 and p73 ($p53^{+/-};p73^{+/-}$) developed more metastatic tumors and showed a higher tumor burden than heterozygous p53 mutant ($p53^{+/-}$) mice (Flores et al., 2005). Although p63 and p73 are not mutated in human tumors, the tumors often show an altered expression of p63 and p73, respectively. Some human tumors express higher levels of p73 compared to the normal tissue, like colorectal carcinoma (Sunahara et al., 1998), bladder cancer (Yokomizo et al., 1999), lung cancer (Mai et al., 1998), breast cancer (Zaika et al., 1999), ovarian cancer (Concin et al., 2004), neuroblastoma (Kovalev et al., 1998), rhabdomyosarcoma (Cam et al., 2006), hepatocellular carcinoma (Tannapfel et al., 1999b), glioma (Wager et al., 2006) and cholangiocellular carcinoma (Tannapfel et al., 1999a), for example. Also downregulation of p73 could be observed in lymphoblastic leukemias and Burkitt´s lymphoma (Corn et al., 1999; Kawano et al., 1999). Likewise, p63 was shown to be overexpressed in human tumors

such as head and neck squamous cell carcinoma (Sniezek et al., 2004; Weber et al., 2002), lung cancer (Massion et al., 2003), cervical carcinoma (Wang et al., 2001), lymphoma (Pruneri et al., 2005) and bladder carcinoma (Park et al., 2000). But also loss of p63 expression was detected in bladder cancer (Koga et al., 2003; Urist et al., 2002). These findings suggest that p63 and p73 definitively play a role in cancer development and progression. However, it is controversially discussed if p63 and p73 can be denoted tumor suppressors (Davis and Dowdy, 2001; Deyoung and Ellisen, 2007; Flores et al., 2005; Kaelin, 1999; Melino et al., 2002; Pietsch et al., 2008; Rosenbluth and Pietenpol, 2008).

1.2.1 p53

p53 was discovered as a protein, which binds to the oncogenic T antigen from the SV40 virus (DeLeo et al., 1979; Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero et al., 1979). It is one of the most important tumor suppressor proteins. The *p53* gene is mutated or inactivated in over 50 % of human cancers (Hollstein et al., 1996; Hollstein et al., 1991; Joerger and Fersht, 2007; Olivier et al., 2002). p53 mutant protein often accumulates in tumor cells, since mutant p53 is not able to induce the expression of its negative regulator Mdm2 (see chapter 1.2.1.2) (Midgley and Lane, 1997). Moreover, heterotetramerization of wildtype and mutant p53 leads to dominant-negative activities of mutant p53 protein because the wildtype p53 protein is inhibited and not able to activate transcription of target genes (de Vries et al., 2002; Harms and Chen, 2006; Ko and Prives, 1996). Germ-line mutations of one allele of *p53* occur in the Li-Fraumeni syndrome, a disorder where affected persons are highly tumor-prone (Li and Fraumeni, 1969; Li et al., 1988; Malkin, 1993; Srivastava et al., 1990). Furthermore, mice deficient for p53 ($p53^{-/-}$) show also a very high risk to develop spontaneous tumors (Donehower et al., 1995; Donehower et al., 1992).

p53 is a transcription factor that regulates the expression of genes which play a role in apoptosis, cell cycle arrest and senescence, thereby preventing the division of cells containing damaged DNA and thus cancer formation. Under normal conditions p53 is present at a very low level within a cell but after cellular stress, like DNA damage, p53 becomes rapidly activated which leads to apoptosis, senescence or cell cycle arrest (reviewed in Boehme and Blattner, 2009; el-Deiry, 1998; Ko and Prives, 1996; Levine, 1997; Oren, 1999; Prives and Hall, 1999; Vousden and Lu, 2002). Therefore, p53 is also called the „guardian of the genome" (Lane, 1992).

p53 is well established as a transcriptional activator, however it was shown that p53 is also able to suppress the transcription of particular genes (reviewed in(el-Deiry, 1998; Laptenko and Prives, 2006). Furthermore, p53 is not only involved in central mechanisms as apoptosis and cell cycle arrest that are important to preserve the genomic integrity. It coordinates several other processes which are also important for the p53 stress response and contribute to cancer formation and progression as angiogenesis (Menendez et al., 2006; Teodoro et al., 2006), metabolism (Green and Chipuk, 2006; Matoba et al., 2006) or immune response (Taura et al., 2008).

1.2.1.1 Structure and isoforms of the p53 protein

The human, mouse and zebrafish *p53* gene is composed of 11 exons and gives rise to a protein of 393, 387 and 373 amino acids, respectively, with a modular structure. The N-terminal transactivation domain (TAD) of the p53 protein is separated from the central DNA-binding domain (DBD) by a proline-rich domain (PR). The DBD is followed by a nuclear localization signal (NLS) and by a C-terminal oligomerization/tetramerization domain (OD, TET), a nuclear export signal (NES) and by a basic regulatory domain (REG) (Figure 7) (Boehme and Blattner, 2009). These different domains exhibit distinct functions. The N-terminal TAD (activation domain 1, AD1) activates transcription after binding of p53 to a specific promoter and associates with the acetylase and co-activator p300/CBP, factors of the basal transcriptional machinery, Mdm2 (see chapter 1.2.1.2), Mdm2-homologue MdmX/Mdm4 and many others. Acetylation of lysines in the C-terminus of p53 by p300/CBP leads to a stronger binding of p53 with the DNA binding domain to promoters of target genes thereby increasing the proapoptotic activity (Boehme and Blattner, 2009; Knights et al., 2006). The PR contains a weak second transactivation domain AD2 (activation domain 2), which is a weaker transcriptional activator for p53 target genes than AD1. AD1 seems to be important for transactivation and cell cycle arrest, whereas AD2 seems to be necessary for p53-dependent apoptosis. The PR itself is required for induction of apoptosis, accounts for growth suppression and plays a role as a protein binding site (Candau et al., 1997; Harms and Chen, 2005, 2006; Lin et al., 1994; Sakamuro et al., 1997; Scoumanne et al., 2005; Venot et al., 1998; Venot et al., 1999; Walker and Levine, 1996; Zhu et al., 1998b). As a sequence-specific transcription factor p53 binds to specific p53 responsive elements via its DBD. The central DBD is highly conserved among different species and most of the mutations of the *p53* gene in cancer are missense mutations occurring in the DBD (Harms and Chen, 2006; Scoumanne et al., 2005). Due to the NLS and NES p53 is able to shuttle between the nucleus and the cytoplasm (Harms and Chen, 2006; O'Keefe et al., 2003;

Scoumanne et al., 2005). p53 binds to the DNA as a homotetramer, which is crucial for the function of p53. This oligomerization is mediated through the OD or TET (reviewed in Chene, 2001). The REG is supposed to be an important regulatory domain affecting p53 activities both negatively and positively. It is subjected to various posttranslational modifications and interacts with several other proteins (Harms and Chen, 2005, 2006; Scoumanne et al., 2005).

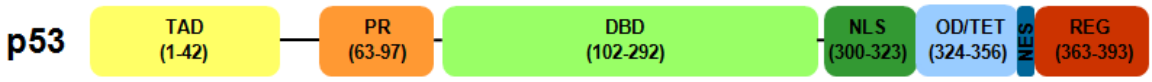


Figure 7: Schematic drawing of the human p53 protein.
The p53 protein has a modular structure and consists of several functional domains, namely a N-terminal transactivation domain (TAD), a proline-rich domain (PR), a central DNA-binding domain (DBD), a nuclear localization signal (NLS), a C-terminal oligomerization/tetramerization domain (OD/TET), a nuclear export signal (NES) and a regulatory domain (REG). The numbers indicate the amino acid residues.

Due to alternative splicing, alternative translation initiation sites and usage of distinct promoters, at least 9 different isoforms of the human p53 protein could be identified. The N-terminal isoform Δ40p53 (also called ΔNp53) is produced by either alternative splicing of intron 2 or by alternative initiation of translation. There are two other N-terminal isoforms, Δ133p53, which is generated by an alternative promoter located in intron 4 and usage of AUG 133, and the full-length p53 protein (TAp53). Alternative splicing of exon 9 results in the β and γ isoforms, where the ODs are replaced by 10 and 15 additional residues, respectively. Combination of the three N- and three C-terminal isoforms leads to 9 different p53 isoforms in all (TAp53α (full-length p53), TAp53β, TAp53γ, Δ40p53α, Δ40p53β, Δ40p53γ, Δ133p53α, Δ133p53β and Δ133p53γ). Recent findings suggest the existence of a 10th p53 isoform which is called Δp53. This isoform is generated by a noncanonical splicing between exon 7 and 9, which leads to the deletion of 198 nucleotides being part of the DBD and NLS. There was also an additional ΔN isoform identified, Δ160p53. This isoform is generated by using the internal promoter in intron 4 and AUG 160.
TA-isoforms contain the entire TAD and α-isoforms posses the longest C-terminal domain. Δ40p53 isoforms lack the first TAD and Δ133p53 and Δ160p53 lack the entire TAD and part of the DBD. β and γ isoforms lack the OD (Figure 8) (Bourdon et al., 2005; Courtois et al., 2002; Flaman et al., 1996; Ghosh et al., 2004; Khoury and Bourdon, 2010; Marcel et al., 2011; Marcel et al., 2010; Rohaly et al., 2005; Yin et al., 2002). The different isoforms have distinct functions and the ΔN isoforms can act in a dominant negative manner towards full-

length isoforms (De Laurenzi and Melino, 2000; Pietsch et al., 2008). Furthermore, the ΔN isoforms support proliferation whereas the TA isoforms promote apoptosis, senescence and cell cycle arrest (Dotsch et al., 2010). Thus, the different isoforms can have distinct activities in which TA isoforms act as tumor suppressors and ΔN isoforms act as oncogenes (Stiewe and Putzer, 2002; Stiewe et al., 2002). Therefore, the balance of the different isoforms within a cell may determine the fate of this cell (Dotsch et al., 2010).

Besides the full-length zebrafish p53 protein, only the N-terminally truncated isoforms Δ113p53, which is a homologue to human Δ133p53, and ΔNp53, which is a homologue to human Δ40p53, could be identified in zebrafish until now (Figure 8) (Chen et al., 2005; Davidson et al., 2010; Marcel et al., 2011).

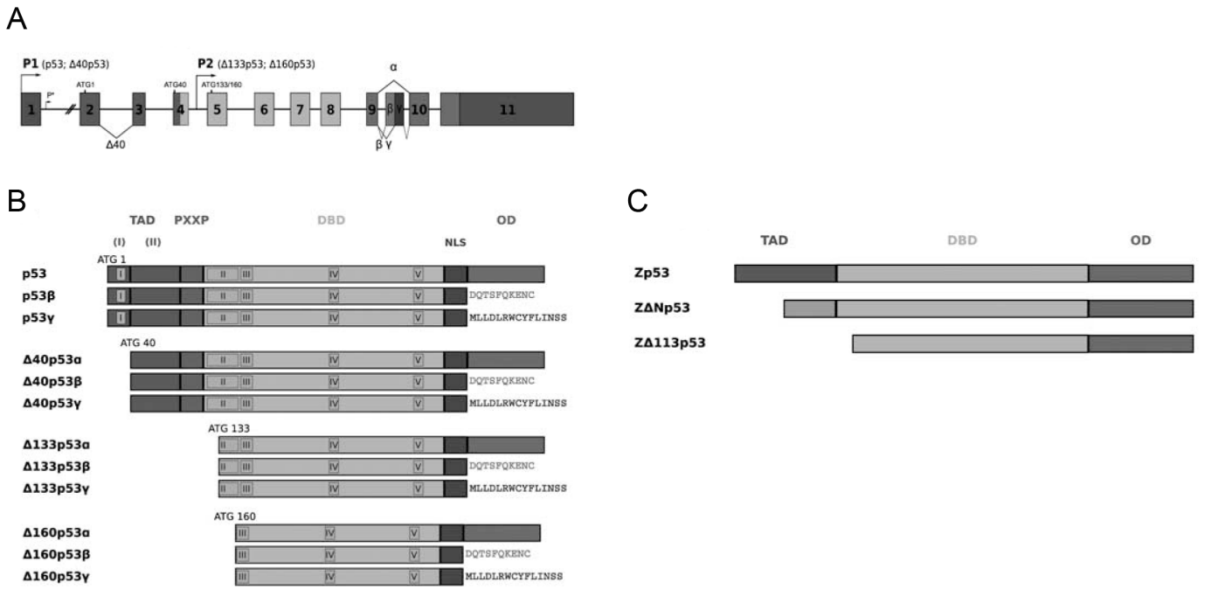


Figure 8: Schematic drawing of human and zebrafish p53 protein isoforms.

(A) Structure of the human p53 gene consisting of 11 exons. Several p53 isoforms are expressed due to alternative splicing and the usage of alternative translation initiation sites and promoters. (B) The different human p53 protein isoforms. (C) The different zebrafish p53 protein isoforms. For a detailed explanation of the characteristics of the different isoforms and how they are generated see text above. Modified from (Marcel et al., 2011).

1.2.1.2 Regulation of p53

Since p53 possesses an anti-proliferative capacity and is able to induce apoptosis, the activity of p53 has to be strictly regulated. p53 and its main regulator the RING-finger E3

ubiquitin ligase and oncoprotein mouse double minute-2 (Mdm2) are connected in an autoregulatory feedback loop (Wu et al., 1993). In normal, unstressed cells where p53 is not required, the p53 protein has a very short half-life (about 30 minutes) and the p53 level is kept low because p53 is rapidly degraded. Mdm2 binds to p53 at the N-terminal transactivation domain and inhibits the transcription of p53 target genes (Kussie et al., 1996; Momand et al., 1992). Furthermore, p53 becomes ubiquitinated by Mdm2 (Honda et al., 1997) and a few other ubiquitin ligases as constitutively photomorphogenic 1 (COP1) (Dornan et al., 2004) and Pirh2 (Leng et al., 2003), which results in the degradation of the p53 protein by the 26S proteasome (Haupt et al., 1997; Kubbutat et al., 1997). However, after cellular stress, like DNA damage caused by ultraviolet (UV) or ionizing irradiation, hypoxia or oncogene activation, when the activity of p53 is needed, p53 and Mdm2 dissociate. In addition p53 becomes also posttranslationally modified. Thus p53 is activated, rescued from degradation and is able to accumulate to high levels. Subsequently the transcription factor p53 can activate its target genes, which leads to cell cycle arrest (*p21*), apoptosis (Bcl-2-associated X protein (*Bax*), p53-up-regulated modulator of apoptosis (*Puma*) and *noxA*) or senescence (Figure 9). p53 is also capable of inducing DNA repair (Gatz and Wiesmuller, 2006; Helton and Chen, 2007). After damage cell cycle arrest allows a cell to repair the caused damage and if the damage is too severe apoptosis is induced. Moreover, p53 additionally induces the transcription of its own negative regulator *Mdm2* (Barak et al., 1993; Juven et al., 1993). Therefore, Mdm2 facilitates the degradation of p53 whereas p53 induces the transcription of its target gene and negative regulator Mdm2 (Barak et al., 1993; Bond et al., 2005; Haupt et al., 1997; Honda et al., 1997; Juven et al., 1993; Kubbutat et al., 1997; Kussie et al., 1996 ; reviewed in Boehme and Blattner, 2009). The importance of Mdm2 as negative regulator of p53 is demonstrated by the fact that *Mdm2* knock-out mice show early embryonal lethality because of p53-mediated apoptosis. When p53 is inactivated simultaneously this lethal phenotype is rescued and the embryos develop normal (Jones et al., 1995; Montes de Oca Luna et al., 1995). Furthermore, it was shown that p53 residing in the cytoplasm is able to induce apoptosis transcription-independently as well by interacting with Bcl-2 family proteins located at the membranes of mitochondria (Galluzzi et al., 2008).

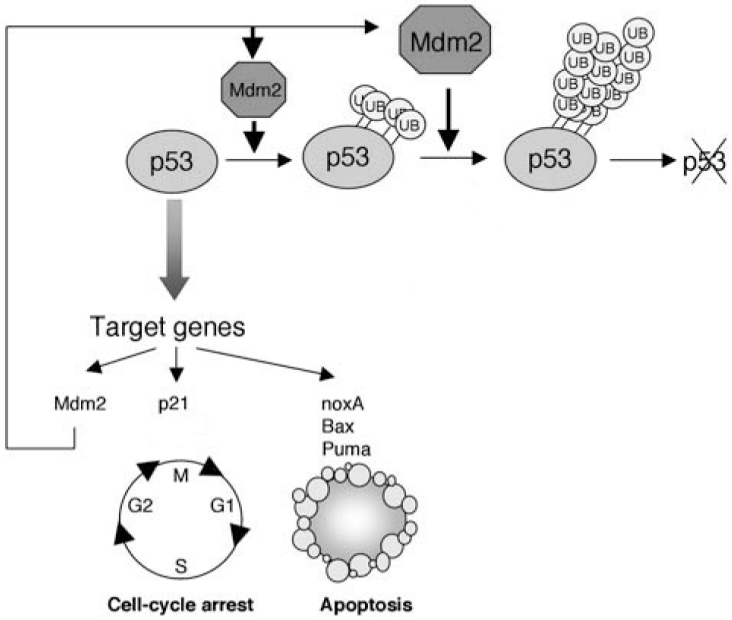


Figure 9: The p53-Mdm2 autoregulatory feedback loop.

Under normal conditions p53 becomes ubiquitinated by Mdm2, which targets p53 for degradation. After cellular stress p53 becomes activated, accumulates and is able to activate target genes, which leads to the induction of cell cycle arrest (*p21*) or apoptosis (*noxA*, *Bax*, *Puma*). p53 also induces its own negative regulator *Mdm2*. Modified from (Marine and Lozano, 2010).

Not only the stability and therefore the amount of the p53 protein is regulated but also the activity of p53 molecules present in a cell. This regulation is achieved by interaction of p53 with other proteins and by post-translational modifications, like phosphorylation, methylation, ribosylation and acetylation. The best-studied post-translational modification of p53 is phosphorylation and besides few sites that are phosphorylated in resting cells, most of phosphorylation occurs after cellular stress, mainly on serines and threonines. Phosphorylation of p53 after ionizing or UV irradiation is mediated by several kinases, as Ataxia telangiectasia mutated (ATM), Ataxia telangiectasia and Rad3-related protein (ATR), mitogen-activated protein (MAP)-kinases and checkpoint kinase 2 (Chk2) for example. Which kinases phosphorylate p53 on distinct residues depends on the sort of stimulation of p53 and leads to different responses. Furthermore it was demonstrated that acetylation of lysines has major influence on p53 activity and that acetylation is increased as well after cellular stress. Most acetylations are necessary for induction of pro-apoptotic genes and are performed by p300/CBP.

The majority of proteins that interact with p53 bind to the N-terminal transactivation or the DNA binding domain, thereby modulating the transcriptional activity of p53. Proteins binding to the DBD either inhibit or promote p53 activity (reviewed in Boehme and Blattner, 2009; Pietsch et al., 2008). However, IASSP binds to the proline-rich domain of p53 as an inhibitor.

Furthermore, Mdm2 heterodimerizes with its homologue MdmX (Mdm4), which results in a better ubiquitin ligase for p53 than Mdm2 homodimers. Additionally, MdmX binds to the N-terminus of p53 and antagonizes transactivation, like Mdm2 (Kawai et al., 2007; Linares et al., 2003; Marine et al., 2006; Okamoto et al., 2009). In contrast, it was shown that binding of MdmX stabilizes p53 because Mdm2-mediated degradation is delayed (Jackson and Berberich, 2000; Sharp et al., 1999).
Another means to regulate p53 is to interact with Mdm2. p14ARF (p19ARF in mouse) binds to Mdm2 which inhibits ubiquitination of p53 by Mdm2 (Honda and Yasuda, 1999; Midgley et al., 2000; Pomerantz et al., 1998). It was also suggested, that in unstressed cells p53 protein is able to negatively regulate the translation of *p53* mRNA by binding to the 5´UTR (Mosner et al., 1995).

1.2.1.3 p53 in zebrafish (*Danio rerio*)

Zebrafish p53 is 48% identical to human p53 (Cheng et al., 1997) and it is also negatively regulated by Mdm2 (Thisse et al., 2000). Furthermore, *p21* is a target gene of p53 in zebrafish as well (Langheinrich et al., 2002). Zebrafish p53 exhibits the same anti-proliferative and pro-apoptotic functions like human p53 after cellular stress (Storer and Zon, 2010). Therefore, p53 plays an important role in induction of apoptosis in zebrafish, like in mammals and other model organisms (Langheinrich et al., 2002; Storer and Zon, 2010).
p53 is mostly present in zygotes and early embryos. In the early zebrafish embryo p53 is ubiquitously expressed (Thisse et al., 2000). At 1 hour post fertilization (hpf) p53 expression starts to decrease, at 24-48 hpf (pharyngula stage) p53 is predominantly expressed in the head region and it is barely detectable at 48 hpf (Cheng et al., 1997; Thisse et al., 2000). As observed in mice, enhanced expression of p53 in zebrafish embryos caused by knock-down of Mdm2 via morpholinos, results in severe developmental defects. These defects can be rescued by co-injection of p53-specific morpholinos (Langheinrich et al., 2002).
The expression of p53 in older zebrafish embryos can be induced by cellular stress and p53-activating chemicals. It is enhanced following treatment with R-roscovitine, a cyclin-dependent kinase inhibitor that downregulates Mdm2 expression thereby activating p53 (Kotala et al., 2001; Lu et al., 2001), or ionizing irradiation, for instance. At 2 days post fertilization (dpf) the expression of p53 can be induced in several tissues of the zebrafish embryo. Whereas at 5 dpf a much more restricted expression of p53 can be observed. The induction of p53 in zebrafish embryos depends on the inducing signal and is tissue-specific and p53 seems to be regulated in a temporal- and spatial-specific manner. Furthermore, also p53 target genes, like *p21* or *Mdm2*, are up-regulated following treatment of embryos with

p53-activating agents. In contrast to mammals where p53 protein but not mRNA levels increase rapidly after DNA damage due to post-translational modifications, the *p53* mRNA is up-regulated as well after DNA damage in zebrafish. This suggests that there are other mechanisms additional to posttranslational modifications in zebrafish that may regulate the activity of p53 after cellular stress (Lee et al., 2008). Furthermore, translational control of zebrafish p53 was also reported. It was shown by Zhao et al. that zebrafish p53 protein is able to bind to the 3´UTR of endogenous *p53* mRNA in zebrafish embryos, which enhances the translational efficiency of *p53* mRNA following treatment with camptothecin, an anti-cancer compound that inhibits topoisomerase I (Langheinrich et al., 2002), or irradiation with UV. This results in delayed development of these zebrafish embryos (Zhao et al., 2012). The most prominent consequence of p53 up-regulation during zebrafish development is neural apoptosis (Langheinrich et al., 2002; Robu et al., 2007).

Berghmans et al. demonstrated that zebrafish containing a point mutation in the DNA binding domain of p53 (*tp53*^{M214K}) at a site, which is often mutated in human tumors, develop normally but the embryos do not induce expression of target genes or apoptosis after irradiation since the transcriptional activity of p53 is inactivated. As knock-down of p53 by morpholinos does also not lead to developmental defects p53 seems to be dispensable for early zebrafish development (Langheinrich et al., 2002). Like p53-deficient mice, *tp53*^{M214K} mutant zebrafish are also tumor-prone and they start to develop malignant peripheral nerve sheath tumors (MPNSTs) at the age of 8.5 months (Berghmans et al., 2005). Moreover it was shown, that very high levels of mutant p53 protein persisted in *tp53*^{M214K} mutant zebrafish embryos after irradiation, because mutant p53 is not able to induce its negative regulator Mdm2 and it accumulates until it is degraded by other processes. Additionally, mutant p53 protein accumulated in spontaneous tumors of adult *tp53*^{M214K} mutant zebrafish (Guo et al., 2013).

As already mentioned above (see chapter 1.2.1.1), two isoforms of the p53 protein could be identified in zebrafish, Δ113p53 and ΔNp53 (Chen et al., 2005; Davidson et al., 2010; Marcel et al., 2011). It was demonstrated that the expression of Δ113p53 is regulated by digestive-organ expansion factor (def) (Chen et al., 2005; Tao et al., 2013). Δ113p53 regulates the activity of p53 both during development and after cellular stress. It was shown that the induction of Δ113p53 after genotoxic stress in zebrafish embryos is dependent on full-length p53 protein and that the apoptotic activity of p53 is antagonized by Δ113p53 by up-regulating expression of anti-apoptotic genes like Bcl-2L. Protein-protein interaction between full-length p53 and Δ113p53 is required for the anti-apoptotic function of Δ113p53 (Ou et al., 2014). It could be speculated that the function of the Δ113p53 isoform is protection of zebrafish embryos against environmental stress conditions by inhibition of p53-dependent apoptosis (Chen et al., 2009; Chen and Peng, 2009). Guo et al. demonstrated that the induction of

p53-dependent transcription of target genes, like *Mdm2*, *Puma* and *p21*, is reduced by expression of Δ113p53. This indicates a novel feedback loop where Δ113p53 is induced by full-length p53 in response to DNA-damaging or developmental signals and regulates the p53 response by activating anti-apoptotic genes (Chen et al., 2009; Guo et al., 2010). Furthermore, the ΔNp53 isoform is also induced by ionizing irradiation in zebrafish embryos and counteracts the lethal effects of full-length p53. Contrary to the Δ113p53 isoform, overexpression of ΔNp53 leads to developmental defects of zebrafish embryos due to full-length p53-dependent activation of p21 (Davidson et al., 2010).
Moreover, zebrafish models for neurodegenerative diseases exist showing that p53 plays a role in these processes. DJ-1, which is one of the genes involved in Parkinson´s disease (Abeliovich and Flint Beal, 2006), protects neurons against oxidative stress (Kim et al., 2005; Taira et al., 2004; Yang et al., 2005). In zebrafish, loss of DJ-1 expression results in enhanced susceptibility of dopaminergic neurons to H_2O_2 (hydrogen peroxide). This leads to death of neurons caused by increased apoptotic activity of p53 and Bax (Bretaud et al., 2007). p53-dependent apoptosis plays a role in a zebrafish model of Alzheimer´s disease as well, where presenilin enhancer (Pen-2) is necessary to control neuronal cell survival and protection from apoptosis (Campbell et al., 2006). Furthermore, a zebrafish model for the Li-Fraumeni syndrome does also exist (Parant et al., 2010).

1.2.1.4 p53 in the developing mammalian nervous system

p53 is expressed in proliferative regions, like the SVZ and SGZ of the DG, the RMS and the cells of the external granular layer of the cerebellum, during embryonic and postnatal development of rodents (Jori et al., 2003; van Lookeren Campagne and Gill, 1998). Additionally to the proliferative regions, p53 is also expressed in post-mitotic neurons of the cerebral cortex and the hippocampus in the developing brain. During postnatal development the amount of p53 protein decreases in the brain (Medrano and Scrable, 2005; van Lookeren Campagne and Gill, 1998). Forsberg et al. showed *in vivo* that p53 is highly expressed in the embryonic telencephalon during neurogenesis and after this period p53 is present to a lesser extent. The expression of p53 persists in proliferating neuronal precursor cells of the adult SVZ (van Lookeren Campagne and Gill, 1998).
As already mentioned above (see chapter 1.2.1), some female p53-deficient mice show developmental defects like exencephaly, which results from a lack of apoptosis of progenitor cells (Jacobs et al., 2006; Jacobs et al., 2004). Therefore, p53 seems to be important for a normal neuronal development (Frenkel et al., 1999; Tedeschi and Di Giovanni, 2009). During development of the nervous system there are two phases where apoptosis occurs and is

essential for the correct development of the nervous system. The first phase takes place just before the onset of neurogenesis to eliminate progenitors that have not differentiated properly and to guarantee the generation of the correct number of cells in the distinct structures like the cerebral cortex (Blaschke et al., 1996; Jacobs et al., 2004; Miller et al., 2000). It was suggested that p53, probably together with full-length p63 and/or p73 isoforms, is important for elimination of neural progenitors and new-born neurons that did not differentiate properly (Jacobs et al., 2006; Jacobs et al., 2004; Miller et al., 2000). The second period of naturally occurring neuronal death occurs primarily during the first 2 weeks after birth and it was suggested that p53 plays a pro-apoptotic role in sympathetic neurons during development (Jacobs et al., 2006; Jacobs et al., 2004; Murase et al., 2011). During this second period of neuronal death differentiated neurons migrate to their target location and compete for nerve growth factor (NGF) and other trophic factors. Neurons that do not obtain enough trophic factors undergo apoptosis (Jacobs et al., 2006; Jacobs et al., 2004). By apoptosis of up to one-half of the present neurons, the number of neurons is matched to the size of the target tissue (Jacobs et al., 2004; Oppenheim, 1991). The neurons, that survive this neuronal death period during development, become comparatively invulnerable to injury leading to a long-term survival (Jacobs et al., 2006; Jacobs et al., 2004). In contrast to numerous observations, where p53 is important for developmental neuronal death, D'Sa-Eipper et al. claim, that p53 is not crucial for apoptosis of neural progenitor cells during development.

It has been hypothesized that p53 plays a role in cell fate decisions and differentiation during development of the nervous system but this role is controversial. It was reported, that *p53* mRNA is increased during differentiation of early neuronal precursor cells in the brain and it decreases during terminal differentiation (Rogel et al., 1985; Schmid et al., 1991; Tedeschi and Di Giovanni, 2009). Accordingly, it was illustrated by Ferreira and Kosik that p53 is expressed in fetal proliferating cerebellar neuroblasts and when the migrating neurons reach their final location p53 becomes down-regulated during terminal differentiation. Loss of p53 results in an accelerated terminal differentiation of the neurons, which suggests that p53 probably inhibits neuronal differentiation (Ferreira and Kosik, 1996). Liu et al. showed *in vitro* and *in vivo* that there is a high level of p53 expression during neuronal differentiation and p53 most likely prevents proliferation and neural differentiation of NSCs but it positively regulates gliogenesis. The BMP-Smad1 pathway and inhibitor of DNA binding 1 (Id1), which is a target gene of this pathway, seem to play a role in this process. p53 is able to repress the expression of Smad1 and also Id1 and p53 deficiency leads to an enhanced expression of Smad1 and Id1, which results in increased proliferation and neuronal differentiation of NSCs but less gliogenesis (Liu et al., 2013). In contrast to other observations, where Id1 reacts as a negative regulator of neural differentiation of NSCs (Miyazono and Miyazawa, 2002),

enhanced expression of Id1 results in increased neuronal differentiation of NSCs deficient for p53 (Liu et al., 2013). However, another *in vitro* study demonstrated that the level of expression of p53 is not changed during differentiation of NSCs but the activity of p53 is increased. p53 might be necessary for early neuronal differentiation via suppression of Forkhead box O3a (FOXO3A)/Id1 signaling since knock-down of p53 delays neuronal differentiation, which is in contrast to other observations where p53 suppresses differentiation (Aranha et al., 2009).

Furthermore, p53 plays a role in the development of oligodendrocytes and the differentiation of OPCs since the number of oligodendrocytes is decreased and the number of OPCs is increased in optic nerves of p53-deficient mice (Billon et al., 2004). Moreover, it was shown that opposite actions of the p19ARF (p14ARF in humans)-p53 and Myc pathway are necessary to regulate self-renewal, proliferation and cell fate choices of neural stem cells during development (Nagao et al., 2008). Additionally, it was demonstrated by Armesilla-Diaz et al. that p53 is important for differentiation and proliferation of stem cells in the olfactory bulb of mouse embryos *in vitro*. Stem cells lacking p53 show an increased proliferation and tend to produce more neurons than wildtype cells at the expense of astrocytes (Armesilla-Diaz et al., 2009). Thus, p53 seems to play a role in the differentiation of progenitor cells in which it is able to both inhibit and permit differentiation in distinct situations.

1.2.1.5 p53 in the adult mammalian nervous system

In the adult mammalian brain the tumor suppressor p53 is expressed in SVZ cells (Jori et al., 2003; van Lookeren Campagne and Gill, 1998) and it is also highly expressed in the RMS and the DG (Medrano and Scrable, 2005). Furthermore, the expression of p53 can be observed in few glial cells in the parenchyma (Martin et al., 2001) and in Purkinje cells of the cerebellum (Wood and Youle, 1995). It was shown that p53 expression in the adult cerebellum is essential for rapid walking synchronization (Campana et al., 2003). Meletis et al. detected *p53* mRNA expression in ependymal cells, which line the ventricle, in astrocytes and in progenitor cells. Differentiating neuroblasts in the RMS show a weak *p53* expression. Therefore, p53 shows a higher expression in the neural stem cell lineage compared to other cells and it is especially expressed in stem and progenitor cells (Meletis et al., 2006). Under normal conditions the amount of *p53* mRNA in the SVZ is very low but it increases after irradiation, particularly along the lateral walls of the ventricles (Gil-Perotin et al., 2006).

In the adult brain p53 plays a critical role in regulating the number of cells in the SVZ because it modulates self-renewal, differentiation and proliferation of these cells (Gil-Perotin et al., 2006). It was shown *in vitro* and *in vivo*, that proliferation of brain stem cells is highly

increased in p53-deficient mice. This enhanced proliferation leads to regions of hyperplasia in the subventricular zone of the brain since the number of neuroblasts (A cells) and adult neural stem cells (B cells) (see chapter 1.1.1.1) is increased by loss of the anti-proliferative p53 protein (Gil-Perotin et al., 2006). Due to the rapid differentiation of these SVZ cells the number of new oligodendrocytes and neurons is enhanced by loss of p53 (Gil-Perotin et al., 2006; Li et al., 2008).

Meletis et al. demonstrated *in vitro* and *in vivo* that p53 negatively regulates self-renewal, that means survival and proliferation, of adult neural stem cells but it does not influence their differentiation. However, a role for p53 in neural cell differentiation *in vitro* has been proposed by Jori et al. and also Gil-Perotin et al. could observe an increased differentiation without p53. Furthermore, it was shown by Meletis et al. that there are more proliferating cells detectable in p53 knock-out mice in comparison to wildtype mice, as observed by Gil-Perotin et al. It was suggested that it could be crucial to suppress self-renewal of stem cells by p53 for long-term maintenance of the stem cell population since enhanced proliferation could lead to premature senescence (Meletis et al., 2006). Moreover, it was hypothesized, that p53 prevents post-mitotic neurons to re-enter the cell cycle, at least *in vitro*, thereby preserving the post-mitotic character of differentiated neurons (Miller et al., 2003).

In a model of aging using mice that over-express ΔNp53 (also called Δ40p53 or p44) (Maier et al., 2004), these aged mice show a reduced number of NSCs in the SVZ and the number of neurons in the OB is decreased due to a reduced ability of NSCs to proliferate. Over-expression of ΔNp53 results in a constitutive activation of p53 and subsequent expression of p21 in NSCs and thereby to a reduction in proliferation. Thus, p53 is important for maintaining neurogenesis of the adult brain because it controls the proliferation of progenitor and stem cells (Medrano et al., 2009). p53 seems to be crucial for the right balance between proliferation and quiescence of NSCs since loss of p53 leads to increased and over-expression of p53 results in decreased proliferation. Furthermore it was shown that the level of p44 in the mouse brain increases with age and that p44 stimulates the phosphorylation of tau, that plays a role in Alzheimer´s disease, thereby leading to a modified tau metabolism which is symptomatic for aging (Pehar et al., 2013).

Neuronal apoptosis does not only occur during development but also following excitotoxic, ischemic and traumatic injury of the nervous system or neurodegenerative conditions (Beattie et al., 2000; Bengzon et al., 2002; Graham and Chen, 2001; Wood and Youle, 1995; Yuan et al., 2003). p53 is up-regulated and crucial for the induction of apoptosis after injury of the mature nervous system and over-expression of p53 in cultured postmitotic neurons is sufficient to induce apoptosis (Slack et al., 1996). Therefore, p53 is an essential factor for neuronal death following distinct forms of acute damage, caused by treatment with ionizing irradiation, camptothecin, glutamate or kainate for example, or chronic neurodegenerative

diseases (Culmsee and Mattson, 2005; Miller et al., 2000; Morrison and Kinoshita, 2000; Morrison et al., 2003; Napieralski et al., 1999; Xiang et al., 1998; Yonekura et al., 2006). p53 is constitutively expressed at low levels in neurons but it becomes up-regulated in injured adult neuronal cells by DNA damage, for instance. It is predominantly located in the nucleus in injured cells in contrast to uninjured cells where it shows a weak cytoplasmic staining (Inamura et al., 2001; Plesnila et al., 2007). Neuronal injury leads to the production of reactive oxygen species (ROS) which causes accumulation of DNA strand breaks that activate p53 by phosphorylation by ATM and Chk2 kinase, for example. A severe damage leads to the induction of apoptosis by the mitochondrial/intrinsic pathway. p53 induces Puma, which interacts both with anti-apoptotic proteins like Bcl-2 or Bcl-X_L and with pro-apoptotic proteins like Bax. Bax translocates to the mitochondria which leads to release of cytochrome *c* from the mitochondria and activation of caspases. The mitochondrial pathway also functions in developing neurons and p53 induces apoptosis after damage of neurons during development as well (Cregan et al., 1999; Culmsee and Mattson, 2005; D'Sa et al., 2003; Jacobs et al., 2006; Jacobs et al., 2004; Johnson et al., 1998; Martin et al., 2001; Morris et al., 2001; Morrison and Kinoshita, 2000; Niizuma et al., 2009; Xiang et al., 1998). Moreover, apoptosis inducing factor (AIF) can be released from the mitochondria triggered by p53, translocate to the nucleus, degrade DNA and induce cell death independent of caspases (Cregan et al., 2002). p53 is also able to induce apoptosis of neural progenitors via the extrinsic apoptotic pathway. It was shown *in vitro* that Fas/CD95, a death receptor, plays a p53-dependent role in the response after exposure of neural progenitors to γ-irradiation (Semont et al., 2004). p53 is also capable of inducing apoptosis of neurons transcription-independently after ischemia via translocation to the mitochondria where it interacts with Bcl-X_L followed by release of cytochrome *c in vivo* (Endo et al., 2006). Geng et al. showed *in vitro* that cytoplasmic p53 can trigger apoptosis of neural precursor cells transcription-independently by interacting with Bax. Furthermore Martin et al. demonstrated *in vitro*, that differentiated neurons are less sensitive to DNA damaging agents than immature neurons. The kind of stress stimulus determines which stress signaling pathway is activated after neuronal damage and finally p53-mediated apoptosis is induced (Akhtar et al., 2006). Loss of p53 protects neurons from cell death after induced damage also *in vivo* (Culmsee and Mattson, 2005; Martin et al., 2001). Furthermore, ΔNp73 and perhaps ΔNp63 take care that apoptosis is only induced if the damage of the adult neuron is severe (Jacobs et al., 2004).
Moreover, p53-mediated neuronal death may also play a role in neurodegenerative disorders like Alzheimer´s disease (AD), Parkinson´s disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington´s disease (Bae et al., 2005; Culmsee and Mattson, 2005; de la Monte et al., 1997; Eve et al., 2007; Jacobs et al., 2006; Martin, 2000; Mogi et al., 2007; Nair et al., 2006; Yuan and Yankner, 2000). In contrast to these observations, Tomasevic et al. claim, that p53

is in fact activated after neuronal injury but it may not be responsible for the death of neurons *in vivo* and it was shown by Maeda et al. that p53-deficiency worsens brain injury after ischemia *in vivo*. In addition, p53 appears to play a role in apoptosis of glial cells caused by oxidative stress *in vitro* as well (Bonini et al., 2004; Kitamura et al., 1999).
Mutations of p53 often occur in brain tumors and they lead to tumor development and growth (Sidransky et al., 1992). Furthermore, it was shown that p53 is often mutated or deleted in glial tumors (Hayashi et al., 2004; Sung et al., 2000; von Deimling et al., 1992; Watanabe et al., 1996). However, loss of p53 is not sufficient for the development of spontaneous glial tumors (Donehower et al., 1992; Philipp-Staheli et al., 2004). But as it leads to areas of hyperplasia due to a proliferative advantage of slow- and fast-proliferating SVZ cells followed by their rapid differentiation, an additional mutagenic stimulus could then lead to tumor development (Gil-Perotin et al., 2006).

In summary, the transcription factor and tumor suppressor p53 is expressed in a similar pattern in the mammalian brain during development and in adulthood. Additionally, p53 is involved in similar cellular processes in the developing and in the adult central nervous system such as proliferation and differentiation of precursor cells and apoptotic death of neurons.

1.2.1.6 p53 in the Medaka (*Oryzias latipes*) brain

Like zebrafish (see chapter 1.1.2.1), medaka contain several proliferation zones in the adult brain (Kuroyanagi et al., 2010) and it was demonstrated that p53 is expressed in most of them (Isoe et al., 2012). In contrast to the observations that were made in the adult mammalian brain, where p53 suppresses proliferation of neural stem cells (see chapter 1.2.1.5), Isoe et al. showed that a p53 null mutation (Taniguchi et al., 2006) does not increase but inhibit neurogenesis in the telencephalon of medaka. Two different mutants were used in this study, $p53^{E241X}$ and $p53^{Y186X}$. Both mutations lead to a stop codon in the DNA binding domain that results in the expression of a truncated non-functional p53 protein (Taniguchi et al., 2006). Wildtype medaka exhibit a greater number of BrdU-positive, thus proliferating, progenitor cells that migrate from proliferation zones in the telencephalon than p53 mutant fish one week after BrdU administration. However, the number of BrdU-positive cells 4 hours after BrdU treatment was not affected by mutant p53. Thus, in contrast to stem cells in the adult mammalian brain, p53 deficiency does not affect highly proliferating stem cells in medaka probably because p63 and/or p73 could take over the functions of p53. Furthermore, the number of apoptotic cells is not different between wildtype and mutant

medaka which indicates that the discrepancy in proliferating progenitor cells one week after BrdU administration is not due to increased cell death but to repressed proliferation. Therefore, contrary to the mammalian brain, p53 seems to positively regulate neurogenesis in the medaka brain (Isoe et al., 2012). However, the positive role of p53 in regulating neurogenesis in medaka has to be further investigated.

1.2.2 p63

The transcription factor p63 has a crucial function during development and plays the most prominent role of the three members of the p53 family during development. p63-deficient mice show severe developmental defects. These mice are born without stratified epithelia (skin, urothelia, breast, prostate) and they also lack hair follicles, teeth, mammary, salivary and lachrymal glands. In addition their limbs are missing or the limbs are truncated due to a defective apical ectodermal ridge and they exhibit craniofacial malformations. p63-deficient mice normally die shortly after birth because of dehydration (Mills et al., 1999; Yang et al., 1999). In humans germ-line mutations of p63 lead to the Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome (EEC syndrome) and nonsyndromic split hand-split foot malformation (SHFM) amongst others (Celli et al., 1999; Rinne et al., 2007; van Bokhoven et al., 2001). p63 may be necessary for ectodermal differentiation and/or maintenance of progenitor cells during development. However, it is not clear if p63 plays a role in both differentiation and stem cell self-renewal or just in one of these processes (Koster et al., 2004; Mills et al., 1999; Su et al., 2009; Yang et al., 1999).

Moreover, p63 is able to induce the expression of p53-responsive genes like *p21*, *Mdm2*, *Bax*, *noxA* and *Puma* and thus to cause cell cycle arrest and apoptosis. (De Laurenzi and Melino, 2000; Levrero et al., 2000; McKeon and Melino, 2007; Murray-Zmijewski et al., 2006; Pietsch et al., 2008). p63 is capable of inducing apoptosis via intrinsic or extrinsic apoptotic pathways (Candi et al., 2007; Gressner et al., 2005). Furthermore, the level of p63 protein also increases *in vitro* after treatment with DNA-damaging agents which is most likely due to post-translational modifications of p63 (Katoh et al., 2000; Okada et al., 2002). Notably it was demonstrated that p53 requires p63 or p73 to function properly after DNA damage and to induce several target genes associated with apoptosis like *Bax* or *NoxA* for example. Contrary, the induction of *p21* or *Mdm2* does not require the presence of p63 or p73 (Flores et al., 2002). Furthermore, p63 does not interact with Mdm2 as p53 (see chapter 1.2.1.2) but nevertheless it may be degraded by proteasomes in the absence of cellular stress (Okada et al., 2002).

1.2.2.1 Structure and isoforms of the p63 protein

Human and mouse *p63* consists of 14 and zebrafish *p63* consists of 12 exons. It gives rise to a protein of 680 or 588 amino acids, respectively. The structure of p63 is very similar to that of p53 (see chapter 1.2.1.1). The p63 protein also contains a N-terminal TAD, several PRs that are scattered throughout the coding region (Irwin and Kaelin, 2001b), a central DBD and a C-terminal OD. However, unlike p53, p63 does not possess a basic REG but a sterile alpha motive (SAM) domain (Thanos and Bowie, 1999), which is a protein-protein interaction domain, and a transcription inhibition domain (TID), which inhibits the transcriptional activity of TAp63 by interacting with the TA domain (Serber et al., 2002), at the C-terminus (Benard et al., 2003; Bourdon, 2007; Danilova et al., 2008a; De Laurenzi and Melino, 2000; Levrero et al., 2000). p63 also contains a NLS and a NES (Harms and Chen, 2006).
As it is the case for p53, there are also different p63 isoforms expressed caused by two different promoters and alternative splicing of the C-terminus. 6 distinct p63 isoforms could be identified, 3 isoforms that contain the TAD and 3 ΔN variants: TAp63α (full-length), TAp63β, TAp63γ, ΔNp63α, ΔNp63β, ΔNp63γ. The promoter upstream of exon one produces the TA isoforms containing the TAD. Whereas usage of an alternative promoter located in intron 3 leads to N-terminally truncated isoforms (ΔNp63) lacking the TAD. Alternative splicing at the C-terminus results in 3 different splice variants. The α isoform contains the whole C-terminus including the SAM and TID whereas the β isoform lacks exon 13 and the γ isoform lacks exons 11 to 14 but contains an additional exon 15 (Figure 10) (Levrero et al., 2000; Marcel et al., 2011; Murray-Zmijewski et al., 2006; Pietsch et al., 2008).

