POSTMITOTIC STATE OF A CELL AS A CHALLENGE FOR REGENERATION: INNER EAR AS A MODEL

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ACADEMIC DISSERTATION

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Helsinki 2017
Cover image: (UP) Whole mount preparation of cochlea from ICR mouse at age P12. Phalloidin labeling (violet) of filamentous actin shows cellular organization of hair cells and β-tubulin staining (green) marks microtubule network of supporting cells. (DOWN) Whole mount preparation of utricle from ICR mouse at age P55. Parvalbumin staining (violet) marks hair cells and Sox2 staining (green) marks supporting cells.
“The true delight is in the finding out rather than in the knowing”

Isaac Asimov
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (referred to in the text by their Roman numerals):


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CONTRIBUTIONS

I The author contributed to the planning of the experiments and conducted all of the experiments. The author participated in the writing of the manuscript.

II The author contributed to the planning of the experiments and conducted the majority of the experiments. The author contributed to the writing of the manuscript.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AdβGal</td>
<td>Adenovirus encoding β-galactosidase</td>
</tr>
<tr>
<td>AdcD1</td>
<td>Adenovirus encoding cyclin D1</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia mutated</td>
</tr>
<tr>
<td>Atoh1</td>
<td>Atonal homolog 1</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BIO</td>
<td>6-bromoindirubin-3'-oxime</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>βGal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>cD1</td>
<td>cyclin D1</td>
</tr>
<tr>
<td>Chk2</td>
<td>Checkpoint kinase 2</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DAPT</td>
<td>N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in vitro</td>
</tr>
<tr>
<td>DII</td>
<td>Delta-like</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>DSB</td>
<td>DNA double-strand break</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2'-deoxyuridine</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fzd</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GER</td>
<td>Greater epithelial ridge</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>HRR</td>
<td>Homologous recombination repair</td>
</tr>
<tr>
<td>HC</td>
<td>Hair cell</td>
</tr>
<tr>
<td>H2AX</td>
<td>H2A variant X</td>
</tr>
<tr>
<td>γH2AX</td>
<td>H2A variant X phosphorylated at serine 139</td>
</tr>
<tr>
<td>IHC</td>
<td>Inner hair cell</td>
</tr>
<tr>
<td>Jag</td>
<td>Jagged</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Lgr5</td>
<td>Leucine-rich repeat-containing, G-protein-coupled receptor 5</td>
</tr>
<tr>
<td>LRPS5/6</td>
<td>Low-density lipoprotein receptor-related protein 5/6</td>
</tr>
<tr>
<td>MAML</td>
<td>Mastermind-like</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Mouse double minute 2 homolog</td>
</tr>
<tr>
<td>MRN</td>
<td>Mre11-Rad50-Nbs1</td>
</tr>
<tr>
<td>Myo6</td>
<td>Myosin 6</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end-joining</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>OHC</td>
<td>Outer hair cell</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>Rbpj</td>
<td>Recombining binding protein suppressor of hairless</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SC</td>
<td>Supporting cell</td>
</tr>
<tr>
<td>SNHL</td>
<td>Sensorineural hearing loss</td>
</tr>
<tr>
<td>TACE</td>
<td>Tumor necrosis factor-$\alpha$-converting enzyme</td>
</tr>
<tr>
<td>53BP1</td>
<td>p53-binding protein 1</td>
</tr>
</tbody>
</table>

Gene names are written in *italics* and protein names in Roman.
ABSTRACT

Supporting cells (SCs) of the mammalian inner ear are differentiated, postmitotic cells that hold promise as a platform in therapeutic interventions to replace lost sensory cells, the hair cells (HCs). However, SCs exhibit an age-dependent decline in their responsiveness to regenerative manipulations that aim to trigger cell cycle re-activation or to stimulate transdifferentiation into HCs. The aim of this PhD thesis project was to identify barriers restricting the therapeutic potential of auditory and vestibular SCs. Mammalian SCs were studied with the aid of organotypic cultures of mouse inner ear sensory epithelia, viral-mediated gene transfer in vitro, mouse models with targeted gene inactivation, immunohistochemistry, and imaging. Using viral-mediated ectopic expression of cyclin D1 (cD1) in organotypic cultures to force postmitotic SCs to re-enter the cell cycle, the efficiency of the cells to complete cell cycles was shown to decrease with maturation. Unscheduled cell cycle re-activation was found to be associated with accumulation of DNA double-strand breaks (DSBs), indicated by the upregulation of the serine 139 phosphorylated form of histone H2AX (γH2AX). By studying the dynamics of the DNA repair protein Rad51, the underlying reason for age-related restrictions in proliferative plasticity was shown to be delayed or inefficient DNA repair. Furthermore, delayed repair of DNA damage was found to lead to SC death. To study the possible involvement of DNA damage in stimulated SC-to-HC transdifferentiation, the pharmacological inhibitor of Notch signaling that triggers SC transdifferentiation was applied to inner ear explant cultures. It was shown that unlike forced cell cycle re-activation, stimulated transdifferentiation does not trigger DNA damage, suggesting that it might be a “safe” way generate HCs.

p53 has been shown to antagonize cell proliferation and regenerative events in other contexts. Thus, it was investigated here whether this is the case also in the inner ear. Using a loss-of-function mutant mouse model, p53 was shown to be dispensable for inner ear development. By applying various growth-promoting manipulations on cochlear explants from these mutant mice in vitro, inactivation of p53 was shown to not confer regenerative potential to SCs. Excess levels of p53 that are generally associated with cellular stress response have been shown to direct cells to cell death. Thus, p53 levels are under a tight control. The significance of controlled levels of p53 in different developmental situations and in a tissue context is poorly understood. Thus, inner ear was used here as a model to study the consequences of p53 overexpression in various developmental contexts: proliferation; differentiation; and homeostasis. Mutant mouse models in which the interaction between p53 and its negative regulator Mouse double minute 2 (Mdm2) was abolished demonstrated that p53 accumulation is lethal to both proliferating HC, SC and neuronal progenitors, and quiescent, differentiating SCs and HCs. More thorough analysis that focused on SCs showed that their sensitivity to p53 decreases with postnatal maturation. The data presented here thus suggests that epigenetic signaling and maturation-related mechanisms that regulate chromatin conformation might limit p53’s pro-apoptotic functions in maturing SCs.
This PhD work has revealed DNA damage signaling and inefficient DNA repair as important barriers restricting the proliferative potential of mammalian SCs. This work also demonstrates the importance of controlled levels of p53 for the survival of cells of the auditory organ. Furthermore, the data obtained from this work can be extrapolated to other postmitotic, differentiated cell types when evaluating their potential for regenerative therapies.
**SUMMARY (KOKKUVÕTE)**


Kirjanduses on arvukalt näiteid olukordadest kus kasvaja supressor p53 takistab raku jagunemist ja regeneratiivseid protsess. Et uurida p53 rolli sisestöö, kasutati transgeenset hiiremudelit, kus p53 ei ole funktsionaalne. Selle hiiremudeli abil näidati,


1. INTRODUCTION

The inner ear is a fascinating organ that allows us to experience the wonderful world of sounds and to position ourselves in space. Unfortunately, the mammalian inner ear is very sensitive to insults and has only limited capacity to restore its function following traumas. Significant hearing loss affects 5% of the world’s population. The worldwide prevalence of balance dysfunctions is unclear due to difficulties in its accurate diagnosis. In the US, a significant 35% of the adult population has experienced some form of balance dysfunction. Importantly, both hearing and balance deficiencies are highly common in growing elderly populations.

Currently, no therapeutic interventions are available to restore sensory deficiencies associated with the inner ear. However, mechanical devices are available for people suffering from hearing dysfunction, such as: hearing aids that amplify sounds; and cochlear implants that bypass the defective sensory HCs and directly stimulate auditory nerve fibers. Cochlear implants allow profoundly deaf individuals to perceive sound, however, these devices can restore the functionality of the hearing organ only partially. Vestibular implants are currently under development and clinical trials have commenced to evaluate their safety and efficacy. Altogether, a biological therapeutic intervention to restore sensory deficiencies is very much needed.

Intense research efforts on the regenerative potential of the mammalian inner ear were fueled by discoveries almost three decades ago that non-mammalian vertebrates have the capacity to regenerate sensory HCs in both their hearing and balance epithelia (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Decades of work have elucidated that mammalian inner ear sensory epithelia exhibit limited regenerative plasticity and that it is mediated by non-sensory postmitotic SCs, like in non-mammalian species. Unfortunately, the regenerative plasticity of these cells in mammals rapidly declines during early postnatal development. The underlying mechanisms for this decline have remained largely unexplored.

Postnatal mammalian inner ear SCs represent an interesting population of postmitotic, differentiated epithelial cells that during early postnatal development progress from a plastic, responsive state to a non-responsive state. This rapid age-associated decline in regenerative plasticity is an interesting phenomenon and elucidating the underlying mechanisms could be useful for understanding the maturation-associated decline of plasticity in other postmitotic, differentiated cell types.
2. LITERATURE REVIEW

2.1 Postmitotic, differentiated cell types and regenerative plasticity

2.1.1 Cell cycle

Activation of cell cycle leads to DNA replication and production of two daughter cells. Cell cycle progresses from G1 (protein synthesis for DNA replication) to S (DNA replication), to G2 (protein synthesis for cell division), and M (mitosis) phase (Fig. 1). Mitosis consists of prophase, metaphase, anaphase, telophase, and cytokinesis. During prophase, chromatin condenses, nuclear membrane and nucleoli disappear, and mitotic spindle becomes evident. During metaphase, chromosomes are aligned at the equatorial plane. In anaphase, chromatids are separated to the opposite sides of the mitotic spindle. Finally, during telophase chromosomes decondense into diffuse chromatin and the nuclear membranes are formed around the two new nuclei, after which the cytokinesis takes place and cytoplasm is divided between the daughter cells. Proliferation and differentiation are not usually compatible and, therefore, the onset of cell differentiation is preceded by cell cycle exit. Differentiated cells are permanently maintained in G0 phase.