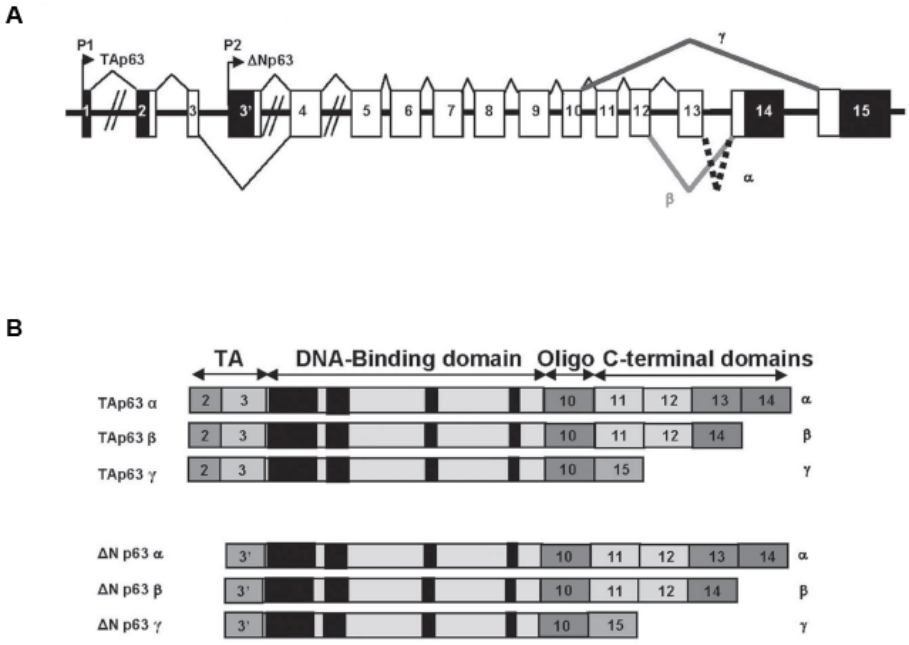


Figure 10: Schematic drawing of human p63 protein isoforms.

(A) Structure of the human p63 gene consisting of 14 exons. Usage of an alternative promoter in intron 3 and alternative splicing at the C-terminus leads to the expression of 6 p63 isoforms. (B) The 6 different human p63 protein isoforms are depicted. Numbers indicate the exons present in the corresponding isoforms. For a detailed explanation of the characteristics of the different isoforms and how they are generated see text. Modified from (Bourdon, 2007).

The TAp63γ isoform, which lacks the SAM and TID, is most similar to p53 (Deyoung and Ellisen, 2007; Petitjean et al., 2008) and is a more potent transactivating isoform than TAp63α containing both domains (Danilova et al., 2008a; Serber et al., 2002). Furthermore, dominant-negative ΔNp63 isoforms do not contain the TAD but nevertheless they are able to transactivate target genes by using an alternative transactivation domain in their N-terminus including the PR (Dohn et al., 2001; Helton et al., 2006). ΔNp63α and ΔNp63β contain an additional proline-rich domain at the C-terminus that enhances the transcriptional activity of these isoforms (Candi et al., 2007; Ghioni et al., 2002; Helton et al., 2006). It has been shown that ΔNp63β possesses the strongest transcriptional activity of the ΔNp63 isoforms (Helton et al., 2006). Liefer et al. demonstrated that dominant-negative ΔNp63 isoforms are down-regulated and TAp63 isoforms increase after irradiation with UV light. Thus the ΔNp63 isoforms are not able to inhibit the apoptotic function of p53.

Both TAp63 and ΔNp63 isoforms are necessary for a normal epidermal development (Danilova et al., 2008a; Koster et al., 2004). TAp63 isoforms are expressed earlier than ΔNp63 isoforms but ΔNp63 isoforms are predominantly expressed during development (Candi et al., 2007; Koster et al., 2004; Mikkola, 2007). Moreover it was suggested that p63 plays two distinct roles during development and in the mature epidermis. During development it induces epithelial stratification and in the mature epidermis p63 sustains proliferation of basal keratinocytes (Koster et al., 2004). Accordingly, p63 is highly expressed in proliferating basal cells of several epithelial structures and ΔNp63α is the major isoform expressed in the mature epidermis (Liefer et al., 2000; Yang et al., 1998). Though, TAp63 is critical for maintaining stem cells of the adult skin and preventing premature aging of tissues by regulation of genomic stability and cellular senescence.

1.2.2.2 Regulation of p63

Like p53 (see chapter 1.2.1.2), p63 is also regulated by ubiquitin ligases. The HECT E3 ubiquitin ligase Itch binds to the p63 protein, ubiquitinates p63 and promotes its degradation by proteasomes. However until now any negative feedback-loop like between Mdm2 and p53 has been identified for p63 and an E3 ubiquitin ligase (Rossi et al., 2006; Watson and Irwin, 2006). Furthermore, p63 induces the expression of Mdm2 but Mdm2 does not target p63 for degradation by the proteasome as p53. Though, the effects of the interaction between Mdm2 and p63 are controversial. It was demonstrated that Mdm2 binds to p63 thereby stabilizing p63 and enhancing its transactivation function (Calabro et al., 2002). However, it was also reported that Mdm2 does not interact with p63 (Okada et al., 2002), that overexpression of Mdm2 inhibits the transcriptional activity of p63 (Kadakia et al., 2001) or that there are no effects of Mdm2 on transcriptional activity and stability of p63 (Little and Jochemsen, 2001).
As p53, p63 is regulated by post-translational modifications, like phosphorylation, as well (Danilova et al., 2008a; Okada et al., 2002; Petitjean et al., 2008). Moreover, Ankirin repeats, SH3 domain, proline-rich protein (ASPP1) and ASPP2 interact with p63 and enhance its apoptotic activity by promoting the induction of *Bax* and *Puma* by p63 (Bergamaschi et al., 2004). Furthermore, ΔNp63 can be positively and negatively regulated by both p53 and ΔNp63α itself, respectively (Harmes et al., 2003) or by TAp63γ (Li et al., 2006). Ratovitski et al. demonstrated that p53 binds to ΔNp63 and targets it for degradation by caspases. Additionally it was shown that the expression of dominant negative ΔNp73 is induced by TAp63 after genotoxic stress which could be a hint for a regulatory loop that controls the activity of p63 (Petitjean et al., 2008).

1.2.2.3 p63 in zebrafish (*Danio rerio*)

During early development ΔNp63 is expressed in the somites, eyes, otic vesicle and epidermis. Later in development the expression of ΔNp63 is restricted to the eye epithelium and epidermis (Lee and Kimelman, 2002). ΔNp63α is the major isoform expressed during development and the expression of ΔNp63α in zebrafish is first detected during gastrulation. It is needed in non-neural ectoderm for ventral specification where it acts as a transcriptional repressor activated by BMP signaling to prevent neural specification. Later in development the expression of ΔNp63α can be detected in the epithelium where it is essential for the formation of fins and skin. It antagonizes p53 thereby stimulating the proliferation of epidermal cells but ΔNp63 seems to be dispensable for epidermal differentiation (Bakkers et al., 2002; Lee and Kimelman, 2002). p63-deficient zebrafish show similar developmental defects as mice since the fin and skin development is impaired (Lee and Kimelman, 2002). Over-expression of ΔNp63 promotes the overgrowth of fins and other non-neural structures and inhibits neural structures of zebrafish embryos (Bakkers et al., 2002).

1.2.2.4 p63 in the mammalian nervous system

During development p63 is expressed in Cajal-Retzius cells of the cerebral cortex and hippocampus and in some SVZ cells. In contrast to p53 the expression of p63 increases during development and in the adult brain it is almost ubiquitously expressed. In the adult brain expression of both ΔNp63 and TAp63 could be detected in the cortex, cerebellum, SVZ and olfactory bulb. p63 is also expressed in neurons and precursor cells of the hippocampus. In the adult cortex and hippocampus p63 is expressed in pyramidal neurons for example (Hernandez-Acosta et al., 2011; Jacobs et al., 2005).

TAp63 is the main isoform expressed in the nervous system during development and the amount of TAp63 increases in sympathetic neurons during developmental neuronal cell death (Jacobs et al., 2005). Over-expression of TAp63 leads to neuronal apoptosis and apoptosis of sympathetic neurons was inhibited *in vivo* by p63 deficiency. TAp63 together with p53 and TAp73 may be crucial for elimination of newly born neurons and neural progenitors in the nervous system of embryos that failed to differentiate properly (Jacobs et al., 2006; Jacobs et al., 2004). Therefore, TAp63 is an important proapoptotic factor that induces death of sympathetic neurons during embryonal development (Jacobs et al., 2005). The survival of neurons during developmental cell death may depend on the balance between pro-survival (ΔN) and pro-apoptotic (TA) isoforms of p53, p63 and p73 (Jacobs et al., 2004). Furthermore, Bax is necessary for TAp63, as for p53, to induce apoptosis of

sympathetic neurons (Jacobs et al., 2005). Additionally, it was shown that p53 requires TAp63 to induce neural apoptosis during the development of mouse embryos while TAp63 is able to induce apoptosis on its own (Jacobs et al., 2005).

Furthermore it was demonstrated *in vitro* and *in vivo* that ΔNp63 is crucial for the survival of newly born neurons and precursor cells in the cerebral cortex of embryos since it antagonizes the pro-apoptotic functions of p53 and probably TAp63. It was suggested that ΔNp63 stimulates survival by impeding the intrinsic cell death pathway (Dugani et al., 2009; Jacobs et al., 2005). In contrast to these observations, it was shown *in vitro* and *in vivo* using p63 knock-out mice by Holembowski et al. that p63 is of no importance for the correct development of the central nervous system and that it does not play a role in the maintenance or differentiation of embryonic neural stem and progenitor cells. This is in line with the observation that p63 knock-out mice do not show any obvious malformation of the brain (Hernandez-Acosta et al., 2011; Holembowski et al., 2011). It was suggested that p63 may be more important in the adult brain. Likewise, Cancino et al. showed *in vitro* and *in vivo* that ΔNp63 promotes the survival of adult NPCs and adult newborn neurons in the olfactory bulb and in neurogenic regions of the lateral ventricles and hippocampus as well by inhibition of p53- and Puma-dependent apoptosis. Loss of ΔNp63 results in increased apoptosis of newborn neurons and NPCs. This leads to deficits in hippocampus-dependent memory formation (Cancino et al., 2013b). In a recent paper it was shown that both p63 and p73 play a role in maintaining NPCs in the adult brain by regulating p53. p63 supports NPC survival through antagonizing p53 and p73 controls the self-renewal of NPCs and decides if NPCs undergo apoptosis mediated by p53 or senescence. Thus both proteins are crucial for self-renewal and long-term maintenance of NPCs in the adult forebrain and hippocampus (Fatt et al., 2014). In contrast to p53 and p73, p63 seems not to play a role in oligodendrocyte development (Billon et al., 2004).

1.2.3 p73

p73, the third member of the p53 family of transcription factors, is crucial for normal development. p73-deficient mice exhibit several defects like hydrocephalus, hippocampal dysgenesis, chronic infections and inflammation. In addition they also show problems in pheromone sensory pathways, and thus infertility due to abnormal mating. In contrast to p63-deficient mice (see chapter 1.2.2), p73-deficient mice are more viable and do not die shortly after birth. Contrary to p53-deficient mice (see chapter 1.2.1), p73-deficient mice do not develop spontaneous tumors (Yang et al., 2000). Compared to p53 and p63, no human

disorder has been linked until now to a germ line mutation of the *p73* gene (Danilova et al., 2008a; Murray-Zmijewski et al., 2006). Moreover, it was demonstrated that in most human cell lines and tissues the expression level of p73 is very low.

1.2.3.1 Structure and isoforms of the p73 protein

The human, mouse and zebrafish *p73* gene is composed of 14 exons, which give rise to a protein with 636, 631 and 640 amino acids, respectively. Like p53 (see chapter 1.2.1.1), the p73 protein contains a N-terminal TAD, a central DBD, a C-terminal OD, and like p63 (see chapter 1.2.2.1) several PRs (Irwin and Kaelin, 2001a, b), a SAM (Thanos and Bowie, 1999) and a TID (Chi et al., 1999; Danilova et al., 2008a; Kaghad et al., 1997; Murray-Zmijewski et al., 2006; Pietsch et al., 2008). p73 possesses also a NLS and a NES (Harms and Chen, 2006; Inoue et al., 2002).

There are 7 different C-terminal isoforms expressed due to alternative splicing of the C-terminus: α, β, γ, δ, ε, ζ and η. Alternative splicing at the N-terminus and usage of different promoters leads to the expression of further isoforms. Transcription from the promoter upstream of exon 1 produces TAp73 isoforms containing the TAD. Whereas usage of an internal promoter located in intron 3 generates ΔNp73 isoforms lacking the TAD. Alternative splicing of exon 2 causes Ex2p73 isoforms, which lack a part of the TAD. Ex2/3p73 isoforms are generated by alternative splicing of exons 2 and 3. These isoforms lack the entire TAD. In addition, the ΔN`p73 isoform could be identified, at least at the mRNA level. The corresponding protein could either be a very short protein or an isoform identical to ΔNp73. The ΔN`p73 isoform is produced by alternative splicing of exon 3` contained in intron 3. It could be transcribed from promoter 1 until a stop codon in exon 3`. Thus it could produce a very short protein consisting only of the TAD. Or it could be translated from a third AUG present in exon 3` which leads to a p73 protein identical to the ΔNp73 isoform. In all, 29 different p73 protein isoforms can be generated (Figure 11) (Bourdon, 2007; Murray-Zmijewski et al., 2006; Pietsch et al., 2008). TAp73α resembles the full-length protein. β isoforms lack exon 13, γ isoforms lack exon 11 and in δ isoforms exons 11 to 13 are spliced out. Furthermore ε isoforms do not contain exons 11 and 13, ζ isoforms lack exons 11 and 12 and η isoforms lack exon 14 (Bourdon, 2007; De Laurenzi et al., 1998; Levrero et al., 2000; Melino et al., 2002).

The TAp73 isoforms are able to activate the transcription of target genes to induce apoptosis and cell cycle arrest. Whereas the ΔNp73 isoforms are able to act in a dominant-negative and anti-apoptotic manner to p53, p63 and p73 (Murray-Zmijewski et al., 2006). However, since ΔNp73 isoforms contain an alternative TAD including the PR at their N-terminus they

are able to transactivate target genes, such as *p21*, and induce cell cycle arrest or apoptosis as well. They are also capable of activating genes that are not induced by TAp73 isoforms (Liu et al., 2004; Murray-Zmijewski et al., 2006).

The distinct C-terminal isoforms differ in their ability to activate target genes (Grob et al., 2001). The α C-terminal isoforms containing the SAM and TID are less potent to induce the transcription of target genes than other isoforms lacking the SAM and TID (Harms and Chen, 2006; Liu et al., 2004). The SAM and TID block the interaction of p73 with p300/CBP thereby inhibiting the transcriptional activity of p73 (see chapter 1.2.3.2) (Liu and Chen, 2005). p73β isoforms show a strong transcriptional activity contrary to p73γ isoforms which are less efficient in transactivation (De Laurenzi et al., 1998). Moreover, the TAp73δ isoform is most similar to p53 (De Laurenzi et al., 1998; Levrero et al., 2000) and the Ex2p73 isoforms is only expressed in several types of tumors but not in normal tissues (Melino et al., 2002) .

The knock-out of either the TA or ΔN isoforms of p73 causes different phenotypes compared to the simultaneous knock-out of all isoforms described above (see chapter 1.2.3). It was shown that *TAp73*-null mice are tumor-prone and infertile due to defective oocytes (Tomasini et al., 2008). Additionally, they exhibit characteristics of premature aging caused by mitochondrial dysfunction (Rufini et al., 2012). Moreover, TAp73 is important for the development of the hippocampus and TAp73-deficient mice exhibit hippocampal dysgenesis (Tomasini et al., 2008). In contrast to TAp73-deficient mice, cells from ΔNp73-deficient mice show enhanced p53-dependent apoptosis and are more sensitive to DNA-damaging agents. It was shown that ΔNp73 plays an inhibitory role in the DNA damage response pathway. ΔNp73 is able to localize to the site of DNA damage and to prevent phosphorylation of p53 by inhibition of ATM kinase (Wilhelm et al., 2010). During development ΔNp73 isoforms are the major isoforms expressed (Danilova et al., 2008a). However, over-expression of ΔNp73 in embryos is lethal maybe due to dysregulation of p53, p63 and p73 (Erster et al., 2006). Furthermore, adult ΔNp73-deficient mice exhibit signs of neurodegeneration since ΔNp73 plays an anti-apoptotic role in neurons (Pozniak et al., 2000). Additionally it was hypothesized, that the hydrocephalus of p73-deficient mice is caused by extensive loss of neurons since p73 is necessary for the survival of neurons (see chapter 1.2.3.4). Furthermore, in humans TA isoforms are mostly expressed during development and foetal tissues express more p73 than the equivalent adult tissue (Grob et al., 2001).

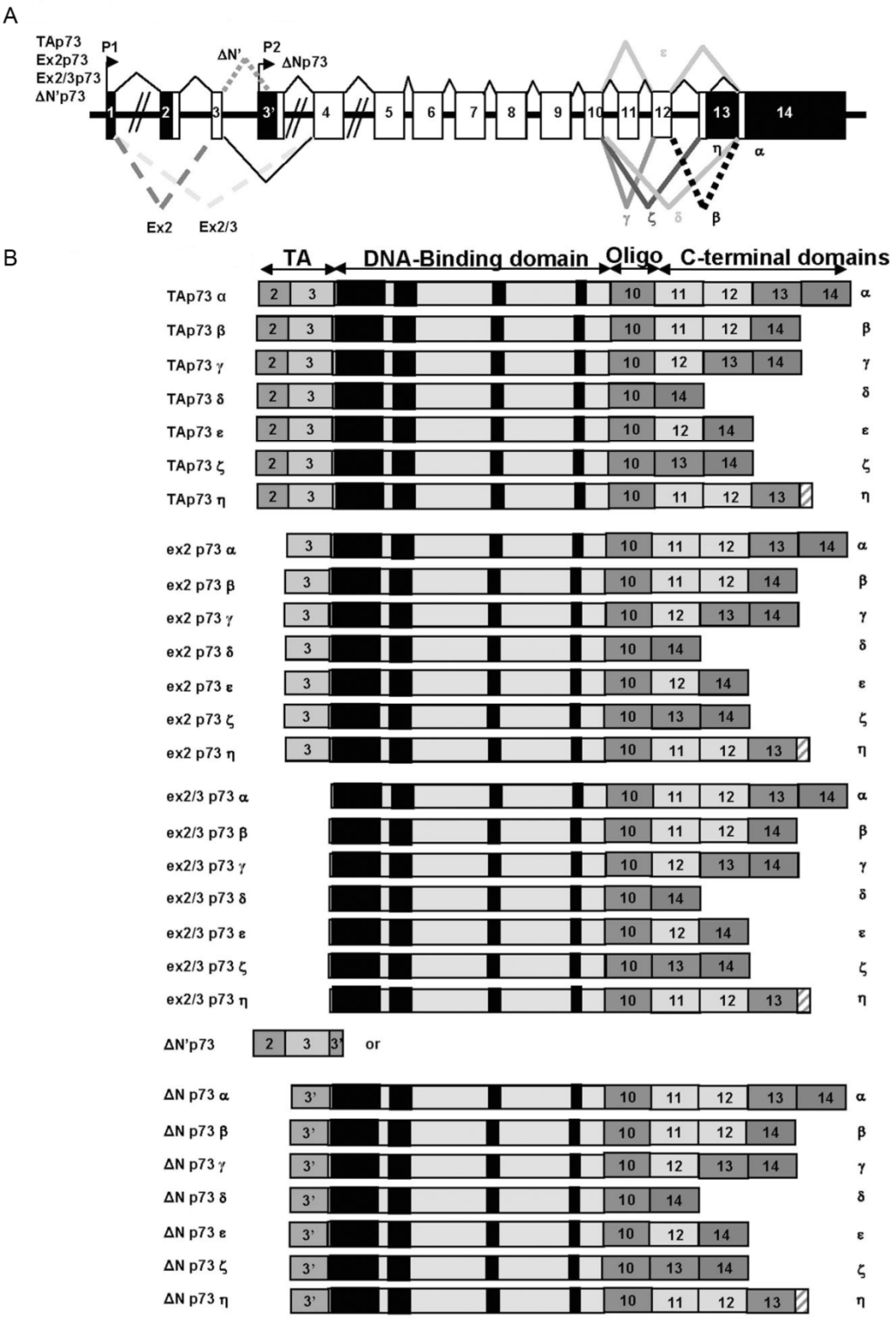


Figure 11: Schematic drawing of human p73 protein isoforms.

(A) Structure of the human p73 gene consisting of 14 exons. Due to usage of an alternative promoter located in intron 3 and alternative splicing at the N- and C-terminus 29 different p73 isoforms can be generated. (B) The 29 p73 protein isoforms are illustrated. Numbers indicate the exons present in the corresponding isoforms. For a detailed explanation of the characteristics of the different isoforms and how they are generated see text. Modified from (Bourdon, 2007).

1.2.3.2 Regulation of p73

In accordance with p53 (see chapter 1.2.1.2), the level of p73 in a cell is strictly regulated (Oberst et al., 2005). Under normal conditions p73 is degraded ubiquitin-independently by the 20S proteasome (Asher et al., 2005), by calpains (Munarriz et al., 2005) or by the 26S proteasome (Rossi et al., 2005). As p63 (see chapter 1.2.2.2), p73 is also ubiquitinated by Itch and thereby targeted for degradation by 26S proteasomes (Rossi et al., 2005). After cellular stress like DNA damage, there is a rapid accumulation of p73 but the way how p73 becomes activated differs from that of p53 (Melino et al., 2002). It was demonstrated that after genotoxic stress as DNA-damage or oncogenes or oxidative stress, p73 becomes transcriptionally activated by p53 and p73 also up-regulates its own expression (Chen et al., 2001; Wang et al., 2007a). This leads to the induction of cell cycle arrest or apoptosis. The induction of apoptosis by p73 is mediated by transactivation of pro-apoptotic p53-target genes like *NoxA*, *Puma* and *Bax* or the death receptor *CD95*. However, p73 is able to induce some of the p53 target genes, like *Mdm2*, *p21* or *Bax*, but not all (Zhu et al., 1998a). It also induces genes that are not target genes of p53 (Harms et al., 2004). Furthermore it was demonstrated that p73 is able to induce apoptosis transcription-independently, as p53 (see chapter 1.2.1.2). p73 is cleaved by caspases and localizes to mitochondria where it induces release of cytochrome *c* (Sayan et al., 2008). Furthermore p73 is able to induce genes that play a role in DNA repair (Lin et al., 2009).

As it is the case for p53 (see chapter 1.2.1.2), p73 is regulated by interaction with other proteins and by post-translational modifications, such as phosphorylation, ubiquitination or acetylation, as well (Pietsch et al., 2008). The tyrosine kinase c-Abl phosphorylates and stabilizes p73 after DNA damage thereby enhancing the transactivating and apoptotic potential of p73 (Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999). Furthermore, the activity of p73 is also regulated by transcriptional co-activators like p300 and CBP. It was shown *in vitro* and *in vivo* that p300/CBP bind to and increase the transcriptional activity of p73 (Zeng et al., 2000). In addition p73 becomes acetylated by p300 after DNA damage in a c-Abl-dependent manner. Due to acetylation the ability of p73 to induce the transcription of pro-apoptotic target genes is increased (Costanzo et al., 2002; Pietsch et al., 2008; Zeng et al., 2000). Moreover, the peptidyl-prolyl cis/trans-isomerase Pin1 interacts with p73 in a c-Abl dependent manner and stimulates the acetylation of p73 by p300. In general, these events lead to the stabilization and an increased apoptotic activity of p73 (Mantovani et al., 2004). It was also demonstrated *in vitro* and *in vivo* that ASPP1 and ASPP2 bind to p73, as to p53 and p63, and enhance also the apoptotic potential of p73 (Bergamaschi et al., 2004). Moreover, it was shown that some p53 mutants are able to bind to and inactivate the transcriptional activity of several p73 isoforms (Strano et al., 2000).

Furthermore, p73 is not activated by a variety of DNA damaging agents like p53 but only by a subset of agents. Actinomycin D or UV irradiation do not affect p73 for example (Kaghad et al., 1997). Additionally, the reaction of p73 to different types of DNA-damaging agents varies and accumulation and phosphorylation of p73 occurs only after specific types of cellular stress. For example, treatment of cells with cisplatin, which is a cytostatic drug, results in an increased half-life of p73 but p73 is not tyrosine-phosphorylated (Gong et al., 1999). Whereas ionizing irradiation does not lead to p73 stabilization (Levrero et al., 2000). However, ionizing irradiation induces activation of c-Abl by ATM kinase, which leads to the phosphorylation of p73 by c-Abl. Therefore it depends both on the cell type and on the DNA-damaging agent if c-Abl phosphorylates or stabilizes p73 (Davis and Dowdy, 2001; Levrero et al., 2000; Yuan et al., 1999). Phosphorylation and/or accumulation of p73 is crucial for induction of apoptosis by p73 after DNA-damage (Levrero et al., 2000). Moreover, Costanzo et al. showed that after treatment with doxorubicin p73 becomes stabilized by acetylation.

In addition, ΔNp73 is degraded via proteasomes, while TAp73 is up-regulated after DNA damage in order to permit apoptosis and cell cycle arrest (Maisse et al., 2004; Oberst et al., 2005). TAp73 induces the ubiquitin ligase p73-induced Ring finger protein 2 (PIR2), which binds to, ubiquitinates and degrades ΔNp73 (Sayan et al., 2010).

Additionally, feedback-loops could be identified that regulate the activity of p73 and several other proteins. p53 and TAp73 are capable of binding to the second promoter of ΔNp73 in intron 3 and induce its transcription. ΔNp73 on the other hand impairs the function of TAp73 and p53, also on its own promoter, via binding the proteins (TAp73) or competing for promoter sites (p53) thereby creating a negative feedback loop (Grob et al., 2001; Kartasheva et al., 2002; Nakagawa et al., 2002; Stiewe et al., 2002). p73 is also able to induce the expression of *p21* (Zhu et al., 1998a) and *Mmd2*. Mdm2 is activated by p73 and in turn inhibits the function of p73 but does not affect its stability (Zeng et al., 1999). In contrast to p53 (see chapter 1.2.1.2), Mdm2 binds to p73 but does not target it for degradation by proteasomes (Rossi et al., 2005; Zeng et al., 1999). It was also shown that p73 binds to MdmX (Ongkeko et al., 1999). Mdm2 and MdmX compete with p73 for binding to p300/CBP thereby inhibiting its function (Melino et al., 2002; Zeng et al., 1999; Zeng et al., 2000). However, Ongkeko et al. pointed out that induction of *p21* and growth suppression functions and induction of apoptosis by p73 were increased in presence of Mdm2. Furthermore they also showed that binding of Mdm2 and MdmX to p73 enhances the half-life of p73 (Ongkeko et al., 1999). Another feedback-loop has been identified between p73 and TRIM32. TAp73 induces the expression of TRIM32 and TRIM32 interacts with TAp73 and promotes its ubiquitination and degradation. ΔNp73 inhibits the expression of TRIM32 (Gonzalez-Cano et al., 2013).

1.2.3.3 p73 in zebrafish (*Danio rerio*)

Zebrafish p73 is 70 to 95% identical to p73 of other vertebrates (Pan et al., 2003). It seems to be essential for the first 24 hours during zebrafish development (Davidson et al., 2008). In contrast to other observations, where p73 expression was already observed at the one-cell stage (Pan et al., 2003), the expression of p73 could not be detected until midgastrulation onwards by others. Contrary to p53 (see chapter 1.2.1.3) and according to ΔNp63 (see chapter 1.2.2.3), p73 is not expressed during early gastrulation (Rentzsch et al., 2003). In contrast to p53 and p63, which are ubiquitously expressed, the expression of p73 is more restricted. Compared to mice and in accordance with human embryos, TAp73 isoforms are mainly expressed during the development of zebrafish. Their expression is very restricted during development and can be detected in specific neurons in the telencephalon, dorsal diencephalon, olfactory epithelium, and hypothalamus, in differentiating slow muscle cells of the somites, pharyngeal arches, endodermal pouches and in the pronephric ducts (Danilova et al., 2008b; Pan et al., 2003; Rentzsch et al., 2003; Satoh et al., 2004). In the adult zebrafish p73 is expressed in the brain (telencephalon, hypothalamus, olfactory bulbs and optic tectum), skin, kidney, gill, eye and muscles (Pan et al., 2003; Satoh et al., 2004). Additionally, expression of p73 could be observed in the ovary, testis and fin (Pan et al., 2003). Although the expression of *p73* in the adult zebrafish was investigated by RT-PCR and rough whole-mount ISH, a detailed characterization of *p73* expression was not provided by these studies. Down-regulation of TAp73 leads to developmental defects of the telencephalon, pharyngeal arches and olfactory system. Thus TAp73 is required for a proper craniofacial and neuronal development in zebrafish similar to mammals. Whereas, over-expression of TAp73α does not increase apoptosis in zebrafish embryos, contrary to p53. Therefore, in contrast to mammals, TAp73 seems not to induce the same pro-apoptotic target genes in zebrafish embryos as p53 (Rentzsch et al., 2003).

It is not clear if there is a ΔNp73 isoform expressed in zebrafish, which is similar to the ΔNp73 isoforms in mammals (Rentzsch et al., 2003). However, Satoh et al. were able to detect ΔNp73 at least in a subset of zebrafish tissues. Furthermore, in zebrafish the p73θ isoform could be identified, which has not been detected in mammals. This isoform lacks the SAM domain but contains additional 55 amino acids at the C-terminus. p73θ exhibits a strong transcriptional activity and over-expression leads to developmental defects (Satoh et al., 2004).

1.2.3.4 p73 in the mammalian nervous system

p73 is expressed in the embryonic and adult vomeronasal organ, the olfactory epithelium and in the preplate layer of the telencephalic ventricle. As p63 (see chapter 1.2.2.4), p73 is expressed in Cajal-Retzius neurons in the hippocampus and in the marginal zone of the developing cerebral cortex (Hernandez-Acosta et al., 2011; Yang et al., 2000). p73-deficient mice show a defective neuronal development caused by loss of Cajal-Retzius cells (Hernandez-Acosta et al., 2011). In postnatal mice, p73 is expressed in cells of the SVZ and p73 seems to be necessary for the proliferation in the SVZ. During development the expression of p73 in the brain is restricted but in the adult brain p73 is almost ubiquitously expressed. The expression of p73 can be observed in pyramidal neurons of the adult human cortex and hippocampus for example (Hernandez-Acosta et al., 2011).

The different isoforms of the p73 protein possess distinct functions in the brain and both TAp73 and ΔNp73 isoforms are crucial for a normal functional brain. Both TAp73 and ΔNp73 isoforms are present in neurons in the adult human cerebral cortex and hippocampus. ΔNp73 is mainly located in the nucleus but it is also present in the cytoplasm whereas TAp73 is predominantly expressed in the cytoplasm in cortical neurons. The same holds true for cortical neurons of mice (Cabrera-Socorro et al., 2006). ΔNp73 isoforms are the predominant isoforms present in Cajal-Retzius cells (Hernandez-Acosta et al., 2011) and in the fetal human brain ΔNp73α shows a high expression in these cells (Meyer et al., 2002). In the developing mouse brain ΔNp73 is predominantly expressed and functions antagonistically to p53 and TAp63 thereby preventing neuronal death. Removal of NGF induces p53-mediated neuronal cell death (see chapter 1.2.1.4) and a decrease of ΔNp73 expression. Over-expression of ΔNp73 protects sympathetic neurons from cell death. Therefore ΔNp73 plays a crucial pro-survival role in sympathetic neurons during development (Jacobs et al., 2005; Jacobs et al., 2006; Jacobs et al., 2004; Pozniak et al., 2000). Tissir et al. demonstrated *in vivo* that ΔNp73 is essential for neuronal survival. In postnatal and adult cortical neurons p73 is crucial for their survival as well but in the survival of embryonic cortical neurons it is not involved (Pozniak et al., 2002; Pozniak et al., 2000). Furthermore, ΔNp73 does not control the survival of precursor cells in the cortex (Dugani et al., 2009). Therefore, ΔNp73 is essential for the survival of neurons in both the central (cortical neurons) and the peripheral (sympathetic neurons) nervous system and it is crucial both for the survival of sympathetic neurons during developmental neuronal death and for the long-term maintenance of adult neurons (Pozniak et al., 2002). Adult neurons are relatively invulnerable after treatment with DNA damaging agents or after injury. It was hypothesized that this is achieved by increasing the ratio of ΔNp73 to p53 (Jacobs et al., 2004; Walsh et al., 2004). Thus, if a neuron lives or

dies depends on the relative levels of pro-survival (ΔNp63, ΔNp73) and pro-apoptotic (p53, TAp63, TAp73) proteins (Jacobs et al., 2004).
Furthermore, TAp73 is a crucial regulator of neural stem cell maintenance and self-renewal in embryos as well as in adults (Agostini et al., 2010; Fujitani et al., 2010; Talos et al., 2010) and as already mentioned above (see chapter 1.2.3), TAp73-deficient mice exhibit hippocampal dysgenesis. Neural stem cells express a much higher level of TAp73 compared to ΔNp73 (Talos et al., 2010). It was shown *in vitro* and *in vivo* that TAp73 is crucial for self-renewal and long-term maintenance of adult neural precursors in the dentate gyrus of the hippocampus and the SVZ via induction of bHLH *Hey2* (Sakamoto et al., 2003) and other target genes that control asymmetric cell division and proliferation. Hey2 inhibits premature differentiation thereby promoting the maintenance of neural precursors (Fujitani et al., 2010; Talos et al., 2010). Accordingly, it was demonstrated *in vitro* that TAp73 positively regulates self-renewal of embryonic neural stem cells and that loss of p73 depletes their number due to premature neuronal differentiation (Agostini et al., 2010; Gonzalez-Cano et al., 2010). In addition, it was reported by Talos et al. that p73 promotes proliferation and self-renewal and prevents premature senescence of neural stem and progenitor cells. Thus p73 is crucial for maintaining a stem cell pool both in adult and in embryonic neurogenesis (Agostini et al., 2010; Talos et al., 2010).
Moreover, TAp73 is able to induce neuronal differentiation *in vitro* in a neuroblastoma cell line (De Laurenzi et al., 2000). As p53 (see chapter 1.2.1.4), p73 plays a role in the differentiation of OPCs (Billon et al., 2004). Accordingly, it was shown that the expression of TAp73 increases and is also required for differentiation of precursor cells into oligodendrocytes or neurons (Agostini et al., 2010; Talos et al., 2010).
As for p53 (see chapter 1.2.1.5), neurodegenerative diseases as Alzheimer´s disease have been linked to p73 (Cancino et al., 2013a; Wetzel et al., 2008). In hippocampal pyramidal Alzheimer´s disease neurons increased p73 levels show a nuclear localization whereas in a healthy brain p73 is located in the cytoplasm (Wilson et al., 2004). However, it was also observed that in some cases of Alzheimer´s disease the expression of p73 is decreased (Li et al., 2004). Additionally, p73 is involved in responses to cortical ischemia (Bui et al., 2009).

In summary, the transcription factor p73 is crucial for normal development, also of the brain. It is involved in survival of embryonic and adult neurons of the central and peripheral nervous system. Furthermore, p73 is essential for self-renewal and maintenance of neural stem cells both in embryos and in adult mice. Additionally, p73 plays a role in the differentiation of precursor cells.

1.3 Aims of the project

Both p53 and p73 play crucial roles in mammalian adult neurogenesis. p53 suppresses the self-renewal of adult neural stem cells and is involved in apoptotic death of neurons. p73 is relevant for the survival of neurons, self-renewal and maintenance of neural stem cells and differentiation of precursor cells. Given the high constitutive and regenerative neurogenic capacity of the zebrafish, a crucial question is whether the two genes play similar roles during these processes in the adult zebrafish brain. This may give insights into the underlying mechanisms and how they compare to the mammalian system. Such comparative approaches are key to extrapolate the knowledge of mechanisms underlying regeneration in the zebrafish to a potential application in humans for development of therapeutic approaches.

One objective of my work was to characterize the overall expression of the two transcription factors p53 and p73 in the adult zebrafish brain and to assess whether the expression correlates with known neurogenic niches. Moreover by focusing on the adult zebrafish telencephalon I asked first whether the expression can be mapped to specific cell types such as neurons, OPCs and the different types of stem and progenitor cells in the telencephalon and whether the expression pattern is changed in response to injury. Finally, I assessed whether the neurogenic properties of the healthy and injured brain is changed in p53 mutants. The results are discussed in relation to the known functions of p53 and p73 in the mammalian systems.

Chapter 2

Material and Methods

2.1 Consumables and instruments

Borosilicate glass capillary tubes	Warner instruments
Cell culture flasks	Greiner
Compound microscope	Leica DM5000
Confocal microscope	Leica TCS2 SP5
Developing machine	Kodak
Dissecting microscope	Nikon
ECL films	GE Healthcare Europe
Electroporator	Bio-Rad GenePulser II
ELISA reader	Biotek instruments
Gel dryer	BioRad
Incubator cell culture	Heraeus
Incubator fish	Binder
Microinjector	Eppendorf Femtojet express
Micropipette puller	Sutter Instrument Company
PCR machine	Perkin Elmer
PVDF Transfer membrane Imobilon P	Millipore
Spectrometer	Thermo Scientific NanoDrop
Stereo microscope	Leica stereo MZ16F
Vibratome	Leica VT1000S

2.2 Kits and reagents

DIG-RNA labeling mix	Roche
Gel extraction kit	Peqlab
Maxiprep kit	Qiagen

2.3 Antibodies

Primary antibodies:

Anti-p53 zebrafish	rabbit, AnaSpec (1:200)
Anti-GFP	chicken, Aves Labs (1:1000)
Anti-HuC/D	mouse, Molecular probes (1:300)
Anti-BrdU	mouse, DAKO (1:400)
Anti-PCNA	mouse, DAKO (1:500)
Anti-PSA-NCAM	mouse, Chemicon (1:500)
Anti-S100β	rabbit, DAKO (1:400)
AP-anti-DIG	Roche (1:4000)
Anti-DIG-poly-POD	Roche (1:1000)

Secondary antibodies:

Anti-chicken Alexa Fluor 488	Invitrogen (1:500)
Anti-mouse Alexa Fluor 488	Invitrogen (1:500)
Anti-rat Alexa Fluor 488	Invitrogen (1:500)
Anti-mouse Alexa Fluor 680	Invitrogen (1:500)
Anti-mouse Alexa Fluor 594 IgM	Invitrogen (1:500)
Anti-rabbit Alexa Fluor 546	Invitrogen (1:500)
Anti-rabbit-HRP	DAKO (1:1000)
Anti-rat-HRP	DAKO (1:1000)
Anti-mouse-HRP	DAKO (1:1000)

Tyramide Cy3 solution	Perkin Elmer (1:50)
Tyramide Cy5 solution	Perkin Elmer (1:100)
DRAQ5	Biostatus (1:500)

2.4 Buffers, media, solutions, chemicals

Acrylamide solution	Roth
APS	Sigma-Aldrich
BCIP	Roche
Blocking Buffer	1x PBS, 0.1% (v/v) Tween 20, 0.2% (w/v) BSA, 1% (v/v) DMSO

Blotting Buffer	25 mM Tris, 192 mM Glycin, 10% MeOH
BT-Fix	4% (w/v) PFA, 4% (w/v) sucrose, 0.12 mM $CaCl_2$, 0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4
Coomassie staining solution 250 ml	150 mg Coomassie Brilliant Blue R-250, 100 ml MeOH, 25 ml glacial acetic acid, 125 ml H_2O
Coomassie discolouration solution 500 ml	200 ml MeOH, 250 ml H_20, 50 ml glacial acetic acid
DMEM	Invitrogen
Dyalisis tubes	G-Biosciences
Fetal calf serum (FCS)	PAA
Freund´s Adjuvant Complete	Sigma-Aldrich
Freund´s Adjuvant Incomplete	Sigma-Aldrich
Glycerol	Roth
GST-elution buffer	1 M Tris pH 8.0, L-Glutathion reduced
HBS	280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.05
HYB buffer	50% (v/v) formamide, 5x SSC, 500 µg/ml yeast RNA, 50 µg/ml Heparin, 0.1% (v/v) Tween 20 9 mM Citric acid
IPTG	Peqlab
LB medium	10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl
Low-fat milk powder	Saliter
NBT	Roche
PBS	Invitrogen
Penicillin/ Streptomycin	Invitrogen
Proteinase K	Sigma-Aldrich
PTW	1x PBS, 0.1% (v/v) Tween 20
Running Buffer SDS-PAGE	25 mM Tris, 192 mM Glycin, 0.1% SDS
20x SSC	0.3 M sodium citrate, 3 M NaCl, pH 7,0

Staining buffer	100 mM Tris pH 9.5, 50 mM $MgCl_2$ 100 mM NaCl, 0.1% (v/v) Tween 20
TAE 50x	2 M Tris, 1 M glacial acetic acid, 0.05 M EDTA
TEMED	Roth
Trypsin-EDTA 0.25%	Invitrogen
Wash buffer 1 ISH	50% (v/v) formamide, 1x SSC, 0.05% (v/v) Tween 20
Wash buffer 2 ISH	2x SSC, 0.1% (v/v) Tween 20
Wash buffer 3 ISH	0.2x SSC, 0.1% (v/v) Tween 20
Wash buffer 4 ISH	0.1x SSC, 0.05% (v/v) Tween 20, 50% (v/v) PTW

2.5 Molecular biological and microbiological methods

2.5.1 Polymerase chain reaction (PCR)

The PCR was performed according to the protocols of the manufacturers of the reaction mixes. *Pfu*-polymerase (Fermentas) was used when the correct sequence of the amplicon was required. The PCR-programm was as follows: a single denaturation step for 5 min at 95℃, followed by 27 cycles of 95℃ for 30 sec, ann ealing temperature according to the melting temperature of the primers for 30 sec and 72℃ for an extension time according to the length of the amplicon, followed by a final extension step at 72℃ for 10 min.
Standard PCR was performed with GoTaq DNA polymerase (Promega). The PCR-programm was as follows: a single denaturation step for 2 min at 95℃, followed by 35 cycles of 95℃ for 30 sec, annealing temperature according to the melting temperature of the primers for 30 sec and 72℃ for an extension time according to the length of the amplicon, followed by a final extension step at 72℃ for 15 min.
Primers (Metabion) used for the cloning of the C-terminus of *zfp53* and *zfp73* in the pGEX-4T-2 vector for the production of the polyclonal antibodies:
BamHI-zfp53_neu_for: 5´-ccgcgtggatccccacaagagaccaaaaccatggcgaaaaccaccact-3´
NotI-zfp53-Reverse: 5´-cgatgcggccgctcgttaatcagagtcgcttcttcc-3´
BamHI-zfp73_neu_for: 5´-ccgcgtggatccccacagagtgtgtaccaccttcagactcttact-3´

NotI-zfp73-rev2: 5´-cgatgcggccgctcgtcagtgaacgtcgccttc-3´
As templates plasmids containing wildtype *zfp53* and *zfp73*, respectively, were used. The last 342 base pairs (bp) of *zfp53* and the last 363 bp of *zfp73* were amplified.
Primers used for the cloning for *in situ* probes:
BamHI-zfp53-Forward: 5´-ccgcgtggatccccaatggcgcaaaacgacagccaagag-3´
NotI-zfp53-Reverse: 5´- cgatgcggccgctcgttaatcagagtcgcttcttcc-3´
As template cDNA from zebrafish embryos was used.
Primers used for the cloning of *p53* in the pCS2:GFP vector:
EcoRI-p53-for: 5`-ttcgaattcaagatggcgcaaaacgacagc-3´
XhoI-p53-rev: 5´-ggctcgagaggcatcagagtcgcttcttcc-3´
As template a pcDNA3 vector with *myc-zfp53* was used.

2.5.2 Agarose gel electrophoresis and gel extraction

DNA molecules were separated by size via agarose gel electrophoresis. The concentration of agarose varied depending on the size of the DNA fragments. For small DNA fragments (<500 bp) a 2% (w/v) and for larger fragments (>500 bp) a 1 or 1.5% (w/v) agarose gel was used. The agarose was melted in an appropriate amount of 1x TAE buffer. After cooling down to 60℃ 0.1 µg/ml ethidium bromide (Roth) was added. The samples were supplemented with 6x DNA loading dye (Fermentas) and the gel was run in 1x TAE buffer at 10 V/cm. GeneRuler DNA ladder mix (Fermentas) was used as a size marker. For extracting the gels the PeqGOLD gel extraction kit was used according to the manufacturer´s instructions.

2.5.3 Restriction digest

The restriction digest of DNA was performed by using FastDigest restriction enzymes (Fermentas) or HF restriction enzymes (NEB) as recommended by the manufacturers. The appropriate amount of DNA (PCR product or plasmid) was mixed with 1x buffer and 1 µl of the corresponding enzymes in a total volume of 40 µl. The digestion was carried out at 37℃ for up to 1h.

2.5.4 Ligation

For the ligation of DNA into linearized plasmids 100 ng of the linearized vector was mixed with the appropriate amount of insert (1:4 molar ratio vector:insert), 10x ligase buffer and 1µl of T4 DNA ligase (Fermentas) in a total volume of 20 µl. The mixture was incubated at room temperature for 2 to 3 hours. Afterwards it was directly used for transformation of competent bacteria.

2.5.5 Transformation of competent bacteria

100 µl of frozen competent *Escherichia coli* DH5α, BL21 or XL1-Blue were thawed on ice for several minutes. After adding the corresponding DNA the mixture was incubated on ice for 20 min. Subsequently the bacteria were heatshocked for 30 sec at 42℃ and incubated afterwards for 10 min on ice again. After adding 500 µl of 1x LB medium (without antibiotics) the transformation mixture was shaken at 37℃ for 30 min. 20-200 µl of the bacteria were plated on selective plates containing LB medium with 50 µg/ml ampicillin (or other antibiotics as required by the plasmids). The plates were incubated overnight at 37℃.

2.5.6 Small-scale (Miniprep) and large-scale (Maxiprep) DNA preparation

In order to identify positive clones containing the plasmid of interest 2, 5 ml of 1x LB medium with the appropriate antibiotics were inoculated with single colonies picked from the selective LB plates. The cultures were shaken overnight at 37℃. On the next day the bacterial culture was centrifuged at 13000 rpm for 30 sec at room temperature. The bacterial pellet was resuspended in 250 µl of P1 buffer (Buffers P1-3 are from the Maxiprep kit from Qiagen) by vortexing. Afterwards 250 µl of P2 buffer were added and the samples were inverted 7 times to lyse the bacteria. Adding of P3 buffer and inverting the tubes 7 times stopped the lysis. After centrifugation at room temperature at 13000 rpm for 25 min the supernatant containing the DNA was transferred into new vials and 800 µl of isopropanol (1 volume) was added to precipitate the DNA. The samples were mixed before incubating them at -80℃ for 30 min. Afterwards they were centrifuged for 25 min at 4℃ at 13000 rpm. The supernatant was removed and the pellet was washed by adding 500 µl 75% (v/v) EtOH and centrifugation for 5 min at 4℃ at 13000 rpm. The DNA pellet was dried at room temperature for about 15 min and then resuspended into 50 µl of water.