The cell cycle is orchestrated by an intricate network of positive and negative regulators that respond to extracellular (mitogens) and intracellular cues (DNA damage, nutrients) and mediate cell cycle checkpoint pathways. Eukaryotic cell cycle exhibits three main checkpoints that regulate cell cycle progression: G1/S, G2/M, and spindle checkpoint (Fig. 1). Core cell cycle regulators comprise mitogen-responsive cyclins that bind and activate their respective serine/threonine cyclin-dependent kinases (CDKs) (Fig. 1). CDKs are responsible for regulating the transition from one phase of the cell cycle to the next, only after the cell has completed the previous phase. Cell cycle activating cyclin-CDK complexes are suppressed by two families of cyclin-dependent kinase inhibitors (CKIs): the INK4 family (p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c} and p19\textsuperscript{INK4d}) that inhibits cyclin D-CDK4/6 complexes and Cip/Kip family (p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2}) that suppresses all other cyclin-CDK complexes. Cell cycle entry or G1-to-S transition is regulated by the tumor suppressor retinoblastoma protein (pRb) that belongs to a retinoblastoma protein family together with p107 and p130 proteins. pRb is maintained in an active, hypo-phosphorylated state by CKI-mediated negative regulation of cyclin-CDK activity. In its active state, pRb inhibits E2F transcription factors that activate the expression of genes required for cell cycle progression. Normally cyclins and CDKs function at a particular phase of the cell cycle. However, remarkably, they can substitute for each other’s functions in different phases. The unidirectionality of the cell cycle is safeguarded by ubiquitin ligase complexes that in a stage-specific manner target the core cell cycle regulators for degradation (Ang and Wade Harper, 2005).

In addition to above-mentioned core cell cycle regulators, the cell cycle is also regulated by the p53 tumor suppressor that coordinates cellular responses to DNA
damage by regulating G1/S and G2/M checkpoint pathways. More detailed description of the DNA damage-activated DNA damage response is given in chapter 2.2.

Figure 1. Cell cycle and cell cycle checkpoints. The G1/S checkpoint examines the cell size, nutrient status, growth factors and DNA integrity to decide whether conditions are right for cell division. If the right conditions are not met, the cell can enter a resting phase, called G0. The G2/M checkpoint ensures that the DNA is intact and that all the DNA has been replicated before it allows mitosis to commence. The spindle checkpoint during mitosis assures that all the chromosomes have attached to the spindle before it allows anaphase to start. The checkpoint pathways communicate with cyclin (cyc)-CDK complexes and their negative regulators, CKIs. According to the classical model of cell cycle regulation, at G1 phase, upon mitogenic signals, cyclin D binds and activates CDK4 and CDK6, leading to hyper-phosphorylation and inactivation of pRb (Sherr and Roberts, 1999). Phosphorylation-mediated inactivation of pRb leads to activation of E2F transcription factors and transcription of genes required for the G1-to-S phase transition, including cyclin E. In late G1, accumulating cyclin E activates CDK2 that further phosphorylates pRb, leading to progression into S phase. During S phase, cyclin A complexes with CDK2 and regulates DNA replication. Cyclin A/CDK1 complex is important for G2 phase and G2/M transition is regulated by cyclin B/CDK1 complex. The activity of cyclin-CDK complexes is suppressed by INK4 family of CKIs (inhibits cyclin D-CDK4/6 complexes) and Cip/Kip family of CKIs (suppresses all other cyclin-CDK complexes).
2.1.2 Cell cycle exit and differentiation

Most cells in adult animals are in a differentiated state that is established following permanent exit from the cell cycle during development. The differentiated state is associated with specialized morphological and physiological features that enable the cell to perform unique functions. In this state, cells are maintained in G0 phase and are generally refractory to cell cycle promoting mitogens due to high levels of negative cell cycle regulators. It is generally believed that a cell must exit the cell cycle before it can differentiate and conversely that a differentiated cell is unable to proliferate unless it dedifferentiates and loses its specific molecular and morphological features. The fact that differentiated state is incompatible with proliferation is demonstrated, for example, in neurodegenerative diseases, such as Alzheimer’s disease, where differentiated neurons that ectopically activate their cell cycles succumb to apoptosis (Yang et al., 2001). The importance of dedifferentiation for the proliferation of a postmitotic cell is also demonstrated in regenerating zebrafish retina, where it allows postmitotic differentiated Müller glia to proliferate and to replace lost neurons (Fausett and Goldman, 2006). Interestingly, however, there are examples showing that differentiated state can be maintained during proliferation. For example, proliferating adult mouse retinal neurons maintain their morphological features following retinoblastoma family ablation-mediated cell cycle re-activation (Ajioka et al., 2007).

Whether permanent cell cycle exit and differentiation are even separate events is unclear, as cell cycle regulators can also regulate differentiation. To add to the complexity, the components of cell cycle machinery can also be re-purposed for non-cell cycle associated functions following permanent cell cycle exit, shown for example in differentiated neurons (Herrup, 2013). One example of such a case is negative cell cycle regulator CKI p57kip2 that during postnatal retinal development promotes retinal neuron differentiation (Dyer and Cepko, 2000). Altogether, it remains unclear how cell cycle exit and differentiation are coordinated. Different tissues seem to utilize different mechanisms at different stages of development.

2.1.3 Regenerative potential of postmitotic, differentiated cells

Mammals, including humans, show poor capacity to regenerate tissues following injury. Tissue regeneration is mediated either via activation of somatic stem cells or dedifferentiation and transdifferentiation of differentiated cells (Jopling et al., 2011). Somatic stem cells maintain tissue homeostasis by proliferating and giving rise to progeny that differentiates into tissue-specific cell types. Some tissues in mammals, such as the hematopoietic system, skin and intestinal epithelium, contain multipotent somatic stem cells and are thus able to continuously renew their populations of differentiated, functional cell types.

In addition to somatic stem cells, differentiated cell types can also contribute to tissue homeostasis. Dedifferentiation allows differentiated cells to revert back to a less
differentiated stage of their own lineage. By acquiring a more immature state, these cells are able to re-enter the cell cycle and replenish lost cells before redifferentiating. This process is used for example in regenerating amphibian limb. The cells that are close to the wound dedifferentiate, forming a blastema of undifferentiated proliferating cells that eventually redifferentiate, providing all the cell types necessary for the limb reconstruction. Natural dedifferentiation is also found in the mammalian peripheral nervous system, where Schwann cells dedifferentiate and proliferate following an injury to the nerve they are associated with (Chen et al., 2007). Tissue homeostasis can also be mediated by transdifferentiation or lineage conversion of differentiated cells. One of the rare examples of natural transdifferentiation in mammals comes from the inner ear, where postmitotic, fully differentiated SCs can transdifferentiate into sensory HCs following toxin-induced trauma to HCs (Golub et al., 2012).

Altogether, despite the rare examples of regenerative plasticity, the majority of adult mammalian tissues show only limited capacity to restore their functional cells following trauma. Examples of such easily depleted functional cell types include cardiomyocytes, neurons and inner ear HCs. At the other end of the spectrum are non-mammalian vertebrates that are able to regenerate many tissues (teleost fish, such as zebrafish; urodele amphibians, such as salamanders).

2.1.3.1 Cardiomyocyte regeneration

Cardiomyocytes of the adult mammalian heart were previously considered postmitotic. However, by using radiocarbon dating, adult human cardiomyocytes have been shown to exhibit constant, albeit very low turnover that declines with aging (Bergmann et al., 2009). Cardiomyocytes in neonatal mouse heart are able to proliferate following injury, but their regenerative plasticity declines by the end of the first postnatal week (Porrello et al., 2011). Remarkably, cardiomyocytes also from newborn human fetus have recently been shown to exhibit proliferative plasticity following myocardial infarction (Haubner et al., 2016). In addition, there are indications that a low number of cardiac progenitors exist in the adult mammalian heart (Beltrami et al., 2003; Ellison et al., 2013), but their capacity to restore cardiac function after infarction is limited (van Berlo et al., 2014). Thus, unlike in adult zebrafish heart, where differentiated cardiomyocytes replace lost cardiomyocytes following cardiac injury by dedifferentiation and cell cycle re-activation (Jopling et al., 2010; Poss et al., 2002), their counterparts in mammalian heart are incapable of doing so (Graham and Bergmann, 2017). Thus, cardiac injury in adult mammals leads to the deposition of fibrotic scar tissue and decreased contractile function.

Regenerative approaches to augment postnatal mammalian cardiomyocyte populations have taken advantage of manipulations of cell cycle regulators. Negative cell cycle regulators pRb (encoded by \( Rb \)) and p130 (encoded by \( Rbl2 \)) have shown to direct heterochromatin accumulation to cell cycle promoting E2F target genes in postnatal postmitotic cardiomyocytes (Sdek et al., 2011). Thus, \( Rb/Rbl2 \)-ablated adult cardiomyocytes are able to re-activate their cell cycle and re-express some of the fetal
cardiac genes. However, the number of these cardiomyocytes that are able to progress through the cell cycle is low. In addition, the overexpression of a positive cell cycle regulator cyclin D2 triggers cell cycle re-activation of mature cardiomyocytes and improves healing from myocardial infarction (Pasumarthi et al., 2005). However, cardiomyocyte production is limited following such manipulations and full functional restoration of the mammalian heart remains out of reach.

One of the possible reasons for inefficient cell cycle progression of mammalian postmitotic differentiated cardiomyocytes might be their inability to dedifferentiate and to lose their morphologically complex characteristics (sarcomeric contractile structures, binucleated state) that impose obstacles for cell division.

2.1.3.2 Neuronal regeneration

Neurons are postmitotic, differentiated cells that are unable to divide after they have undergone terminal mitoses during development. New hippocampal neurons are produced in the adult mammalian brain, but it is based on divisions of stem cells in the stem cell niche of the hippocampal dentate gyrus and never on cell cycle re-activation and division of differentiated neurons (Altman and Das, 1965; Goncalves et al., 2016).

Under certain conditions, such as following neurotrophic factor deprivation and DNA damage, differentiated neurons have been shown to re-activate their cell cycle, but it generally leads to cell cycle arrest before S-phase and apoptosis (Farinelli and Greene, 1996; Park et al., 1997). Differentiated neurons can also initiate cell cycle during early stages of neurodegenerative pathologies, such as Alzheimer’s disease, but these cells arrest in cell cycle after replicating their DNA and become tetraploid (Yang et al., 2001). These tetraploid neurons are proposed to gradually die over a longer period of time. Spinal cord injury has also been shown to trigger neuronal cell cycle re-initiation that is followed by apoptotic death (Di Giovanni et al., 2003). The importance of the maintenance of the postmitotic state for neuronal survival is underlined by a study with transgenic mice whose neurons lacking the negative cell cycle regulator pRb re-enter the cell cycle and massively die (Lee et al., 1992). Under certain conditions, however, differentiated neurons can progress through the entire cell cycle. Retinal horizontal neurons lacking pocket proteins pRb and p130 re-initiate and complete the cell cycle while remaining fully differentiated (Ajioka et al., 2007).