The Maxipreps were performed as described in the manufacturer´s protocol (Qiagen).

2.5.7 Sequencing

The sequencing was performed by Qiagen Sequencing services and Microsynth AG.

2.6 Generation of the GST-zfp53-C-terminus and GST-zfp73-C-terminus proteins

2.6.1 Protein expression in bacteria and purification of GST proteins

After transformation of competent BL21 bacteria with a pGEX-4T-2 vector (GE Healthcare) containing the C-terminus of *zfp53* and *zfp73*, respectively, single colonies were used to inoculate 100 ml of 1x LB medium with ampicillin (100 µg/ml). The cultures were shaken over night at 37℃ and at the next day the cultures were diluted to 1 l and shaken again for 1.5 h at 37℃. By adding 1ml of 1 M Isopropylthiogalactopyranosid (IPTG) and shaking for additional 6 h at 37℃ the expression of zfp53-C-terminus and zfp73-C-terminus proteins fused to glutathione-S-transferase (GST) was induced. Afterwards the bacterial cultures were centrifuged for 10 min at 6000 rpm and 4℃ and the pellets were resuspended in 25 ml 1x PBS. These suspensions were sonicated on ice 2 times for 30 sec. After adding 250 µl of PMSF (Sigma-Aldrich) and Triton X-100 (Sigma-Aldrich) the samples were shaken for 30 min at 4℃. Then the samples were centrifuged for 30 min at 9000 rpm and 4℃. The supernatants were incubated overnight at 4℃ with 200 µl Glutathione Sepharose (GE Healthcare), which was washed with PBS before added to the samples. On the next day the samples were put on columns for protein purification (BioRad). After washing 2 times with PBS the GST-zfp53-C-terminus and GST-zfp73-C-terminus proteins were eluted from the Glutathione Sepharose with different amounts of GST-elution buffer.

2.6.2 Dialysis of the GST-zfp53-C-terminus and GST-zfp73-C-terminus proteins

To further purify the GST-zfp53-C-terminus and GST-zfp73-C-terminus proteins and to remove the glutathione from the protein solutions, the solutions were dialysed against PBS overnight at 4℃. On the next day the PBS was chang ed and the protein solutions were dialysed again for several hours.

2.6.3 SDS-PAGE

10% acrylamide gels were prepared (separating gel: 4 ml H_2O, 3.3 ml Acrylamide, 2.5 ml 1.5 M Tris pH 8.8, 100 µl 10% SDS, 100 µl 10% APS, 4µl TEMED; stacking gel: 3.4 ml H_2O, 830 µl Acrylamide, 630 µl 1 M Tris pH 6.8, 50 µl 10% SDS, 50 µl 10% APS, 5 µl TEMED). The protein samples were supplemented with 2x loading buffer (160 mM Tris pH 6.8, 4% SDS, 20% Glycerin, 4% β-mercaptoethanol, 0.02% bromphenolblue) and incubated at 95℃ for 5 min. The protein samples were loaded onto the acrylamide gel. As a standard to determine the concentration of the GST-zfp53-C-terminus and GST-zfp73-C-terminus proteins different volumes of a BSA solution (1µg/µl) were also loaded onto the gel. For the cell lysates 30 µg protein was used, 500 ng of GST protein and 30 µl telencephalon lysate were loaded onto the gels. PageRuler Prestained protein ladder (Fermentas) was used as a size marker. The gels were run in running buffer at 150 V.

2.6.4 Staining, discolouration and drying of polyacrylamide gels

Polyacrylamide gels were stained overnight in a Coomassie-staining solution. On the next day the gels were discoloured in a Coomassie-discolouration solution until the protein bands were clearly visible. The discolouration solution was changed several times. Afterwards the gels were dried for 2 h at 80℃ in a gel dryer.

2.6.5 Estimation of the protein concentration

To estimate the concentration of the GST-zfp53-C-terminus and GST-zfp73-C-terminus proteins the BSA standard was used. Thereby the bands of the produced proteins were compared to the bands of the BSA standard. This is an example how the concentration of the proteins was estimated:
30 µl of GST-zfp53-C-terminus or GST-zfp73-C-terminus protein were loaded onto the gel and correlate to 1 µl of BSA with a concentration of 1µg/µl. Therefore in 30 µl protein solution 1 µg protein is contained and thus the protein concentration is 0,033 µg/µl.
Both the GST-zfp53-C-terminus and the GST-zfp73-C-terminus protein have a size of about 39 kDa (13 kDa of zfp53-C-terminus or zfp73-C-terminus protein plus 26 kDa GST tag).

2.7 Generation of polyclonal antibodies

2.7.1 Immunization of rats

For the generation of the polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus proteins 4 female BD10 rats were injected intraperitoneal 6 times (twice a week) with 15 µg (ca. 300 µl solution) of GST-zfp53-C-terminus and GST-zfp73-C-terminus protein (see chapter 2.6), respectively. The injections were performed according to the following scheme:

Injection 1	15 µg protein plus Freund´s Adjuvant Complete
Injection 2	15 µg protein plus Freund´s Adjuvant Incomplete
Injection 3	15 µg protein in PBS
Injection 4	15 µg protein in PBS
Injection 5	15 µg protein in PBS
Injection 6	15 µg protein in PBS

The Freund´s Adjuvant Complete and Freund´s Adjuvant Incomplete were used as described in the manufacturer´s protocol.

2.7.2 Extraction of the polyclonal antibodies

After finishing the immunization process the rats were sacrificed by an overdose of CO_2. Afterwards the blood of the rats was gained by heart puncture. The amount of blood of the distinct rats varied between 4 and 8 ml. The blood was incubated at room temperature for 30 min and then at 4°C for 6 h. To obtain the serum, which contained the polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus proteins, the blood was centrifuged at room temperature for 10 min at 3500 g.

2.7.3 Affinity purification of the polyclonal antibodies

For the affinity purification pieces of a nitrocellulose membrane (Piercenet) with a size of 9 cm^2 were wetted with PBS and 100 µg of GST-zfp53-C-terminus or GST-zfp73-C-terminus protein were added to the membranes. The membranes were incubated at 4°C for 5 h. Afterwards 5 ml 100 mM Glycin/HCl pH 2.5 were added for 5 min. Then the membranes were washed twice with 5 ml TBS for 2 min. After that the membranes were blocked with 2 ml 5% (w/v) low-fat milk for 1 h at 4°C. The membranes were washed 3 times with PBS before 200 µl polyclonal antibodies in 2 ml 5% milk were added. The membranes plus the antibodies were incubated overnight at 4°C to let the antibodies bind to the membrane. On the next day the membranes were washed 5 times for 5 min with PBS. Then the antibodies were eluted with 500 µl elution buffer (0.2 M Glycin pH 2.5, 150 mM NaCl, 0.1% (w/v) BSA) and 125 µl Tris HCl pH 7.9 were added to the polyclonal antibodies. Afterwards the specificity of the antibodies was tested.

2.8 Cell culture methods

2.8.1 Cell line

The H1299 cell line (ATCC no. HTB-96TM) used in all experiments is a p53-negative adherent human non-small cell lung carcinoma cell line derived from a lymph node.

2.8.2 Cell culture maintenance

H1299 cells were maintained in Dulbeco´s modified eagle medium (DMEM) plus 10% FCS and 1% Penicillin/Streptomycin. They were passaged twice a week by trypsinization at a ration of 1:8 to 1:10 and were cultivated in 175 cm^2 cell culture flasks at 37℃ in a 5% CO_2 humidified incubator.

2.8.3 Transient transfections

Transient transfections with *myc-zfp53* (in pcDNA3 vector, Invitrogen) were performed with calcium-phosphate precipitation. H1299 cells were passaged by trypsinization at a ratio of 1:8 to 1:10 and incubated in petridishes for 4 to 6 h at 37℃. For immunohistochemistry small round coverslips were put into the petridishes. Afterwards the transfection mix was added to the cells: 0.125 M $CaCl_2$, 5 µg DNA, 1x HBS in a volume of 1ml. The cells were incubated overnight at 37℃. The next day, a glycerol shock was performed by removing the maintenance medium from the cells and adding 5 ml of 15% (v/v) Glycerol for 3 min. After washing with PBS the cells were incubated in maintenance medium at 37℃ until they were harvested 6 h later.
For transfection of cells with *myc-zfp73α* (in pcDNA3 vector) Effectene (Qiagen) was used according to the manufacturer´s protocol

2.8.4 Cell lysis

Attached cells were removed from the bottom of the petridishes by a scraper and transferred into vials. After centrifugation at 13000 rpm at 4°C for 1 min the cell pellet was washed with

PBS. Then the cells were lysed in 100 µl NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA pH 8.0, 1 mM PMSF) for 20 min on ice. Afterwards the samples were centrifuged for 20 min at 13000 rpm and 4°C. The supernatant containing the proteins was transferred into a new vial.

To determine the protein concentration of the cell lysates a Bradford assay was performed. For the standard curve 1 ml Bradford solution (100 mg Coomassie Brilliant Blue, 50 ml EtOH, 100 ml 85% phosphoric acid in 1l) was mixed with 2 µl lysis buffer and 0, 2, 4, 6, 8 or 10 µl BSA (1 µg/µl). 2 µl of the cell lysates were added to 1 ml Bradford solution. 150 µl of these samples were transferred into a 96 well plate and measured at the ELISA reader at 595 nm.

The protein concentration was calculated as follows:

Protein concentration = x * extinction of the protein

x = extinction (BSA-standard) / concentration (BSA-standard)

2.9 Western blot

For the western blot the PVDF membranes were activated in methanol before setting up the blot device. The device was set up in a way that the proteins from the polyacrylamide gel migrate onto the membrane. The western blot was performed overnight in Blotting buffer at 30 Volt.

On the next day the membranes were blocked for 30 min in 5% milk (5% (w/v) milk powder in PBS plus 0.2% (v/v) Tween). For the commercially available anti-p53 antibody the membranes were blocked in milk without Tween. Afterwards the membranes were incubated with the primary antibodies. The primary antibodies were diluted in 5% milk as follows: polyclonal antibodies against GST-zfp53-C-terminus or GST-zfp73-C-terminus 1:1000, anti-myc antibody (supernatant of 9E10 hybridoma cells) 1:50, commercially available anti-p53 antibody 1:500. The primary antibodies were incubated at room temperature for 1.5 h or overnight at 4°C. Then the membranes were washed 3 to 5 times for 10 min in PBS plus 0.2% (v/v) Tween (PTW) before incubating them for 1.5 h at room temperature with the secondary antibodies diluted 1:1000 in 5% milk (polyclonal antibodies: anti-rat-HRP, myc antibody: anti-mouse-HRP). For the commercially available anti-p53 antibody an anti-rabbit IRDye800 secondary antibody was used diluted 1: 5000 in 5% milk. This antibody was incubated for 1.5 h at room temperature in the dark. Afterwards the membranes were washed 5 times for 10 min in PTW and 2 times for 10 min in PBS.

The membranes incubated with the polyclonal or the myc antibodies were incubated with ECL solution 1 (100 mM Tris-HCl pH 8.5, 400 µM cumaric acid 2.5 M Luminol) and 2 (100

mM Tris-HCl 8.5, 0.02% H_2O_2) for 2 min. Afterwards the membranes were exposed to an ECL-film. The exposure time depended on the intensity of the signals. The films were developed in a developing machine.
The membranes incubated with the commercially available anti-p53 antibody were scanned at the Odyssey system.

2.10 Immunohistochemistry on cells

For the immunohistochemistry on cells, cells cultured on cover slips were used (see chapter 2.8.3). The coverslips were transferred into a 24-well plate and washed with PBS. The cells were fixed on ice for 8 min with 800 µl Aceton/Methanol (1:1) and afterwards the cells were dried at room temperature. Then the cells were washed 3 times with PBS and treated with PBS with 0.5% (v/v) Triton X-100 for 10 min. Before the cells were blocked in blocking buffer (1% BSA and 1% goat serum in PBS) for 30 min they were washed 3 times in PBS. Afterwards the cells were washed with PBS and the primary antibodies were added to the cells diluted in blocking buffer (polyclonal antibodies 1: 200 and anti-myc antibody 1:50). The antibodies were incubated for 1.5 h. Then the cells were washed 3 times with PBS before incubated with the secondary antibodies diluted in blocking buffer (Anti-rat Alexa Fluor 488 and Anti-mouse Alexa Fluor 488 1:1000). To label the nuclei the nuclear marker DRAQ5 was also added diluted 1:1000. The cells were incubated for 1.5 h in the dark. Afterwards the cells were washed 3 times with PBS, mounted with Hydromount (National diagnostics) on glas slides and analysed at the confocal microscope.

2.11 Histological methods on adult zebrafish brains

2.11.1 Fish strains used

Experiments were performed on adult 6-12 month old wildtype (AB), transgenic *Tg(olig2:EGFP)* (Shin et al., 2003), *Tg(gfap:GFP)* (Bernardos and Raymond, 2006) and *Tg(-3.9nestin:GFP)* (Lam et al., 2009) or *tp53*^{M214K} mutant (Berghmans et al., 2005) zebrafish (*Danio rerio*). The fish were maintained on a 14 h/ 10 h light-dark cycle at 28.5℃ in

recirculation systems (Schwarz Ltd Germany, Müller and Pfleger Ltd Germany) and fed commercial food and in-house hatched brine shrimp as described (Westerfield, 2007).

2.11.2 Dissection and fixation of brains

Fish were anesthetized with 0.02% (w/v) Tricaine (Ethyl 3-aminobenzoate methanesulfonate salt, Sigma) before being killed in ice water (Westerfield, 2007). Brains were carefully removed and fixed in 4% paraformaldehyde over night at 4℃. They were then step wise dehydrated in a methanol/ PBS concentration series and stored at -20℃ (Adolf et al., 2006).

2.11.3 *In situ* hybridization (ISH)

Digoxigenin labelled anti-sense riboprobes were used from a repertoire described in (Armant et al., 2013). The Digoxigenin labelled p53 anti-sense riboprobe was generated according to (Armant et al., 2013; Lam et al., 2009). Digoxigenin labelled sense riboprobes were generated with suitable restriction enzymes and RNA polymerases according to (Armant et al., 2013; Lam et al., 2009). Briefly, the *in situ* templates were cloned by performing a PCR with the GoTaq polymerase kit (see chapter 2.5.1). The PCR products were subcloned into the pGEM-T Easy vector (p53 and p73, Promega) or modified pBluescript II SK(-)vector (p21 and Mdm2, Invitrogen, Addgene) as described by the manufacturer. The templates for the synthesis of DIG-labelled anti-sense and sense riboprobes were linearized plasmids. The plasmids were linearized with the following restriction enzymes: p53 anti-sense SalI (T7), p53 sense SacII (Sp6), p73 anti-sense ApaI (New England Biolabs) (Sp6), p73 sense SacI (T7), Mdm2 and p21 anti-sense XhoI (T3). The linearized plasmids were purified by phenol/chloroform extraction and precipitated with isopropanol. For the synthesis of Digoxigenin labelled anti-sense and sense riboprobes the following mixture was prepared: 1 µl linearized template, 0.75 µl RNAse inhibitor (Fermentas), 0.75 µl corresponding RNA polymerase (Fermentas), 1 µl DIG labeling mix (Roche) and 1x transcription buffer (Fermentas) in a total volume of 10 µl. The mixture was incubated at least 3 h at 37℃. The transcribed RNA was precipitated by adding sodium acetate and ethanol and incubation at -80℃ for 30 min and centrifugation afterwards at 4° C for 25 min. The Digoxigenin labelled riboprobes were stored in HYB buffer at -20℃.
ISH on adult brains was performed according to (Adolf et al., 2006). Brains stored in 100% MeOH were step wise rehydrated in a decreasing methanol/PBS concentration series and

washed afterwards 4 times for 5 min in PTW. Then the brains were treated for 30 min with ProtK PTW buffer (10 mg/ml Proteinase K in PTW) and fixed again for 20 min in BT-Fix. Afterwards the brains were washed 5 times for 5 min in PTW before they were rinsed in HYB buffer. Subsequently the brains were prehybridized in HYB buffer at 67℃ for at least 3 h. Then the brains were hybridized overnight at 67℃ with the Digoxigenin labelled anti-sense and sense riboprobes in HYB buffer. The following day the brains were washed at 67℃ 2 times for 30 min with wash buffer 1, 15 min with wash buffer 2, 2 times for 30 min with wash buffer 3 and 5 min with wash buffer 4. Afterwards the brains were washed for 5 min at room temperature in blocking buffer before embedding the brains in 2% (w/v) agarose in PBS (Schmidt et al., 2014). Using a vibratome 50 µm sections were cut, collected in blocking buffer and blocked for at least 1 h at room temperature (Schmidt et al., 2014). The brain sections were incubated overnight at 4℃ with Anti- DIG-AP diluted 1:4000 in blocking buffer. On the next day sections were washed 5 times for 15 min with PTW and 2 times with staining buffer. Afterwards the sections were stained in the dark with NBT/ BCIP staining solution. To stop the staining, sections were washed at least 3 times with PTW. The experiments with sense probes were stopped at the same time as the experiments with the antisense probes. The sections were imaged on a compound microscope.

For fluorescent *in situ* hybridization (FISH) the following modifications of the ISH protocol were performed. Signal amplification was performed by using tyramide amplification (TSA plus Cyanine 5 System, Perkin Elmer). After rehydration and washing of the brains, they were incubated in 3% (v/v) H_2O_2 in PBS for 35 min for quenching of endogeneous peroxidase. The 50 µm sections were incubated overnight with Anti-DIG-poly-POD antibody (Roche) diluted 1:1000 in blocking buffer at 4℃. The next day, after washing with PTW the brain sections were washed 2 times for 10 min in PTW plus 0.002% (v/v) H_2O_2. Afterwards they were stained in the dark with tyramide Cy5 solution 1:100 in PTW plus 0.002% (v/v) H_2O_2 for 2 h. Then the sections were washed 3 times for 5 min in PTW. Afterwards the sections were processed for immunohistochemistry using the anti-p53 antibody and tyramide Cy3.

All fluorescent pictures were acquired using a laser scanning confocal microscope (Leica TCS2 SP5) and processed using Leica software. Pictures were adjusted for brightnes and contrast and panels were created in Adobe Photoshop CS4.

All brains were oriented as dorsal up, ventral down. In brains with stab wounds, the lesioned telencephalic hemisphere was always located to the left.

2.11.4 Immunohistochemistry

Immunostainings were performed on free-floating vibratome sections as described in (Adolf et al., 2006). Brains stored in 100% MeOH were step wise rehydrated in a decreasing methanol/PBS concentration series and washed afterwards 4 times for 5 min in PTW. Afterwards the brains were embedded in 2% (w/v) agarose in PBS. Sections were cut with a vibratome to a thickness of 50 µm (Schmidt et al., 2014). The sections were blocked in blocking buffer for at least 2 h at room temperature. The self-made polyclonal antibodies were incubated overnight at 4℃ diluted 1:10 in blo cking buffer and the rabbit anti-S100β antibody was incubated overnight at 4℃ diluted 1:4 00 in blocking buffer. Afterwards the sections were washed 3 times for 10 min with PTW before they were incubated in the dark for 2 h with the secondary antibody from the Alexafluor series (anti-rat Alexa 488 and anti-rabbit Alexa 546 1:500). Then the sections were washed 3 times for 10 min in PTW and mounted on glass slides in Aqua Polymount (Polyscience).

For the commercially available rabbit anti-p53 antibody a different protocol was used. After rehydration, washing and cutting sections with a vibratome the sections were collected in PBS. Then an antigen retrieval was performed by incubating the sections for 30 min at 80℃ in sodium citrate pH 6, 0. Afterwards the sections were washed in PBS for 5 min at room temperature before they were incubated for 30 min in PBS plus 3% (v/v) H_2O_2 for quenching of endogeneous peroxidase. Then the brain sections were washed 2 times for 5 min in PBS plus 0.2% (v/v) Tween20 and blocked in 5% (w/v) milk in PBS plus 0.2% (v/v) Tween20 for at least 45 min. The primary rabbit anti-p53 antibody was incubated overnight at room temperature diluted 1: 200 in 5% (w/v) milk in PBS plus 0.2% (v/v) Tween20. The next day, sections were washed 2 times for 10 min with PBS plus 0.2% (v/v) Tween20 before they were incubated with the secondary antibody (anti-rabbit-HRP 1:1000 in 5% (w/v) milk in PBS plus 0.2% (v/v) Tween20). Afterwards the sections were washed 2 times for 10 min with PBS plus 0.2% (v/v) Tween20 and then they were incubated for 7 min in the dark with tyramide Cy3 solution (1:50 in 1x plus amplification diluent from the TSA plus Cyanine 3 System kit, Perkin Elmer). Then the sections were washed 3 times for 5 min with PBS plus 0.2% (v/v) Tween20. For co-staining with PSA-NCAM Cy5 instead of Cy3 was used.

Co-stainings were performed after finishing the staining for p53. Therefore, after removing the Cy3 solution and washing, the sections were again incubated for 2 h at room temperature in blocking buffer. The primary antibodies were incubated overnight at 4℃. Primary antibodies were: chicken anti-GFP (1:1000), mouse anti-HuC/D (1:300), mouse anti-BrdU (1:400), mouse anti-PCNA (1:500), mouse anti-PSA-NCAM (IgM, 1:500). Before incubation with the anti-BrdU antibody the sections were treated for 30 min with 2 N HCl, washed afterwards 2 times for 10 min in 0.1 M Na-tetraborate buffer pH 8.5 and then washed 3 times

for 5 min in PTW. The next day, sections were washed 3 times for 10 min in PTW and were then incubated with the secondary antibodies from the Alexafluor series (Alexa 488, Alexa 594 IgM, Alexa 680 1:500). Nuclear staining was performed with DRAQ5 (1:500 in PBS, Biostatus). DRAQ5 solution was incubated for 10 min and then the sections were washed 3 times for 10 min with PTW. The sections were mounted on glass slides in Aqua Polymount.

2.11.5 BrdU administration

Intraperitoneal injection of BrdU (5-Bromo-2`deoxyuridine) into *Tg(olig2:EGFP)* adult zebrafish (Shin et al., 2003) was performed as described in (Chapouton et al., 2006) cumulatively for 3 days and followed by a chase of 2, 3 and 4 weeks, respectively. For the injections fish were anesthetized with 0.02% (w/v) Tricaine and the BrdU solution was mixed with methylene blue for injection control. At the corresponding time points fish were sacrificed and the brains were processed as described in chapter 2.11.2.

2.11.6 Stab wounds

Fish were anesthetized with 0.02% (w/v) Tricaine and placed afterwards into a slit in a block of tricaine-soaked foam. Under a dissecting microscope with light from the top a syringe needle (30 G) was inserted vertically through the scull into the medial region of the right telencephalic hemisphere. The contralateral unlesioned left hemisphere served as the control. Afterwards fish were transferred to a tank with fresh fish water and were removed at different time points after lesioning (Schmidt et al., 2014).

2.11.7 TUNEL staining

For detection of apoptosis via TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) the ApopTag Red in situ apoptosis detection kit (Chemicon) was used according to the manufacturer´s instructions. Briefly, brains were step wise rehydrated in a decreasing methanol/PBS concentration series and washed afterwards 4 times for 5 min in PTW. Then the brains were treated for 30 min with ProtK PTW buffer (10 mg/ml Proteinase K in PTW) and fixed again for 20 min in BT-Fix. Afterwards the brains were washed 4 times for 5 min in PTW before embedding the brains in 2% (w/v) agarose in PBS. Using a vibratome 50 µm

sections were cut. Then the brain sections were washed for 2 min in equilibration buffer and incubated for 1 h in working strength TdT enzyme at 37℃. Afterwards the sections were treated with working strength stop/wash buffer for 10 min at room temperature. Then the sections were washed in PBS 3 times for 1 min and incubated for 30 min in the dark with working strength Anti-digoxigenin conjugate. Afterwards the sections were washed 4 times for 5 min with PBS and mounted on glass slides in Aqua Polymount.

2.12 Lysis of adult zebrafish telencephala

Adult wildtype fish were anesthetized with 0.02% (w/v) Tricaine before being killed in ice water. Brains were carefully removed and the telencephala were transferred in lysis buffer (PBS plus 0.1% (v/v) Triton-X 100, 1 mM DTT, 1 mM PMSF, 1x protease inhibitor mix (Roche)) on ice. The telencephala were homogenized and sonicated. Afterwards the solution was centrifuged at 13000 rpm for 10 min at 4℃ and the supernatant was transferred to a new vial.

2.13 Microinjection

Injection mixes plus 0.1% (w/v) phenol red as an injection control were prepared. Injection needles were obtained from borosilicate glass capillary tubes by pulling the heated capillaries on a micropipette puller. Wildtype zebrafish eggs were transferred directly after spawning into petridishes with fishwater. The injection mixes were loaded into the needles and the tip of the needles was opened. The fishwater was completely removed from the eggs and approximately 4 nl of the injection mix was injected into one cell stage zebrafish eggs with a microinjector as described by (Muller et al., 1999). After injection zebrafish water plus methylene blue (1mg/ml) as a fungizide was added to the injected embryos. Unfertilized or dead eggs were removed around the sphere stage and the embryos were kept at 28℃ until the experiments were performed.

20 ng/µl of *p53-GFP* RNA was injected. The RNA was produced from the pCS2 vector with p53-GFP by using a mMessage mMachine Sp6 Transcription kit (Ambion) according to the manufacturer´s protocol. At 8 hpf GFP-positive embryos were selected and fixed at 4℃ over

night in BT-Fix. The next day the embryos were dechorionated manually with forceps and stored in methanol at -20°C.

2.14 Immunohistochemistry of embryos

The embryos were step wise rehydrated in a decreasing methanol/PBS concentration series and washed afterwards 4 times for 15 min in PTW. Then the embryos were rinsed in water and the water was immediately removed. Pre-cooled aceton was added and incubated for 7 min at -20°C. The aceton was removed and the embryo s were rinsed in water. The water was immediately removed and the embryos were washed 5 times for 20 min in PTW. Then an antigen retrieval was performed by incubating the embryos for 30 min at 80°C in sodium citrate pH 6.0. Afterwards the embryos were washed in PBS for 5 min at room temperature before they were incubated for 30 min in PBS plus 3% (v/v) H_2O_2 for quenching of endogeneous peroxidase. Then the embryos were washed 2 times for 5 min in PBS plus 0.2% (v/v) Tween20 and blocked in 5% (w/v) milk in PBS plus 0.2% (v/v) Tween20 for at least 45 min. The primary antibodies were incubated overnight at room temperature (rabbit anti-p53 1:200, chicken anti-GFP 1:1000 diluted in 5% (w/v) milk in PBS plus 0.2% (v/v) Tween20). The next day, embryos were washed 2 times for 10 min with PBS plus 0.2% (v/v) Tween20 before they were incubated with the secondary antibody (anti-rabbit-HRP 1:1000, Alexa 488: 1:500 in 5% (w/v) milk in PBS plus 0.2% (v/v) Tween20). Afterwards the embryos were washed 2 times for 10 min with PBS plus 0.2% (v/v) Tween20 and then they were incubated for 7 min in the dark with tyramide Cy3 solution (1:50 in 1x plus amplification diluent from the TSA plus Cyanine 3 System kit, Perkin Elmer). Then the embryos were washed 3 times for 5 min with PBS plus 0.2% (v/v) Tween20. The embryos were embedded in 0.5% (w/v) agarose for imaging them at the confocal microscope.

2.15 BAC recombineering

The strategy for modifying a BAC clone for transgenesis is adapted from (Lam et al., 2009; Shin et al., 2003). The first exon of the *p53* coding region in the BAC clone CHORI CH211-92I1 and the first exon of the *p73* coding region in the BAC clone CHORI CH211-69A12 were replaced by EGFP. Furthermore, the *p53* and *p73* coding regions were fused to GFP. 500 bp

of the 5´ and 3´ flanking regions of the first exon and the C-terminus of p53 and p73, respectively, were amplified by PCR from the BAC and cloned into pPCR-EGFP.

The following primers and enzymes were used for cloning:

Replacing the first exon of *p53*:

5´arm forward: 5´-cgcgtcgacttgtgtgttgtattattgtaacaaac-3´

5´arm reverse: 5´-gcgaagctttgctgtgaaattataaacacgaaagtc-3´

Enzymes: SalI and HindIII

3´arm forward: 5´-cgcccgcgggtaagttcgcaagggtcgcactcctgatac-3´

3´arm reverse: 5´-gcggagctcgcctaaatccatgatcgccgggatagtcgc-3´

Enzymes: SacII and SacI

Fusion construct *p53*:

5´arm forward: 5´-cgcgtcgactaccttcagtatttgagatgacctaagaag-3´

5´arm reverse: 5´-gcgaagcttgatcagagtcgcttcttccttcgtcctt-3´

Enzymes: SalI and HindIII

3´arm forward: 5´-cgcccgcggttatggattggatgtctaaatatgagc-3´

3´arm reverse: 5´-gcggagctcgaacaaataatatgggagtcgttgggataa-3´

Enzymes: SacII and SacI

Replacing the first exon of *p73*:

5´arm forward: 5´-cgcgtcgaccatcatatactgtgatgaaaacaagcc-3´

5´arm reverse: 5´-gcgaagcttcttggatgttggacaatccaccgc-3´

Enzymes: SalI and HindIII

3´arm forward: 5´-cgcccgcgggtgagttcattcacactcattaaatac-3´

3´arm reverse: 5´-gcggagctccattatcgtagtgaaaaatgaattgttgcg-3´

Enzymes: SacII and SacI

Fusion construct *p73*:

5´arm forward: 5´-cgcgtcgacaatagtgaagtccactaaaggaggttgagc-3´

5´arm reverse: 5´-gcgaagcttggtgaacgtcgccttcggcaaactc-3´

Enzymes: SalI and HindIII

3´arm forward: 5´-cgcgcggccgctttgtgcaattgtgtatgatattg-3´

3´arm reverse: 5´-gcggagctcgtagactactttacggattttgcctattgc-3´

Enzymes: NotI and SacI

The BAC clones were electroporated into temperature sensitive EL250 cells. The targeting cassettes were cut out of the pPCR vector with SacI and KpnI. Then the targeting cassettes were electroporated into the EL250 cells containing the BAC clones followed by a heat-shock at 42℃ for 15 min to induce the production of reco mbination proteins. After positive selection clones that were kanamycin-resistant were further verified by PCR for successful recombination. The following primers were used:

Replacing the first exon of *p53*:
Upstream forward primer: 5´-gcagatttaaatcgtctacgcgacctg-3´
Fusion construct *p53*:
Upstream forward primer: 5´-gcacatttacagctttgtgcgtacgcaatg-3´
Replacing the first exon of *p73*:
Upstream forward primer: 5´-caactgaatcgattcacaggtgtgaaagc-3´
Fusion construct *p73*:
Upstream forward primer: 5´-atacgtagaacttagcaggctacatcactg
EGFP reverse primer: 5´-ccgtccagctcgaccag-3´
Afterwards the kanamycin cassette was removed by induction with 1% arabinose. To allow transposon-mediated BAC transgenesis the Tol2 cassette was amplified from the iTol2 plasmid (Suster et al., 2011) using the following primers:
Bac-tol2 forward: 5´- gcgtaagcggggcacatttcattacctctttctccgcacccgacatagatccctgctcgagccgggc ccaagtg-3`
Bac-tol2 reverse: 5´-cgcggggcatgactattggcgcgccggatcgatccttaattaagtctactaattatgatcctctagatc agatct-3´
The Tol2 cassette was electroporated as described for the targeting cassette. Integration of the Tol2 cassette was checked by PCR. The following primers were used:
Outside loxP forward: 5´-tggccaatatggacaacttct-3´
Inside Amp reverse: 5´-cacccaactgatcttcagca-3´
For stable integration into the genome 30 ng/µl Tol2-BAC DNA and 30 ng/µl transposase mRNA were co-injected into one-cell stage zebrafish embryos. Fish were raised and outcrossed to identify stable integration of the transgene.

2.16 Homozygous *tp53*^{M214K} mutant zebrafish

To obtain homozygous *tp53*^{M214K} mutant zebrafish (Berghmans et al., 2005), heterozygous fish were bred and raised. About 2 months later the raised fish were genotyped by fin clipping to identify the homozygous ones. Briefly, fish were anesthetized with 0.02% (w/v) Tricaine and a small piece of the tail fin was cut off and put into tail buffer (80 mM Tris-HCl pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 100 µg/ml Proteinase K). The fin clips were incubated over night at 63℃. The next day, isoprop anol was added to precipitate genomic DNA and the samples were centrifuged at 13000 rpm for 10 min. The pellet was washed with 70% (v/v) ethanol and centrifuged at 13000 rpm for 10 min. Then the pellet was dried and resuspended in water. A PCR was carried out to reveal the genotypes of the fish.

Primers used for genotyping from (Berghmans et al., 2005):

p53 genotyping forward: 5´-gatagcctagtgcgagcacactctt-3´

p53 genotyping mutant reverse: 5´-agctgcatgggggggaa-3´

p53 genotyping wildtype reverse: 5´-agctgcatgggggggat-3´

The homozygous fish were further bred and raised.

Chapter 3

Results

3.1 Characterization of the expression of p53 and p73 in the adult zebrafish brain

My overall aim was to investigate the role of p53 and p73 in the adult zebrafish brain since it was shown that both p53 and p73 play important roles in adult neurogenesis in the mammalian brain. p53 suppresses the self-renewal of adult neural stem cells (Meletis et al., 2006) and is involved in neuronal death following damage (Culmsee and Mattson, 2005; Miller et al., 2000; Morrison and Kinoshita, 2000). Furthermore, p73 is essential both for the survival of neurons (Pozniak et al., 2002) and for the self-renewal and maintenance of neural stem cells (Agostini et al., 2010; Talos et al., 2010) in mammals.
There are differences between the mammalian and the zebrafish brain regarding the capacity of adult neurogenesis and the regeneration of damaged brain areas (Adolf et al., 2006; Chapouton et al., 2007; Grandel et al., 2006; Kaslin et al., 2008; Zupanc, 2001; Zupanc and Zupanc, 2006). Therefore, I intended to examine the expression of p53 and p73 in the adult zebrafish brain and to detect potential differences to the mammalian brain.

To examine the role of p53 and p73 in the brain of the adult zebrafish I first characterized in which regions of the brain p53 and p73 mRNAs are expressed by performing *in situ* hybridizations (ISH). Additionally, I analyzed the expression of the p53 protein in the adult zebrafish brain by immunohistochemistry.

3.1.1 Analysis of *p53* mRNA expression in the adult zebrafish brain

To investigate the expression of *p53* mRNA in the adult zebrafish brain, an ISH on cross sections through the whole brain with a *p53* antisense probe was performed. As a control a sense probe for *p53* was used. As shown in Figure 12 the *p53* mRNA is broadly expressed in many parts of the adult zebrafish brain.

In the telencephalon *p53* mRNA expression was observed in the periventricular zone of the telencephalic ventricle (TelV) (Figure 12 A-C) as well as in the central (Vc) (Figure 12 B), dorsal (Vd) (Figure 12 A-B), ventral (Vv) (Figure 12 B) and postcommissural (Vp) (Figure 12 C) nuclei of the ventral telencephalic area (V). Furthermore, *p53* mRNA was expressed in the medial (Dm) (Figure 12 B-C), lateral (Dl) (Figure 12 B-C), dorsal (Dd) (Figure 12 C) and posterior (Dp) (Figure 12 B-C) zone of the dorsal telencephalic area (D) and in the sulcus ypsiloniformis (SY) (Figure 12 B-C).

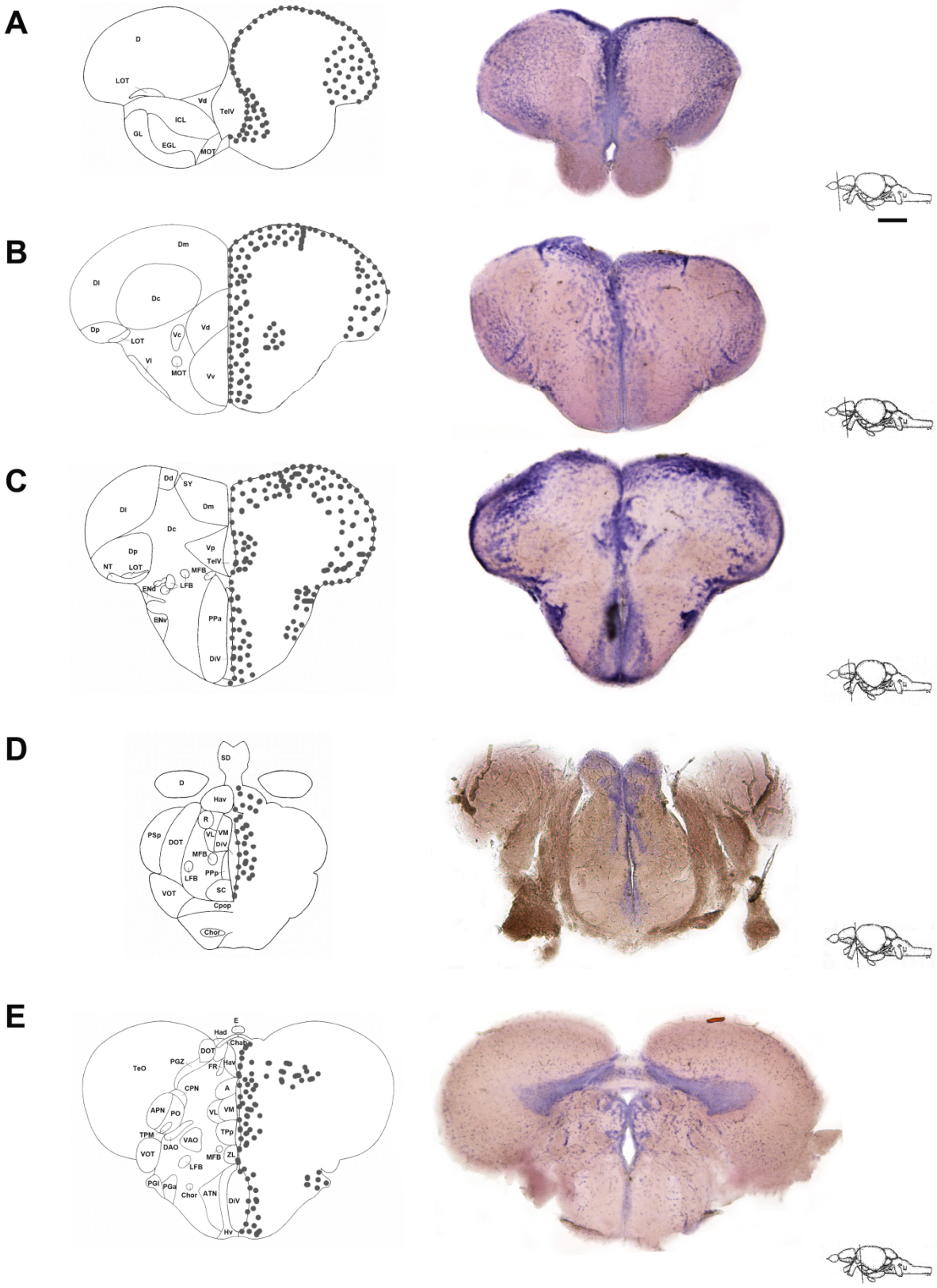
The diencephalon showed an expression of *p53* mRNA in the periventricular zone of the diencephalic ventricle (DiV) (Figure 12 C-F) and in the anterior (PPa) (Figure 12 C) and posterior (PPp) (Figure 12 D) part of the parvocellular preoptic nucleus. *p53* mRNA was also expressed in the dorsal (ENd) (Figure 12 C) and ventral (ENv) (Figure 12 C) part of the entopeduncular nucleus, in the anterior (PGa) (Figure 12 E) and lateral (PGl) (Figure 12 E) preglomerular nucleus and in the ventromedial (VM) (Figure 12 D-E) and anterior (A) (Figure 12 E) thalamic nucleus. Additionally, *p53* expression was detectable in the caudal (Hc) (Figure 12 G), dorsal (Hd) (Figure 12 F-G) and ventral (Hv) (Figure 12 E-F) zone of the periventricular hypothalamus and in the corpus mamillare (CM) (Figure 12 F-G). Moreover, *p53* mRNA could be detected in the ventral habenular nucleus (Hav) (Figure 12 D-E) and in the periventricular nucleus of the posterior tuberculum (TPp) (Figure 12 E).

In the mesencephalon an expression of *p53* mRNA could be observed in the periventricular zone of the tectal ventricle (TeV) (Figure 12 F-G), in the periventricular grey zone of the optic tectum (PGZ) (Figure 12 E-G) and in the torus longitudinalis (TL) (Figure 12 F-G).

In the hindbrain *p53* mRNA was expressed in the periventricular zone of the rhombencephalic ventricle (RV) (Figure 12 H), in the lateral division of the valvula cerebelli (Val) (Figure 12 F-G), in the corpus cerebelli (CCe) (Figure 12 H), in the crista cerebellaris (CC) (Figure 12 H) and in the eminentia granularis (EG) (Figure 12 H).

Figure 13 shows an ISH with a *p53* sense probe as a negative control. As there is no staining visible in the brain regions that showed an obvious staining with the *p53* antisense probe, the staining for *p53* is specific and *p53* mRNA is expressed in the regions described above.

p53 antisense



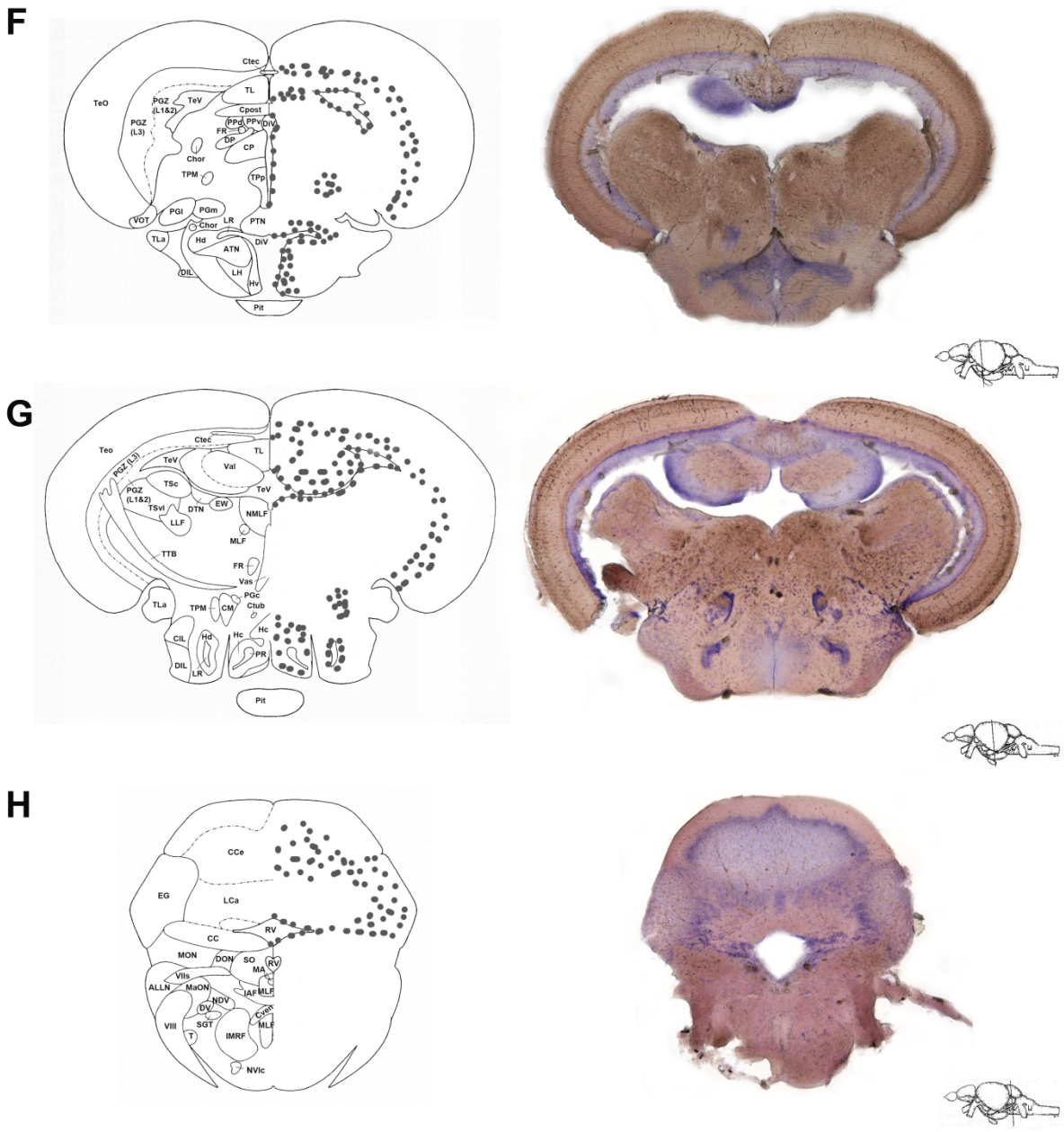


Figure 12: *p53* mRNA is expressed in many regions of the adult zebrafish brain.

(A-H) *In situ* hybridization with a *p53* antisense probe on cross sections through the whole adult zebrafish brain to investigate the expression of *p53* mRNA. The schematics on the left side (modified from Wullimann et al., 1996) show the anatomical subdomains of the examined cross sections. Grey dots indicate areas of *p53* mRNA expression. The small inserts on the right side indicate the anterior-posterior levels of the sections. The *p53* mRNA is broadly expressed in many parts of the adult zebrafish brain.

Expression of *p53* mRNA in (A) Vd, D, periventricular zone of the TelV; (B) Vv, Vd, Vc, Dm, Dl, Dp, SY, periventricular zone of the TelV; (C) PPa, Vp, SY, Dm, Dd, Dl, Dp, ENd, ENv, periventricular zone of the TelV and DiV; (D) PPp, VM, Hav, periventricular zone of the DiV; (E) PGZ, Hv, TPp, VM, A, PGl, PGa, periventricular zone of the DiV; (F) PGZ, TL, Val, Hv, Hd, CM, periventricular zone of the DiV

and TeV; (G) PGZ, TL, Val, Hd, Hc, CM, periventricular zone of the TeV; (H) CCe, EG, CC, periventricular zone of the RV.