All the above-mentioned examples, with rare exceptions, indicate that the differentiated status of a cell presents a formidable obstacle on the road to neuronal regeneration. In the majority of cases, cell cycle re-initiation in differentiated neurons seems to be associated with a stress-response to an insult or with a pathological process.
2.2 DNA damage and repair

All cellular DNA continuously accumulates lesions arising from a wide array of endogenous (reactive oxygen species (ROS) accumulating from cellular metabolism) and exogenous sources (UV light and ionizing radiation). Resulting mutations and chromosomal re-arrangements can lead to an aberrant activation of oncogenes and inactivation of tumor suppressors, and eventually lead to cancer development. Cells have evolved an elaborate signaling network termed DNA damage response (DDR) to detect and repair DNA damage (Jackson and Bartek, 2009). DDR signaling communicates with cell cycle machinery and can arrest the cell cycle in G1, S, or G2 phases upon detection of genotoxic lesions, to prevent DNA-damaged cells from entering mitosis and propagating the damage to their progeny.

2.2.1 DNA damage response

DDR signaling components can be grouped into damage sensors, mediators, signal transducers and effectors that mediate DNA repair and cell fate decisions. Depending on the type of DNA damage, different arms of DDR signaling are activated (Shiloh, 2003). In case of DNA double-strand breaks (DSBs), the most severe form of DNA damage, damage-sensing Mre11-Rad50-Nbs1 (MRN) complex detects the damage and recruits phosphoinositide 3-kinase related kinase Ataxia-telangiectasia mutated (ATM) to the lesion sites (Fig. 2). ATM phosphorylates histone H2A variant X (H2AX) on serine 139 in megabase-sized regions surrounding the break. Phosphorylated H2AX (γH2AX) acts as a docking site for mediators, such as p53-binding protein 1 (53BP1) and transducers, such as Checkpoint kinase 2 (Chk2) kinase that amplify the signal. Activated DDR signaling converges onto p53 that is post-translationally modified and activated (its half-life increases and the transcriptional activity is enhanced), and activates also DNA damage repair signaling. Depending on the severity of the DNA lesion, the phase of the cell cycle and success of DNA repair, activated p53 can direct cells either to transient cell cycle arrest (via transcriptional activation of p21^{Cip1} CKI), apoptosis (via transcriptional and non-transcriptional activation of pro-apoptotic B-cell lymphoma 2 (Bcl-2) family members) or senescence (via transcriptional activation of tumor suppressor p19^{ARF} and CKI p16^{Ink4a}) (Green and Kroemer, 2009; Riley et al., 2008).
Figure 2. DNA damage response to double strand breaks.
2.2.2 DNA repair in postmitotic cells

DNA accumulates various kinds of lesions, such as: single-and double-strand breaks; inter-and intra-strand crosslinks; and DNA base damage. DSBs are the most severe type of DNA damage, as they can lead to chromosomal rearrangements. DSBs are repaired by non-homologous end-joining (NHEJ) and homologous recombination repair (HRR) pathways (Chapman et al., 2012). NHEJ is an error-prone DNA repair pathway that functions mainly during G0/G1 phases of the cell cycle and relies on DNA-dependent protein kinase (DNA-PK) complex. As NHEJ functions by direct ligation of DSB ends, nucleotides are often lost or added during the repair process. HRR is a high-fidelity DNA repair pathway that relies on Rad51-family proteins and functions during S/G2/M phases of the cell cycle when a homologous DNA duplex is present and can be used as a template to recover all the lost sequences. During HRR, the DSB is resected, generating a 3’ single-stranded DNA overhang that invades an intact homologous sister chromatid and uses it as a template to repair the DSB.

When cells exit the cell cycle and differentiate, their physiology and properties of their genome change, leading to changes in their DNA repair properties. Transcriptionally silenced chromatin, heterochromatin, that constitutes a large part of chromatin in terminally differentiated cells poses difficulties for DNA repair (Goodarzi et al., 2008). As postmitotic, differentiated cells are not supposed to replicate their DNA, they can afford to accumulate lesions to non-transcribed regions of the DNA. Thus, to save energy, a differentiation-associated repair mechanism that effectively repairs only transcribed regions of the genome becomes prominent in these cells (Nouspikel and Hanawalt, 2002). In addition, error-prone NHEJ becomes the major mechanism of DSB repair in postmitotic cells, as homologous template strand required for HRR is unavailable, as shown in differentiating neurons, for example (Orii et al., 2006). Age-associated attenuation of DNA repair capacity has been demonstrated in mouse hepatocytes and human fibroblasts (Guedj et al., 2016; Li et al., 2016). Furthermore, human premature aging syndromes, such as Werner syndrome, are caused by mutations in DNA repair proteins (Bohr, 2005), linking deficiencies in DNA repair to aging.

2.2.3 DNA damage response and regenerative manipulations

Considering the above-mentioned properties of postmitotic, differentiated cells (inefficient DNA repair and accumulation of heterochromatin that restricts DNA repair) it is no surprise that regenerative manipulations that trigger cell cycle re-activation are inefficient, often leading to cell cycle arrest and/or apoptosis. Indeed, cell cycle re-activated postmitotic pancreatic β-cells and cochlear HCs accumulate DNA damage and arrest in cell cycle or succumb to apoptosis (Rieck et al., 2012; Sulg et al., 2010). DNA DSB accumulation and DDR activation also occur during somatic cell reprogramming to pluripotent stem cells, even if non-integrative methods to deliver reprogramming factors are used (Gonzalez et al., 2013). Pluripotent stem cells obtained via somatic cell
reprogramming contain genetic abnormalities, suggesting that DDR is not able to orchestrate complete DNA repair (Laurent et al., 2011). Still, the expression of key regulators of DSB repair is indispensable for efficient reprogramming (Gonzalez et al., 2013). Furthermore, the co-expression of DNA repair components, such as HRR component Rad51 together with reprogramming factors increases the efficiency of reprogramming (Lee et al., 2016). It has been proposed that DNA damage accumulates during reprogramming due to DNA replication stress caused by oncogenic reprogramming factors (Bartkova et al., 2006) or due to global epigenetic remodeling that takes place concomitantly with the reprogramming process.

2.3 Cell death

Like cell cycle and differentiation, cell death is a core biological process that is indispensable for normal development and maintenance of homeostasis. Throughout life, cell death is necessary to remove injured, stressed, and genomically aberrant cells from the organism. The importance of cell death is underlined by the fact that deregulated cell death leads to aberrant development and different pathological conditions, such as cancer and neurodegenerative diseases.

Based on the extent of regulation, cell death can be broadly categorized as non-physiological necrosis and regulated, physiological apoptosis.

Necrosis is a cellular death response to traumatizing factors that are in a non-physiological range (such as toxins, infections, or injury). It is manifested as a cellular swelling and rupture of the plasma membrane, followed by the release of cytoplasmic components to the extracellular space. Necrosis can have severe consequences, even death of an organism, as it triggers an inflammatory response in the surrounding tissue.

Apoptosis, on the other hand, is a highly orchestrated process that is triggered by internal (DNA damage, cell cycle checkpoint defects, ROS accumulation, loss of survival factors, activation of stress signaling pathways) or external stimuli (growth factor withdrawal, pathogens, hormones, cytokines). Apoptosis is manifested as cellular shrinkage, chromatin condensation, DNA fragmentation, and formation of apoptotic bodies that are engulfed by phagocytes. Apoptosis is a “safe” form of cell death as the cytotoxic and inflammatory components of the dying cell do not come into contact with the surrounding tissue.

2.3.1 Programmed cell death

Programmed cell death (PCD) is a developmentally regulated type of death that was initially thought to occur mainly by apoptosis (Fuchs and Steller, 2011). However, when the core pro-apoptotic regulators are genetically inactivated in the mouse, the developmental phenotype of these animals is relatively normal (Lindsten and Thompson, 2006). This suggests that non-apoptotic mechanisms of cell death (such as
necroptosis and autophagic cell death) are also involved in PCD, perhaps eliminating cells when apoptosis is prevented (Yuan and Kroemer, 2010).

PCD has an extensive array of functions during development (Fuchs and Steller, 2011). It regulates cell numbers (for example in the developing nervous system where neurons are initially overproduced), eliminates defective cells, remodels tissues, and removes structures that are no longer needed. Furthermore, caspases, the core mediators of apoptosis, can also directly regulate development independent of their apoptosis-related functions (Fuchs and Steller, 2011).

2.3.2 Apoptosis

Most of the morphological changes (such as DNA fragmentation and chromatin condensation) observed in cells undergoing apoptosis are induced by a family of cysteine proteases, caspases that in normal healthy cells are expressed as inactive precursors. The caspase family consists of initiator caspases (caspase-2,-8,-9-10) that initiate apoptosis and effector caspases (caspase-3,-6,-7) that orchestrate the destruction of the cell (Cohen, 1997). Following death-inducing stimuli, initiator procaspases go through autoproteolytic processing and initiate apoptosis by proteolytically cleaving and activating the effector pro-caspases.

Apoptosis can occur via an extrinsic death receptor pathway (stimulated for example by pathogens) or intrinsic mitochondrial pathway (stimulated by intracellular stress stimuli, such as DNA damage) (Elmore, 2007). Both extrinsic and intrinsic apoptotic pathways converge at the effector caspase-3 mediated execution pathway. During the execution phase of apoptosis, the activated effector caspase-3 and other effector caspases cleave cellular substrates, and activate cytoplasmic nuclease that degrades DNA and proteases that degrade nuclear and cytoskeletal proteins. The execution phase leads to DNA fragmentation, protein degradation, and formation of apoptotic bodies. The engulfment and degradation of apoptotic bodies are mediated by phagocytes that are activated by exposed phosphatidylserine on the extracellular surface of the dying cell membrane.

The extrinsic pathway is induced by the activation of transmembrane death receptors leading to the autoproteolytic processing and activation of initiator procaspase-8. Activated caspase-8 cleaves and activates effector pro-caspase-3, initiating the execution phase.