Abbrevations: A: anterior thalamic nucleus; CC: crista cerebellaris; CCe: corpus cerebelli; CM: corpus mamillare; D: dorsal telencephalic area; Dd: dorsal zone of D; DiV: diencephalic ventricle; Dl: lateral zone of D; Dm: medial zone of D; Dp: posterior zone of D; EG: eminentia granularis; ENd: entopeduncular nucleus, dorsal part; ENv: entopeduncular nucleus, ventral part; Hav: ventral habenular nucleus; Hc: caudal zone of periventricular hypothalamus; Hd: dorsal zone of periventricular hypothalamus; Hv: ventral zone of periventricular hypothalamus; PGa: anterior preglomerular nucleus; PGl: lateral preglomerular nucleus; PGZ: periventricular grey zone of optic tectum; PPa: parvocellular preoptic nucleus, anterior part; PPp: parvocellular preoptic nucleus, posterior part; RV: rhombencephalic ventricle; SY: sulcus ypsiloniformis; TeV: tectal ventricle; TelV: telencephalic ventricle; TL: torus longitudinalis; TPp: periventricular nucleus of posterior tuberculum; V: ventral telencephalic area; Val: lateral division of valvula cerebelli; Vc: central nucleus of V; Vd: dorsal nucleus of V; VM: ventromedial thalamic nucleus; Vp: postcommisural nucleus of V; Vv: ventral nucleus of V. Scale bar: 100 µm.

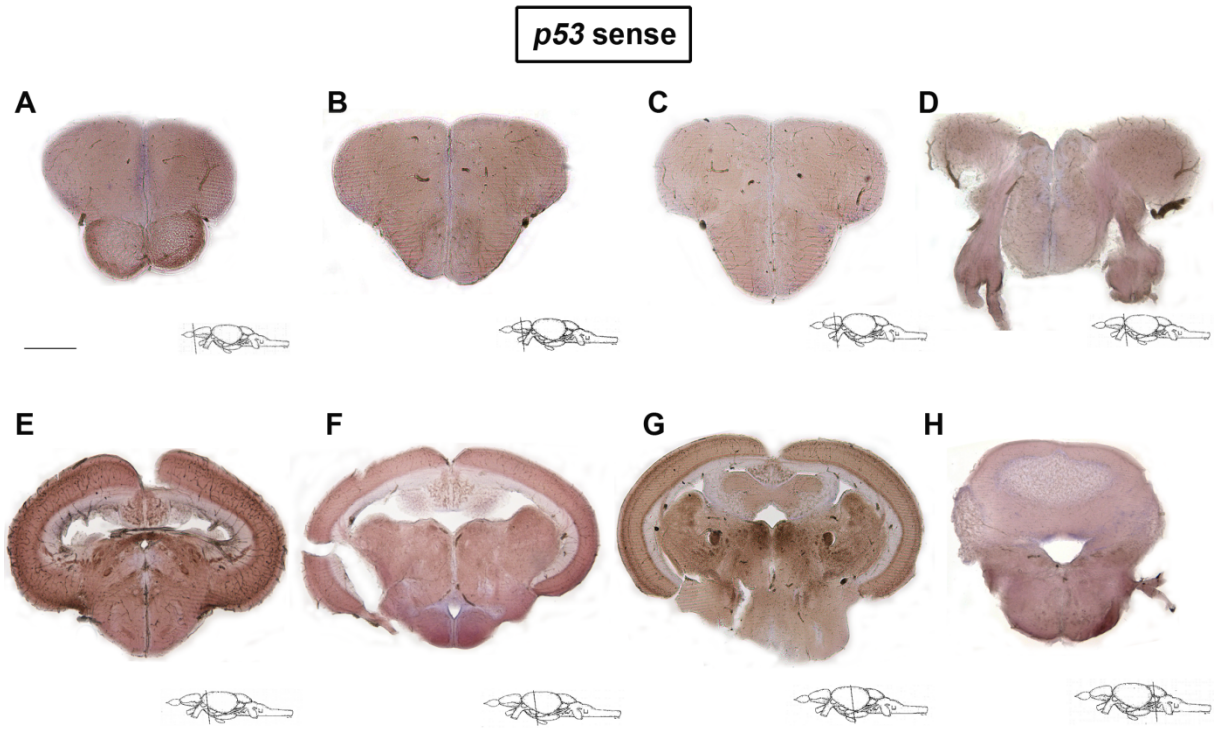


Figure 13: No staining with the *p53* sense probe.

(A-H) *In situ* hybridization with a *p53* sense probe on cross sections through the whole adult zebrafish brain as a negative control. The small inserts indicate the anterior-posterior levels of the sections. No staining could be observed with the *p53* sense probe in all sections examined. Scale bar: 100 µm

3.1.2 Analysis of *p73* mRNA expression in the adult zebrafish brain

In order to analyze the expression of *p73* mRNA in the adult zebrafish brain an ISH on cross sections through the whole brain with a *p73* antisense probe was performed. A sense probe for *p73* was used as a control. The *p73* mRNA is broadly expressed in many parts of the adult zebrafish brain (Figure 14).

p73 mRNA expression could be observed in the telencephalon in the periventricular zone of the telencephalic ventricle (TelV) (Figure 14 A-C) as well as in the central (Vc) (Figure 14 B), dorsal (Vd) (Figure 14 A-B), ventral (Vv) (Figure 14 B) and postcommissural (Vp) (Figure 14 C) nuclei of the ventral telencephalic area (V). Furthermore *p73* mRNA was expressed in the medial (Dm) (Figure 14 B-C), lateral (Dl) (Figure 14 B-C), dorsal (Dd) (Figure 14 C) and posterior (Dp) (Figure 14 B-C) zone of the dorsal telencephalic area (D) and in the sulcus ypsiloniformis (SY) (Figure 14 B).

In the diencephalon *p73* mRNA was expressed in the periventricular zone of the diencephalic ventricle (DiV) (Figure 14 C-F) and in the anterior (PPa) (Figure 14 C) and posterior (PPp) (Figure 14 D) part of the parvocellular preoptic nucleus. *p73* mRNA could also be detected in the dorsal (ENd) (Figure 14 C) and ventral (ENv) (Figure 14 C) part of the entopeduncular nucleus, in the torus lateralis (TLa) (Figure 14 F-G) and in the ventromedial (VM) (Figure 14 D-E) and anterior (A) (Figure 14 E) thalamic nucleus. Moreover, *p73* was expressed in the caudal (Hc) (Figure 14 G), dorsal (Hd) (Figure 14 F-G) and ventral (Hv) (Figure 14 E-F) zone of the periventricular hypothalamus and in the corpus mamillare (CM) (Figure 14 F-G). Furthermore, *p73* mRNA was detected in the ventral habenular nucleus (Hav) (Figure 14 D) and in the periventricular nucleus of the posterior tuberculum (TPp) (Figure 14 E).

In the mesencephalon an expression of *p73* mRNA was observed in the periventricular zone of the tectal ventricle (TeV) (Figure 14 F-G), in the periventricular grey zone of the optic tectum (PGZ) (Figure 14 E-G) and in the torus longitudinalis (TL) (Figure 14 F-G).

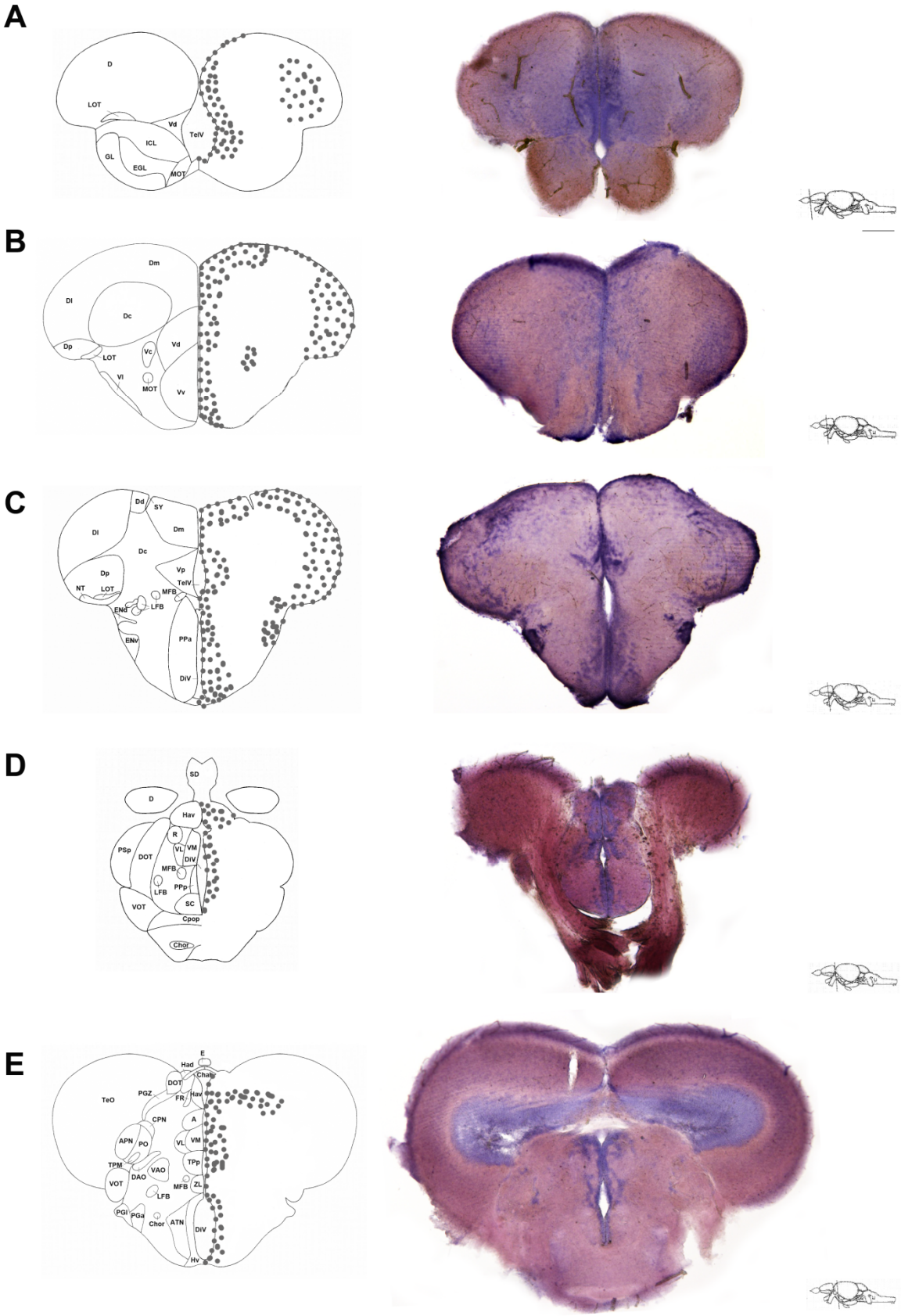
In the hindbrain *p73* mRNA was expressed in the periventricular zone of the rhombencephalic ventricle (RV) (Figure 14 H), in the lateral division of the valvula cerebelli (Val) (Figure 14 F-G), in the corpus cerebelli (CCe) (Figure 14 H), in the crista cerebellaris (CC) (Figure 14 H) and in the eminentia granularis (EG) (Figure 14 H).

In Figure 15 an ISH with a *p73* sense probe as a negative control is shown. There is no staining detectable in the brain regions that showed a clear staining with the *p73* antisense probe. Thus, the staining for *p73* is specific.

When the expression patterns of *p53* (Figure 12) and *p73* (Figure 14) mRNA are compared, it is obvious that both are expressed in a similar manner with few exceptions. In the diencephalon *p53* but not *p73* mRNA was expressed in the anterior (PGa) and lateral (PGl) preglomerular nucleus and in the torus lateralis (TLa) *p73* but not *p53* mRNA was expressed.

In mammals p53 and p73 were shown to interact (Chen et al., 2001; Grob et al., 2001; Kartasheva et al., 2002; Wang et al., 2007a) and p73 is able to transactivate the same target genes as p53 (see chapter 1.2.3.2) (Kaghad et al., 1997; Levrero et al., 2000; Murray-Zmijewski et al., 2006; Zhu et al., 1998a). The co-expression seen in many tissues of the adult zebrafish brain indicates that the two proteins may similarly interact in this context.

p73 antisense



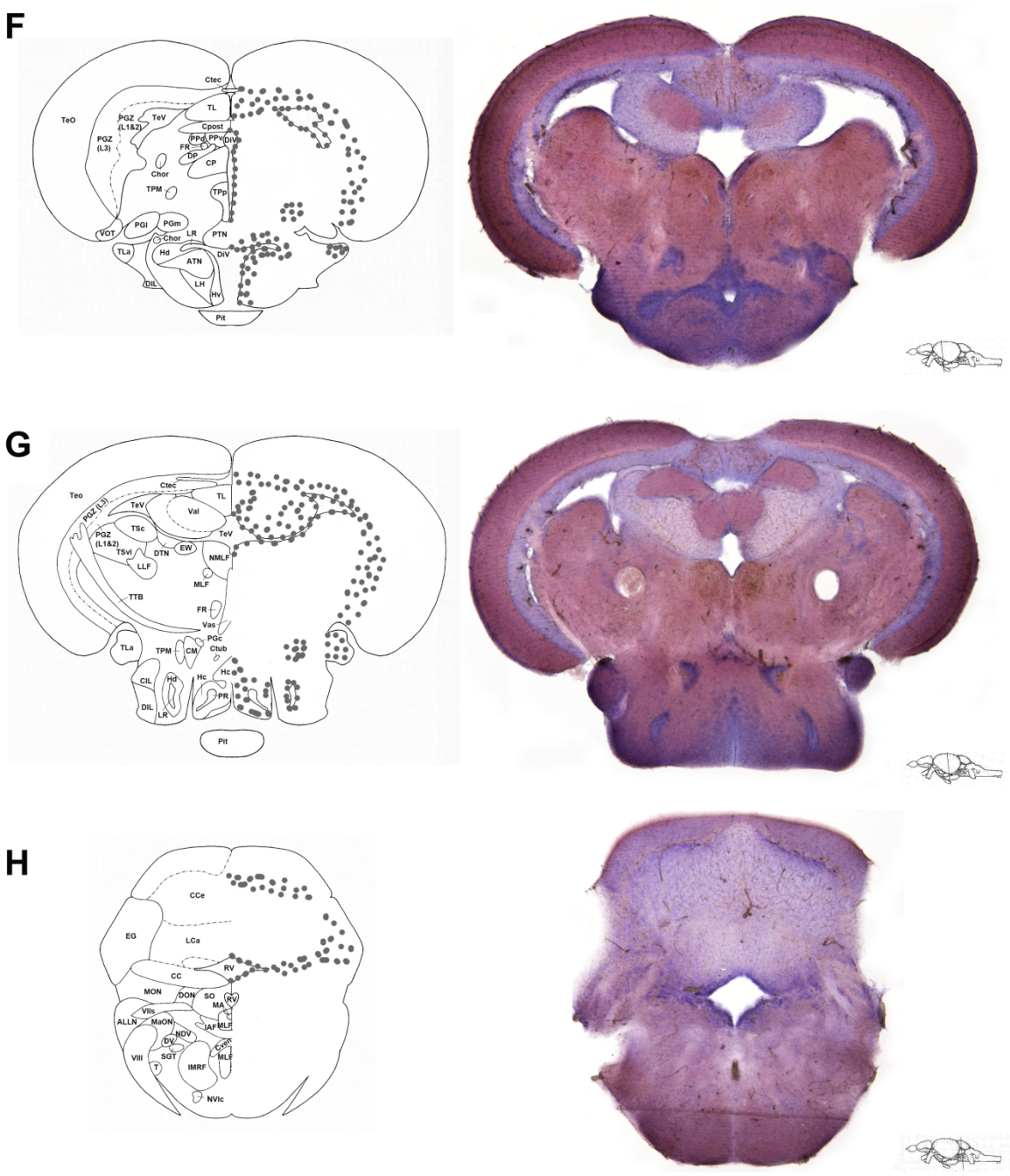


Figure 14: *p73* mRNA is expressed in many regions of the adult zebrafish brain.

(A-H) *In situ* hybridization with a *p73* antisense probe on cross sections through the whole adult zebrafish brain to analyze the expression of *p73* mRNA. The schematics on the left side (modified from Wullimann et al., 1996) show the anatomical subdomains of the examined cross sections. Grey dots indicate areas of *p73* mRNA expression. The small inserts on the right side indicate the anterior-posterior levels of the sections. The *p73* mRNA is expressed in many parts of the adult zebrafish brain.

Expression of *p73* mRNA in (A) Vd, D, periventricular zone of the TelV; (B) Vv, Vd, Vc, Dm, Dl, Dp, SY, periventricular zone of the TelV; (C) PPa, Vp, Dm, Dd, Dl, Dp, ENd, ENv, periventricular zone of the TelV and DiV; (D) PPp, VM, Hav, periventricular zone of the DiV; (E) PGZ, Hv, TPp, VM, A,

periventricular zone of the DiV; (F) PGZ, TL, Val, Hv, Hd, CM, TLa, periventricular zone of the DiV and TeV; (G) PGZ, TL, Val, Hd, Hc, CM, TLa, periventricular zone of the TeV; (H) CCe, EG, CC, periventricular zone of the RV.

Abbrevations: A: anterior thalamic nucleus; CC: crista cerebellaris; CCe: corpus cerebelli; CM: corpus mamillare; D: dorsal telencephalic area; Dd: dorsal zone of D; DiV: diencephalic ventricle; Dl: lateral zone of D; Dm: medial zone of D; Dp: posterior zone of D; EG: eminentia granularis; ENd: entopeduncular nucleus, dorsal part; ENv: entopeduncular nucleus, ventral part; Hav: ventral habenular nucleus; Hc: caudal zone of periventricular hypothalamus; Hd: dorsal zone of periventricular hypothalamus; Hv: ventral zone of periventricular hypothalamus; PGZ: periventricular grey zone of optic tectum; PPa: parvocellular preoptic nucleus, anterior part; PPp: parvocellular preoptic nucleus, posterior part; RV: rhombencephalic ventricle; SY: sulcus ypsiloniformis; TeV: tectal ventricle; TelV: telencephalic ventricle; TL: torus longitudinalis; TLa: torus lateralis; TPp: periventricular nucleus of posterior tuberculum; V: ventral telencephalic area; Val: lateral division of valvula cerebelli; Vc: central nucleus of V; Vd: dorsal nucleus of V; VM: ventromedial thalamic nucleus; Vp: postcommisural nucleus of V; Vv: ventral nucleus of V. Scale bar: 100 µm.

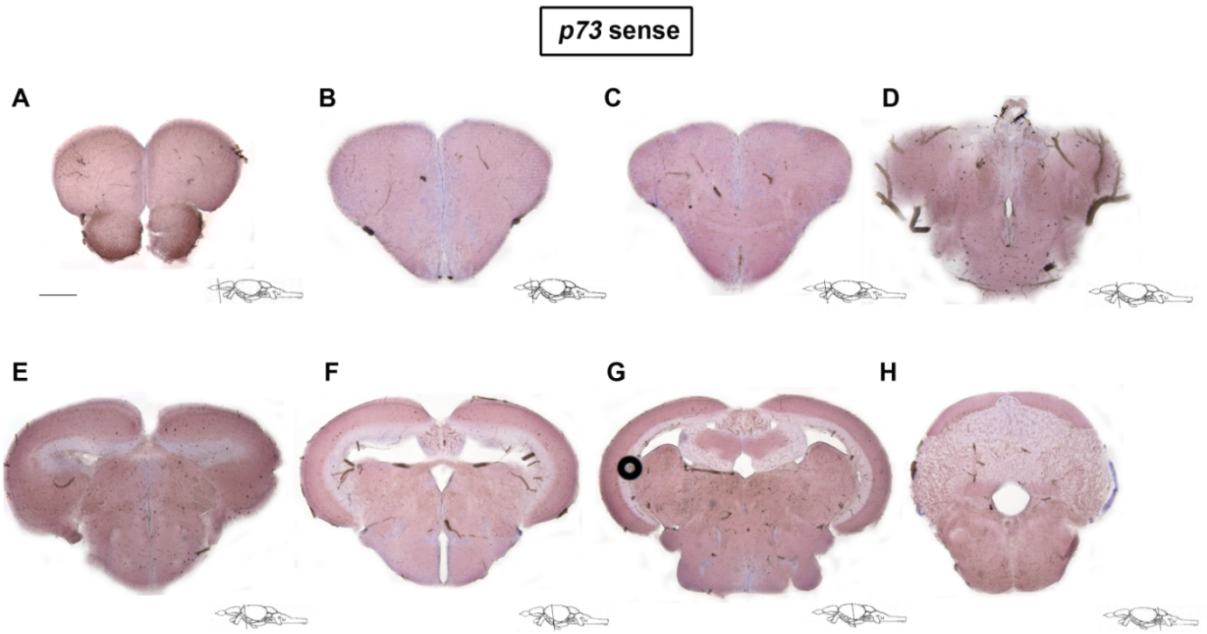


Figure 15: No staining with the *p73* sense probe.

(A-H) *In situ* hybridization with a *p73* sense probe on cross sections through the whole adult zebrafish brain as a negative control. The small inserts indicate the anterior-posterior levels of the sections. No staining could be detected with the *p73* sense probe in all sections examined. Scale bar: 100 µm.

3.1.3 Generation of polyclonal antibodies against the C-terminus of the zebrafish p53 and p73 protein and testing of a new antibody against the zebrafish p53 protein

Specific antibodies are important tools for characterizing the expression of proteins and to compare their expression to the expression of the mRNA. When I started to work on zebrafish p53 and p73 there were no antibodies against the two proteins available. Therefore, I first generated polyclonal antibodies against the zebrafish p53 and p73 protein.
Since p53 (see chapter 1.2.1) and p73 (see chapter 1.2.3) have very similar sequences, especially in the DNA binding domain (Danilova et al., 2008a; De Laurenzi and Melino, 2000; Levrero et al., 2000; Murray-Zmijewski et al., 2006), I produced polyclonal antibodies only against the variable C-terminus of these proteins to avoid cross reactivity of the antibodies.
For producing the polyclonal antibodies I cloned the C-terminus of zebrafish *p53* and *p73*, respectively, in a vector (pGEX-4T-2) for expression of the proteins fused to Glutathion-S-Transferase (GST) in bacteria. Afterwards the purified p53 and p73 proteins were used to immunize rats in order to produce the polyclonal antibodies. At the end, the serum of the immunized rats was recovered which contained the polyclonal antibodies against the C-terminus of the zebrafish p53 or p73 protein. Subsequently, the specificity of the polyclonal antibodies was tested by western blot and immunohistochemistry.

3.1.3.1 The polyclonal antibodies are functional in western blot

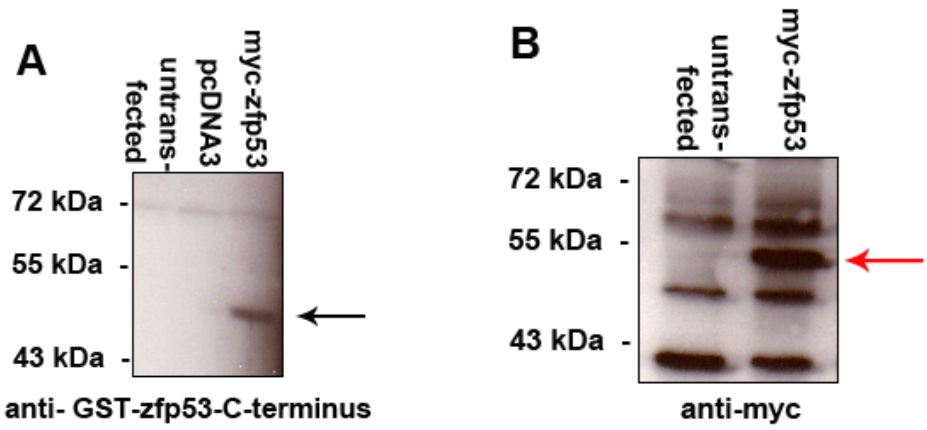
After producing, extracting and purifying the polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus protein they were first tested by western blot if they are able to detect the zebrafish p53 and p73 protein. Therefore, H1299 cells, a p53 negative cell line, were transfected with a pcDNA3 vector that contained either full-length zebrafish *p53* or *p73α* plus a myc tag (myc-zfp53 and myc-zfp73α). The myc tag was used to confirm that the transfection was successful. As negative controls H1299 cells were transfected with an empty pcDNA3 vector or they stayed untransfected. Subsequently the cells were lysed and the cell lysates were loaded onto a polyacrylamide gel. For the following western blot the self-made polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus protein or an anti-myc antibody were used.
In Figure 16 A a band of 53 kDa (black arrow) can be observed for the cells transfected with myc-zfp53. This suggests that the polyclonal antibodies against the GST-zfp53-C-terminus protein are able to recognize the zebrafish p53 protein in western blot. As expected, there are any bands visible for the untransfected cells and for the cells transfected with an empty

vector since these cell lysates should not contain any zebrafish p53 protein. But there are unspecific bands present in all three different cell lysates with a size of about 72 kDa.

Figure 16 B shows the western blot with an antibody against the myc tag for the cell lysates of untransfected cells and cells transfected with myc-zfp53. These lysates are the same lysates than the ones that were used in Figure 16 A. The band at 53 kDa (red arrow) for the cells transfected with myc-zfp53 indicates that the transfection was successful. There is any band present for the untransfected cells because these cells do not contain a p53 protein with a myc tag. There are also several unspecific bands visible.

Figure 16 C shows that the polyclonal antibodies against the GST-zfp73-C-terminus protein are capable of detecting the zebrafish p73α protein in western blot (black arrow). As for p53, there are also unspecific bands visible and there is only a band for p73 at ca. 73 kDa detectable in the lysate of cells that were transfected with myc-zfp73α. Moreover, the transfection with myc-zfp73α also worked since there is a band present at 73 kDa in the lysate of cells transfected with myc-zfp73α (Figure 16 D).

In summary, the self-made polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus protein are both able to detect the zebrafish p53 and p73α protein, respectively, demonstrating that they work in western blot.



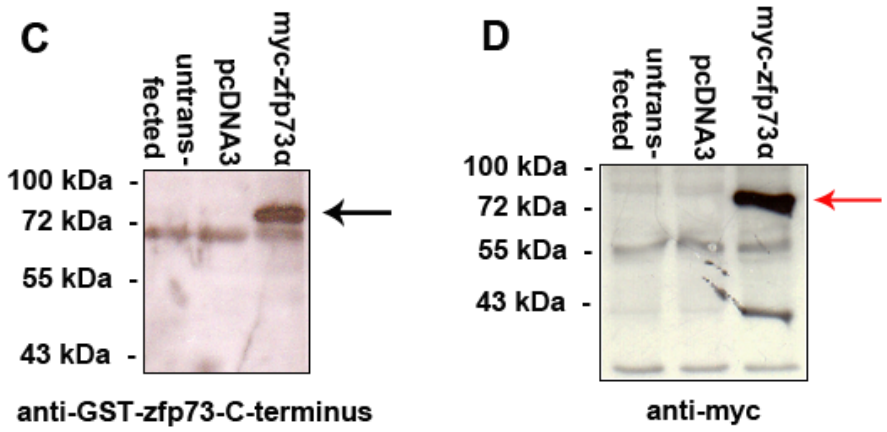


Figure 16: The self-made polyclonal antibodies against the GST-zfp53-C-terminus and the GST-zfp73-C-terminus protein are functional in western blot.

(A-D) To investigate if the polyclonal antibodies against the GST-zfp53-C-terminus and the GST-zfp73-C-terminus protein are working, H1299 cells were transfected with a pcDNA3 vector containing either full length zebrafish *p53* (A, B) or *p73α* (C, D) plus a myc tag (myc-zfp53 and myc-zfp73α). As negative controls cells were transfected with an empty pcDNA3 vector or they stayed untransfected. The myc tag was used to proof that the transfection was successful (B, D). Cells were lysed and the lysates were separated by a polyacrylamide gel. For the western blot the polyclonal antibodies against the GST-zfp53-C-terminus (A) and the GST-zfp73-C-terminus (C) protein or an antibody against the myc tag (B, D) were used. The polyclonal antibodies are able to detect zebrafish p53 (A, black arrow) and p73 (C, black arrow) protein in western blot. Furthermore, the myc control shows that the transfection was successful (B, D; red arrows).

Additionally, it was tested by western blot if the polyclonal antibodies are also able to detect the GST protein since the GST tag was not cleaved off from the p53 and p73 protein before they were injected into rats to produce the antibodies. Thus, there is the possibility that the rats produced not only antibodies against zebrafish p53 and p73 but also against GST. To test this option, also cell lysate of H1299 cells either transfected with a pcDNA3 vector that contained full length zebrafish *p53* or *p73α* plus a myc tag (myc-zfp53 and myc-zfp73α) as a positive control and additionally GST protein was loaded onto a polyacrylamide gel. For the following western blot the polyclonal antibodies against the GST-zfp53-C-terminus or GST-zfp73-C-terminus protein were used.

Figure 17 A and B show that, as already observed before, the polyclonal antibodies detect the zebrafish p53 and p73 protein (black arrows). But in addition they also recognize the GST protein (red arrows) which is indicated by the band at 26 kDa. Since the antibodies detect not only the zebrafish p53 and p73 protein but additionally also the GST protein this

could lead to problems in further experiments because the antibodies are not exclusively specialized to the proteins of interest. This could lead to false positive results.

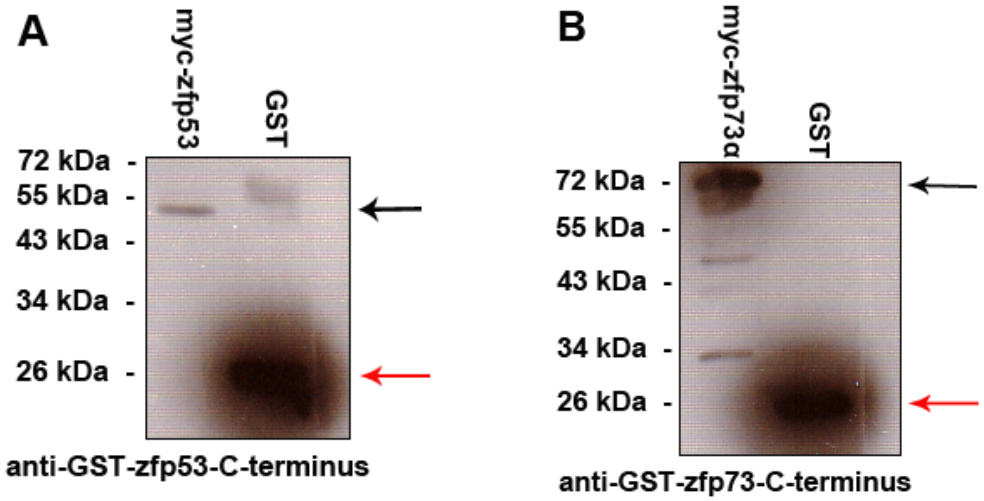


Figure 17: The self-made polyclonal antibodies against the GST-zfp53-C-terminus and the GST-zfp73-C-terminus protein also recognize the GST protein.

(A and B) To investigate if the polyclonal antibodies against the GST-zfp53-C-terminus and the GST-zfp73-C-terminus protein are also able to detect GST protein, cell lysate from H1299 cells transfected with a pcDNA3 vector containing either full length zebrafish *p53* (A) or *p73α* (B) plus a myc tag (myc-zfp53 and myc-zfp73α) as a positive control and GST protein were loaded onto a polyacrylamide gel. For the western blot the polyclonal antibodies against the GST-zfp53-C-terminus (A) and the GST-zfp73-C-terminus (B) protein were used. The polyclonal antibodies detect the zebrafish p53 (A, black arrow) and the zebrafish p73 (B, black arrow) protein. But they are also able to detect the GST protein (A, B; red arrows).

3.1.3.2 The polyclonal antibodies are functional in Immunohistochemistry on cells

The self-made polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus protein were also tested whether they worked in immunohistochemistry. Therefore, as for the western blot, H1299 cells were transfected with a pcDNA3 vector that contained either full length zebrafish *p53* or *p73α* plus a myc tag (myc-zfp53 and myc-zfp73α). As negative controls H1299 cells were transfected with an empty pcDNA3 vector or they stayed untransfected. The cells were cultured on coverslips to perform the immunostaining with the polyclonal antibodies or with an antibody against the myc tag to ensure that the transfection was successful. Moreover, the nuclei were stained with the nuclear marker DRAQ5.

Figure 18 shows that the polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus protein do not only detect the zebrafish p53 and p73 protein in western blot but also in immunohistochemistry. The nuclei stained with DRAQ5 are depicted in red and the p53 or p73 protein is shown in green.

Figure 18 A suggests that the polyclonal antibodies against the GST-zfp53-C-terminus protein detect the p53 protein in immunohistochemistry (white arrows). Additionally, the polyclonal antibodies against the GST-zfp73-C-terminus protein are able to recognize the p73 protein (Figure 18 C, white arrows). Furthermore, this figure shows that the transfection of the cells was effective indicated by the positive staining with the antibody against the myc tag (Figure 18 B and D, yellow arrows). However, not all cells are positive for myc. This indicates that not all of the cells were transfected effectively and therefore not all of the cells could be positive for p53 or p73.

Of course also some negative controls were performed where H1299 cells were transfected with an empty pcDNA3 vector or they stayed untransfected. There was any staining visible for the negative controls neither with the polyclonal antibodies nor with the antibody against the myc tag (data not shown).

These results suggest that the self-made polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus protein are functional both in western blot and immunohistochemistry when the experiments were performed with cells.

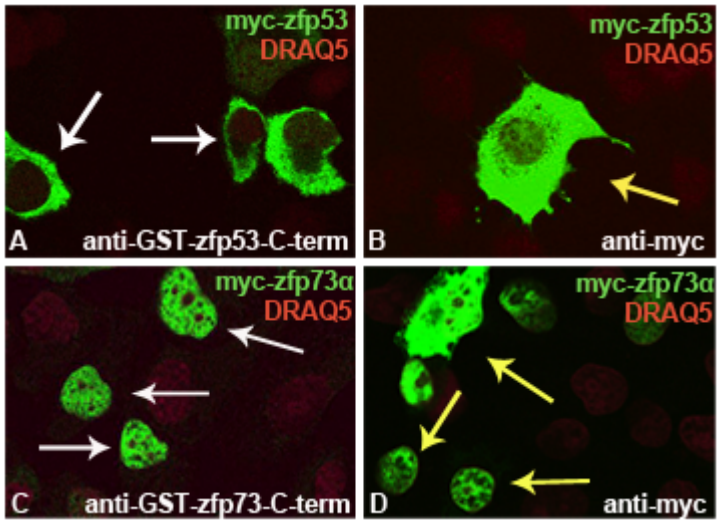


Figure 18: The self-made polyclonal antibodies against the GST-zfp53-C-terminus and the GST-zfp73-C-terminus protein are functional in immunohistochemistry on cells.

(A-D) To examine if the polyclonal antibodies against the GST-zfp53-C-terminus and the GST-zfp73-C-terminus protein are working in immunohistochemistry, H1299 cells were transfected with a pcDNA3 vector containing either full length zebrafish *p53* (A, B) or *p73α* (C, D) plus a myc tag (myc-zfp53 and

myc-zfp73α). The myc tag was used to proof that the transfection was successful (B, D). Cells were seeded on coverslips to perform the immunostainings with the antibodies against the GST-zfp53-C-terminus (A) and the GST-zfp73-C-terminus (C) protein or with an antibody against the myc tag (B, D). Additionally, the nuclei of the cells were stained with the nuclear marker DRAQ5. The myc control shows that the transfection was effective (B, D; yellow arrows). Furthermore, the polyclonal antibodies are able to detect zebrafish p53 (A, white arrows) and p73 (C, white arrows) protein in immunohistochemistry.

3.1.3.3 The polyclonal antibodies are not functional in Immunohistochemistry on brain sections

Since I was interested in the expression of p53 and p73 in the adult zebrafish brain, the polyclonal antibodies were tested if they also specifically detect the p53 and p73 protein, respectively, in immunohistochemistry on sections of the adult zebrafish brain. To address this, cross sections through the telencephalon of adult zebrafish were stained with the polyclonal antibodies.

Figure 19 A shows a section of a zebrafish telencephalon stained with the antibodies against p53. Since there is any staining visible for p53 this demonstrates that the self-made antibodies did not work in the immunostaining and they did not detect the p53 protein. The same holds true for the antibodies against p73. In Figure 19 B there is also any staining present for p73. Thus, the polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus protein do not work in immunohistochemistry on brain sections.

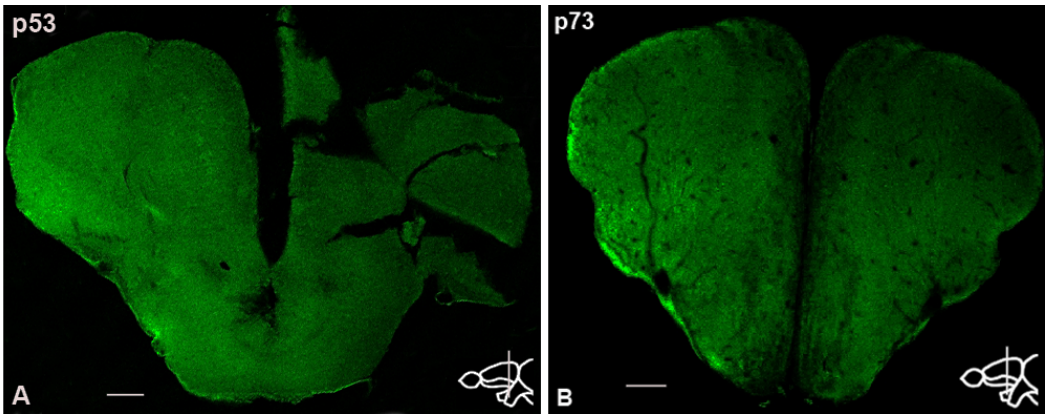


Figure 19: The self-made polyclonal antibodies against the GST-zfp53-C-terminus and the GST-zfp73-C-terminus protein are not functional in immunohistochemistry on brain sections.
(A-B) To find out if the polyclonal antibodies against the GST-zfp53-C-terminus and the GST-zfp73-C-terminus protein are working in immunohistochemistry on adult zebrafish brain sections, an immunostaining of cross sections through the telencephalon of adult wildtype zebrafish with the antibodies against the p53 (A) or p73 (B) protein was performed. The inserts indicate the anterior-posterior positions. The absent staining for both p53 (A) and p73 (B) denotes that the polyclonal antibodies against the GST-zfp53-C-terminus and the GST-zfp73-C-terminus protein are not working in immunohistochemistry on brain sections. Scale bars: 100 µm in A and B.

In summary, the self-made polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus protein are working in western blot and in immunohistochemistry when the experiments were performed with cells. However, they are not working in immunohistochemistry on zebrafish brain sections. Furthermore, the antisera are not only specific to p53 and p73 because they also detect GST. For this reason I decided not to use the self-made polyclonal antibodies for the following experiments.

3.1.3.4 A commercially available antibody against the zebrafish p53 protein detects p53 in Immunohistochemistry and western blot

As the self-made polyclonal antibodies against the GST-zfp53-C-terminus protein are not working properly (see chapter 3.1.3.3), an antibody against the zebrafish p53 protein, which was launched in the meantime, was tested. This antibody is a rabbit polyclonal antibody called "Anti-Tp53, ZFish^{TM}" (further referred to as anti-p53 antibody). It is available from AnaSpec and is directed against the full length p53 protein. I investigated if this antibody is able to detect the p53 protein and if this antibody is specific.
First, the anti-p53 antibody was tested if it works in western blot. Telencephala of adult wildtype zebrafish were lysed and the lysate was loaded, together with lysate from H1299 cells transfected with a pcDNA3 vector that contained full length zebrafish *p53* plus a myc tag (myc-zfp53) (see chapter 3.1.3.1) as a positive control, onto a polyacrylamide gel. For the following western blot the anti-p53 antibody was used.
As shown in Figure 20 there are bands visible at around 53 kDa which suggests that the anti-p53 antibody is able to detect the p53 protein both in the cell lysate and in the telencephalon lysate (Figure 20, red box). There are also unspecific bands visible for the telencephalon lysate. This result indicates that on the one hand the antibody works in western blot and on the other hand that the p53 protein is present in the telencephalon of adult zebrafish.

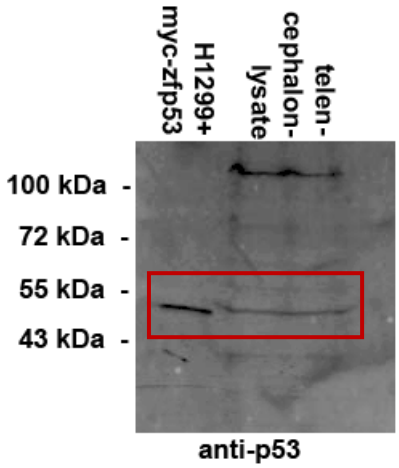


Figure 20: The new anti-p53 antibody detects the p53 protein in telencephalon-lysate.
To investigate if the new anti-p53 antibody is working in western blot, telencephala of adult zebrafish were lysed and the lysate was loaded onto a polyacrylamide gel together with lysate of H1299 cells transfected with myc-zfp53 as a positive control. For the western blot the new anti-p53 antibody was used. This antibody is able to detect the p53 protein in the positive control as well as in the telencephalon lysate (red box).

Furthermore, cross sections through the telencephalon of adult zebrafish were stained with the anti-p53 antibody to find out if this antibody is working in immunohistochemistry on brain sections. The nuclei were stained with the nuclear marker DRAQ5.
As shown in Figure 21, in contrast to the self-made polyclonal antibodies, the new anti-p53 antibody is able to detect the p53 protein on brain sections. The p53 protein is expressed along the ventricular zone (Figure 21 C', white arrow) and in the parenchyma (Figure 21 C'', white arrow) of the adult zebrafish telencephalon. Furthermore, p53 shows a nuclear expression. As a negative control brain sections were stained only with the secondary antibody. There was no staining present without the first anti-p53 antibody (data not shown), which suggests that the staining is specific.

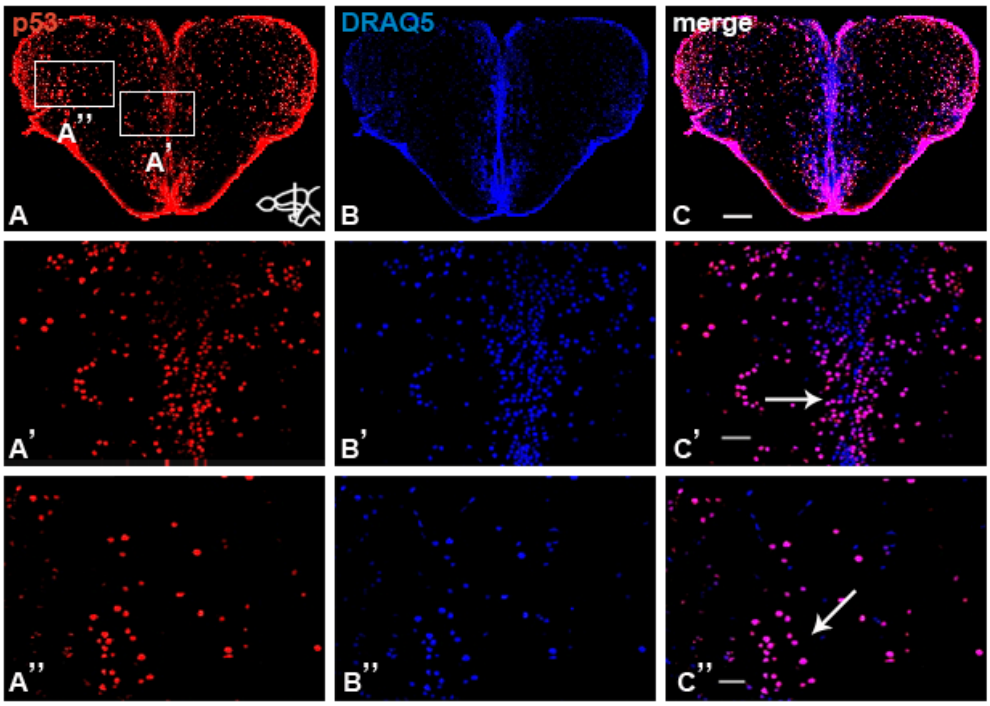


Figure 21: The new anti-p53 antibody detects the p53 protein in immunohistochemistry on brain sections.

(A-C'') To examine if the new anti-p53 antibody is working in immunohistochemistry on adult zebrafish brain sections cross sections through the telencephalon of adult wildtype zebrafish were immunostained with this antibody (A-A''), the nuclei were stained with the nuclear marker DRAQ5 (B-B''). (C-C'') show the merged panels. These pictures display a representative vibratome section through the medial telencephalon. The insert in (A) indicates the anterior-posterior position and the boxes in (A) show the regions depicted in higher magnification in (A'-C', right box) and (A''-C'', left box). The new anti-p53 antibody detects the p53 protein in a brain section of the adult zebrafish telencephalon. p53 is expressed along the ventricular zone (C', white arrow) and in the parenchyma (C'', white arrow) of the telencephalon and it shows a nuclear expression. Scale bars: 100 µm in C; 20 µm in C' and C''.

To further verify that the observed staining is specific and that the detected protein is p53, I performed a fluorescent *in situ* hybridization (FISH). A *p53* antisense probe and the anti-p53 antibody were used to compare the expression of *p53* mRNA with the expression of the protein detected by the anti-p53 antibody in the adult zebrafish telencephalon. If the detected protein is p53, there should be an overlap in the expression of *p53* mRNA and immunostaining.

In Figure 22 it is shown that the *p53* mRNA is expressed in almost the same areas of the section of the telencephalon as the protein which is detected by the anti-p53 antibody. The *p53* mRNA is also expressed along the ventricular zone and partially in the parenchyma.

This suggests that there is an overlap in the expression of *p53* mRNA and detected protein (Figure 22 C', white arrows). Thus it is most likely that the protein detected by the antibody is p53. There are also some regions where the protein is present but the mRNA is not, as in the ventral telencephalic area. This is most likely a technical problem of the probe.

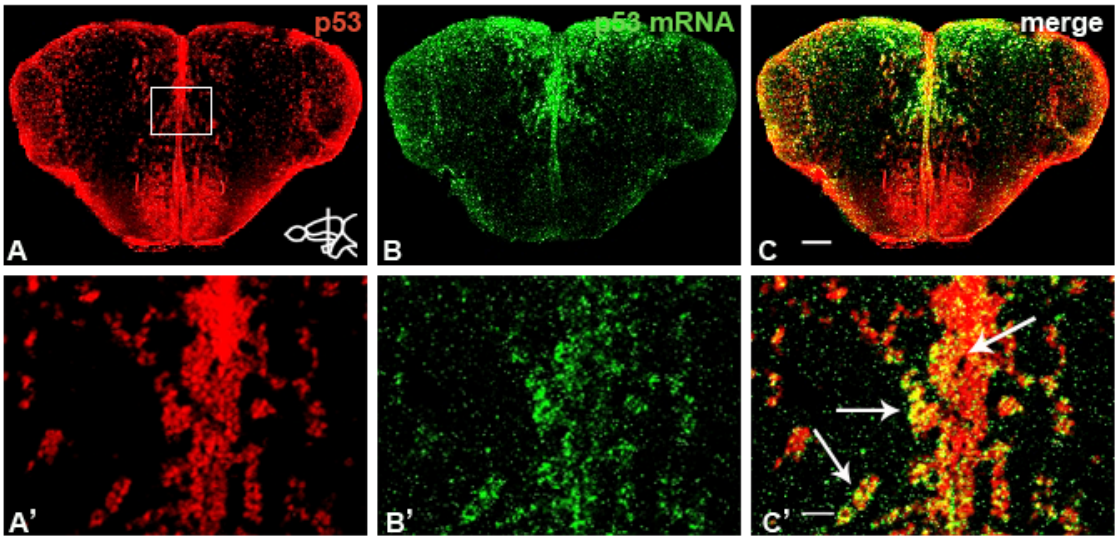


Figure 22: The expression of *p53* mRNA overlaps with the immunostaining for p53.
(A-C') Fluorescent *in situ* hybridization (FISH) with a *p53* antisense probe (B-B') and the anti-p53 antibody (A-A') was performed on cross sections through the telencephalon of adult wildtype zebrafish in order to investigate if there is an overlap in the expression of *p53* mRNA with the protein detected by the new antibody. The merged panels are shown in (C-C'). These pictures display a representative vibratome section through the medial telencephalon. The insert in (A) indicates the anterior-posterior position and the box in (A) shows the region depicted in higher magnification in (A'-C'). There is an overlap in the expression of *p53* mRNA and the potential p53 protein which is detected by the anti-p53 antibody (C', white arrows). Scale bars: 100 µm in C; 20 µm in C'.

Another proof that the commercially available anti-p53 antibody specifically detects the p53 protein was provided by immunostaining embryos. Therefore *p53-GFP* RNA was injected into one-cell stage wildtype zebrafish embryos. p53-GFP positive embryos were fixed at 8 hours post fertilization (hpf) and afterwards the embryos were co-immunostained with the anti-p53 antibody and an antibody against GFP. Since a p53-GFP fusion construct was injected, p53 expression should be detected everywhere where GFP expression can be observed.
Figure 23 indicates that this is the case because the representative embryo shows expression of the injected p53-GFP construct and all cells that are positive for GFP show

also a staining for p53 (Figure 23 C, white arrows). Consequently, this experiment supports the assumption that the staining with the new anti-p53 antibody is specific.

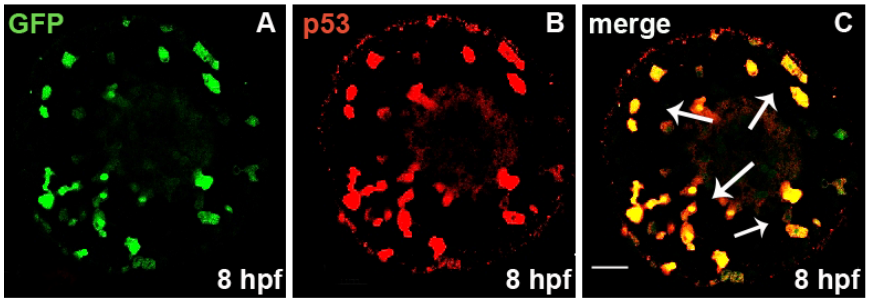


Figure 23: The staining with an anti-GFP antibody overlaps with the staining for p53 in the embryo.

(A-C) *p53-GFP* RNA was injected into one-cell stage wildtype zebrafish embryos. p53-GFP positive embryos were fixed at 8 hpf and were co-immunostained afterwards with an anti-GFP and the anti-p53 antibody. This figure shows a representative embryo stained with an anti-GFP (A) and the anti-p53 antibody (B). (C) shows the merged view of the staining. All cells of the embryo that are positive for GFP are also positive for p53 (C, white arrows). Scale bar: 50 µm.

In summary these results suggest that the new anti-p53 antibody was working very well. It detected the p53 protein both in western blot and, in contrast to the self-made polyclonal antibodies, also in immunohistochemistry on brain sections. Moreover, the staining with the antibody is specific. Furthermore, these results lead to the assumption that the p53 protein is expressed in the adult zebrafish brain which will be further investigated in the following paragraphs. Since the new antibody was working properly I used it for the following experiments.

Unfortunately there is no antibody against the zebrafish p73 protein commercially available and therefore I could not investigate the expression of the p73 protein.

3.1.4 Analysis of p53 protein expression in the adult zebrafish brain

With this new p53 antibody as tool, I wanted to investigate the expression of the p53 protein not only in the telencephalon but also in the other parts of the adult zebrafish brain. In addition, I intended to find out if the p53 protein is expressed in the same areas of the adult

zebrafish brain as the mRNA (see chapter 3.1.1). The p53 protein is tightly regulated by Mdm2 and other components (Boehme and Blattner, 2009; Levine et al., 2006) (see chapter 1.2.1.2). Therefore it could be possible that the mRNA of p53 is expressed in a region of the brain but the protein is not present in this region. To examine the expression of the p53 protein in the adult zebrafish brain, cross sections through the whole brain were immunostained with the anti-p53 antibody. The nuclei were stained with the nuclear marker DRAQ5. In Figure 24 it is shown that the p53 protein is broadly expressed in many parts of the adult zebrafish brain, like the *p53* mRNA.

In the telencephalon the p53 protein was expressed in the periventricular zone of the telencephalic ventricle (TelV) (Figure 24 A-C) and in the central (Vc) (Figure 24 B), dorsal (Vd) (Figure 24 A-B), ventral (Vv) (Figure 24 B) and postcommisural (Vp) (Figure 24 C) nuclei of the ventral telencephalic area (V). Furthermore, the p53 protein was expressed in the medial (Dm) (Figure 24 B-C), lateral (Dl) (Figure 24 B-C), dorsal (Dd) (Figure 24 C) and posterior (Dp) (Figure 24 B-C) zone of the dorsal telencephalic area (D). Unlike the mRNA, the p53 protein could not be detected in the sulcus ypsiloniformis (SY).

In the diencephalon p53 protein expression was observed in the periventricular zone of the diencephalic ventricle (DiV) (Figure 24 C-F) and in the anterior (PPa) (Figure 24 C) and posterior (PPp) (Figure 24 D) part of the parvocellular preoptic nucleus. Moreover, the p53 protein was also expressed in the dorsal (ENd) (Figure 24 C) and ventral (ENv) (Figure 24 C) part of the entopeduncular nucleus, in the anterior (PGa) (Figure 24 E) and lateral (PGl) (Figure 24 E) preglomerular nucleus and in the ventromedial (VM) (Figure 24 D-E) and anterior (A) (Figure 24 E) thalamic nucleus. p53 could also be detected in the caudal (Hc) (Figure 24 G), dorsal (Hd) (Figure 24 F-G) and ventral (Hv) (Figure 24 E-F) zone of the periventricular hypothalamus and in the corpus mamillare (CM) (Figure 24 G). Additionally, the p53 protein was expressed in the ventral habenular nucleus (Hav) (Figure 24 D) and in the periventricular nucleus of the posterior tuberculum (TPp) (Figure 24 E-F).

In the mesencephalon an expression of p53 protein was observed in the periventricular zone of the tectal ventricle (TeV) (Figure 24 F-G), in the periventricular grey zone of the optic tectum (PGZ) (Figure 24 E-G) and in the torus longitudinalis (TL) (Figure 24 F-G).

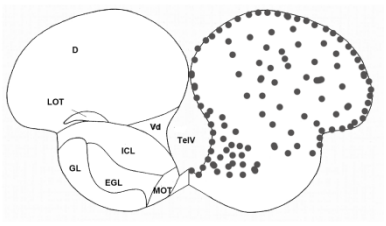
In the hindbrain the p53 protein was expressed in the periventricular zone of the rhombencephalic ventricle (RV) (Figure 24 H), in the corpus cerebelli (CCe) (Figure 24 H), in the crista cerebellaris (CC) (Figure 24 H) and in the eminentia granularis (EG) (Figure 24 H). In contrast to *p53* mRNA, the p53 protein was not expressed in the lateral division of the valvula cerebelli (Val).

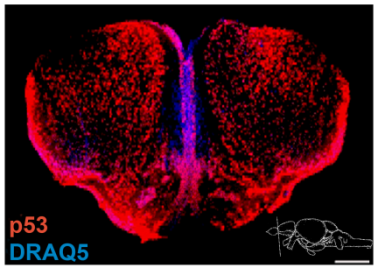
When the expression of *p53* mRNA (Figure 12) and p53 protein (Figure 24) are compared it is obvious that both are expressed in the same regions of the adult zebrafish brain, with few exceptions. There are some small differences in the expression pattern but nevertheless it is

evident that the p53 mRNA and protein are broadly expressed in many regions of the adult zebrafish brain and the p53 protein is expressed in most of the regions where an expression of *p53* mRNA was observed.

p53 protein

A





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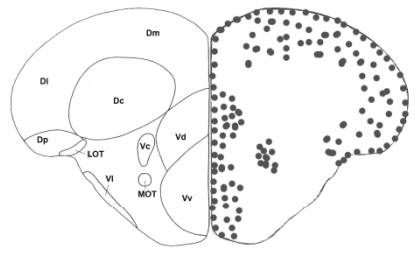

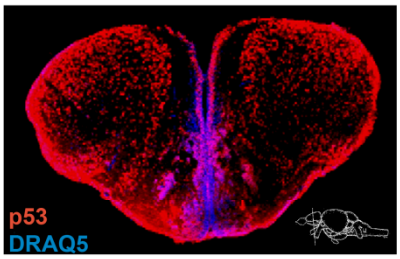

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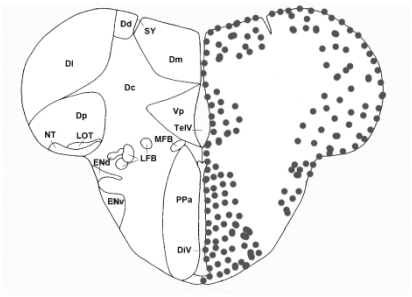

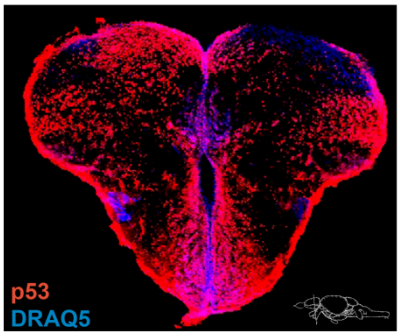

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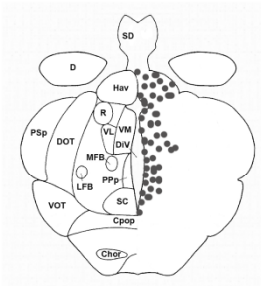

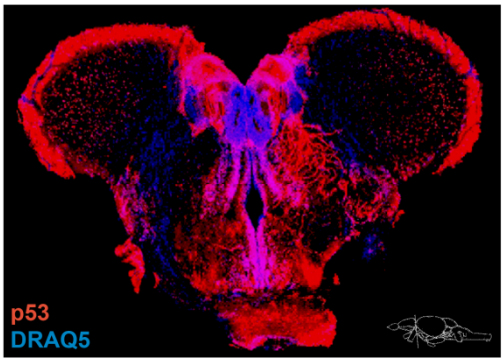

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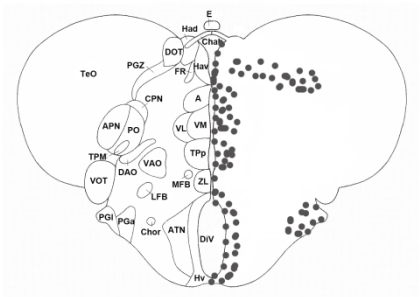

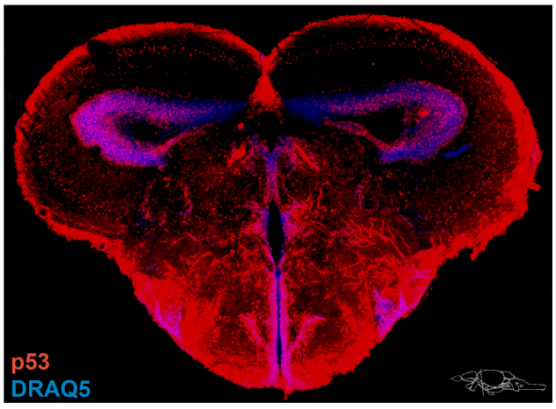

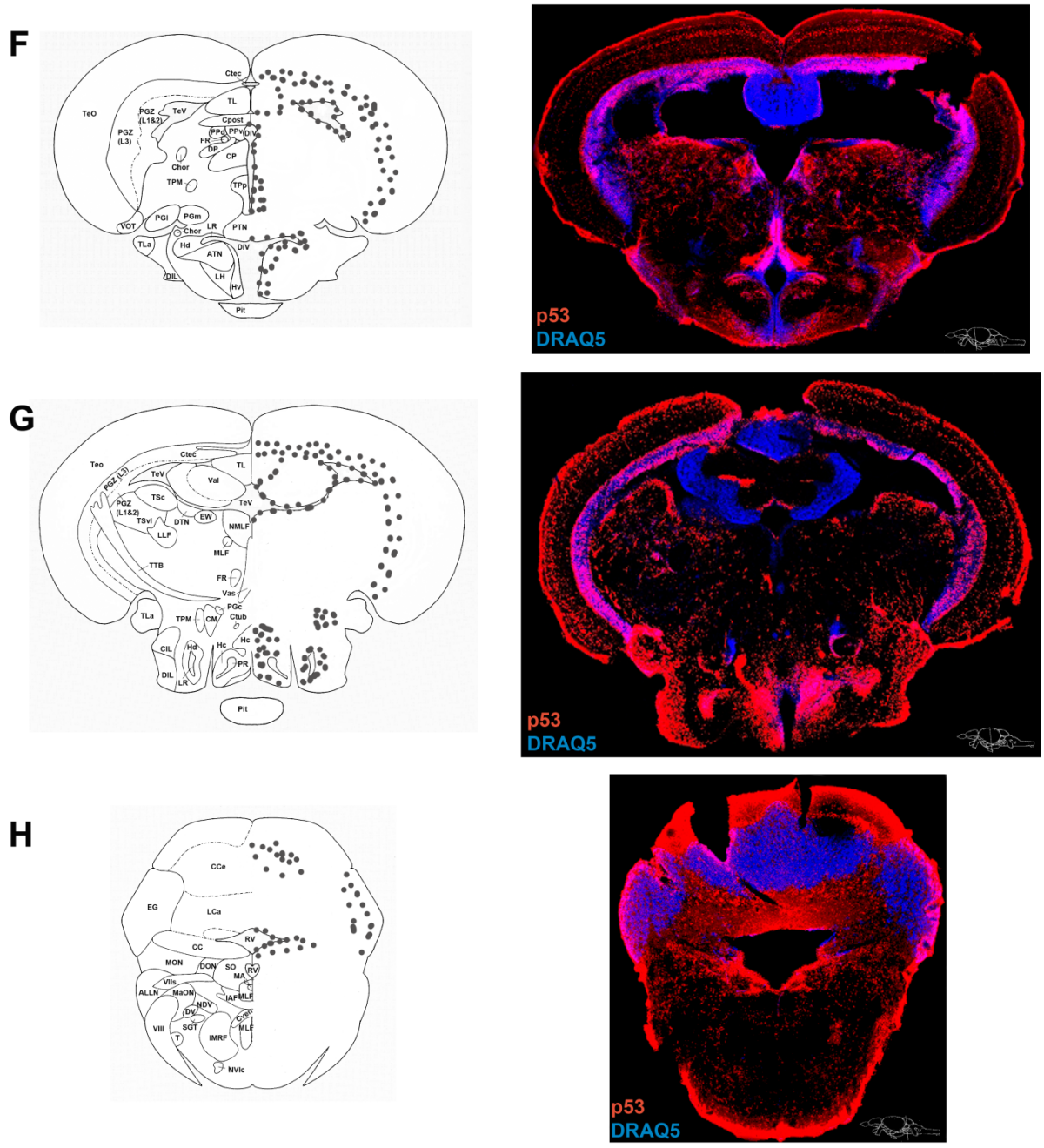

Figure 24: The p53 protein is broadly expressed in many regions of the adult zebrafish brain.
(A-H) Immunohistochemistry on cross sections through the whole adult zebrafish brain with the anti-p53 antibody to examine the expression of the p53 protein. The nuclei were stained with the nuclear marker DRAQ5. The schematics on the left side (modified from Wullimann et al., 1996) show the anatomical subdomains of the examined cross sections. Grey dots indicate areas of p53 protein expression. The small inserts on the right side indicate the anterior-posterior levels of the sections. Like the *p53* mRNA, the p53 protein is broadly expressed in many parts of the adult zebrafish brain. Expression of the p53 protein in (A) Vd, D, periventricular zone of the TelV; (B) Vv, Vd, Vc, Dm, Dl, Dp, periventricular zone of the TelV; (C) PPa, Vp, Dm, Dd, Dl, Dp, ENd, ENv, periventricular zone of the TelV and DiV; (D) PPp, VM, Hav, periventricular zone of the DiV; (E) PGZ, Hv, TPp, VM, A, PGl,

PGa, periventricular zone of the DiV; (F) PGZ, TL, Hv, Hd, TPp, periventricular zone of the DiV and TeV; (G) PGZ, TL, Hd, Hc, CM, periventricular zone of the TeV; (H) CCe, EG, CC, periventricular zone of the RV. Abbreviations: see Figure 12 and Figure 14. Scale bar: 100 µm.

3.1.5 Generation of p53 and p73 transgenic lines

Since any antibody against the zebrafish p73 protein was available it was not possible to perform any experiments regarding the expression of the p73 protein. But as I wanted to examine the expression of the p73 protein as well, I started to generate transgenic lines for p73. At the same time I also tried to generate transgenic lines for p53. I attempted to generate two different transgenic lines for p53 and p73, respectively. On the one hand I tried to generate the transgenic lines *Tg(p53:EGFP)* and *Tg(p73:EGFP)* by using a BAC clone of the zebrafish p53 or p73 locus to drive the expression of enhanced green fluorescent protein (EGFP). On the other hand, *Tg(p53-EGFP)* and *Tg(p73-EGFP*) fusion lines should be generated by using BAC clones harboring the zebrafish p53 or p73 locus. After injection of the different constructs into one-cell stage zebrafish embryos, the fish were raised to adulthood and outcrossed to identify a stable integration of the transgene. Unfortunately, any positive founder has been found for the different lines.

In summary, it was shown in this chapter that both *p53* mRNA and p53 protein are broadly expressed in many regions of the adult zebrafish telencephalon, as well as *p73* mRNA. Furthermore, it turned out that the self-made polyclonal antibodies against the GST-zfp53-C-terminus and GST-zfp73-C-terminus protein are not functional in immunohistochemistry on zebrafish brain sections but the commercially available anti-p53 antibody detects specifically the p53 protein both in western blot and immunohistochemistry.

3.2 The p53 protein is expressed in different cell types in the adult zebrafish telencephalon

After mapping the overall expression of p53 protein in the various subdivisions of the adult zebrafish brain (see chapter 3.1.4). I focus in this chapter on the results of the high resolution mapping of expression of p53 to determine in which cell types exactly the p53 protein is expressed. I performed co-immunostainings with specific markers for the different cell types that are present in the adult zebrafish brain to further characterize the expression of p53 in these cell types. I focused my work in this and the following chapters on the telencephalon. The telencephalon, like the rest of the zebrafish CNS, shows a very high proliferative and regenerative capacity in comparison to the mammalian brain (Adolf et al., 2006; Chapouton et al., 2007; Grandel et al., 2006; Kaslin et al., 2008; Zupanc, 2001; Zupanc and Zupanc, 2006) In addition it was shown that the adult zebrafish telencephalon harbours homologs of the SVZ and SGZ, the two mammalian neurogenic regions (Adolf et al., 2006; Grandel et al., 2006; März et al., 2010a). To know in which cell types p53 is expressed could be a first hint to its role in the adult zebrafish telencephalon in general and to its potential role in constitutive neurogenesis.

3.2.1 p53 expression in cells located in the parenchyma of the adult zebrafish telencephalon

3.2.1.1 The p53 protein is not expressed in *Tg(olig2:EGFP)*- positive cells

As shown in chapter 3.1.4, the p53 protein is expressed in the parenchyma of the adult zebrafish telencephalon (Figure 24 A-C). Therefore I investigated whether p53 is expressed in cells of the oligodendrocyte lineage, which are located in the parenchyma of the adult zebrafish telencephalon and express the basic helix-loop-helix (bHLH) transcription factor Olig2 (März et al., 2010b). I made use of the *Tg(olig2:EGFP)* line (Shin et al., 2003) that faithfully marks these cell types (März et al., 2010b). Cross sections through the telencephalon of *Tg(olig2:EGFP)* fish were co-immunostained with an anti-GFP and the anti-p53 antibody. The nuclei were stained with the nuclear marker DRAQ5.

The p53 protein is not expressed in *Tg(olig2:EGFP)*-positive cells (Figure 25). Instead p53 is expressed in cells that are located next to *Tg(olig2:EGFP)*-positive cells (Figure 25 D' and D'', white arrows). It was shown that *Tg(olig2:EGFP)*-positive cells can often be found in

close association with cells that express the neuronal marker HuC/D (März et al., 2010b). Therefore, the p53 expressing cells residing next to the *Tg(olig2:EGFP)*-positive cells could be neurons.

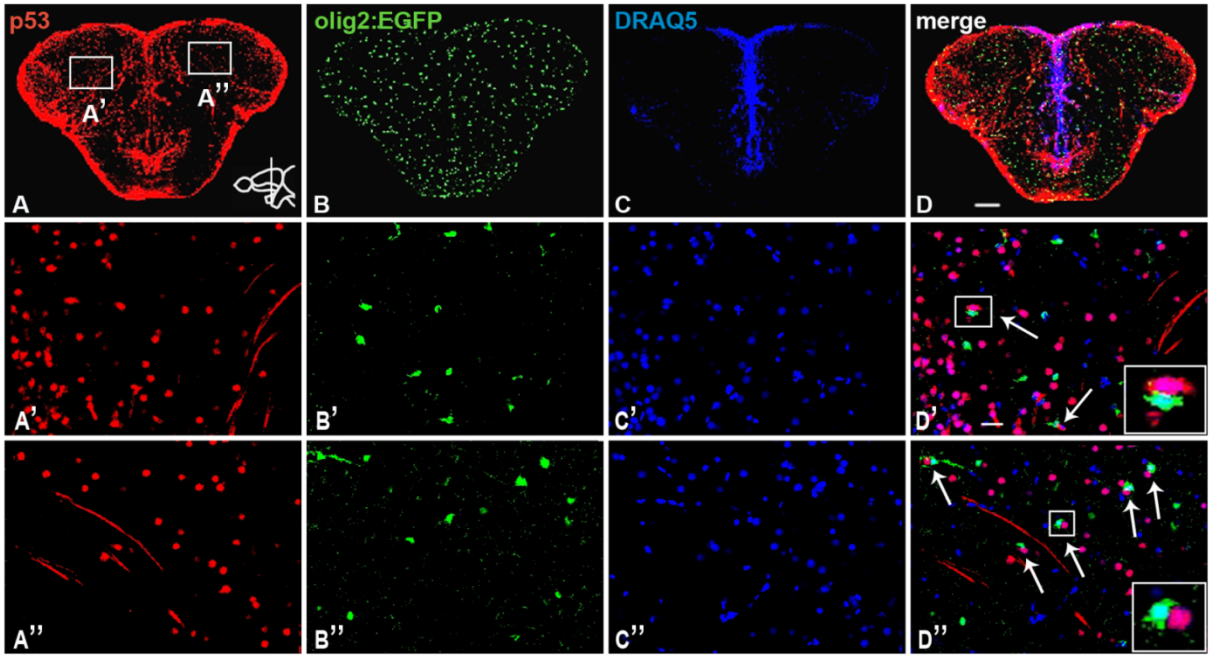

Figure 25: The p53 protein is not expressed in *Tg(olig2:EGFP)*-positive cells in the adult zebrafish telencephalon.

(A-D'') To investigate if the p53 protein is expressed in cells of the oligodendrocyte lineage, cross sections through the telencephalon of adult *Tg(olig2:EGFP)* zebrafish were co-immunostained with the anti-p53 antibody (A-A'') and with an anti-GFP antibody (B-B''). The nuclei were stained with the nuclear marker DRAQ5 (C-C''). (D-D'') show the merged panels. These pictures display a representative vibratome section through the medial telencephalon. The insert in (A) indicates the anterior-posterior position and the boxes in (A) show the regions depicted in higher magnification in (A'-D', left box) and (A''-D'', right box). *Tg(olig2:EGFP)*-positive cells in the parenchyma do not express p53, p53- positive cells are located next to *Tg(olig2:EGFP)*-positive cells ((D') and (D''), white arrows). The boxes in (D') and (D'') show magnified p53 positive cells next to *Tg(olig2:EGFP)*-positive cells. Scale bars: 100 µm in D; 20 µm in D' and D''.

3.2.1.2 The p53 protein is expressed in HuC/D-positive neurons

The result of the immunostaining performed in chapter 3.2.1.1 led to the assumption that the p53 protein could be expressed in neurons. In order to prove if this hypothesis is correct, I performed co-immunostainings of cross sections through the telencephalon of adult wildtype

zebrafish with antibodies against p53 and HuC/D, which is a RNA binding protein and labels cells that show a neuronal differentiation state (Mueller and Wullimann, 2002). The nuclei were stained with the nuclear marker DRAQ5.

The p53 protein is indeed expressed in cells of the neuronal lineage in the adult zebrafish telencephalon (Figure 26). There are many p53-positive cells that co-express the neuronal marker HuC/D (Figure 26 D' and D'', white arrows). In mammals p53 preserves the post-mitotic character of neurons (see chapter 1.2.1.5) (Miller et al., 2003). Thus, p53 could have the same anti-proliferative role in the adult zebrafish brain. However, HuC/D does not discriminate between mature neurons and neuroblasts/immature neurons since it is expressed from early stages of neuronal differentiation onwards (Kroehne et al., 2011).

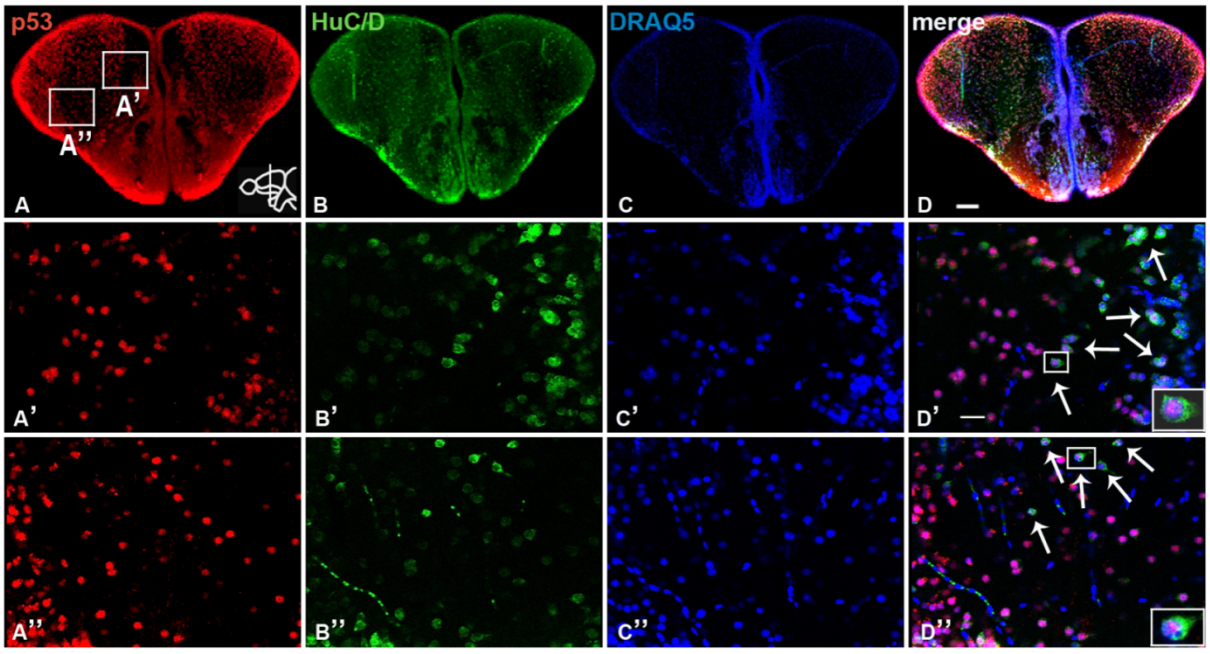

Figure 26: The p53 protein is expressed in neurons in the adult zebrafish telencephalon.
(A-D'') Co-immunostainings of cross sections through the telencephalon of adult wildtype zebrafish with antibodies against p53 (A-A'') and the neuronal marker HuC/D (B-B'') were performed to examine if the p53 protein is expressed in neurons. The nuclei were stained with the nuclear marker DRAQ5 (C-C''). (D-D'') show the merged panels. These pictures display a representative vibratome section through the medial telencephalon. The insert in (A) indicates the anterior-posterior position and the boxes in (A) show the regions depicted in higher magnification in (A'-D', upper box) and (A''-D'', lower box). p53- positive cells co-express the neuronal marker HuC/D ((D') and (D'')), white arrows) which indicates that the p53 protein is expressed in neurons. The boxes in (D') and (D'') show magnified triple-positive cells for p53, HuC/D and DRAQ5. Scale bars: 100 µm in D; 20 µm in D' and D''.

3.2.1.3 The p53 protein is expressed in HuC/D-positive neurons located next to *Tg(olig2:EGFP)*-positive cells

In chapter 3.2.1.2 it was shown that the p53 protein is expressed in HuC/D-positive neurons in the adult zebrafish telencephalon. Therefore, the cells located next to *Tg(olig2:EGFP)*-positive cells could be p53-positive neurons (see chapter 3.2.1.1). To verify this speculation I co-immunostained cross sections through the telencephalon of *Tg(olig2:EGFP)* zebrafish with an anti-GFP, an anti-HuC/D and with the anti-p53 antibody.

The p53-positive cells residing next to the *Tg(olig2:EGFP)*-positive cells are also HuC/D-positive (Figure 27). These results suggest that in the adult zebrafish telencephalon there are p53-positive neurons present that are located close to cells that belong to the oligodendrocyte lineage (Figure 27 D' and H', white arrows). As already mentioned in chapter 3.2.1.2, the p53-positive neurons could be early neurons since oligodendrocytes are myelinating new-born neurons (März et al., 2010b). This could be the reason why these neurons are located next to cells of the oligodendrocyte lineage.

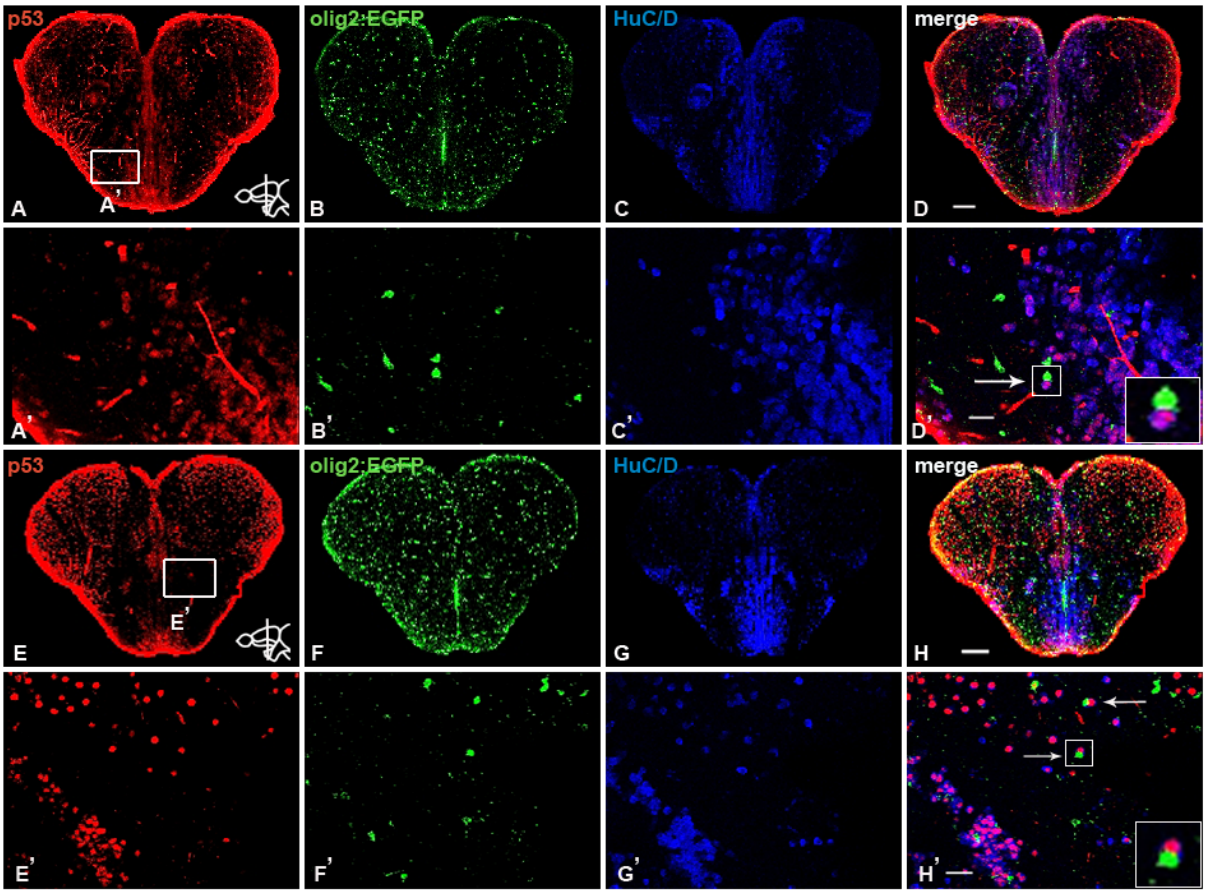

Figure 27: The p53 protein is expressed in neurons located next to *Tg(olig2:EGFP)*-positive cells in the adult zebrafish telencephalon.

(A-H') To investigate if the p53 protein is expressed in HuC/D-positive neurons that are located next to *Tg(olig2:EGFP)*-positive cells in the parenchyma of the adult zebrafish telencephalon, cross sections through the telencephalon of adult *Tg(olig2:EGFP)* zebrafish were co-immunostained with the anti-p53 antibody (A-A' and E-E'), an anti-GFP antibody (B-B' and F-F') and with an anti-HuC/D antibody (C-C' and G-G'). (D-D' and H-H') show the merged panels. These pictures display representative vibratome sections through the medial telencephalon. The inserts in (A) and (E) indicate the anterior-posterior positions and the boxes in (A) and (E) show the regions depicted in higher magnification in (A'-D') and (E'-H'), respectively. HuC/D-positive cells co-express p53 and are located next to *Tg(olig2:EGFP)*-positive cells ((D') and (H'), white arrows). This denotes that the p53 protein is expressed in neurons which are residing next to *Tg(olig2:EGFP)*-positive cells in the adult zebrafish telencephalon. The boxes in (D') and (H') show magnified p53-positive neurons next to *Tg(olig2:EGFP)*-positive cells. Scale bars: 100 µm in D and H; 20 µm in D' and H'.

3.2.1.4 The p53-positive neurons located next to *Tg(olig2:EGFP)*-positive cells are not positive for BrdU

In order to investigate if the p53-positive neurons located next to *Tg(olig2:EGFP)*-positive cells are new-born neurons (see chapter 3.2.1.3) I injected the thymidine analogue BrdU intraperitoneally on 3 consecutive days into *Tg(olig2:EGFP)* zebrafish to label the DNA of proliferating cells. This was followed by a chase of 2, 3 and 4 weeks, respectively. Afterwards a triple staining of cross sections through the telencephalon of BrdU injected *Tg(olig2:EGFP)* zebrafish was performed with an anti-GFP, an anti-BrdU and with the anti-p53 antibody.
Two weeks after a BrdU pulse, BrdU-positive cells have migrated from the ventricular zone into the adjacent parenchyma. Furthermore, 74% of the BrdU-positive cells are also positive for the neuronal marker Hu (Adolf et al., 2006) which demonstrates that these cells are new-born neurons. These data indicate that new neurons are generated at the ventricular zone by neural stem cells and these new-born neurons then migrate into the parenchyma. However, in constitutive neurogenesis new-born neurons settle mainly at the subventricular zone or in the adjacent parenchyma. Whereas in regenerative neurogenesis newborn neurons migrate longer distances into the injured parenchyma (Baumgart et al., 2012; Kroehne et al., 2011).
If the p53-positive neurons located next to *Tg(olig2:EGFP)*-positive cells are new-born neurons that become myelinated they should be labeled by BrdU.
For all three time points there are no p53 and BrdU double-positive cells present that are located next to *Tg(olig2:EGFP)*-positive cells in the parenchyma of the adult zebrafish telencephalon (Figure 28). Whereas at the ventricular zone BrdU and p53 double-positive cells could be observed next to *Tg(olig2:EGFP)*-positive cells (Figure 28 H', yellow arrow). This result leads to the assumption that the p53-positive neurons located next to *Tg(olig2:EGFP)*-positive cells in the parenchyma are not new-born but already differentiated neurons. This is in accordance with the assumption that p53 expression retains the post-mitotic character of neurons in the adult zebrafish brain (see chapter 3.2.1.2). These data are also in agreement with the findings that new-born neurons settle mainly at the subventricular zone or in the adjacent parenchyma and do not migrate deep into the parenchyma under normal conditions.

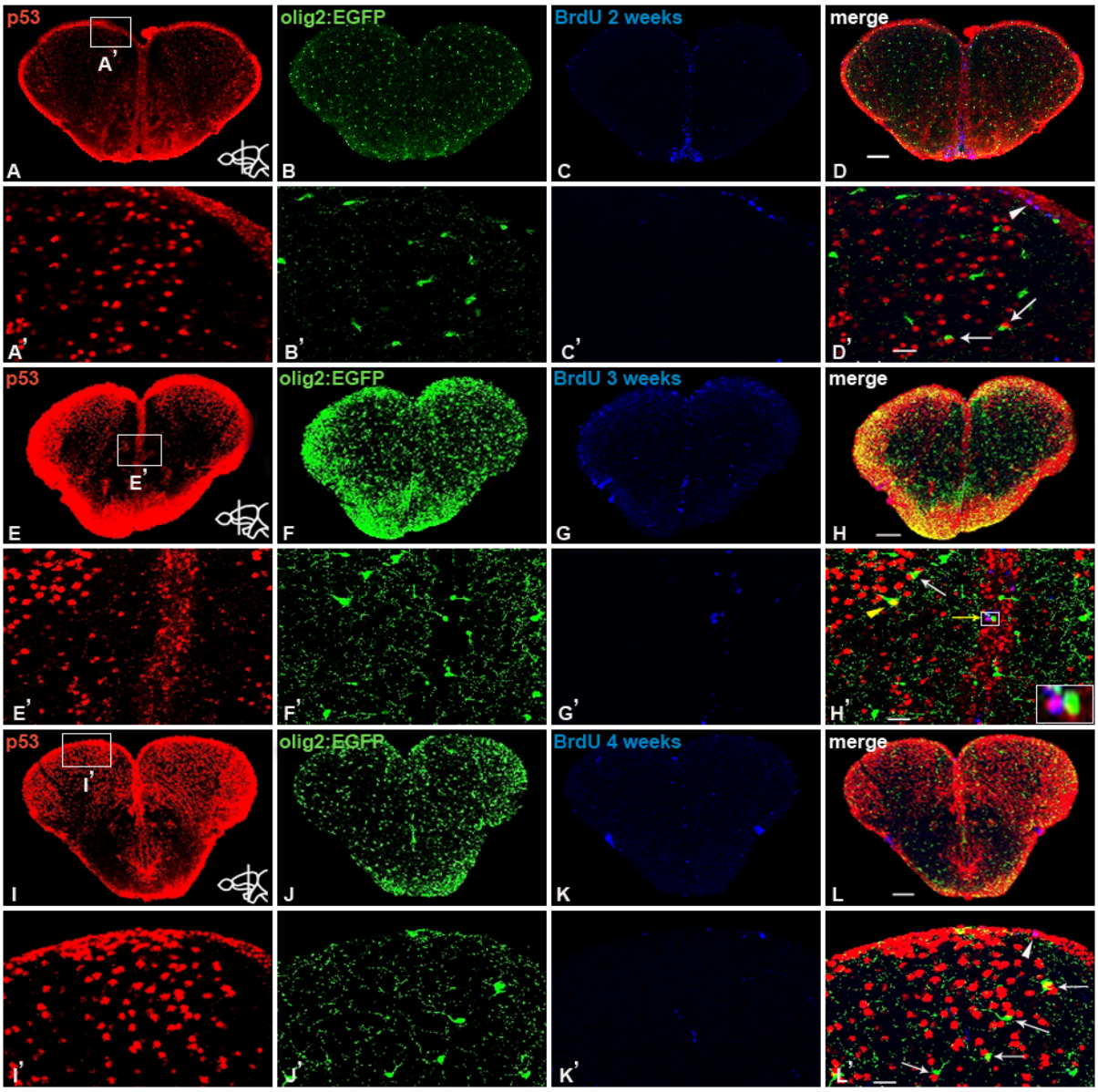

Figure 28: The p53-positive neurons located next to *Tg(olig2:EGFP)*-positive cells are not BrdU-positive in the adult zebrafish telencephalon.

(A-L') To examine if the p53-positive neurons located next to *Tg(olig2:EGFP)*-positive cells are early neurons, BrdU was injected on 3 consecutive days into *Tg(olig2:EGFP)* zebrafish followed by a chase of 2, 3 and 4 weeks, respectively. Subsequently a triple immunostaining was performed on cross sections through the telencephalon of BrdU-injected *Tg(olig2:EGFP)* zebrafish with antibodies against p53 (A-A',E-E' and I-I'), GFP (B-B', F-F' and J-J') and BrdU (C-C', G-G' and K-K'). (D-D', H-H' and L-L') show the merged panels. The inserts in (A), (E) and (I) indicate the anterior-posterior positions and the boxes in (A), (E) and (I) show the regions depicted in higher magnification in (A'-D'), (E'-H') and (I'-L'), respectively. No p53 and BrdU double-positive cells located next to *Tg(olig2:EGFP)*-positive cells in the parenchyma could be observed. But at the ventricle a p53 and BrdU double-positive cell located next to a *Tg(olig2:EGFP)*-positive cell could be detected ((H'), yellow arrow). The box in (H') shows a magnification of these cells. This indicates that the p53-positive neurons located next to *Tg(olig2:EGFP)*-positive cells in the parenchyma are not new-born neurons. In the parenchyma p53-

positive but BrdU-negative cells were located next to *Tg(olig2:EGFP)*- positive cells ((D'), (H') and (L'), white arrows). Also some p53 and BrdU double-positive cells located at the ventricular zone could be observed ((D') and (L'), white arrowheads). A *Tg(olig2:EGFP)*-positive cell interfered with a p53-positive cell which looked like as if they were one double-positive cell ((H'), yellow arrowhead). Scale bars: 100 µm in D, H and L; 20 µm in D', H' and L'.

3.2.2 p53 expression in cells located at the ventricular zone of the adult zebrafish telencephalon

3.2.2.1 The p53 protein is expressed in *Tg(gfap:GFP)*-positive cells

As shown above, the p53 protein is not only expressed in the parenchyma of the adult zebrafish telencephalon but also along the ventricular zone (see chapter 3.1.4 and Figure 24 A-C). März et al. showed that there are distinct progenitor cell types present at the ventricular zone of the adult zebrafish telencephalon which are heterogenic in terms of their proliferation rate (see chapter 1.1.2.1). The different cell types are characterized by the expression of a specific set of markers, which classifies them into at least four cell types. Type I cells, which are non-dividing RGCs, and Type II cells, which are dividing RGCs, express radial glial markers like S100β and GFAP and the progenitor marker Nestin. Additionally, the dividing Type II cells express the proliferation marker PCNA. Type IIIa cells are characterized by a low expression of glial markers and they also express PCNA. Moreover, they express PSA-NCAM which is a neuronal progenitor marker. Type IIIb cells are neuroblasts and lack expression of all glial markers but they are positive for PCNA and PSA-NCAM (März et al., 2010a).

In order to investigate if p53 is expressed in the different cell types located at the ventricular zone in the adult zebrafish telencephalon I first examined if p53 is expressed in GFAP-positive cells. Cross sections through the telencephalon of *Tg(gfap:GFP)* fish (Bernardos and Raymond, 2006) were co-immunostained with an anti-GFP and the anti-p53 antibody. The nuclei were stained with the nuclear marker DRAQ5.

The p53 protein is expressed in *Tg(gfap:GFP)*-positive cells (Figure 29). Only a very low number of *Tg(gfap:GFP)*-positive cells show co-expression with p53 (Figure 29 D' and D'', white arrows). These p53 and *Tg(gfap:GFP)* double-positive cells are most likely either Type I or Type II cells (RGCs). But since also Type IIIa cells show a low expression of glial markers they could also be neural progenitors.