The intrinsic pathway is initiated upon the activation of the pro-apoptotic members of a Bcl-2 protein family (Elmore, 2007). Bcl-2 protein family regulates apoptosis by controlling the localization of mitochondrial cytochrome c. Cytosolic localization of cytochrome c triggers apoptosis. The balance between the pro- and anti-apoptotic members of the Bcl-2 family determines whether cytochrome c is released from mitochondria. Pro-apoptotic Bcl-2 proteins mediate its cytoplasmic release by triggering the permeabilization of the mitochondrial outer membrane or by forming pore channels into the mitochondrial membrane. Cytosolic cytochrome c activates
initiator pro-caspase-9 that cleaves and activates the executioner pro-caspase-3 leading to initiation of the execution phase.

Tumor suppressor and transcription factor p53 is the main regulator of cellular responses to stress (more details in chapter 2.4), regulating both intrinsic and extrinsic pathways of apoptosis. Stress-stabilized p53 activates the transcription of pro-apoptotic genes of the Bcl-2 family (such as Bax, Bak, Puma) that regulate the intrinsic pathway of apoptosis. In addition, p53 directly interacts with cytoplasmic and mitochondrial pro (Bax) and anti-apoptotic (Bcl-2, Bcl-xL) members of the Bcl-2 family that regulate the release of cytochrome c from mitochondria.

2.3.3 Apoptosis and regeneration

Apoptosis has been shown to play an important role in regeneration (Fuchs and Steller, 2011). Cells that are undergoing apoptosis can stimulate the proliferation of neighboring cells, contributing to the proliferative component of regeneration. For example, in the regenerating tail bud of the Xenopus tadpole, apoptotic activity is indispensable for regeneration, as inhibition of effector caspase-3 abolishes the regenerative response (Tseng et al., 2007). The abolished regenerative response is attributed to lack of proliferation in the growth zone of the amputated tail bud. In Hydra, the apoptotic response that follows the amputation of its upper half is necessary and sufficient for its head regeneration (Chera et al., 2009). Regenerative proliferation is stimulated by apoptotic cells that secrete Wnt3 ligand. Finally, in effector caspase-3 and -7 deficient mice, both wound healing and liver regeneration are abolished (Li et al., 2010). The underlying reason for the abolished regenerative response is attributed to the suppression of proliferation at the wound site, due to the lack of caspase-regulated secretion of prostaglandin E2. Hence, the apoptosis-induced compensatory proliferation of neighboring cells that is important for maintaining proper tissue homeostasis following injury seems to be dependent on apoptosis-mediating caspases.

2.4 p53-Mdm2 signaling

p53 is a transcription factor (encoded by Trp53 in mouse, TP53 in humans) that regulates a diverse set of cellular processes, spanning from the coordination of responses to a variety of stresses (such as oncogene activation, genotoxic stress, hypoxia, and nutrient deprivation) and metabolic homeostasis, to regulation of development and differentiation (Kruiswijk et al., 2015). With aging, the function of p53 has been shown to decrease, and shift into the regulation of homeostatic longevity-affecting processes (Argon and Gidalevitz, 2015; Feng et al., 2007). p53 was initially identified as a potent tumor suppressor that suppresses cancer formation and progression by inhibiting proliferation of damaged, malfunctioning cells and by inducing their death if necessary. p53 mediates these functions mainly by acting
as a transcriptional activator of cell cycle suppressive genes (such as CKI $p21^{Cip1}$) and of apoptosis-related genes of the Bcl-2 family (such as Bax, Noxa and Puma). p53 can also activate apoptosis by direct interaction with members of the Bcl-2 family.

A good indication of p53’s importance as a tumor suppressor arises from the fact that it is mutated in over a half of human tumors and that $Trp53$ inactivation leads to susceptibility to tumor formation (Donehower et al., 1992). Patients suffering from autosomal dominant Li-Fraumeni syndrome consistently carry a germline mutation in $TP53$, rendering them highly susceptible to tumor formation (Merino and Malkin, 2014).

p53 knockout mice appear to be mostly normal (Donehower et al., 1992), however, a proportion of female $Trp53$ knockout embryos suffer from defects in neural tube closure (Sah et al., 1995). It is currently unclear why this defect is restricted to only a subset of female embryos and why the number of affected females depends on the mouse strain. It has been proposed that p53 might be activated only when a developmental process is dysregulated, explaining the incomplete penetrance of developmental defects in $Trp53$ knockout mice (Shin et al., 2013). In contrast, p53 inactivation in $Xenopus$ embryo disrupts its normal development, suggesting that unlike in mammals, frogs lack redundant regulators of development that would compensate for the lack of p53 (Wallingford et al., 1997).