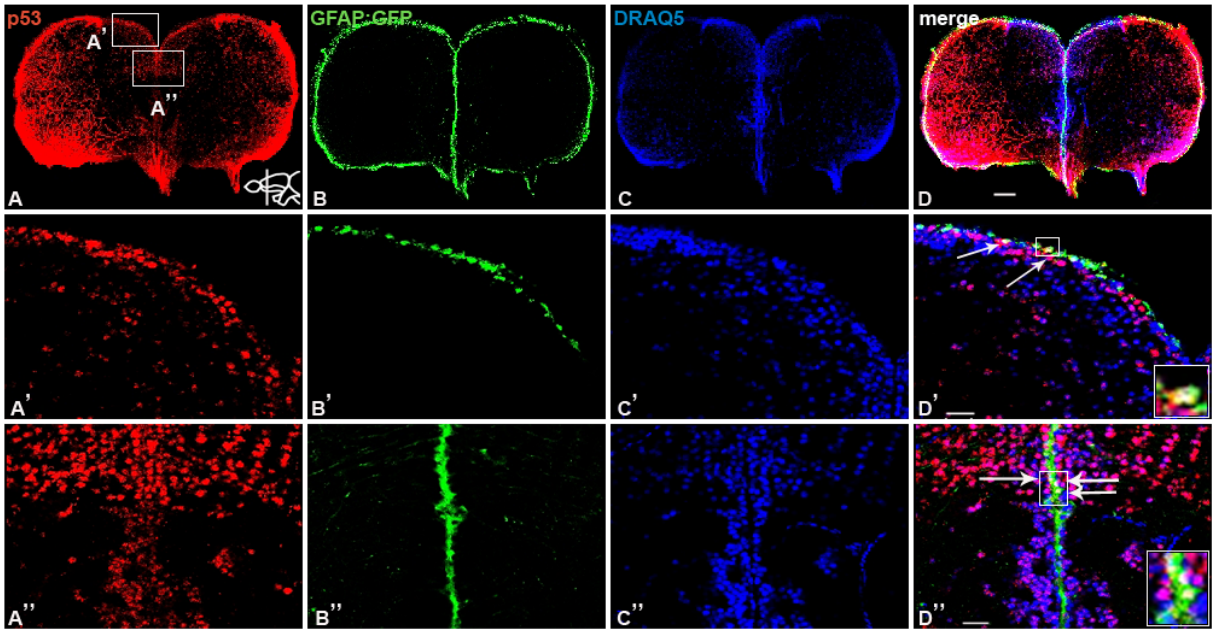

Figure 29: The p53 protein is expressed in *Tg(gfap:GFP)*-positive cells in the adult zebrafish telencephalon.

(A-D'') Co-immunostaining of cross sections through the telencephalon of adult *Tg(gfap:GFP)* zebrafish with antibodies against p53 (A-A'') and GFP (B-B'') was performed to investigate if the p53 protein is expressed in *Tg(gfap:GFP)*-positive cells. The nuclei were stained with the nuclear marker DRAQ5 (C-C''). (D-D'') show the merged panels. These pictures display a representative vibratome section through the anterior telencephalon. The insert in (A) indicates the anterior-posterior position and the boxes in (A) show the regions depicted in higher magnification in (A'-D', upper box) and (A''-D'', lower box). The p53 protein is expressed in some *Tg(gfap:GFP)*-positive cells ((D') and (D'')), white arrows). The boxes in (D') and (D'') show magnified triple-positive cells for p53, GFP and DRAQ5. Scale bars: 100 µm in D; 20 µm in D' and D''.

3.2.2.2 The p53 protein is expressed in Type I but not in Type II cells

The p53 protein is expressed in *Tg(gfap:GFP)*-positive cells (see chapter 3.2.2.1 and Figure 29), which could be Type I (non-dividing RGCs) or Type II (dividing RGCs) cells. In order to investigate in which of these two cell types p53 is expressed, a triple immunostaining of cross sections through the telencephalon of *Tg(gfap:GFP)* fish with antibodies against GFP and p53 and against the proliferation marker PCNA was performed.

The p53 protein is expressed in *Tg(gfap:GFP)*-positive and PCNA-negative cells (Figure 30 D'' and H', white arrows). These cells are Type I cells. Furthermore, it is expressed in PCNA-positive and *Tg(gfap:GFP)*-negative cells (Figure 30 D', D'' and H', white arrowheads), which could be Type IIIb cells. There are also *Tg(gfap:GFP)*-positive cells present that are negative

for p53 and PCNA (Figure 30 D' and H', yellow arrows). Thus, p53 seems not to be expressed in all Type I cells. Since p53 is not expressed in PCNA- and *Tg(gfap:GFP)*-positive cells it is not expressed in proliferating Type II but only in quiescent Type I cells. In the mouse brain p53 is expressed in neural stem and progenitor cells as well and suppresses the self-renewal of adult neural stem cells. (Gil-Perotin et al., 2006; Li et al., 2008). It was suggested that it could be crucial to suppress the self-renewal of stem cells by p53 for long-term maintenance of the stem cell population (Meletis et al., 2006). The expression of the p53 protein in quiescent Type I cells in the adult zebrafish telencephalon is in accordance with the antiproliferative capacities of p53.

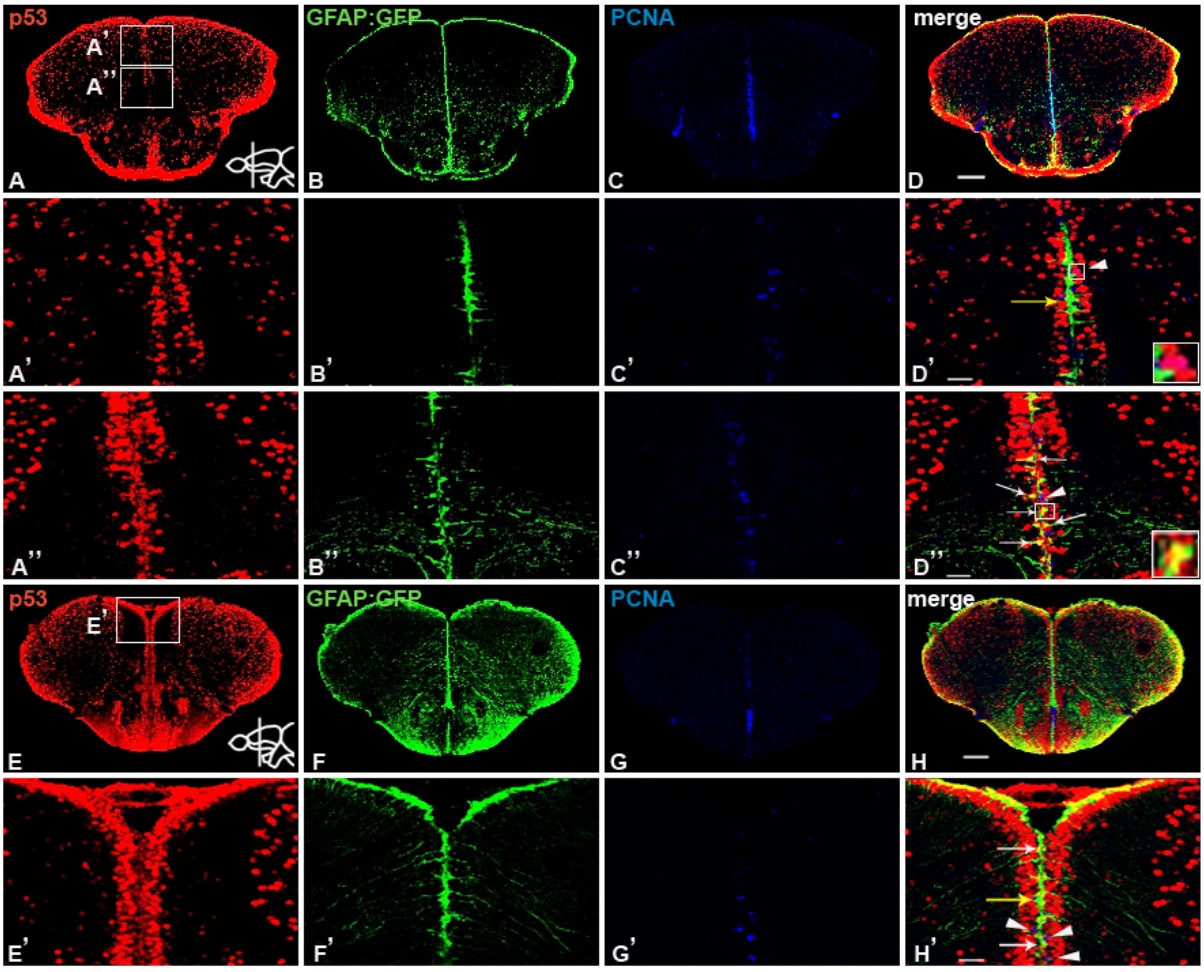

Figure 30: The p53 protein is expressed in Type I cells in the adult zebrafish telencephalon.
(A-H') To investigate if the p53 protein is expressed in Type I and/or Type II cells a triple immunostaining of cross sections through the telencephalon of adult *Tg(gfap:GFP)* zebrafish with antibodies against p53 (A-A'' and E-E'), GFP (B-B'' and F-F') and the proliferation marker PCNA (C-C'' and G-G') was performed. (D-D'' and H-H') show the merged panels. The inserts in (A) and (E) indicate the anterior-posterior positions and the boxes in (A) and (E) show the regions depicted in

higher magnification in (A'-D'), (A''-D'') and (E'-H'), respectively. The p53 protein is expressed in PCNA-positive and *Tg(gfap:GFP)*-negative cells (D', D'' and H', white arrowheads), which could be Type IIIb cells. It is also expressed in *Tg(gfap:GFP)*-positive and PCNA-negative cells (D'' and H', white arrows), which are Type I cells. Since it is not expressed in PCNA- and *Tg(gfap:GFP)*-positive cells, p53 seems not to be expressed in Type II cells. There are also *Tg(gfap:GFP)*- positive cells that do not co-express p53 (D' and H', yellow arrows). The boxes in (D') and (D'') show magnified double-positive cells either for p53 and PCNA (D') or p53 and *Tg(gfap:GFP)* (D''). Scale bars: 100 µm in D and H; 20 µm in D', D'' and H'.

3.2.2.3 The p53 protein is expressed in Type IIIa and Type IIIb cells

The p53 protein is expressed in *Tg(gfap:GFP)*-positive and PCNA-negative cells, which are Type I cells, at the ventricular zone of the adult zebrafish telencephalon (see chapter 3.2.2.1 and 3.2.2.2; see Figure 29 and Figure 30). Furthermore, p53 is expressed in PCNA-positive and *Tg(gfap:GFP)*-negative cells which could be Type IIIb cells (see chapter 3.2.2.2 and Figure 30). To further investigate if p53 is expressed in Type IIIb cells a triple immunostaining of cross sections from *Tg(-3.9nestin:GFP)* fish, which mark progenitor cells, (Lam et al., 2009) with antibodies against GFP, p53 and PSA-NCAM, a marker for early neuronal precursors (Type III cells), was performed.

The p53 protein is expressed in Type IIIa (*Tg(-3.9nestin:GFP)*- and PSA-NCAM-positive) (Figure 31 D', white arrow) and in Type IIIb cells (*Tg(-3.9nestin:GFP)*-negative and PSA-NCAM-positive) (Figure 31 D', white arrowhead) in the area of the RMS, where Type III cells are predominantly located (März et al., 2010a). As demonstrated before (see chapter 3.2.1.4), p53 is expressed in mature neurons in the adult zebrafish telencephalon. Therefore, p53 seems to be expressed both in mature neurons in the parenchyma and in neural progenitor cells at the ventricular zone of the adult zebrafish telencephalon.

Normally, Type IIIa cells show also a weak expression of *Tg(gfap:GFP)* and they are also PCNA-positive. But p53 is not expressed in PCNA- and *Tg(gfap:GFP)*-positive cells, which could lead to the assumption that p53 is not expressed in Type IIIa cells. The staining for *Tg(-3.9nestin:GFP)*, p53 and PSA-NCAM shows, that this assumption is wrong. It is possible that the *Tg(gfap:GFP)*-expression was too weak to be detected and that the PCNA-positive cells in Figure 30 represent both Type IIIa and IIIb cells.

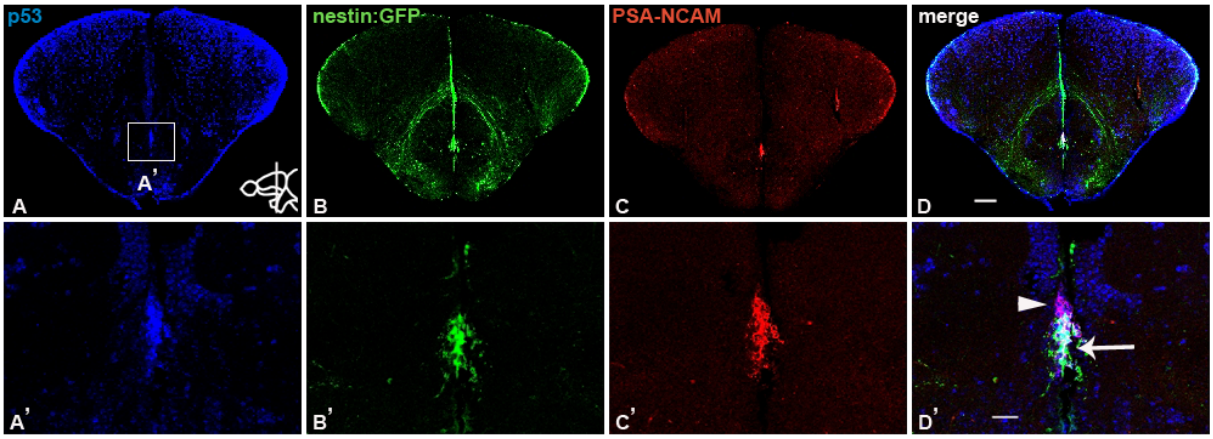

Figure 31: The p53 protein is expressed in Type IIIa and IIIb cells in the adult zebrafish telencephalon.

(A-D') A triple immunostaining of cross sections through the telencephalon of adult *Tg(-3.9nestin:GFP)* zebrafish with antibodies against p53 (A-A'), GFP (B-B') and PSA-NCAM (C-C') was performed to examine if the p53 protein is expressed in Type III cells. (D-D') show the merged panels. These pictures display a representative vibratome section through the medial telencephalon. The insert in (A) indicates the anterior-posterior position and the box in (A) shows the region of the RMS depicted in higher magnification in (A'-D'). The p53 protein is expressed both in Type IIIa cells, which are *Tg(-3.9nestin:GFP)*-positive and PSA-NCAM-positive ((D'), white arrow), and in Type IIIb cells, which are *Tg(-3.9nestin:GFP)*-negative and PSA-NCAM-positive ((D'), white arrowhead). Scale bars: 100 µm in D; 20 µm in D'.

In summary, it was demonstrated in this chapter that p53 is not expressed in *Tg(olig2:EGFP)*-positive cells but in HuC/D-positive mature neurons, some of which are located next to *Tg(olig2:EGFP)*-positive cells in the parenchyma of the adult zebrafish telencephalon. Moreover, it was shown that p53 expression was detected at the ventricular zone of the adult zebrafish telencephalon in Type I, Type IIIa and Type IIIb cells but not in Type II cells.

3.3 Expression of p53 and p73 in the adult zebrafish telencephalon following injury

Zebrafish exhibit a remarkable regenerative potential to repair lesions of the CNS (see chapter 1.1.2.2) (Adolf et al., 2006; Chapouton et al., 2007; Grandel et al., 2006; Kaslin et al., 2008; Zupanc, 2001; Zupanc and Zupanc, 2006). After inflicting a stab wound on the zebrafish telencephalon several processes are started to repair the tissue damage. There is an increase in the expression of GFAP and also in proliferation. Moreover, OPCs accumulate at the site of the lesion. However, this accumulation is, contrary to mammals, only transient. Additionally, also the number of microglia cells at the lesion is increased (März et al., 2011). Kyritsis et al. showed that after a brain injury an active inflammatory response, which is characterized by the activation of leukocytes and microglia, takes place. This inflammation persists only a few days and does not lead to a chronic inflammation. Another early response to injury is cell death (see chapter 1.1.2.2) (Kroehne et al., 2011).
In this chapter I investigated how the expression of p53 and p73 changes in response to a stab wound of the adult zebrafish telencephalon. Therefore *in situ* hybridizations and immunohistochemistry were performed to examine if there is a difference in the expression of p53 and p73, respectively, in an injured brain compared to an intact brain. An altered expression of p53 and p73 as a reaction to an injured telencephalon could be a hint to a potential role for p53 and p73 in regenerative neurogenesis.

3.3.1 The *p53* mRNA is up-regulated in response to stab injury

The *p53* mRNA is broadly expressed in many regions of the adult zebrafish telencephalon (see chapter 3.1.1 and Figure 12). In order to investigate the expression of *p53* mRNA after brain injury an ISH with a *p53* antisense probe on cross sections through the lesioned telencephalon at different time points after injury was performed.
There is an up-regulation of *p53* mRNA in the injured telencephalic hemisphere in comparison to the uninjured site after brain injury at all time points investigated (Figure 32). From 1 to 9 dpl an enhanced expression of *p53* mRNA could be observed at the ventricular zone (black arrowheads) as well as in the region of the lesion canal (black arrows). Especially at 5 dpl the up-regulation of *p53* mRNA at the site of the lesion is very pronounced (Figure 32 C, black arrows). At 1 dpl the up-regulation is not as intense as at the other time points (Figure 32 A). And also at 9 dpl an increased expression of *p53* mRNA was still

observed (Figure 32 E). Therefore, p53 seems to play a role after injury of the brain. In the mammalian brain p53 induces neuronal apoptosis following damage (Miller et al., 2000; Morrison and Kinoshita, 2000). Thus, it could be suggested that p53 induces apoptosis in the damaged brain area around the lesion. Moreover, p53 may prevent over-proliferation of stem cells as a reaction to the injury.

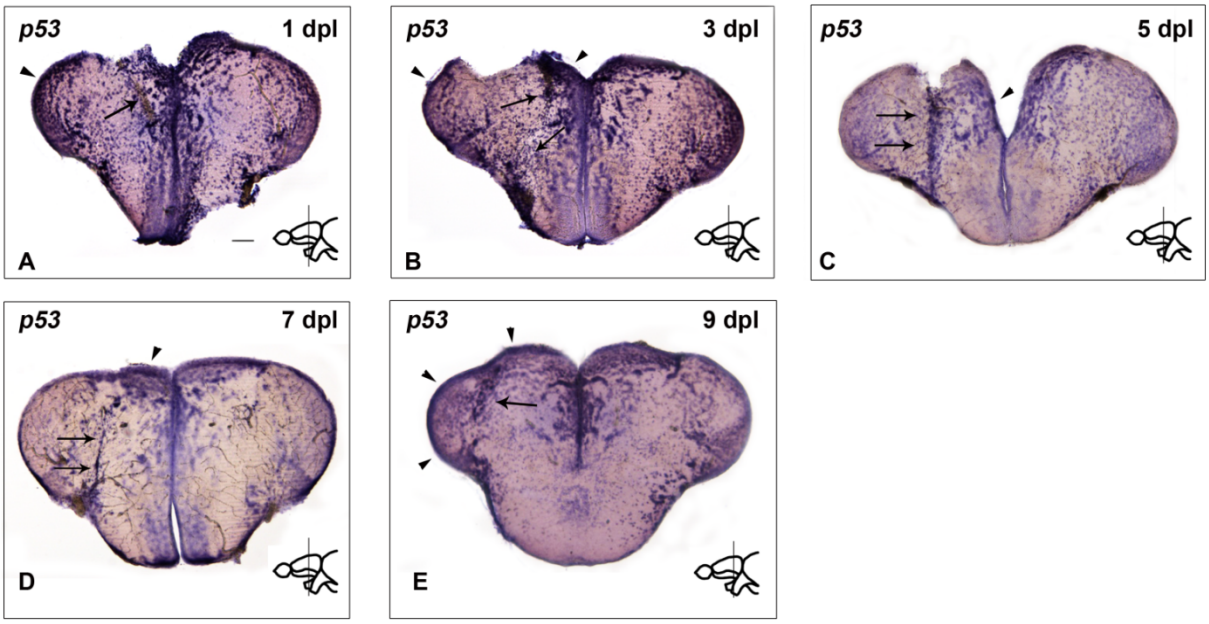

Figure 32: The *p53* mRNA is up-regulated upon stab injury of the adult zebrafish telencephalon. (A-E) *In situ* hybridization with a *p53* antisense probe on cross sections through the telencephalon of adult zebrafish at different time points after inflicting a stab wound to investigate the expression of *p53* mRNA after brain injury. The hemispheres containing the lesion are always depicted on the left side. The right uninjured hemispheres serve as the control. The inserts indicate the anterior-posterior positions of the sections. For all time points investigated (1-9 dpl) an up-regulation of *p53* mRNA both at the site of the lesion ((A-E), black arrows) and at the ventricular zone of the hemisphere containing the lesion ((A-E), black arrowheads) was observed. Scale bar: 100 µm.

3.3.2 The *p73* mRNA is up-regulated in response to stab injury

Like *p53* mRNA, the *p73* mRNA is broadly expressed in many regions of the adult zebrafish telencephalon (see chapter 3.1.2 and Figure 14). Hence, ISH with a *p73* antisense probe on cross sections through the injured telencephalon at different time points after inflicting a stab wound was performed.

There is, in contrast to the control hemisphere, an up-regulation of *p73* mRNA on the telencephalic hemisphere containing the lesion at all time points examined (Figure 33). For 1 to 7 dpl an elevated expression of *p73* mRNA could be observed at the ventricular zone (Figure 33 A-D, black arrowheads) and for 3 to 7dpl there was additionally an increase of *p73* mRNA expression in the region of the lesion canal (Figure 33 B-D, black arrows). At 9 dpl an up-regulation of *p73* mRNA could only be observed at the area of the lesion (Figure 33 E, black arrow). Hence, the *p73* mRNA reacts in a similar way to brain injury as the *p53* mRNA and thus seems to play a role in the response to brain damage as well. In mammals p73 is able to support survival of neurons (ΔNp73 isoforms) or to induce apoptosis (TAp73 isoforms) (see chapter 1.2.3.4) (Pozniak et al., 2002; Pozniak et al., 2000). Thus it could be suggested that in zebrafish p73 acts synergistically to p53 and induces apoptosis and prevents the cells from over-proliferation. And/or p73 acts antagonistically to p53 and inhibits the death of cells thereby preventing a severe damage of the brain due to loss of a high number of cells.

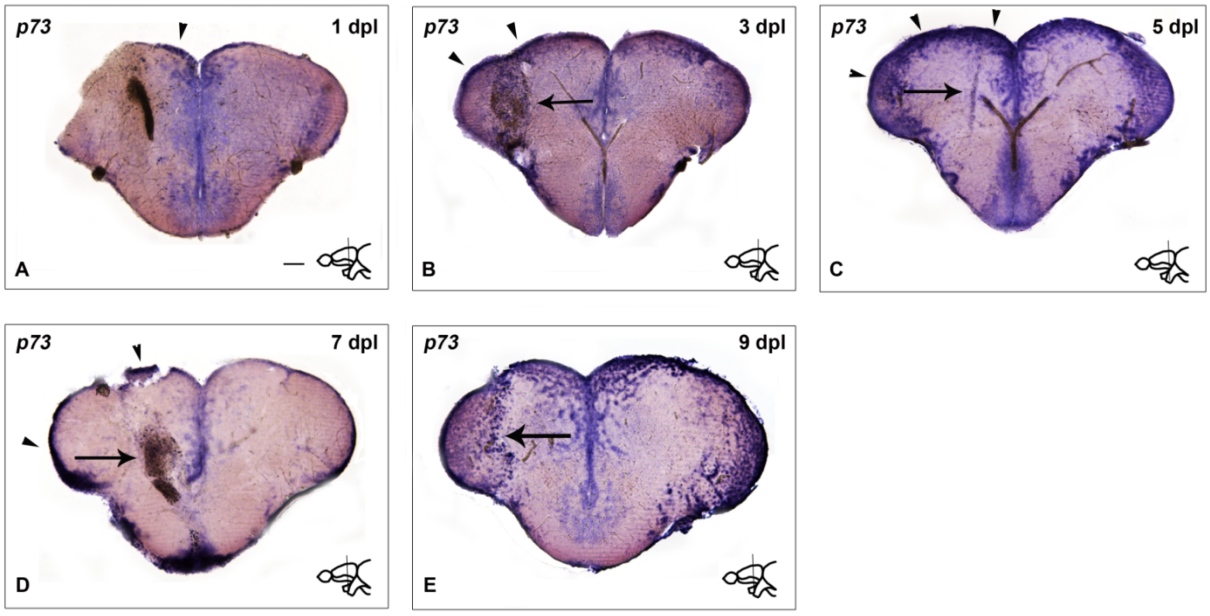

Figure 33: The *p73* mRNA is up-regulated upon stab injury of the adult zebrafish telencephalon. (A-E) *In situ* hybridization with a *p73* antisense probe on cross sections through the telencephalon of adult zebrafish at different time points after stab injury was performed in order to examine the expression of *p73* mRNA in injured brains. The hemispheres containing the lesion are always depicted on the left side. The right uninjured hemispheres serve as the control. The inserts indicate the anterior-posterior positions of the sections. An up-regulation of *p73* mRNA could be observed at the ventricular zone of the injured hemisphere ((A-D), black arrowheads) and/or at the site of the lesion ((B-E), black arrows) at 1 to 9 dpl. Scale bar: 100 µm.

3.3.3 The p53 protein is up-regulated in response to stab injury

p53 is known to be regulated at the protein level (see chapter 1.2.1.2) (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). To investigate if the expression of the p53 protein is also up-regulated after brain injury, an immunostaining of cross sections through the injured telencephalon with the anti-p53 antibody at different time points after inflicting a stab wound was performed. The nuclei were stained with the nuclear marker DRAQ5.

There is an up-regulation of p53 protein on the injured telencephalic hemisphere in comparison to the uninjured site at all time points investigated (Figure 34). For 1 to 9 dpl an elevated expression of p53 protein could be observed at the ventricular zone (Figure 34 A-E, white arrowheads). In contrast to the *p53* mRNA, which shows an up-regulation both at the ventricular zone and at the site of the lesion, there is no overall increase in p53 protein expression in the region of the lesion. Normally, when the mRNA is present also the protein should be detectable. In the region of the lesions at 1, 3 and 5 dpl only an intense DRAQ5 staining was observed (Figure 34 A-C, white arrows). Except the region of the lesion canal, the p53 protein shows almost the same up-regulation following stab injury as the mRNA. At the site of the lesion *p53* mRNA and p53 protein are regulated differentially. Thus, p53 may not play a role at the lesion site following brain injury. However, it cannot be excluded that there are changes in p53 protein expression in individual cells.

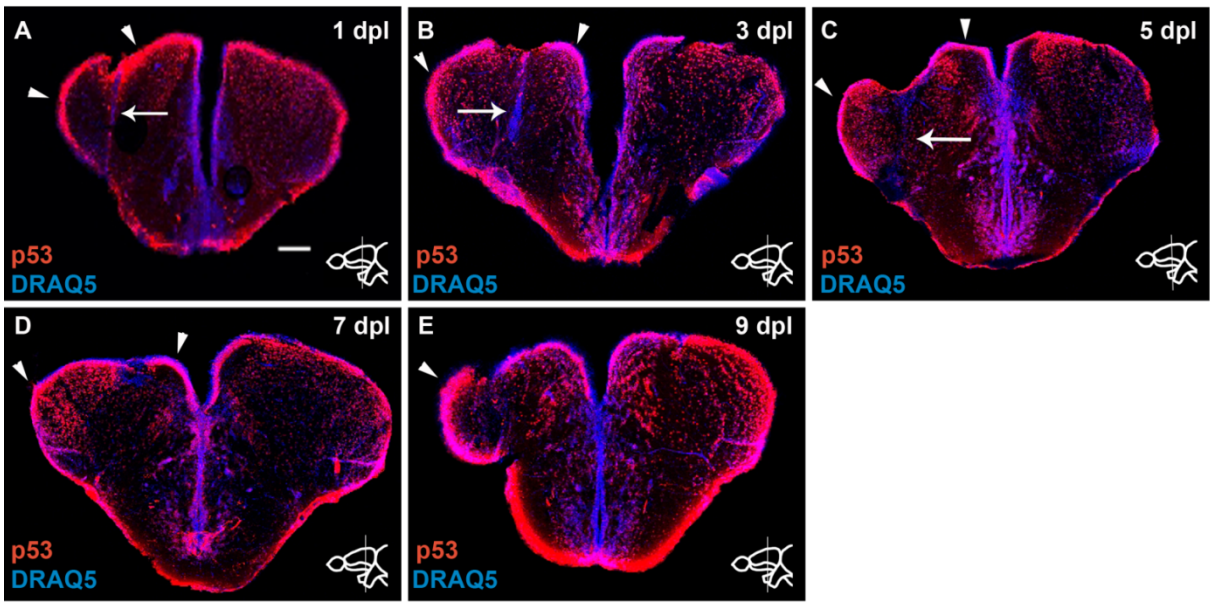

Figure 34: The p53 protein is up-regulated upon stab injury of the adult zebrafish telencephalon.

(A-E) Immunohistochemistry with the anti-p53 antibody on cross sections through the telencephalon of adult zebrafish at different time points after inflicting a stab wound to investigate if the expression of the p53 protein is up-regulated after brain injury. The nuclei were stained with the nuclear marker DRAQ5. The hemispheres containing the lesion are always depicted on the left side. The right uninjured hemispheres serve as the control. The inserts indicate the anterior-posterior positions of the sections. For 1 to 9 dpl an up-regulation of the p53 protein at the ventricular zone of the injured hemisphere could be observed (white arrowheads). In contrast to the *p53* mRNA, no up-regulation of the p53 protein at the site of the lesion was detected. The regions of the lesions show an intense DRAQ5 staining at 1, 3 and 5 dpl (A-C, white arrows). Scale bar: 100 µm.

3.3.4 Expression of the p53 protein in different cell types after stab injury

As shown in chapter 3.2, the p53 protein is expressed in mature neurons, in Type I and Type III cells in the adult zebrafish telencephalon. Moreover, in chapter 3.3.3 it is shown that there is an increase in the expression of p53 following stab injury of the telencephalon. In this chapter I wanted to investigate in which cell types p53 is expressed or up-regulated, respectively, after brain injury and if the expression in different cell types changes after a telencephalic lesion.

3.3.4.1 The expression of p53 in PCNA-positive cells is not changed after stab injury

I showed before that the p53 protein is expressed in PCNA-positive Type III cells and in Type I cells, which are PCNA-negative. It is not expressed in PCNA-positive Type II cells (see chapter 3.2.2.2 and 3.2.2.3; see Figure 30 and Figure 31). Additionally, I could show that the p53 protein is up-regulated at the ventricular zone of the lesioned hemisphere (see chapter 3.3.3 and Figure 34). PCNA is up-regulated after stab injury of the adult zebrafish telencephalon at the ventricle of the injured hemisphere from 3 dpl until at least 14 dpl (März et al., 2011). In order to investigate if the expression of p53 in PCNA-positive cells changes in response to stab injury and if the cells that up-regulate PCNA also show an elevated expression of p53, a co-immunostaining of cross sections through the injured telencephalon at 5 dpl with the anti-p53 and with an anti-PCNA antibody was performed. The nuclei were stained with the nuclear marker DRAQ5. Since at 5 dpl a strong up-regulation of both p53

(see chapter 3.3.3 and Figure 34) and PCNA (März et al., 2011) could be observed, this time point was chosen for the following experiments.

Both p53 and PCNA are up-regulated at the ventricular zone of the lesioned hemisphere in comparison to the control side after stab injury of the adult zebrafish telencephalon (Figure 35). p53 is expressed in a few PCNA-positive cells, which are most likely Type III cells (Figure 35 D' and D'', white arrows), but the majority of PCNA-positive cells is p53-negative (Figure 35 D' and D'', yellow arrows). This indicates that the expression of p53 is not up-regulated in PCNA-positive cells but in a different cell type and that the expression of p53 in PCNA-positive cells is not changed after brain injury. I showed before that p53 is expressed in Type I cells. It is likely that the cells in which p53 is up-regulated after stab injury are Type I cells. The PCNA-positive cells could be Type II cells since these cells show an increased proliferation after brain injury (März et al., 2011). Therefore, p53 is not expressed in Type II cells both in the uninjured telencephalon and in a telencephalon containing a lesion. It could by hypothesized that the up-regulation of p53 in Type I cells prevents an exceeding proliferation of stem cells and thus depletion of the stem cell pool. Thus p53 could ensure the maintenance of the stem cell pool in the adult zebrafish telencephalon after injury.

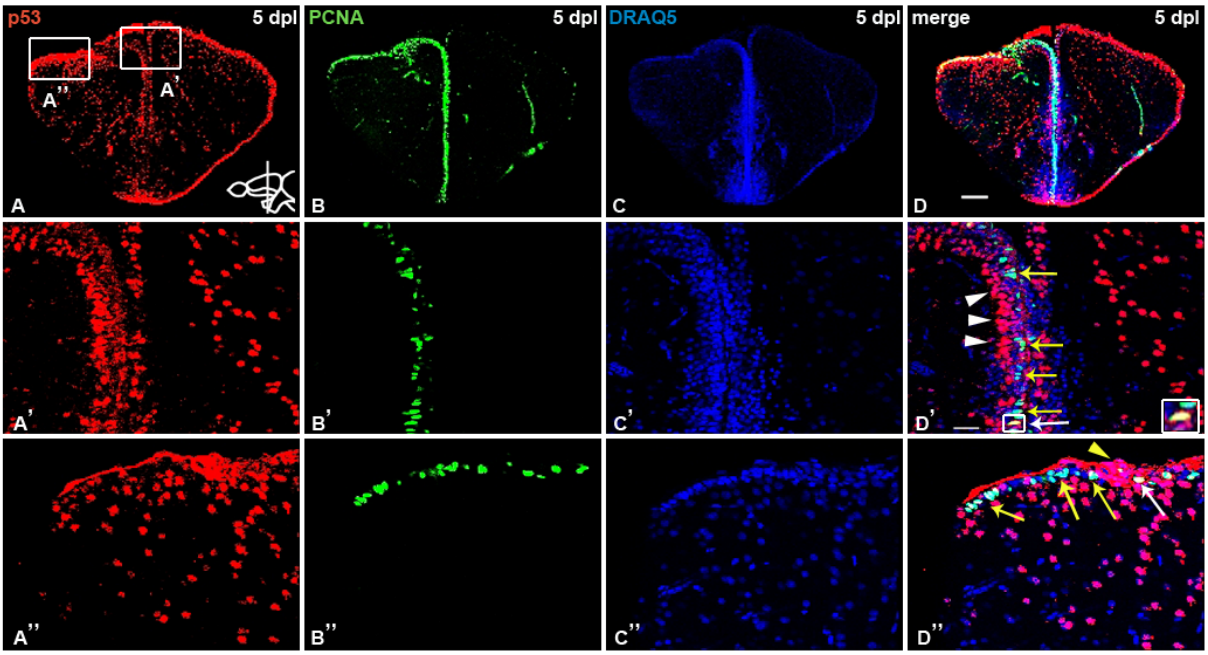

Figure 35: The p53 protein is not up-regulated in PCNA-positive cells after stab injury.

(A-D'') Co-immunostaining with the anti-p53 (A-A'') and with an anti-PCNA (B-B'') antibody of cross sections through the telencephalon of adult zebrafish 5 days post lesion to investigate the expression of p53 and PCNA following brain injury. The nuclei were stained with the nuclear marker DRAQ5 (C-C''). (D-D'') show the merged panels. The hemisphere containing the lesion is oriented to the left side.

The right uninjured hemisphere serves as the control. The insert in (A) indicates the anterior-posterior position of the section and the boxes in (A) show the regions depicted in higher magnification in (A'-D', right box) and (A''-D'', left box). Both the p53 protein (D', white arrowheads) and PCNA are up-regulated in response to brain injury. p53 is expressed in a few PCNA-positive cells (D' and D'', white arrows) but the majority of PCNA-positive cells is negative for p53 (D' and D'', yellow arrows). The box in (D') shows a magnified double-positive cell for p53 and PCNA. Thus the p53 protein is not up-regulated in PCNA-positive cells and therefore the expression of p53 in PCNA-positive cells is not changed after stab injury of the telencephalon. The yellow arrowhead in (D'') points to the region of the lesion. Scale bars: 100 µm in D; 20 µm in D' and D''.

3.3.4.2 The expression of p53 in HuC/D-positive neurons is not changed after stab injury

As demonstrated in chapter 3.2.1.2, the p53 protein is expressed in mature HuC/D-positive neurons in the adult uninjured zebrafish telencephalon (see also Figure 26). Moreover, I showed that p53 is up-regulated at the ventricular zone following brain injury (see chapter 3.3.3 and Figure 34). To analyze if the expression of p53 changes in neurons after inflicting a stab injury, a co-immunostaining of cross sections through the telencephalon at 5 dpl with the anti-p53 antibody and an antibody against the neuronal marker HuC/D was conducted. The nuclei were stained with the nuclear marker DRAQ5.

There is no difference between the uninjured and the injured hemisphere concerning the expression of p53 in HuC/D-positive neurons 5 dpl (Figure 36). Both in the control (Figure 36 D', white arrowheads) and in the injured hemisphere (Figure 36 D'', white arrowheads) p53 is expressed in HuC/D-positive neurons. Furthermore, also in the region of the lesion p53 remains expressed in HuC/D-positive neurons (Figure 36 D''', white arrowheads). This indicates that there is no overall change in the expression of p53 in neurons following stab injury of the telencephalon.

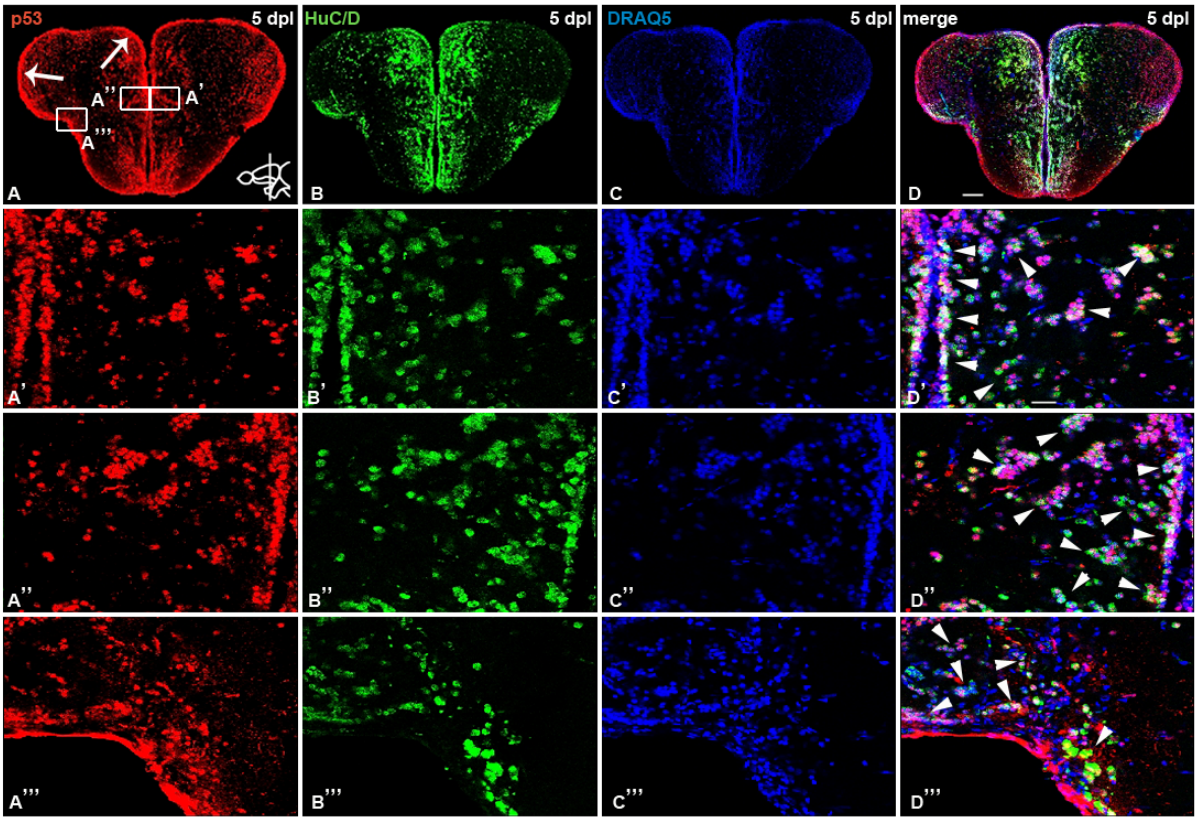

Figure 36: The expression of p53 in neurons is not changed after stab injury.

(A-D''') Co-immunostainig of cross sections through the telencephalon of adult zebrafish 5 dpl with the anti-p53 (A-A''') and with an anti-HuC/D (B-B''') antibody to examine if the expression of p53 in HuC/D-positive neurons changes after brain injury. The nuclei were stained with the nuclear marker DRAQ5 (C-C'''). (D-D''') show the merged panels. The hemisphere containing the lesion is depicted on the left side. The right uninjured hemisphere serves as the control. The insert in (A) indicates the anterior-posterior position of the section and the boxes in (A) show the regions depicted in higher magnification in (A'-D', right box), (A''-D'', left box) and (A'''-D''', lower box). The p53 protein is up-regulated following stab injury (A, white arrows). p53 is still expressed in HuC/D-positive neurons (D'-D''', white arrowheads) after brain injury. Both in the uninjured control (D', white arrowheads) and in the injured (D'', white arrowheads) hemisphere there are a lot of p53- and HuC/D- double-positive neurons visible. At the site of the lesion p53-positive neurons were also observed (D''', white arrowheads). Thus, the expression of p53 in neurons is not changed 5 days after a stab injury. Scale bars: 100 µm in D; 20 µm in D', D'', and D'''.

3.3.4.3 The p53 protein is expressed in *Tg(olig2:EGFP)*-positive cells after stab injury

In chapter 3.2.1.1 and 3.2.1.3 I showed, that in an uninjured telencephalon the p53 protein is not expressed in *Tg(olig2:EGFP)*-positive cells but in HuC/D-positive neurons located next to *Tg(olig2:EGFP)*-positive cells (see also Figure 25 and Figure 27). In addition, it was shown

that *Tg(olig2:EGFP)*-positive OPCs accumulate at the site of the lesion after stab injury. However, this accumulation is only transient (März et al., 2011), which is in contrast to the situation in mammals (Burns et al., 2009; Dimou et al., 2008; Hampton et al., 2004; Magnus et al., 2007). To study the expression of p53 in *Tg(olig2:EGFP)*-positive cells after inflicting a stab injury on the adult zebrafish telencephalon, a triple immunostaining of cross sections through the telencephalon of *Tg(olig2:EGFP)* fish with antibodies against p53, HuC/D and GFP was performed 5 days after brain injury. This time point was chosen since at 5 dpl p53 shows a strong up-regulation and also the accumulation of OPCs is detectable (März et al., 2011).

The expression of p53 in *Tg(olig2:EGFP)*-positive cells changes after inflicting a stab injury on the adult zebrafish telencephalon (Figure 37). The expression of p53 is up-regulated at the ventricular zone of the injured hemispheres compared to the control hemispheres (Figure 37 A and E, white arrows) and *Tg(olig2:EGFP)*-positive cells accumulate at the site of the lesions (Figure 37 B and F, yellow arrows). Under normal conditions p53 is not expressed in *Tg(olig2:EGFP)*-positive cells but in HuC/D-positive neurons residing next to them. After stab injury there were also p53- and HuC/D-double-positive cells located next to *Tg(olig2:EGFP)*-positive cells detectable in the uninjured hemisphere (Figure 37 D' and H', yellow arrowheads). In contrast to the uninjured brain, there were p53- and *Tg(olig2:EGFP)*-double-positive cells present as well in the injured telencephalic hemisphere close to the lesion (Figure 37 D'' and H'', white arrowheads). But also in the control hemisphere single p53- and *Tg(olig2:EGFP)*-double-positive were observed (Figure 37 D', white asterisk). These results indicate that the expression of p53 in *Tg(olig2:EGFP)*-positive cells is changed 5 days after brain injury as p53 is expressed in *Tg(olig2:EGFP)*-positive cells that accumulate at the lesion. Probably p53 prevents OPCs to proliferate at the site of the lesion.

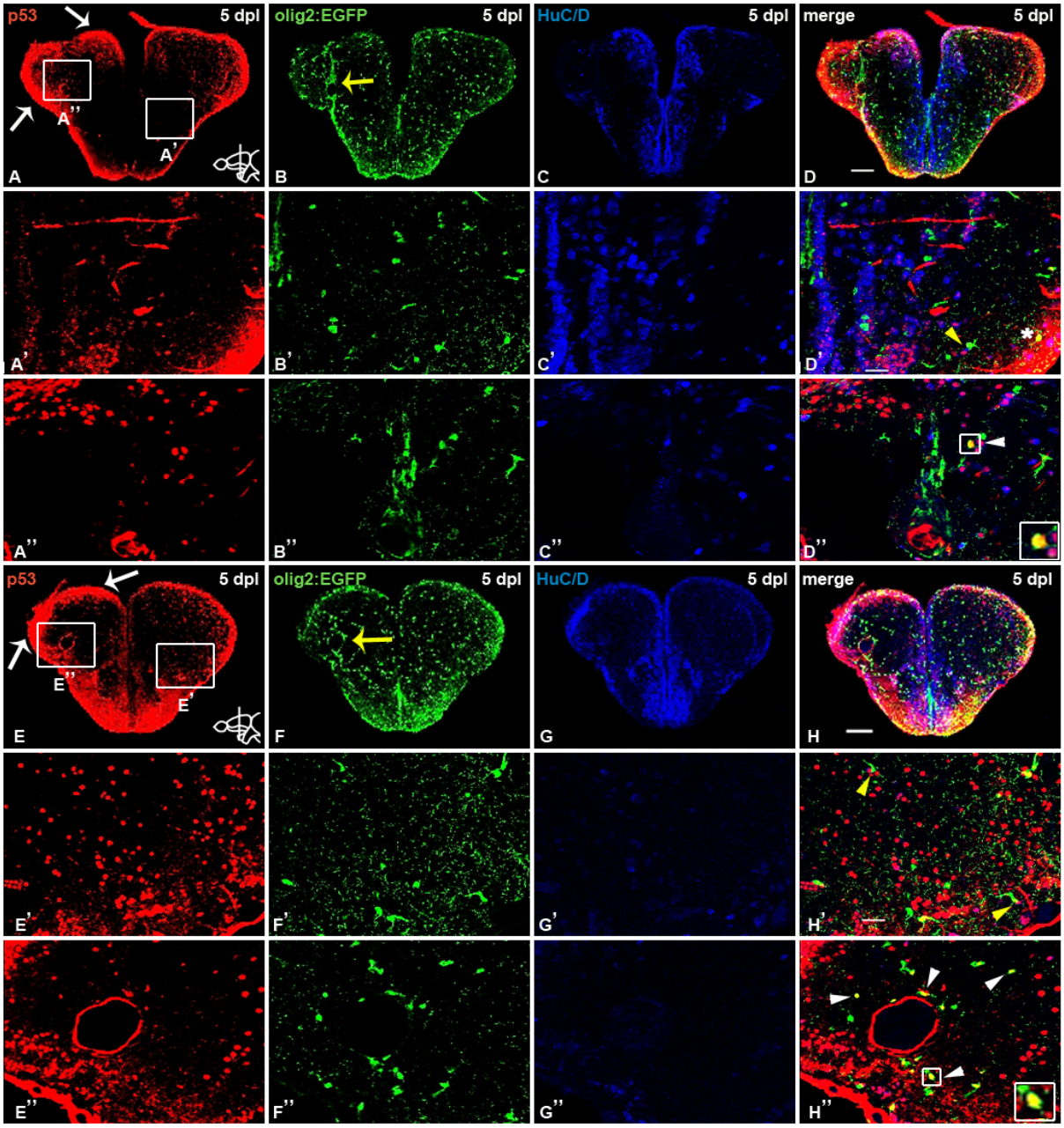

Figure 37: p53 is expressed in *Tg(olig2:EGFP)*-positive cells after brain injury.

(A-H'') A triple immunostaining of cross sections through the telencephalon of adult *Tg(olig2:EGFP)* fish 5 dpl with antibodies against p53 (A-A'' and E-E''), GFP (B-B'' and F-F'') and the neuronal marker HuC/D (C-C'' and G-G'') was performed to analyze the expression of p53 in *Tg(olig2:EGFP)*-positive cells after stab injury. (D-D'' and H-H'') show the merged panels. The inserts in (A) and (E) indicate the anterior-posterior positions and the boxes in (A) and (E) show the regions depicted in higher magnification in (A'-D', right box), (A''-D'', left box), (E'-H', right box) and (E''-H'', left box), respectively. The hemispheres containing the lesion are oriented to the left. The right uninjured hemispheres serve as the controls. The p53 protein is up-regulated at the ventricular zone of the injured hemisphere (A and E, white arrows) and *Tg(olig2:EGFP)*-positive cells accumulate at the site of the lesion (B and F, yellow arrows) after stab injury. In the control hemispheres p53- and HuC/D-double-positive cells are located next to *Tg(olig2:EGFP)*-positive cells (D' and H', yellow arrowheads). In the injured

hemispheres *Tg(olig2:EGFP)*- and p53-double-positive cells could be observed near the lesion (D'' and H'', white arrowheads). The boxes in (D'') and (H'') show magnified double-positive cells for p53 and *Tg(olig2:EGFP)*. Also in the uninjured hemisphere single *Tg(olig2:EGFP)*- and p53-double-positive cells could be detected (D', white asterisk). Thus, the expression of p53 in *Tg(olig2:EGFP)*-positive cells is changed 5 days after stab injury since in an uninjured telencephalon p53 is not expressed in *Tg(olig2:EGFP)*-positive cells. Scale bars: 100 µm in D and H; 20 µm in D', D'', H' and H''.

3.3.5 Expression of p53 target genes after stab injury

As shown before, the expression of p53 is up-regulated after stab injury of the adult zebrafish telencephalon (see chapter 3.3.1 and 3.3.3; see Figure 32 and Figure 34). p53 is a transcription factor that transactivates target genes upon activation by cellular stress, which results in apoptosis, cell cycle arrest or senescence (reviewed in Boehme and Blattner, 2009) (see chapter 1.2.1). Therefore, I intended to find out if there is also an up-regulation of the two best-studied target genes of p53, Mdm2 (Barak et al., 1993; Juven et al., 1993; Kubbutat et al., 1997) and p21 (el-Deiry, 1998; Wang et al., 2007b), after brain injury. If p53 plays a role in regenerative neurogenesis, as apoptosis or inhibition of proliferation, the target genes of p53 should also show a response to stab injury.

3.3.5.1 *Mdm2* mRNA is up-regulated after stab injury

The ubiquitin ligase Mdm2 is a target gene of p53 and the main regulator of p53. Both are linked in an autoregulatory feedback loop (Wu et al., 1993). Under normal conditions p53 is rapidly degraded since Mdm2 ubiquitinates p53 and therefore targets it for degradation by the proteasome. After cellular stress p53 becomes activated and is thereby protected from degradation, which leads to an accumulation of p53 protein. Consequently, p53 is able to induce apoptosis, senescence or cell cycle arrest and also the transcription of Mdm2 (see chapter 1.2.1.2) (Barak et al., 1993; Juven et al., 1993; Kubbutat et al., 1997 ; reviewed in Boehme and Blattner, 2009). To investigate the expression of *Mdm2* mRNA and to find out if *Mdm2* mRNA is up-regulated after stab injury, an ISH with a *Mdm2* antisense probe on cross sections through the uninjured and through the lesioned telencephalon at 4 hours post lesion (hpl) and 1 dpl was performed. The time-points 4 hpl and 1 dpl were chosen because the activation of p53 after cellular stress and thus the activation of target genes of p53 is a

very rapid process (Boehme and Blattner, 2009). Like that, an up-regulation of *p53* mRNA was observed at 1 dpl (Figure 32 A).

Mdm2 mRNA is expressed in several regions of the adult unlesioned zebrafish telencephalon (Figure 38 A-C). The expression of *Mdm2* mRNA was detected in the periventricular zone of the telencephalic ventricle (TelV) (Figure 38 A-C) and the diencephalic ventricle (DiV) (Figure 38 C) as well as in the central (Vc) Figure 38 B), dorsal (Vd) (Figure 38 A-B), ventral (Vv) (Figure 38 B) and postcommisural (Vp) (Figure 38 C) nuclei of the ventral telencephalic area (V). Furthermore, *Mdm2* mRNA was expressed in the central (Dc) zone (Figure 38 B-C) of the dorsal telencephalic area (D), in the anterior (PPa) (Figure 38 C) part of the parvocellular preoptic nucleus and in the sulcus ypsiloniformis (SY) (Figure 38 B). In comparison to the expression of *p53* mRNA (see Figure 12), *Mdm2* mRNA is also expressed in regions where *p53* expression could not be detected, like the central zone of the dorsal telencephalic area, and it is not expressed in regions where *p53* is expressed, like the medial, lateral, dorsal and posterior zone of the dorsal telencephalic area.

In comparison to the control hemisphere, *Mdm2* mRNA is up-regulated at 4 hpl and 1 dpl in the parenchyma of the lesioned hemisphere at the site of the lesion (Figure 38 D-E, black arrowheads). In contrast to *p53,* up-regulation at the ventricular zone of the injured hemisphere was not observed. *p53* and *Mdm2* are co-expressed in several regions of the uninjured adult zebrafish telencephalon and both are up-regulated at the site of the lesion. The up-regulation of *Mdm2* at the lesion site may lead to degradation of p53 protein.

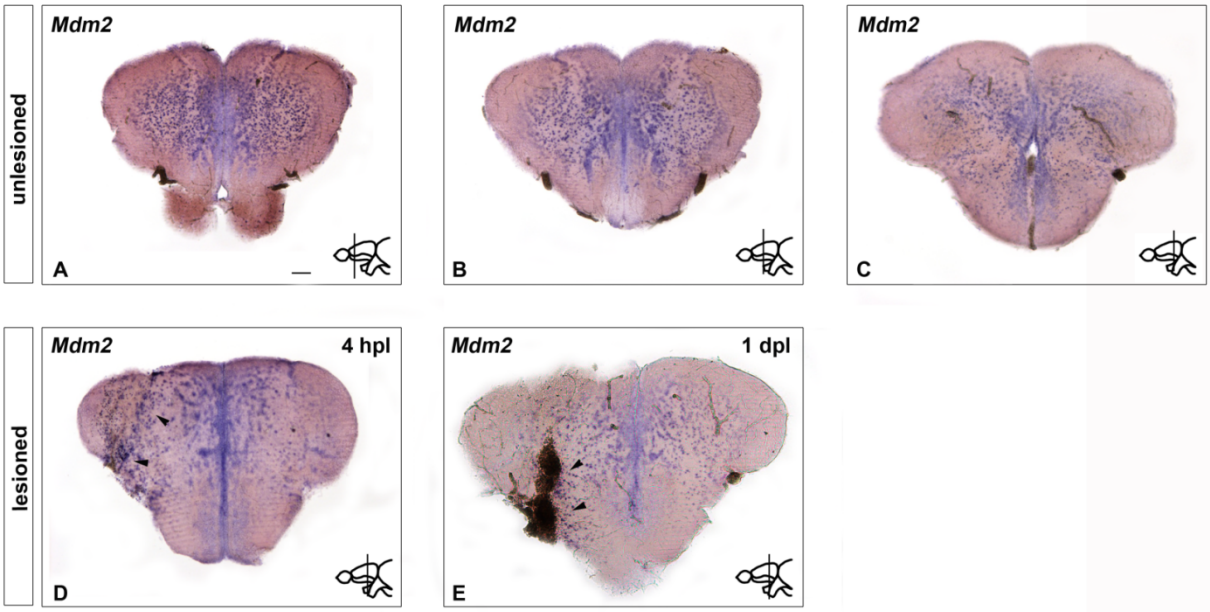

Figure 38: The *Mdm2* mRNA is up-regulated after stab injury of the adult zebrafish telencephalon.

(A-E) *In situ* hybridization with a *Mdm2* antisense probe on cross sections through the unlesioned (A-C) and the lesioned telencephalon of adult zebrafish 4 hpl (D) and 1 dpl (E) to investigate the expression of *Mdm2* mRNA under normal conditions and after stab injury. The hemispheres containing the lesion are oriented to the left side, the right uninjured hemispheres serve as the control (D and E). The inserts indicate the anterior-posterior positions of the sections. *Mdm2* mRNA is expressed in (A) periventricular zone of the TelV, D and Vd; (B) periventricular zone of the TelV, Vv, Vd, Vc, Dc and SY; (C) periventricular zone of the TelV and DiV, PPa, Vp and Dc.

Both at 4 hpl (D) and at 1 dpl (E) an up-regulation of *Mdm2* mRNA in the parenchyma at the site of the lesion was observed (D and E, black arrowheads).

D: dorsal telencephalic area; Dc: central zone of D; DiV: diencephalic ventricle; PPa: parvocellular preoptic nucleus, anterior part; SY: sulcus ypsiloniformis; TelV: telencephalic ventricle; V: ventral telencephalic area; Vc: central nucleus of V; Vd: dorsal nucleus of V; Vp: postcommisural nucleus of V; Vv: ventral nucleus of V. Scale bar: 100µm.

3.3.5.2 *p21* mRNA is only slightly up-regulated after stab injury

p21/CDKN1A (cyclin-dependent kinase inhibitor 1a)/Waf1/Cip1 (further referred to as p21) is an important regulator of the cell cycle. As a target gene of p53 it becomes activated by p53 after cellular stress. p21 is an inhibitor of cyclin-dependent kinases (CDKs) and inactivates cyclin-CDK complexes. This results in the repression of genes that are necessary for proceeding the cell cycle. Therefore, an activation of p53 leads to the activation of p21, which results in cell cycle arrest (see chapter 1.2.1.2) (el-Deiry et al., 1993; Harper et al., 1993;

Jackson et al., 2004). In order to investigate if there is an up-regulation of *p21* mRNA after brain injury and to examine the expression of *p21* mRNA in an uninjured brain, ISH with a *p21* antisense probe on cross sections through the uninjured and through the lesioned telencephalon at 4 hpl and 1 dpl was conducted. Since the activation of p53 target genes takes place very rapidly after activation of p53 by cellular stress, the time-points 4 hpl and 1 dpl were chosen (Boehme and Blattner, 2009).

Expression of *p21* mRNA was detected in several regions of the adult uninjured telencephalon (Figure 39 A-B). *p21* mRNA expression was observed in the periventricular zone of the telencephalic ventricle (TelV) (Figure 39 A-B) and the diencephalic ventricle (DiV) (Figure 39 B) as well as in the central (Vc) (Figure 39 A), dorsal (Vd) (Figure 39 A), ventral (Vv) (Figure 39 A) and postcommisural (Vp) nuclei (Figure 39 B) of the ventral telencephalic area (V). Moreover, *p21* mRNA was expressed in the central (Dc) zone (Figure 39 A-B) of the dorsal telencephalic area (D) and in the anterior (PPa) part of the parvocellular preoptic nucleus (Figure 39 B). *p21* shows a similar expression pattern as *Mdm2*. It is also expressed in regions without *p53* expression, like the central zone of the dorsal telencephalic area, and it is not expressed in regions where *p53* expression is observed, like the medial, lateral, dorsal and posterior zone of the dorsal telencephalic area (see also Figure 12).

p21 mRNA is slightly up-regulated in the parenchyma of the lesioned hemisphere at the site of the lesion both at 4 hpl and at 1 dpl (Figure 39 C and D, black arrowheads). However, this up-regulation is very faint and can only be observed in some single cells near the lesion. Compared to *p53*, up-regulation at the ventricular zone of the lesioned hemisphere was not detected.

It was shown that *Mdm2* and *p21* are also target genes of p73 (Zhu et al., 1998a). *p53*, *p73*, *Mdm2* and *p21* are co-expressed in several regions of the uninjured adult zebrafish telencephalon and all four genes are up-regulated at the site of the lesion. These results indicate that p53 and/or p73 may regulate Mdm2 and p21 in the regions showing co-expression of the genes.

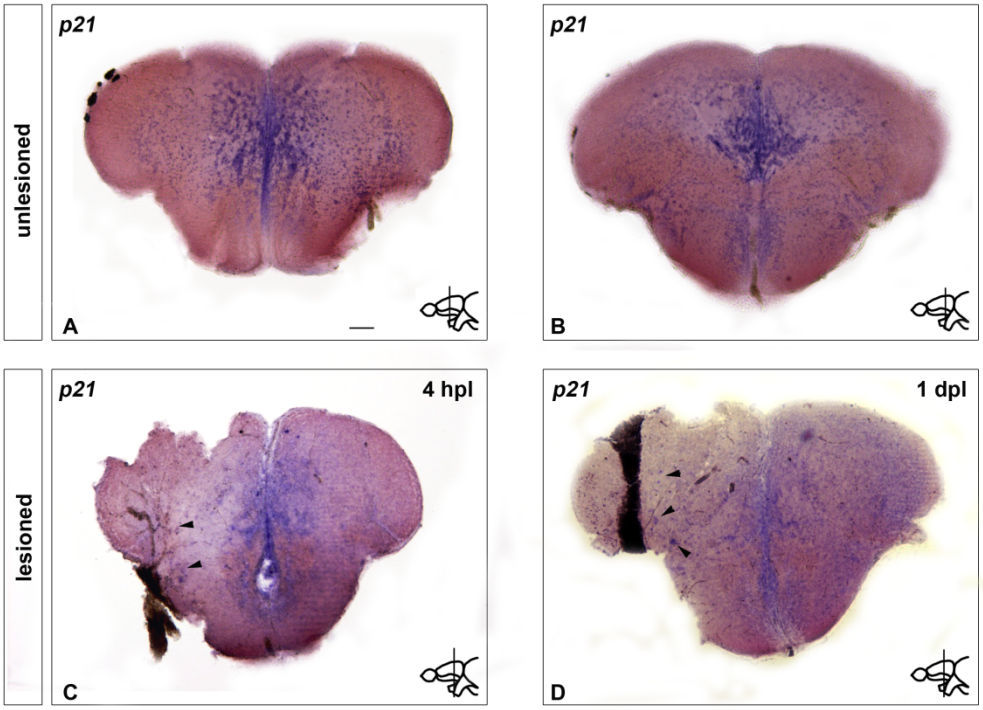

Figure 39: The *p21* mRNA is slightly up-regulated after stab injury of the adult zebrafish telencephalon.

(A-D) *In situ* hybridization with a *p21* antisense probe on cross sections through the unlesioned (A and B) and the lesioned telencephalon of adult zebrafish 4 hpl (C) and 1 dpl (D) to examine if the expression of *p21* mRNA is changed following brain injury and to investigate the expression of *p21* under normal conditions. The hemispheres containing the lesion are oriented to the left side, the right uninjured hemispheres serve as the control (C and D). The inserts indicate the anterior-posterior positions of the sections. *p21* mRNA is expressed in (A) periventricular zone of the TelV, Vv, Vd, Vc and Dc; (B) periventricular zone of the TelV and DiV, PPa, Vp and Dc.

A very slight up-regulation of *p21* mRNA in single cells in the parenchyma at the site of the lesion could be detected at 4 hpl (C, black arrowheads) as well as at 1 dpl (D, black arrowheads).

D: dorsal telencephalic area; Dc: central zone of D; DiV: diencephalic ventricle; PPa: parvocellular preoptic nucleus, anterior part; TelV: telencephalic ventricle; V: ventral telencephalic area; Vc: central nucleus of V; Vd: dorsal nucleus of V; Vp: postcommisural nucleus of V; Vv: ventral nucleus of V. Scale bar: 100 µm.

3.3.6 Induction of Apoptosis after stab injury

The transcription factor p53 is able to induce apoptosis. Under normal conditions p53 is rapidly degraded but after DNA damage or other cellular stress it becomes activated and accumulates. This permits p53 to activate its target genes which results in cell cycle arrest,

senescence or apoptosis to avoid that cells with DNA damage continue to proliferate (reviewed in Boehme and Blattner, 2009). Kroehne et al. showed that cell death occurs early after brain injury of zebrafish. I showed before that p53 is up-regulated at the ventricular zone of the lesioned hemisphere and at the site of the lesion in response to stab injury of the adult zebrafish telencephalon (see chapter 3.3.1 and 3.3.3; see Figure 32 and Figure 34). Therefore, a Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay on cross sections through the lesioned telencephalon was performed in order to investigate if the up-regulation of p53 is correlated with the induction of apoptosis after stab injury. The nuclei were stained with the nuclear marker DRAQ5. Since cell death is an early response to brain injury, the time points 4 hpl and 1 dpl were chosen.

After stab injury there is an induction of apoptosis in the lesioned hemisphere near the site of the lesion at 4 hpl as well as at 1 dpl (Figure 40 A and D, white arrows). For both time points TUNEL-positive cells were observed (see Figure 40 C' and F', white arrowheads). In contrast to the injured hemispheres, no TUNEL-positive cells were detected in the uninjured hemispheres.

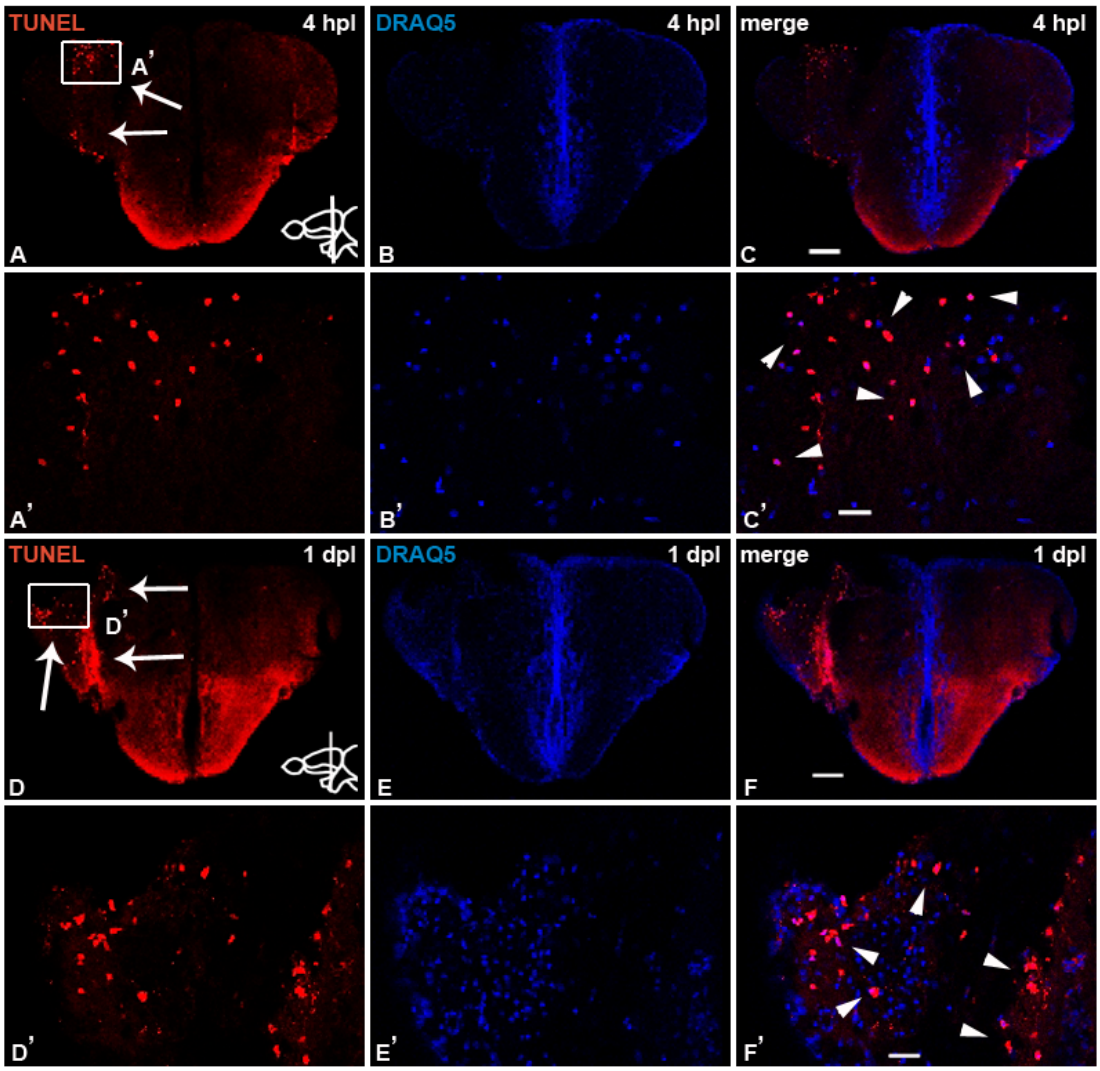

Figure 40: Apoptosis is induced after stab injury of the adult zebrafish telencephalon.
(A-F') TUNEL assay (A-A' and D-D') on cross sections through the lesioned telencephalon of adult zebrafish to investigate if apoptosis is induced at 4 hpl (A-C') and 1 dpl (D-F'). The nuclei were stained with the nuclear marker DRAQ5 (B-B' and E-E'). (C-C' and F-F') show the merged panels. The hemispheres containing the lesion are oriented to the left, the right uninjured hemispheres serve as the control. The inserts in (A) and (D) indicate the anterior-posterior positions of the sections and the boxes in (A) and (D) show the regions depicted in higher magnification in (A'-C') and (D'-F'), respectively. Both at 4 hpl and at 1 dpl TUNEL-positive cells and therefore an induction of apoptosis could be observed in the lesioned hemisphere near the site of the lesion (A and D, white arrows; C' and F', white arrowheads). Scale bars: 100 µm in C and F; 20 µm in C' and F'.

To sum up the results of this chapter, it was shown that *p53* mRNA, p53 protein as well as *p73* mRNA are up-regulated after stab injury of the adult zebrafish telencephalon. In addition it was demonstrated that p53 is not up-regulated in PCNA-positive but in Type I cells following brain injury and that the expression of p53 in HuC/D-positive neurons is not

changed after stab injury. However, after stab injury an expression of p53 was observed in *Tg(olig2:EGFP)*-positive cells, which do not express p53 in an uninjured telencephalon. Furthermore, it was shown that two target genes of p53 and p73, *Mdm2* and *p21*, were up-regulated and that apoptosis was induced following stab injury.

3.3.7 Reactions of *tp53^{M214K}* mutant zebrafish to stab injury

I demonstrated before that wildtype p53 is up-regulated after stab injury of the adult zebrafish telencephalon (see chapter 3.3.1 and 3.3.3; see Figure 32 and Figure 34). Furthermore, I could show that two target genes of p53, *Mdm2* and *p21*, are also up-regulated after brain injury (see chapter 3.3.5.1 and 3.3.5.2; see Figure 38 and Figure 39) and that apoptosis is induced after inflicting a stab wound to the telencephalon (see chapter 3.3.6 and Figure 40). For the following experiments homozygous *tp53^{M214K}* mutant zebrafish (Berghmans et al., 2005), that contain a point mutation where methionin is changed to lysine, were used in order to investigate the reactions following stab injury without a functional p53 protein. This *tp53^{M214K}* mutation is a DNA contact mutation, which is located in the DNA-binding domain. Thus, the mutant p53 transcription factor is not able to bind to p53-specific consensus sequences and therefore an activation of target genes is prevented (Berghmans et al., 2005). It was shown by Berghmans et al. that apoptosis was not induced and that *Mdm2* and *p21* were not up-regulated in homozygous *tp53^{M214K}* mutant zebrafish embryos after γ-irradiation compared to wildtype embryos.

3.3.7.1 The *tp53^{M214K}* mutant zebrafish show the same p53 expression pattern in the telencephalon like wildtype fish

As shown before, the wildtype p53 protein is broadly expressed in many regions of the uninjured adult zebrafish telencephalon (see chapter 3.1.4 and Figure 24). To analyze the expression of tp53^{M214K} mutant protein in the adult zebrafish telencephalon and to compare it to wildtype p53 protein, an immunostaining of cross sections through the uninjured telencephalon of *tp53^{M214K}* mutant zebrafish and wildtype fish with the anti-p53 antibody was performed. The nuclei were stained with the nuclear marker DRAQ5.

The tp53^{M214K} mutant protein (Figure 41 B) is expressed in the same regions of the uninjured adult zebrafish telencephalon as the wildtype p53 protein (Figure 41 A). For a detailed

description in which regions of the telencephalon p53 is expressed, see chapter 3.1.4 and Figure 24 A-C.

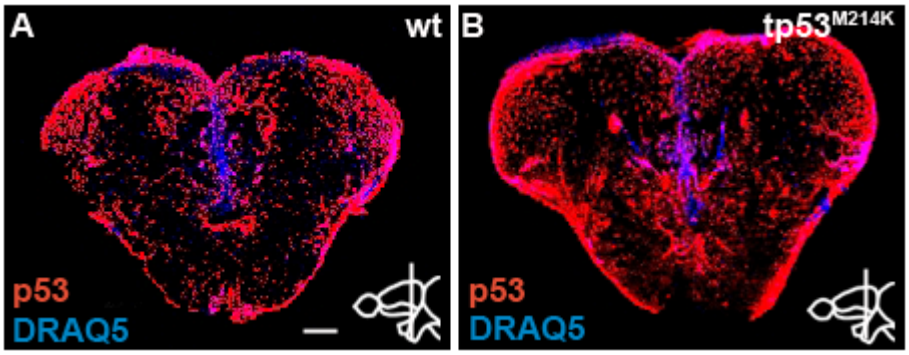

Figure 41: The tp53^{M214K} mutant protein shows the same expression pattern than wildtype p53 protein.

(A-B) Immunohistochemistry on cross sections through the telencephalon of adult wildtype (A) or *tp53^{M214K}* mutant (B) zebrafish with the anti-p53 antibody to compare the expression of wildtype and mutant p53 protein. The nuclei were stained with the nuclear marker DRAQ5. The inserts indicate the anterior-posterior positions of the sections. The tp53^{M214K} mutant protein is expressed in the same regions as the wildtype p53 protein. For a detailed description of p53 expression see also Figure 24. Scale bar: 100 µm.

3.3.7.2 The tp53^{M214K} mutant protein is up-regulated in response to stab injury

The wildtype p53 protein is up-regulated after brain injury (see Figure 34). Furthermore, the tp53^{M214K} mutant protein shows the same expression pattern as wildtype p53 protein (see Figure 41). To find out if the expression of the tp53^{M214K} mutant protein is also up-regulated after stab injury of the telencephalon, an immunostaining of cross sections through the injured telencephalon with the anti-p53 antibody 5 days after inflicting a stab wound was performed. The nuclei were stained with the nuclear marker DRAQ5.

As already demonstrated, there is an up-regulation of wildtype p53 protein at 5 dpl at the ventricular zone of the lesioned hemisphere (Figure 42 A, white arrowheads). In comparison to the uninjured hemisphere, the expression of the tp53^{M214K} mutant protein is also up-regulated at the ventricular zone of the injured hemisphere after stab injury (Figure 42 B, yellow arrowheads). Thus, there is no difference between the reaction of the wildtype and the mutant p53 protein 5 days after stab injury. Interestingly, Berghmans et al. did not observe an up-regulation of mutant *p53* mRNA in homozygous *tp53^{M214K}* mutant embryos after

cellular stress in terms of γ-irradiation. On the contrary, Guo et al. showed that mutant p53 protein accumulated in *tp53*^{M214K} mutant embryos after ionizing radiation. In the adult zebrafish telencephalon the amount of tp53^{M214K} mutant protein is increased after injury although the protein is presumably not functional and is not able to induce the transcription of target genes.

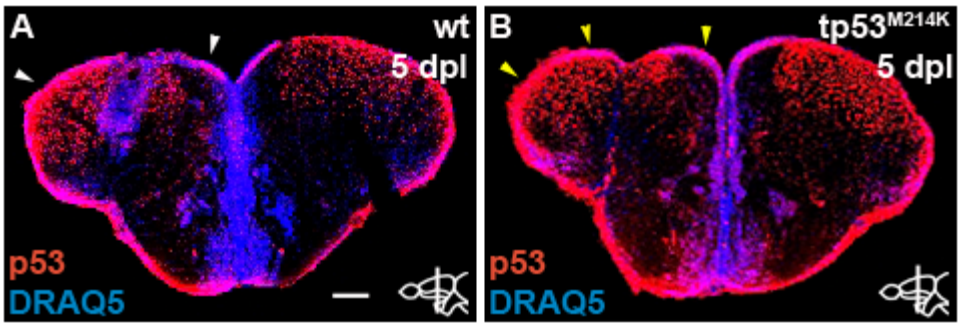

Figure 42: The tp53^{M214K} mutant protein is up-regulated upon stab injury of the adult zebrafish telencephalon.

(A-B) Immunohistochemistry on cross sections through the telencephalon of adult wildtype (A) or *tp53*^{M214K} mutant (B) zebrafish 5 dpl with the anti-p53 antibody to examine if the mutant p53 protein is also up-regulated after brain injury. The nuclei were stained with the nuclear marker DRAQ5. The hemispheres containing the lesion are oriented to the left side, the right uninjured hemispheres serve as the control. The inserts indicate the anterior-posterior positions of the sections. Like the wildtype p53 protein (A, white arrowheads), the tp53^{M214K} mutant protein (B, yellow arrowheads) is up-regulated 5 days after stab injury at the ventricular zone of the lesioned hemisphere compared to the control hemisphere. Scale bar: 100 µm.

3.3.7.3 *Mdm2* mRNA is slightly up-regulated in the telencephalon of *tp53*^{M214K} mutant zebrafish after stab injury

Mdm2 mRNA is up-regulated in the parenchyma of the injured telencephalic hemisphere near the lesion in wildtype zebrafish following brain injury (see chapter 3.3.5.1 and Figure 38). *Mdm2* is the main regulator and a target gene of p53 (see chapter 3.3.5.1; reviewed in Boehme and Blattner, 2009; Wu et al., 1993). It was demonstrated by Berghmans et al. that in homozygous *tp53*^{M214K} mutant embryos *Mdm2* was not up-regulated after γ-irradiation compared to wildtype embryos because the mutant p53 transcription factor is not capable of activating its target genes. In order to study the expression of *Mdm2* mRNA in

tp53^{M214K} mutant zebrafish after stab injury, an ISH with a *Mdm2* antisense probe on cross sections through the lesioned telencephalon of homozygous *tp53*^{M214K} mutant zebrafish at 4 hpl and 1 dpl was performed. The time-points 4 hpl and 1 dpl were chosen because p53 becomes rapidly activated after cellular stress and thus an effect on the target genes of p53 should be detectable at these time points (Boehme and Blattner, 2009). Like that, an up-regulation of *Mdm2* mRNA was observed at 4 hpl and 1 dpl in wildtype zebrafish (see Figure 38).

There is, like in the wildtype fish, an up-regulation of *Mdm2* mRNA in the lesioned hemisphere near the lesion (Figure 43 A and B, black arrowheads) compared to the unlesioned control hemisphere. However, this up-regulation is very weak in comparison to the situation in wildtype zebrafish (compare Figure 43 with Figure 38). An up-regulation of *Mdm2* mRNA was observed in *tp53*^{M214K} mutant zebrafish although there is supposedly no functional p53 protein present that could induce the expression of *Mdm2*. This result suggests that it is either possible that *Mdm2* is activated by another protein than p53 in response to stab injury or that the mutant p53 protein is not completely inoperable. It was shown that p73 is able to induce the expression of *Mdm2* (Zeng et al., 1999). Thus, since p73 is also up-regulated after stab injury of the adult zebrafish telencephalon, it is likely that p73 replaces the inoperable p53 protein and activates p53 target genes.

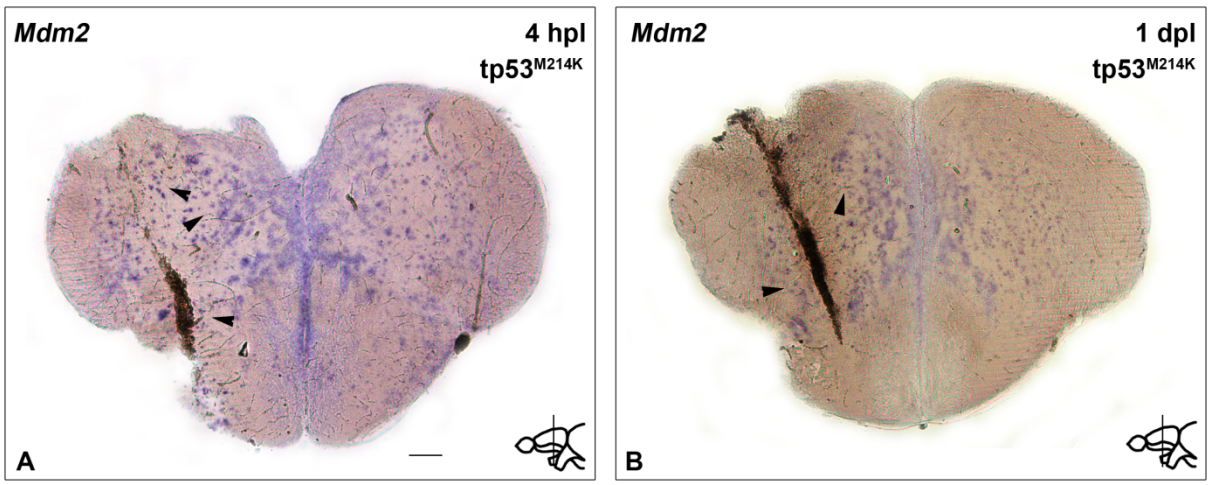

Figure 43: The *Mdm2* mRNA is slightly up-regulated in *tp53*^{M214K} mutant zebrafish after stab injury of the adult zebrafish telencephalon.

(A-B) *In situ* hybridization with a *Mdm2* antisense probe on cross sections through the lesioned telencephalon of adult *tp53*^{M214K} mutant zebrafish 4 hpl (A) and 1 dpl (B) to investigate the expression of *Mdm2* mRNA after stab injury. The hemispheres containing the lesion are oriented to the left side, the right uninjured hemispheres serve as the control. The inserts indicate the anterior-posterior positions of the sections. Both at 4 hpl (A) and at 1 dpl (B) a very weak up-regulation of *Mdm2* mRNA

in the parenchyma at the site of the lesion could be observed (A and B, black arrowheads). Scale bar: 100 µm.

3.3.7.4 *p21* mRNA is slightly up-regulated in the telencephalon of *tp53*^{M214K} mutant zebrafish after stab injury

p21 mRNA is slightly up-regulated in the parenchyma of the adult wildtype zebrafish telencephalon near the lesion in response to stab injury (see Figure 39). Furthermore, I showed that the tp53^{M214K} mutant protein is up-regulated after brain injury (see chapter 3.3.7.2 and Figure 42) and that the p53 target gene *Mdm2* is slightly up-regulated as well in the telencephalon of *tp53*^{M214K} mutant zebrafish after stab injury (see chapter 3.3.7.3 and Figure 43). *p21* is one of the target genes of p53 and becomes activated by p53 after cellular stress which results in cell cycle arrest (see chapter 3.3.5.2; el-Deiry et al., 1993; Harper et al., 1993; Jackson et al., 2004). It was shown by Berghmans et al. that *p21* was not up-regulated in homozygous *tp53*^{M214K} mutant embryos after γ-irradiation compared to wildtype embryos. To investigate if there is an up-regulation of *p21* mRNA in *tp53*^{M214K} mutant zebrafish after stab injury, an ISH with a *p21* antisense probe on cross sections through the lesioned telencephalon of homozygous *tp53*^{M214K} mutant zebrafish at 4 hpl and 1 dpl was performed. Since an up-regulation of *p21* mRNA could be detected at 4 hpl and 1 dpl in wildtype zebrafish (see Figure 39) and p53 becomes rapidly activated after cellular stress (Boehme and Blattner, 2009) the time-points 4 hpl and 1 dpl were chosen.

p21 mRNA is up-regulated in the lesioned hemisphere of *tp53*^{M214K} mutant zebrafish near the lesion (Figure 44 A and B, black arrowheads) compared to the unlesioned control hemisphere. Like in wildtype fish, this up-regulation is very weak (see also Figure 39). As for *Mdm2*, an up-regulation of *p21* mRNA was observed in *tp53*^{M214K} mutant zebrafish in which any functional p53 should be present. This result indicates that *p21* could be activated by another protein. p73 is capable of transactivating some p53 target genes like *p21* (Zhu et al., 1998a).

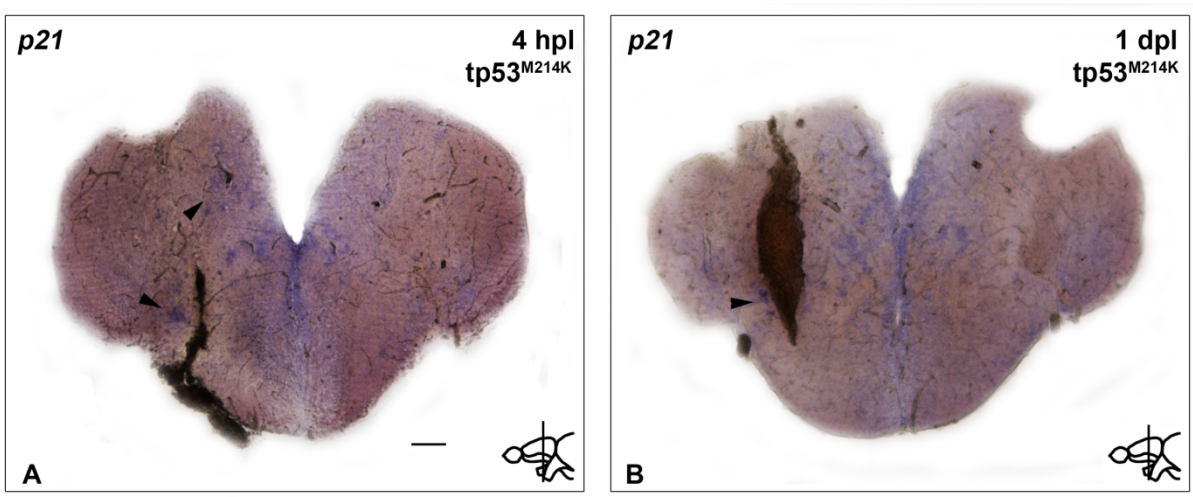

Figure 44: The *p21* mRNA is slightly up-regulated in *tp53*^{M214K} mutant zebrafish after stab injury of the adult zebrafish telencephalon.

(A-B) *In situ* hybridization with a *p21* antisense probe on cross sections through the lesioned telencephalon of adult *tp53*^{M214K} mutant zebrafish 4 hpl (A) and 1 dpl (B) to analyze the expression of *p21* mRNA after stab injury. The hemispheres containing the lesion are oriented to the left side, the right uninjured hemispheres serve as the control. The inserts indicate the anterior-posterior positions of the sections. At 4 hpl (A) as well as at 1 dpl (B) a very faint up-regulation of *p21* mRNA in the parenchyma at the site of the lesion could be observed (A and B, black arrowheads). Scale bar: 100 µm.

3.3.7.5 Induction of apoptosis after stab injury in *tp53*^{M214K} mutant zebrafish

Apoptosis is induced in wildtype zebrafish following stab injury of the telencephalon (see Figure 40). Moreover, I showed that the tp53^{M214K} mutant protein is up-regulated at the ventricular zone of the lesioned hemisphere (see chapter 3.3.7.2 and Figure 42) and that two target genes of p53, *Mdm2* (see chapter 3.3.7.3 and Figure 43) and *p21* (see chapter 3.3.7.4 and Figure 44), are up-regulated at the site of the lesion after brain injury. The transcription factor p53 becomes activated after cellular stress and transactivates target genes. Subsequently, this leads to the induction of apoptosis amongst others (reviewed in Boehme and Blattner, 2009). Kroehne et al. demonstrated that cell death occurs early after brain injury of zebrafish. Furthermore, it was figured out that in homozygous *tp53*^{M214K} mutant embryos the induction of apoptosis after γ-irradiation is suppressed compared to wildtype embryos (Berghmans et al., 2005). In order to examine if apoptosis is induced after stab injury in *tp53*^{M214K} mutant zebrafish a TUNEL assay on cross sections through the lesioned telencephalon of adult *tp53*^{M214K} mutant zebrafish was performed. The nuclei were stained

with the nuclear marker DRAQ5. Because an induction of apoptosis could be observed in wildtype zebrafish at 4 hpl and at 1 dpl (see Figure 40) and since cell death is an early response to brain injury, the time points 4 hpl and 1 dpl were chosen.

Like in wildtype fish, there is an induction of apoptosis in the lesioned hemisphere near the site of the lesion both at 4 hpl (Figure 45 A, white arrowheads) and at 1 dpl (Figure 45 B, white arrowhead). In the uninjured hemispheres TUNEL-positive cells were not observed. This result indicates that apoptosis is induced after stab injury of *tp53*^{M214K} mutant zebrafish in cells residing near the lesion although any functional p53 protein is present. But apoptosis can also be induced independently of p53 transcription via the mitochondria (Galluzzi et al., 2008) or by p73 (Levrero et al., 2000; Murray-Zmijewski et al., 2006).

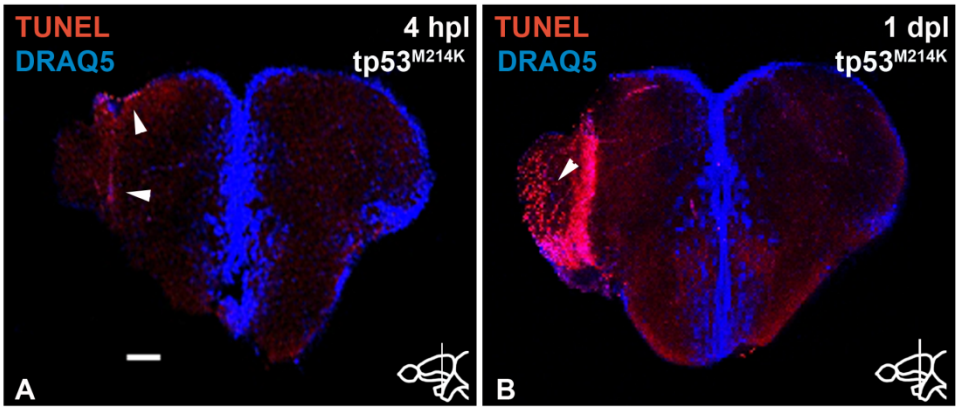

Figure 45: Apoptosis is induced after stab injury of the telencephalon of adult *tp53*^{M214K} mutant zebrafish.

(A-B) TUNEL assay on cross sections through the lesioned telencephalon of adult *tp53*^{M214K} mutant zebrafish to investigate if apoptosis is induced 4 hpl (A) and 1 dpl (B). The nuclei were stained with the nuclear marker DRAQ5. The hemispheres containing the lesion are oriented to the left, the right uninjured hemispheres serve as the control. The inserts in (A) and (B) indicate the anterior-posterior positions of the sections. At 4 hpl (A, white arrowheads) and at 1 dpl (B, white arrowhead) TUNEL-positive cells were observed in the lesioned hemisphere near the site of the lesion. This means that there is an induction of apoptosis following stab injury. Scale bar: 100 µm.

3.3.7.6 No difference in proliferation between *tp53*^{M214K} mutant and wildtype zebrafish after stab injury

Both p53 and PCNA are up-regulated 5 dpl at the ventricular zone of the lesioned hemisphere in response to stab injury though p53 is not up-regulated in PCNA-positive cells but in another cell type, most likely in Type I cells (see also Figure 35). Furthermore, I found out that the tp53^{M214K} mutant protein is up-regulated as well after brain injury (see chapter 3.3.7.2 and Figure 42). März et al. demonstrated that the radial glial marker S100β and the proliferation marker PCNA are up-regulated following brain injury since radial glial cells show a strong increase in proliferation after inflicting a stab wound on the telencephalon. After cellular stress p53 becomes activated and induces the expression of p21, which leads to cell cycle arrest (el-Deiry et al., 1993; Harper et al., 1993). Hence, p53 exhibits anti-proliferative capacities. In order to analyze if proliferation is increased in radial glial cells in *tp53*^{M214K} mutant zebrafish, a co-immunostaining on cross sections through the lesioned telencephalon of adult *tp53*^{M214K} mutant and wildtype zebrafish with antibodies against PCNA and S100β was performed 5 days after stab injury. The nuclei were stained with the nuclear marker DRAQ5.

Both PCNA and S100β, are up-regulated at the ventricular zone of the injured hemisphere in wildtype (Figure 46 A, white arrowheads; Figure 46 B, yellow arrowheads) as well as in *tp53*^{M214K} mutant (Figure 46 E, white arrowheads; Figure 46 F, yellow arrowheads) zebrafish. However, there is no difference in the intensity of the up-regulation visible. This result suggests that proliferation is not increased in radial glial cells in *tp53*^{M214K} mutant zebrafish relative to wildtype fish after stab injury. Therefore, mutant p53 does not lead to an enhanced proliferation. As shown before, there is also an induction of *Mdm2* (see chapter 3.3.7.3 and Figure 43) and more weakly of *p21* (see chapter 3.3.7.4 and Figure 44) following stab injury in *tp53*^{M214K} mutant zebrafish. The induction of p21 after stab injury could still be sufficient to control cell proliferation in *tp53*^{M214K} mutant zebrafish.

Thus even though any functional p53 is present in the telencephalon, the reactions after stab injury are almost the same as in wildtype brains. These results suggest that some other mechanisms are activated in response to brain injury to ensure a proper repair of the caused damage also in the absence of p53. Probably p73 could play a role in these processes.

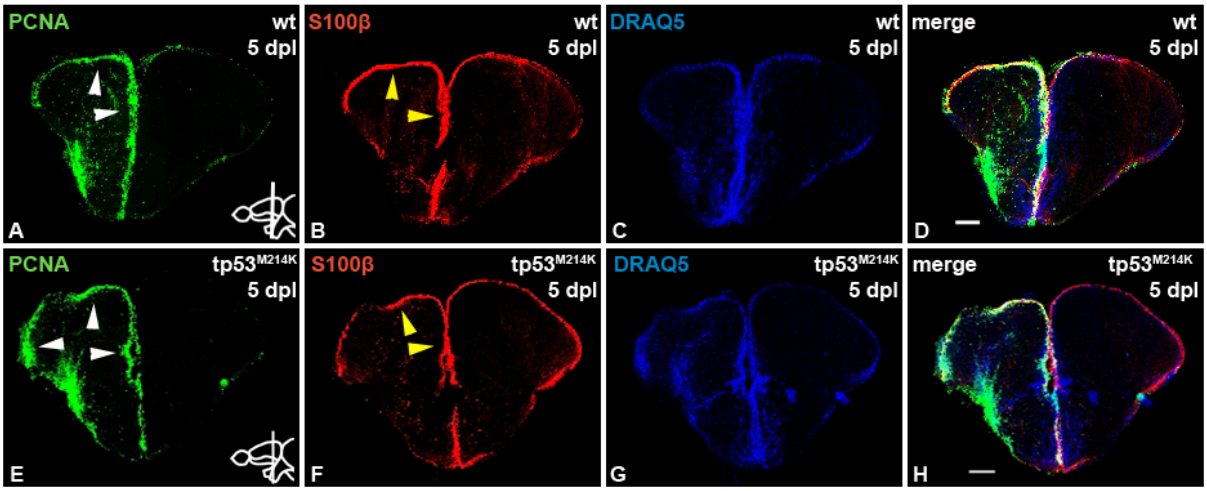

Figure 46: No difference in proliferation after stab injury of the telencephalon of adult *tp53*^{M214K} mutant and wildtype zebrafish.

(A-H) Co-immunostaining of cross sections through the telencephalon of adult wildtype (A-D) and *tp53*^{M214K} mutant (E-H) zebrafish 5 dpl with antibodies against PCNA (A and E) and S100β (B and F) to investigate if proliferation is increased in radial glial cells in *tp53*^{M214K} mutant zebrafish after stab injury. The nuclei were stained with the nuclear marker DRAQ5 (C and G). (D and H) show the merged panels. The hemispheres containing the lesion are oriented to the left side, the right uninjured hemispheres serve as the controls. The inserts in (A and E) indicate the anterior-posterior positions of the sections. Both PCNA (A and E, white arrowheads) and S100β (B and F, yellow arrowheads) are up-regulated at the ventricular zone of the lesioned hemisphere in comparison to the uninjured control hemisphere in wildtype and in *tp53*^{M214K} mutant zebrafish. But there is any difference visible concerning the intensity of the up-regulation of PCNA and S100β between wildtype and *tp53*^{M214K} mutant zebrafish (D and H). This indicates that the mutant p53 protein does not influence the proliferative reactions after brain injury. Scale bar: 100 µm in A and E.

In summary, the tp53^{M214K} mutant protein shows the same expression pattern as wildtype p53 and it is also up-regulated after stab injury. Interestingly, *Mdm2* and *p21* are up-regulated as well and apoptosis occurs in the mutant. Furthermore, there is no difference regarding proliferation after stab injury between wildtype and *tp53*^{M214K} mutant zebrafish. Thus, p53 may either not play a role in adult neurogenesis in zebrafish or there may be redundancy between p53 and p73. The related p73 shows the same expression pattern as p53 in the adult uninjured and injured zebrafish brain.

Chapter 4

Discussion

The adult zebrafish brain exhibits a very high proliferative and neurogenic potential, contrary to the adult mammalian brain. In addition, zebrafish show an extraordinary regenerative potential after lesions of the central nervous system (Adolf et al., 2006; Chapouton et al., 2007; Grandel et al., 2006; Kaslin et al., 2008; Zupanc, 2001; Zupanc and Zupanc, 2006). The two transcription factors p53 and p73 were shown to play crucial roles in the adult mammalian brain. p53 suppresses the self-renewal of adult neural stem cells (Meletis et al., 2006), may preserve the post-mitotic character of differentiated neurons (Miller et al., 2003) and is involved in apoptotic death of neurons following damage (Culmsee and Mattson, 2005; Miller et al., 2000; Morrison and Kinoshita, 2000; Morrison et al., 2003; Napieralski et al., 1999; Xiang et al., 1998; Yonekura et al., 2006). p73 is relevant for the survival of neurons (Jacobs et al., 2004; Pozniak et al., 2002; Pozniak et al., 2000; Tissir et al., 2009), self-renewal and maintenance of neural stem cells (Agostini et al., 2010; Fujitani et al., 2010; Talos et al., 2010) as well as differentiation of precursor cells (Agostini et al., 2010; Billon et al., 2004; De Laurenzi et al., 2000; Talos et al., 2010). In order to assess a possible role of p53 and p73 in neurogenesis in the zebrafish brain I investigated the expression of *p53* and *p73* mRNA and p53 protein in the adult zebrafish brain. Specific emphasis was given to expression in regions known for their constitutive neurogenic potential and during regeneration of injuries focusing on the adult telencephalon as a well-characterized model of both constitutive (Adolf et al., 2006; Grandel et al., 2006; Kaslin et al., 2008; Lam et al., 2009; März et al., 2010a) and regenerative (Baumgart et al., 2012; Kizil et al., 2012a; Kizil et al., 2012b; Kroehne et al., 2011; Kyritsis et al., 2012; März et al., 2011) adult neurogenesis.

4.1 p53 and p73 are expressed in many regions of the adult zebrafish brain in an overlapping pattern

I have first characterized the expression of p53 and p73 in the entire adult zebrafish brain. A detailed mRNA expression map of the entire brain was established (Table 1). *p53* and *p73* mRNAs are expressed in overlapping patterns in most regions in the telencephalon,

mesencephalon and hindbrain. For example in the telencephalon, the two genes are expressed in the parenchyma and more specifically in the ventral nucleus of the ventral telencephalic area (Vv) and in the lateral (Dl) and posterior (Dp) zone of the dorsal telencephalic area. Similarly in the diencephalon, the expression of *p53* and *p73* mRNA overlaps in most of the regions. Exceptions in the diencephalon are the PGa and PGl that express only *p53* and the TLa that expresses only *p73*. This mostly overlapping pattern of expression of the two genes in most regions suggests that the two genes are subject to similar regulatory control. It was shown before that p73 is expressed in the telencephalon, hypothalamus, olfactory bulbs and in the optic tectum of the adult zebrafish brain (Pan et al., 2003). However, in this study the expression of *p73* mRNA was investigated with whole-mount ISH and not with brain sections. Thus, they did not provide a detailed expression map of *p73* mRNA. Accordingly to Pan et al. I observed *p73* mRNA expression in the telencephalon, hypothalamus and optic tectum. However, I did not detect *p73* mRNA in the olfactory bulbs (Table 1).

In the adult mammalian brain, p53 is expressed in the SVZ (Jori et al., 2003; van Lookeren Campagne and Gill, 1998), in the RMS, in the DG (Medrano and Scrable, 2005) and in the cerebellum (Wood and Youle, 1995) while p73 is almost ubiquitously expressed in the adult mammalian brain (Hernandez-Acosta et al., 2011). Thus, the expression of p53 and p73 in the adult mammalian brain seems to be less co-regulated than in the adult zebrafish brain. In mammals, the p53 and p73 proteins are known to interact (Chen et al., 2001; Grob et al., 2001; Kartasheva et al., 2002; Wang et al., 2007a). The observed co-expression of *p53* and *p73* mRNA in many tissues may reflect similar functional interactions of p53 and p73 in the zebrafish brain.

	***p53* mRNA**	***p73* mRNA**	**p53 protein**
Olfactory bulbs			
ECL			
GL			
POF			
ICL			
LOT			
MOT			
Telencephalon			
Periventricular zone of TelV	✓	✓	✓
Vd	✓	✓	✓
Vc	✓	✓	✓
Vl			
Vv	✓	✓	✓
Vs			
Vp	✓	✓	✓

D	✓	✓	✓
Dm	✓	✓	✓
Dl	✓	✓	✓
Dp	✓	✓	✓
Dc			
Dd	✓	✓	✓
Cantd			
Cantv			
SY	✓	✓	
LFB			
MFB			
NT			
Diencephalon			
Periventricular zone of DiV	✓	✓	✓
End	✓	✓	✓
ENv	✓	✓	✓
PPa	✓	✓	✓
PPp	✓	✓	✓
PMg			
PM			
SC			
VM	✓	✓	✓
VL			
I			
A	✓	✓	✓
CP			
DP			
P			
Hav	✓	✓	✓
Had			
Chab			
PSp			
PSm			
SD			
R			
CPN			
DAO			
FR			
Hv	✓	✓	✓
Hd	✓	✓	✓
Hc	✓	✓	✓
APN			
ATN			
E			
PGa	✓		✓
PGl	✓		✓
PGm			
PGc			
PO			
TPM			
TPp	✓	✓	✓

VAO			
ZL			
Cpost			
Ctec			
Ctub			
DIL			
CIL			
LH			
LR			
PCN			
PPd			
PPv			
PTN			
SCO			
TLa		✓	
Pit			
PVO			
RT			
TGN			
SG			
CM	✓	✓	✓
PR			
CO			
ON			
OT			
Chor			
Cpop			
DOT			
VOT			
Mesencephalon			
Periventricular zone of TeV	✓	✓	✓
TeO			
PGZ	✓	✓	✓
TL	✓	✓	✓
TS			
TSc			
TSvl			
LLF			
MLF			
NMLF			
NR			
Vas			
DTN			
EW			
TTB			
TTBc			
TTBr			
Cans			
NLL			
MN V			
N III			

III			
N IV			
IV			
D IV			
V			
V s			
N V md			
N V mv			
N V s			
ND V			
V md			
V mv			
D V			
VI r			
N VI c			
VI c			
VII			
VII s			
N VII m			
L VII			
VIII			
IX			
L IX			
X			
L X			
N X m			
PL			
TMCa			
TMCp			
Nln			
NLV			
GC			
SRF			
SR			
NI			
LC			
Hindbrain			
Periventricular zone of RV	✓	✓	✓
Val	✓	✓	
Vam			
AC			
Cven			
CCe	✓	✓	✓
Ccer			
Cgus			
CC	✓	✓	✓
SGN			
SGT			
ALLN			
EG	✓	✓	✓
PC			

AON			
IMRF			
LCa			
MAC			
MA			
MaON			
MON			
DON			
CON			
IAF			
SO			
T			
PLLN			
TBS			
TVS			
IO			
IR			
IRF			
LRN			
PON			
DV			
Flv			
Fv			
Fd			
Fld			
MFN			
C			
Cinf			
NC			
DH			
DR			
VH			

Table 1: The table lists the different domains of the adult zebrafish brain. Domains showing expression of *p53* and *p73* mRNA and p53 protein are indicated by a check.

Abbreviations: III: oculomotor nerve; IV: trochlear nerve; V: trigeminal nerve; V md: dorsal motor root of the trigeminal nerve; V mv: ventral motor root of the trigeminal nerve; V s: sensory root of the trigeminal nerve; VI r: rostral root of the abducens nerve; VI c: caudal root of the abducens nerve; VII: facial nerve; VII s: sensory root of the facial nerve; VIII: octaval nerve; IX: glossopharyngeal nerve; X: vagal nerve; A: anterior thalamic nucleus; AC: anterior cerebellar tract; ALLN: anterior lateral line nerves; AON: anterior octaval nucleus; APN: accessory pretectal nucleus; ATN: anterior tuberal nucleus; C: central canal; Cans: commissural ansulata; Cantd: commissural anterior, pars dorsalis; Cantv: commissural anterior, pars ventralis; CC: crista cerebellaris; CCe: corpus cerebelli; Ccer: commissural cerebella; Cgus: commissure of the secondary gustatory nuclei; Chab: commissural habenularum; Chor: commissura horizontalis; CIL: central nucleus of the inferior lobe; Cinf: commissural infima of Haller; CM: corpus mamillare; CO: chiasma opticum; CON: caudal octavolateralis nucleus; CP: central posterior thalamic nucleus; CPN: central pretectal nucleus; Cpop: commissura postoptica; Cpost: commissural posterior; Ctec: commissary tecti; Ctub: commissure of

the posterior tuberculum; Cven: commissural ventralis rhombencephali; D: dorsal telencephalic area; D IV: trochlear decussation; D V: descending trigeminal root; DAO: dorsal accessory optic nucleus; Dc: central zone of D; Dd: dorsal zone of D; DH: dorsal horn; DIL: diffuse nucleus of the inferior lobe; DiV: diencephalic ventricle; Dl: lateral zone of D; Dm: medial zone of D; DON: descending octaval nucleus; DOT: dorsomedial optic tract; Dp: posterior zone of D; DP: dorsal posterior thalamic nucleus; DR: dorsal root; DTN: dorsal tegmental nucleus; DV: descending trigeminal root; E: epiphysis; ECL: external cellular layer of olfactory bulb including mitral cells; EG: eminentia granularis; ENd: entopeduncular nucleus, dorsal part; ENv: entopeduncular nucleus, ventral part; EW: Edinger-Westphal nucleus; Fd: funiculus dorsalis; Fld: funiculus lateralis pars dorsalis; Flv: funiculus lateralis pars ventralis; FR: fasciculus retroflexus; Fv: funiculus ventralis; GC: griseum centrale; GL: glomerular layer of olfactory bulb; Had: dorsal habenular nucleus; Hav: ventral habenular nucleus; Hc: caudal zone of periventricular hypothalamus; Hd: dorsal zone of periventricular hypothalamus; Hv: ventral zone of periventricular hypothalamus; I: intermediate thalamic nucleus; IAF: inner arcuate fibers; ICL: internal cellular layer of olfactory bulb; IMRF: intermediate reticular formation; IO: oliva inferior; IR: interior raphe; IRF: inferior reticular formation; L VII: lobus facialis; L IX: lobus glossopharyngeus; L X: lobus vagus; LC: locus coeruleus; LCa: lobus caudalis cerebella; LFB: lateral forebrain bundle; LH: lateral hypothalamic nucleus; LLF: lateral longitudinal fascicle; LOT: lateral olfactory tract; LR: lateral recess of diencephalic ventricle; LRN: lateral reticular nucleus; MA: Mauthner axon; MAC: Mauthner cell; MaON: magnocellular octaval nucleus; MFB: medial forebrain bundle; MFN: medial funicular nucleus; MLF: medial longitudinal fascicle; MN V: mesencephalic nucleus of trigeminal nerve; MON: medial octavolateralis nucleus; MOT: medial olfactory tract; N III: oculomotor nucleus; N IV: trochlear nucleus; N V md: trigeminal motor nucleus, dorsal part; N V mv: trigeminal motor nucleus, ventral part; N V s: primary sensory trigeminal nucleus; N VI c: abducens nucleus, caudal part; N VII m: facial motor nucleus; N X m: vagal motor nucleus; NC: commissural nucleus of Cajal; ND V: nucleus of the descending trigeminal root; NI: nucleus istmi; NLL: nu cleus of the lateral lemniscus; NLV: nucleus lateralis valvulae; NMLF: nucleus of MLF; Nln: nucleus interpeduncularis; NT: nucleus taeniae; NR: nucleus ruber; ON: optic nerve; OT: optic tract; P: posterior thalamic nucleus; PC: posterior cerebellar tract; PCN: paracommissural nucleus; PGa: anterior preglomerular nucleus; PGc: caudal preglomerular nucleus; PGl: lateral preglomerular nucleus; PGm: medial preglomerular nucleus; PGZ: periventricular grey zone of optic tectum; Pit: pituitary; PL: perilemniscal nucleus; PLLN: posterior lateral line nerve; PM: magnocellular preoptic nucleus; PMg: gigantocellular part of magnocellular preoptic nucleus; PO: posterior pretectal nucleus; POF: primary olfactory fiber layer; PON: posterior octaval nucleus; PPa: parvocellular preoptic nucleus, anterior part; PPd: periventricular pretectal nucleus; dorsal part; PPp: parvocellular preoptic nucleus, posterior part; PPv: periventricular pretectal nucleus; ventral part; PR: posterior recess of diencephalic ventricle; PSm: magnocellular superficial pretectal nucleus; PSp: parvocellular superficial pretectal nucleus; PTN: posterior tuberal nucleus; PVO: paraventricular organ; R: rostrolateral nucleus; RT: rostral tegmental nucleus; RV: rhombencephalic ventricle; SC: suprachiasmatic nucleus; SCO: subcommissural organ; SD: saccus dorsalis; SG: subglomerular nucleus; SGN: secondary gustatory nucleus; SGT: secondary gustatory tract; SO: secondary octaval population; SR: superior raphe; SRF: superior reticular formation; SY: sulcus ypsiloniformis; T: tangential nucleus; TBS: tractus bulbospinalis; TeV: tectal ventricle; TelV:

telencephalic ventricle; TeO: tectum opticum; TL: torus longitudinalis; TLa: torus lateralis; TGN: tertiary gustatory nucleus; TMCa: tractus mesencephalocerebellaris anterior; TMCp: tractus mesencephalocerebellaris posterior; TPM: tractus pretectomamillaris; TPp: periventricular nucleus of posterior tuberculum; TS: torus semicircularis; TSc: central nucleus of torus semicircularis; TSvl: ventrolateral nucleus of torus semicircularis; TTB: tractus tectobulbaris; TTBc: tractus tectobulbaris cruciatus; TTBr: tractus tectobulbaris rectus; TVS: tractus vestibulospinalis; V: ventral telencephalic area; Val: lateral division of valvula cerebelli; Vam: medial division of valvula cerebelli; VAO: ventral accessory optic nucleus; Vas: vascular lacuna of area postrema; Vc: central nucleus of V; Vd: dorsal nucleus of V; VH: ventral horn; Vl: lateral nucleus of V; VL: ventrolateral thalamic nucleus; VM: ventromedial thalamic nucleus; VOT: ventrolateral optic tract; Vp: postcommissural nucleus of V; Vs: supracommissural nucleus of V; Vv: ventral nucleus of V; ZL: zona limitans.

p53 and p73 are involved in regulation of neural stem cells in the two neurogenic regions of the adult mammalian telencephalon. p53 negatively regulates the self-renewal of adult neural stem cells and may thereby maintain the stem cell population since enhanced proliferation could lead to premature senescence (Meletis et al., 2006). Thus, p53 may be important for maintaining neurogenesis in the adult mammalian brain because it controls the proliferation of stem and progenitor cells (Gil-Perotin et al., 2006; Medrano et al., 2009). p73 is also a regulator of neural stem cell self-renewal and long-term maintenance. However, p73 positively regulates self-renewal and promotes proliferation, in contrast to p53. Thereby p73 prevents premature differentiation and facilitates the maintenance of stem cells (Agostini et al., 2010; Fujitani et al., 2010; Talos et al., 2010). Although p53 and p73 function in an antagonistic manner, the result of their activity is the same, namely maintenance of the stem cell pool. p53 and p73 may complement and/or regulate one another, depending on whether proliferation or quiescence is required, to ensure proper maintenance of the stem cell pool. In fact, it was recently shown that p73 together with p63, the third member of the p53 family of transcription factors, regulates p53 function to maintain the adult NPC pool (Fatt et al., 2014). In the telencephalon of the adult zebrafish, *p53* and *p73* expression can be observed in regions along the ventricular zone where adult neural stem cells, RGCs, are located (Adolf et al., 2006; Lam et al., 2009; März et al., 2010a). These regions include the homologs of the SVZ and the SGZ of the DG, respectively, the two stem cell niches in the mammalian telencephalon. In addition to the proliferative ventricular zone of the zebrafish telencephalon, *p53* and *p73* mRNAs are also detectable in other neurogenic sites of the adult zebrafish brain (Table 2). Thus *p53* and *p73* expression appear to be closely linked to regions of neurogenesis suggesting similar roles in zebrafish as in mammalian stem cell niches.

	***p53* mRNA**	***p73* mRNA**	**p53 protein**
Olfactory bulbs			
Telencephalon			
Vv	✓	✓	✓
Vd	✓	✓	✓
Ventricular zone of D	✓	✓	✓
Diencephalon			
PPa (only ventral part)	✓	✓	✓
Hav	✓	✓	✓
PP	not determined	not determined	not determined
DT	not determined	not determined	not determined
VM	✓	✓	✓
TPp	✓	✓	✓
Hd	✓	✓	✓
Hv	✓	✓	✓
Hc	✓	✓	✓
PTN			
Pit	not determined	not determined	not determined
Mesencephalon			
PGZ	✓	✓	✓
TL	✓	✓	✓
Hindbrain			
Val	✓	✓	
CCe	✓	✓	✓
LCa			

Table 2: The table lists the different proliferation zones in distinct domains of the adult zebrafish brain (Grandel et al., 2006). Proliferative domains showing expression of *p53* and *p73* mRNA and p53 protein are indicated by a check.

Abbreviations: CCe: corpus cerebelli; D: dorsal telencephalic area; DT: dorsal thalamus; Hav: ventral habenular nucleus; Hc: caudal zone of periventricular hypothalamus; Hd: dorsal zone of periventricular hypothalamus; Hv: ventral zone of periventricular hypothalamus; LCa: lobus caudalis cerebelli; PTN: posterior tuberal nucleus; Pit: pituitary; PP: periventricular pretectal nucleus; PPa: parvocellular preoptic nucleus, anterior part; TeO: tectum opticum; TL: torus longitudinalis; TPp: periventricular nucleus of posterior tuberculum; V: ventral telencephalic area; Val: lateral division of valvula cerebelli; Vd: dorsal nucleus of V; VM: ventromedial thalamic nucleus Vv: ventral nucleus of V.

In mammals p53 protein is subject to regulated protein degradation. Since p53 possesses an anti-proliferative capacity and is able to induce apoptosis, the activity of p53 has to be strictly regulated. Therefore, in normal unstressed cells, where p53 is not required, its level is kept low because it is rapidly degraded. The p53 protein becomes ubiquitinated by its main

regulator Mdm2 (Honda et al., 1997) which targets p53 for degradation by the 26S proteasome (Haupt et al., 1997; Kubbutat et al., 1997). Thus expression of the *p53* mRNA may not provide a comprehensive picture. By systematic analysis of sections through the entire brain by immunohistochemistry, p53 protein was detected in most regions, also in neurogenic sites, in the same pattern as its mRNA (Table 1 and Table 2). Exceptions are the sulcus ypsiloniformis (SY) in the telencephalon and the lateral division of the valvula cerebelli (Val) in the hindbrain where both *p53* and *p73* mRNA are expressed but no p53 protein expression was noted (Table 1). Although the levels of p53 protein are strictly regulated in mammals (Haupt et al., 1997; Kubbutat et al., 1997; Wu et al., 1993) the p53 protein seems to be present in a detectable amount in the same regions as the mRNA suggesting that levels of mRNA reflect protein expression in the brain of the zebrafish. In FISH experiments, I detected the p53 protein but not the *p53* mRNA in the ventral telencephalic area. This is a technical limitation of the double labeling procedure. In ISH experiments with *p53* mRNA alone and colorimetric visualization of the *p53* probe, mRNA expression was clearly detectable in the ventral telencephalic area. The p53 protein showed nuclear localisation, contrary to the mammalian brain where p53 shows a weak cytoplasmic staining in neurons (Inamura et al., 2001; Plesnila et al., 2007). The nuclear localization was confirmed by double staining with the nuclear marker DRAQ5.

When the activity of the p53 protein is not required it is rapidly degraded but when p53 is needed p53 and Mdm2 dissociate and p53 becomes also posttranslationally modified. Thus p53 is activated, rescued from degradation and is able to accumulate to high levels. Subsequently the transcription factor p53 transactivates target genes, which results in apoptosis, cell cycle arrest or senescence (reviewed in Boehme and Blattner, 2009). Cell cycle arrest is controlled by p53 via activation of p21 (el-Deiry, 1998; Wang et al., 2007b). p53 additionally induces the transcription of its own negative regulator Mdm2 (Barak et al., 1993; Juven et al., 1993) because both are connected in an autoregulatory feedback loop (Wu et al., 1993). In zebrafish p53 is negatively regulated by Mdm2 as well (Thisse et al., 2000). It was shown that *Mdm2* and *p21* are also target genes of p73 (Zhu et al., 1998a). To assess whether the pattern of expression of *p53* and *p73* in the zebrafish brain coincides with the target genes known from the mammalian literature I analyzed the expression of the zebrafish homologues in the brain relative to that of *p53* and *p73*. In the telencephalon *Mdm2* and *p21* mRNA had a similar pattern of expression. However, mRNA of the two genes could only be detected in some of the regions which expressed *p53* and *p73* mRNA. For example, *Mdm2* and *p21* were not expressed in the medial, lateral, dorsal and posterior zone of the dorsal telencephalic area (see Table 3 for comparative overview). Hence, p53 and p73 do not always activate the two downstream genes at least in the uninjured brain. In addition, I

observed also *Mdm2* and *p21* expressing regions where *p53* and *p73* expression was not detected, namely the central zone of the dorsal telencephalic area (Table 3). Taken together these results suggest additional and/or alternative mechanisms of regulation of Mdm2 and p21 in different brain areas. As p73, p63 is almost ubiquitously expressed in the adult mammalian brain (Hernandez-Acosta et al., 2011; Jacobs et al., 2005). Moreover, p63 is able to induce the expression of the p53 target genes *p21* and *Mdm2* (De Laurenzi and Melino, 2000; Levrero et al., 2000; McKeon and Melino, 2007; Murray-Zmijewski et al., 2006; Pietsch et al., 2008). Thus, p63 may be an alternative regulator of *Mdm2* and *p21* expression in the adult zebrafish brain.

	p53	***p73***	***Mdm2***	***p21***
TelV	✓	✓	✓	✓
Vc	✓	✓	✓	✓
Vd	✓	✓	✓	✓
Vv	✓	✓	✓	✓
Vp	✓	✓	✓	✓
Dc			✓	✓
Dm	✓	✓		
Dl	✓	✓		
Dd	✓	✓		
Dp	✓	✓		
SY	✓	✓	✓	

Table 3: The table compares the expression of *p53*, *p73*, *Mdm2* and *p21* mRNA in different domains of the adult zebrafish telencephalon. Expression is indicated by a check.
Abbreviations: D: dorsal telencephalic area; Dc: central zone of D; Dd: dorsal zone of D; Dl: lateral zone of D; Dm: medial zone of D; Dp: posterior zone of D; SY: sulcus ypsiloniformis; TelV: telencephalic ventricle; V: ventral telencephalic area; Vc: central nucleus of V; Vd: dorsal nucleus of V; Vp: postcommissural nucleus of V; Vv: ventral nucleus of V.

4.2 p53 and p73 expression in the injured brain

Injury of the adult zebrafish telencephalon by a stab wound leads to dramatic changes which entail cellular stress at the site of lesion followed by apoptosis and necrosis of damaged cells (Kroehne et al., 2011). Blood cells enter the brain at the lesion site and OPCs increase at the site of lesion (März et al., 2011). Within 2 to 3 days the neural stem cells at the ventricular zone start to proliferate and new neurons are born that migrate to the site of lesion (Baumgart et al., 2012; Kroehne et al., 2011; März et al., 2011). p53 (Boehme and Blattner,

2009; Levrero et al., 2000; Murray-Zmijewski et al., 2006; Pietsch et al., 2008) and p73 (Chen et al., 2001; Melino et al., 2002; Wang et al., 2007a) respond to several sorts of cellular stress. In p53-deficient mice proliferation of neural stem cells is highly increased (Gil-Perotin et al., 2006) suggesting that p53 is a negative regulator of neurogenesis. However, due to the anti-proliferative activity of p53 it may maintain the stem cell pool and thus neurogenesis in the adult mammalian brain (Gil-Perotin et al., 2006; Medrano et al., 2009; Meletis et al., 2006). In the medaka brain, p53 seems to positively regulate neurogenesis because a p53 null mutation does not increase but inhibit neurogenesis in the telencephalon of medaka (Isoe et al., 2012). p73 positively regulates self-renewal and promotes proliferation in the adult mammalian brain (Agostini et al., 2010; Fujitani et al., 2010; Talos et al., 2010).

Given these well-documented functions of p53 and p73 in cellular stress responses and neurogenesis in mammals, I investigated if the expression of p53 and p73 is changed at different time points after introducing a stab injury into the adult zebrafish telencephalon by ISH and immunohistochemistry. I observed an up-regulation of *p53* mRNA expression at 1, 3, 5, 7 and 9 dpl at the ventricular zone in the injured telencephalic hemisphere in contrast to the uninjured control hemisphere. In addition, *p53* mRNA was up-regulated at the site of the lesion, particularly at 5 dpl. The up-regulation of *p53* mRNA was less pronounced at 1 dpl. At 9 dpl the expression of *p53* mRNA was still increased. A very similar pattern and kinetics of expression was noted for *p73* mRNA. The p53 protein showed also an up-regulation at the ventricular zone at 1, 3, 5, 7 and 9 dpl. However, the expression of p53 protein was not increased at the site of the lesion, contrary to the mRNA. Thus, p53 protein and *p53* mRNA are regulated differentially at the site of the lesion. Interestingly, the target genes *Mdm2* and *p21* were also up-regulated at the site of lesion but not at the ventricular zone. It is tempting to speculate that p53 protein levels at the site of lesion may be regulated by Mdm2 via degradation resulting at levels of p53 protein beyond detection. As there is no up-regulation of p53 target genes at the ventricular zone after stab injury, p53 may exert its functions at the ventricular zone via other mechanisms.

p53 is able to induce apoptosis and therefore I examined if there is apoptosis following stab injury. TUNEL staining revealed that there is an induction of apoptosis in the lesioned hemisphere near the site of the lesion at 4 hpl as well as at 1 dpl. In the uninjured hemispheres, no apoptotic cells were detected. As the p53 protein is not up-regulated at the site of lesion it is unlikely that p53 induced apoptosis in cells residing close to the lesion following stab injury. In the mammalian brain, necrotic cell death is predominantly observed after injury (Liou et al., 2003; Vajda, 2002) but some apoptosis does also occur in regions surrounding the necrotic injury core (Liou et al., 2003). Necrosis leads to much stronger

inflammatory responses than apoptosis and causes a progressive loss of neural cells and scar formation (Zhang et al., 1997). In the mammalian brain, the pro-apoptotic p53 induces neuronal apoptosis following brain damage (Miller et al., 2000; Morrison and Kinoshita, 2000). However, it was also reported that p53 is activated after neuronal injury but it may not be responsible for the death of neurons and that brain injury is worse after ischemia without p53 (Maeda et al., 2001; Tomasevic et al.). In zebrafish, cell death is an early response to stab injury of the adult zebrafish brain and involves both apoptosis and necrosis (Kroehne et al., 2011). In lesions of the cerebellum of adult fish, apoptosis seems to be the predominant mechanism to eliminate damaged cells (Zupanc et al., 1998). The precise role of p53 at the lesion site remains to be determined - especially as the p53 mutant *tp53*^{M214K} does not show a phenotype. Possible explanations of this lack of a phenotype will be discussed below.

4.3 Expression of the p53 protein in the neural stem cell zone

In response to injury, p53 expression is increased in the neurogenic region of the telencephalon residing in the walls of the ventricles. There are distinct progenitor cell types present at the ventricular zone of the adult zebrafish telencephalon. They can be classified into at least four distinct cell types: Type I, Type II, Type IIIa and Type IIIb cells (März et al., 2010a). These cell types can be distinguished by their expression of specific markers (see chapter 1.1.2.1.1). p53 is expressed in a subset of *Tg(gfap:GFP)*-positive cells in uninjured brains. GFAP is a marker for quiescent Type I as well as proliferating Type II radial glial cells (RGCs) and is also weakly expressed still in Type IIIa neuroblasts (März et al., 2010a; Schmidt et al., 2013). Since p53 was expressed in cells strongly positive for GFAP and negative for the proliferation marker PCNA, I concluded that p53 is expressed in quiescent RGCs. In the adult mammalian brain, p53 is expressed in neural stem cells as well as progenitor cells. It suppresses the self-renewal of adult neural stem cells (Meletis et al., 2006). By modulating self-renewal, differentiation and proliferation, p53 plays a critical role in regulating the number of cells in the mouse SVZ (Gil-Perotin et al., 2006). Proliferation of neuronal stem cells is highly increased in p53-deficient mice and the number of new oligodendrocytes and neurons is enhanced after loss of p53 (Gil-Perotin et al., 2006; Li et al., 2008). It was suggested that it could be crucial to suppress self-renewal of stem cells by p53 for long-term maintenance of the stem cell population (Meletis et al., 2006). The expression of p53 protein in quiescent Type I cells in the adult zebrafish telencephalon is consistent with a possible anti-proliferative role of p53. However, I did not observe a change in proliferation rate in p53 mutants (see below for an in-depth discussion). In contrast p53 is not expressed in proliferating Type II cells. Most of the RGCs in the adult zebrafish telencephalon are Type I

cells and therefore quiescent. However, I noted also *Tg(gfap:GFP)*-positive cells that are negative for p53 and PCNA. Thus, p53 is not expressed in all Type I cells and seems to mark a subpopulation of Type I cells. These p53-positive Type I cells are scattered all over the ventricular zone of the adult zebrafish telencephalon.
Additionally, p53 is expressed in PCNA-positive and *Tg(gfap:GFP)*-negative cells. These cells are Type IIIb cells that express also PSA-NCAM that marks committed neural progenitors (März et al., 2010a). Staining with the neuroblast marker PSA-NCAM and the progenitor marker Nestin revealed that the p53 protein is expressed in Type IIIa (*Tg(-3.9nestin:GFP)*- and PSA-NCAM-positive) as well as in Type IIIb (*Tg(-3.9nestin:GFP)*-negative and PSA-NCAM-positive) cells in the RMS. Type III cells are predominantly located in the RMS (März et al., 2010a). Since p53 is not expressed in PCNA- and *Tg(gfap:GFP)*-positive cells this could suggest that p53 is not expressed in Type IIIa cells. However, the staining for nestin:GFP, p53 and PSA-NCAM shows, that this assumption is wrong and that p53 is also expressed in Type IIIa cells. Probably the staining for *Tg(gfap:GFP)* was just too weak to be detected in Type IIIa cells which normally show a much weaker GFAP staining than Type I or Type II cells (März et al., 2010a). In agreement, differentiating neuroblasts of the RMS of the mammalian brain express also p53 (Meletis et al., 2006). In summary, at the ventricular zone of the adult zebrafish telencephalon, 2 distinct cell types express p53: quiescent Type I RGCs and proliferating neuroblasts. In contrast, proliferating Type II RGCs do not express the p53 protein.

Stab injury leads to a dramatic increase of cell proliferation at the ventricular zone which peaks around 3 to 5 dpl and fades away until around 14 dpl (März et al., 2011). p53 expression is up-regulated at 5 dpl at the ventricular zone. However, most of these p53-expressing cells are PCNA-negative und are thus non-proliferating. Therefore, these cells represent Type 1 RGCs. A very similar profile of expression is presented by the helix-loop-helix protein Id1 in response to injury (Rodriguez Viales et al., in revision). Id1 suppresses proliferation of RGCs and is also up-regulated in wounded brains thus forming a feedback loop bringing proliferating RGCs back to quiescence after expansion in response to injury (Rodriguez Viales et al., in revision). It is tempting to speculate that p53 has a similar role in Type I cells, which show p53 expression in the uninjured brain and up-regulate the expression of p53 following injury to prevent exceeding proliferation of stem cells and thus depletion of the stem cell pool. p53 may maintain the stem cell population in the uninjured adult mammalian brain (Meletis et al., 2006). Therefore p53 may also be crucial to maintain the stem cell pool in the adult zebrafish telencephalon after injury. However, it was shown that in the embryonic murine brain p53 represses the transcription of *Id1* in NSCs. p53 deficiency leads to an enhanced expression of Id1, which results in increased proliferation

and neuronal differentiation of NSCs but less gliogenesis. There is a high level of p53 expression during neuronal differentiation and p53 most likely prevents proliferation and neuronal differentiation of NSCs but it positively regulates gliogenesis (Liu et al., 2013). In contrast to other observations, where Id1 reacts as a negative regulator of neuronal differentiation of NSCs (Miyazono and Miyazawa, 2002), enhanced expression of Id1 results in increased neuronal differentiation of NSCs deficient for p53 (Liu et al., 2013).
In the injured zebrafish brain, the p53 protein was also present in a few PCNA-positive cells, which are most likely Type III cells. The p53 protein was expressed in Type III cells in the uninjured brain as well. After stab injury of the adult zebrafish telencephalon both RGCs and neuroblasts show an increase of proliferation at the ventricular zone (März et al., 2011). After brain injury radial glial progenitor cells residing at the ventricular zone proliferate and give rise to neuroblasts. These neuroblasts migrate to the site of the lesion and differentiate into neurons which express mature neuronal markers and survive for more than 3 months (Kroehne et al., 2011). Contrary to constitutive neurogenesis, where newborn neurons settle mainly at the subventricular zone, in regenerative neurogenesis newborn neurons migrate longer distances into the injured parenchyma (Baumgart et al., 2012; Kroehne et al., 2011).

Glial cells accumulate at the site of lesion and they are also known in mammals to be key to scaring of injured neural tissue. The zebrafish brain does not contain astrocytes as known from the mammalian brain (Adolf et al., 2006; Grandel et al., 2006; März et al., 2010a; März et al., 2011). Abundant OPCs in the zebrafish brain may have taken over their function. I noted scattered p53 cells in the parenchyma of the telencephalon in a pattern characteristic of OPCs. However, mapping with respect to the OPC and mature oligodendrocyte marker olig2 did not reveal expression of p53 in OPCs or myelin basic protein (Mbp)-positive mature oligodendrocytes (Lyons et al., 2005; März et al., 2010b; Roth et al., 1986). Rather, I found p53 expression in scattered neurons located next to the OPCs. These p53-positive neurons located next to olig2-positive cells were not labeled by the proliferation marker BrdU at all 3 time points investigated. This indicates that the p53-positive neurons associated with cells from the oligodendrocyte lineage in the parenchyma are not newborn or immature neurons that become newly myelinated. In the mouse telencephalon similar observations were made for OPCs (Trotter et al., 2010). These slowly cycling OPCs in the adult mammalian brain generate mature oligodendrocytes that take part in myelin repair (Gensert and Goldman, 1997; Nait-Oumesmar et al., 1999; Polito and Reynolds, 2005). In the adult mammalian brain p53 is constitutively expressed at low levels in neurons as well and it becomes up-regulated after injury (Inamura et al., 2001; Plesnila et al., 2007). p53 retains the post-mitotic character of mammalian neurons, at least in cell culture experiments (Miller et al., 2003). Thus, it is tempting to speculate that p53 may have the same function in the adult zebrafish brain. The

anti-proliferative p53 protein may impede the cell cycle re-entry and proliferation of differentiated neurons. In the mammalian brain, it was demonstrated that p53 plays a role in the development of oligodendrocytes and the differentiation of OPCs (Billon et al., 2004). The observation that p53 is not expressed in the OPC lineage in the uninjured brain excludes such a function in the zebrafish brain.

In the injured adult zebrafish telencephalon the expression of p53 in HuC/D-positive neurons is not changed. However, quite in contrast to the uninjured brain, I found expression of p53 protein in *Tg(olig2:EGFP)*-positive cells that have accumulated at the site of lesion at 5 dpl. Importantly these OPCs at the site of lesion proliferate only moderately suggesting that most of the OPCs accumulated at the site of lesion have migrated into the damaged area. In mammals, Olig2 is highly up-regulated in cells at the lesion and these OPCs start to proliferate near the lesion. These events result in impaired neuronal regeneration (Buffo et al., 2005; Dimou et al., 2008; Hampton et al., 2004; Magnus et al., 2007; Robel et al., 2011; Sofroniew, 2009). It is thus possible that p53 has a role in zebrafish to prevent OPCs to proliferate and may thus contribute to the remarkable ability of the zebrafish brain to regenerate without scar formation. In mice p53 is important for the recovery of motor function after spinal cord injury. In p53 $^{-/-}$ mice the spinal cords show a larger scar at the lesion site and an increased number of activated microglia/macrophages (Floriddia et al., 2012). However, the mammalian literature reports also contradictory functions of p53: Deletion of p53 improved the recovery of neuromotor function following experimental brain trauma (Tomasevic et al., 2010) and the time to recover from a lesion is shorter in p53-negative mice than in wildtype mice (Uberti et al., 2001). This may reflect the complexity of the p53 regulated processes and also tissue specific functions including neurogenesis (Uberti et al., 2001), control of OPCs, macrophages and microglia at the site of lesion (Floriddia et al., 2012), axonal regeneration and neurite outgrowth (Di Giovanni et al., 2006; Tedeschi et al., 2009). As p53, p73 plays a role in the differentiation of oligodendrocyte progenitor cells (OPCs) in the mammalian brain (Billon et al., 2004).

4.4 The *tp53^{M214K}* mutant zebrafish

The *tp53^{M214K}* mutation is located in the DNA-binding domain. The mutant p53 transcription factor is not able to bind to the p53 recognition sequences and therefore activation of target genes is prevented (Berghmans et al., 2005). The *tp53^{M214K}* mutant zebrafish are viable. The adult homozygous fish are, however, tumor-prone and start to develop tumors at the age of 8.5 months (Berghmans et al., 2005).

Systematic analysis of neurogenesis, cell proliferation and apoptosis in the intact and injured zebrafish telencephalon did not reveal any deviation from the wildtype situation. Specifically, in *tp53*^{M214K} mutant zebrafish, apoptosis is induced at 4 hpl and 1 dpl, as in the wildtype brain. In contrast, the induction of apoptosis after γ-irradiation is suppressed in homozygous *tp53*^{M214K} mutant embryos (Berghmans et al., 2005). It was shown by Berghmans et al. that *Mdm2* and *p21* were not up-regulated in homozygous *tp53*^{M214K} mutant zebrafish embryos after γ-irradiation compared to wildtype embryos. However, I observed an up-regulation of both *Mdm2* and *p21* mRNA at 4 hpl and 1dpl at the site of the lesion, as in wildtype zebrafish although, the expression of *Mdm2* and *p21* was weaker than in the wildtype brains.
Apoptosis can also be induced by p53 independent of transcription via the mitochondria (Galluzzi et al., 2008) or by p73 (Levrero et al., 2000; Murray-Zmijewski et al., 2006). However, it was also demonstrated that tumor-derived mutants of p53 failed to induce cell death transcription-independently (Pietsch et al., 2008). In mammals p53 exhibits anti-proliferative capacities since p53 becomes activated after cellular stress and induces the expression of p21, which leads to cell cycle arrest (el-Deiry et al., 1993; Harper et al., 1993). One possibility to explain the lack of a phenotype in the adult zebrafish brain is that the mutant p53 protein retained some transcriptional regulatory activity. However with this scenario it is difficult to explain why the target genes *Mdm2* and *p21* are not induced in the mutant embryos after γ-irradiation. Therefore the most likely explanation is that p73 or the related p63 can compensate for the loss of p53. First, p73 is expressed in almost identical patterns in the intact and injured brain as p53. Second, p73 is able to induce apoptosis and the expression of *Mdm2* and *p21* in mammalian systems (Zeng et al., 1999; Zhu et al., 1998a). Furthermore, p63 is able to induce apoptosis (Levrero et al., 2000; Murray-Zmijewski et al., 2006) and to activate *Mdm2* and *p21* as well (Levrero et al., 2000; Murray-Zmijewski et al., 2006). Thus, the lack of clear phenotypes in the adult brain in *tp53*^{M214K} mutants is most likely a reflection of redundancy between p53 and p73 and potentially also p63.

In the intact and injured zebrafish telencephalon, the tp53^{M214K} mutant protein shows the same expression pattern as wildtype p53 protein and was also up-regulated at the ventricular zone of the injured hemisphere at 5 dpl as the wildtype p53 protein. Thus it appears that p53 expression does not require p53 DNA binding activity. Published data from analysis of *tp53*^{M214K} mutant embryos are contradictory with respect to increase of the mutant gene in response to γ-irradiation. Berghmans et al. did not observe an up-regulation of mutant *p53* mRNA in homozygous *tp53*^{M214K} mutant embryos in response to γ-irradiation. However, Guo et al. showed that mutant p53 protein accumulated in *tp53*^{M214K} mutant embryos after ionizing irradiation.

In the mammalian brain pro-apoptotic TAp73 isoforms induce neuronal cell death whereas anti-apoptotic ΔNp73 isoforms support the survival of neurons (Pozniak et al., 2002; Pozniak et al., 2000). It was shown that adult neurons are relatively invulnerable after injury and it was hypothesized that this is achieved by increasing the ratio of ΔNp73 to p53 (Jacobs et al., 2004; Walsh et al., 2004). Thus, if a neuron lives or dies depends on the relative levels of pro-survival (ΔNp63, ΔNp73) and pro-apoptotic (p53, TAp63, TAp73) proteins (Jacobs et al., 2004). Thus the balance of the proteins may play a role and as ΔNp73 isoforms support regeneration with p53 playing a less prominent role this fact may in addition contribute to the lack of a phenotype in regenerating p53 mutant brains.

4.5 Conclusion and future perspectives

I demonstrated that p53 and p73 are co-expressed in many domains of the adult zebrafish brain. In addition, the expression patterns of the p53/p73 target genes *p21* and *Mdm2* overlap in several but not all domains of the telencephalon with the expression patterns of *p53* and *p73.* After stab injury I observed an up-regulation of *p53*, *p73*, *Mdm2* and *p21* as well as induction of apoptosis. I also showed that p53 is expressed in Type I and III cells but not in proliferating Type II cells in the stem cell niches of the adult zebrafish telencephalon. p53 is expressed in mature neurons and some of these p53-positive differentiated neurons are located next to OPCs. Furthermore, OPCs start to express p53 close to the wound suggesting a specific function in these cells after injury. I analysed in depth constitutive and reactive neurogenesis in *tp53*^{M214K} mutant zebrafish. However, there was no difference concerning the reaction to stab injury between wildtype and mutant fish. These results possibly indicate that p53 and p73 act redundantly and that p73 takes over the functions of p53 in case of p53 deficiency. Although my expression analysis suggests that p53 plays crucial roles in various processes during adult neurogenesis, the ultimate experimental evidence was masked by the lack of a phenotype of p53 mutants. The next steps should therefore be the generation of a knock-out of p73 and the analysis of double mutants. With help of a transgenic line for p73 or a p73 specific antibody, the expression of p73 in the different cell types in the adult zebrafish telencephalon should be examined and compared to the expression of p53 to further proof interaction and redundancy of these proteins. Furthermore, it would be interesting to investigate the putative interaction between p53 and Id1 and if p53 indeed confers quiescence to Type I cells by knock-down and overexpression experiments.

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