### 2.4.1 p53 and its regulation by Mdm2 and Mdm4

The activity of p53 is controlled at the transcriptional, translational, and post-translational levels. At the post-translational level, the activity and stability of p53 protein are regulated by an extensive array of modifications (such as phosphorylation, acetylation, ubiquitination, and methylation). Studies in which the target sites of these modifications have been mutated suggest, that rather than behaving as on/off switches, the combinations of these modifications fine-tune the activity of p53 (Toledo and Wahl, 2006). p53 protein has a short half-life. The main regulator of its stability is E3 ubiquitin ligase Mdm2 that suppresses p53 mainly by ubiquitination (Marine et al., 2006) (Fig. 3). Mdm4, a protein structurally closely related to Mdm2, works in concert with Mdm2 to regulate p53’s activity and stability (Marine et al., 2006) (Fig. 3). Upon cellular stress, p53 is phosphorylated by stress-induced kinases (such as ATM), disrupting its interaction with Mdm2 and Mdm4 and leading to the stabilization of its expression levels. Furthermore, cellular stress-associated p53 response is boosted by a self-destructive program of Mdm2 that via ubiquitination targets itself and Mdm4 for proteasomal degradation.
Fig. 3: p53 and its regulation by Mdm2 and Mdm4. Cellular stress response, associated with increased expression levels of p53, is downregulated by a negative auto-regulatory loop, whereby p53 transcriptionally upregulates the expression of its negative regulator, Mdm2. Increased levels of Mdm2 in turn, lead to poly-ubiquitination of p53 that targets it for proteasomal degradation. Mdm2-mediated mono-ubiquitination can serve opposing functions: to shuttle p53 out from the nucleus to suppress its transcriptional activity or to stimulate p53’s non-transcriptional pro-apoptotic mitochondrial function. In addition to ubiquitination, Mdm2 can suppress p53’s transcriptional activity via binding to its transcriptional activation domain and preventing its interaction with the transcriptional machinery. Unlike Mdm2, Mdm4 does not possess a ubiquitin ligase domain, rendering it incapable of directly targeting p53 for degradation. However, Mdm4 can suppress p53 by binding and masking its transcriptional activation domain. Furthermore, Mdm4 has been shown to form a complex with Mdm2 and make it a more efficient ubiquitin ligase.

The importance of the regulated expression of p53 is underlined by the fact that loss of either Mdm2 or Mdm4 is embryonic lethal. In the case of Mdm2 loss, embryos die before implantation and the phenotype is completely rescued by concomitant loss of p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995). This shows that upregulated levels of p53 underlie the phenotype. When Mdm4 is lost, embryos survive up to embryonic day 7.5-8.5 (E7.5-8.5, gastrulation) (Parant et al., 2001). Also in this case, the lethality is rescued by concomitant loss of p53. These studies demonstrate that Mdm2 and Mdm4 act as non-redundant inhibitors of p53.

Consistent with the early embryonic lethality of Mdm2 knockout embryos, both central nervous system- and heart-specific Mdm2 ablation during embryogenesis lead to severe developmental defects due to apoptosis of proliferating neuronal precursors and embryonic cardiomyocytes (Francoz et al., 2006; Grier et al., 2006). Studies where Mdm4 has been ablated in different tissues demonstrate that the phenotype is delayed.
or milder, compared to Mdm2 ablation, suggesting that Mdm2 is the main regulator of p53 activity. For example, Mdm4 ablation in the central nervous system leads to delayed developmental defects compared to Mdm2-ablated mice (Xiong et al., 2006). In contrast to loss of Mdm2, Mdm4 ablation in proliferating embryonic cardiomyocytes does not yield a phenotype (Grier et al., 2006), demonstrating that Mdm4 is dispensable for the proper development of some tissues.

2.4.2 Regulation of p53 in postmitotic cells

Relatively few studies have focused on the relevance of controlled p53 levels in postmitotic cells. Compared to proliferating cells, postmitotic cells are generally believed to be refractory to p53 upregulation. However, tissues that comprise postmitotic, differentiated cells (such as heart and retina) have shown to accumulate dying cells upon Mdm2 ablation (Zhang et al., 2014). Hence, upregulated levels of p53 appear to be lethal also for postmitotic, differentiated cells. In addition, studies on differentiated intestinal smooth muscle cells and neurons of the cerebral cortex have shown that Mdm2-ablation-mediated p53-upregulation leads to apoptosis that it is rescued by concomitant p53 inactivation (Boesten et al., 2006; Francoz et al., 2006). In contrast, Mdm4 ablation in differentiated intestinal smooth muscle cells does not lead to p53 upregulation nor impair the viability, demonstrating that in these cells Mdm2 is the main regulator of p53 stability (Boesten et al., 2006). In differentiated neurons of the cerebral cortex, Mdm2 and Mdm4 work synergistically to regulate p53, as their combined ablation leads to increased levels of apoptosis compared to ablation of either of these regulators (Francoz et al., 2006).

2.4.3 p53 in regeneration

Consistent with its tumor suppressive role, p53 has been shown to antagonize regeneration. For example, during natural regeneration of the amputated salamander limb, downregulation of p53 is required for both cell cycle re-entry of postmitotic mesenchymal cells and subsequent limb blastema formation (Yun et al., 2013). p53 has also been shown to suppress the regenerative potential of mammalian retinal Müller glia and self-renewal of adult neural stem cells (Meletis et al., 2006; Ueki et al., 2012). In addition, the efficiency of somatic cell reprogramming to pluripotent stem cells and lineage conversion of fibroblasts to neurons is increased by suppression of p53 (Jiang et al., 2015; Kawamura et al., 2009).
2.5 Mammalian inner ear sensory epithelia as models to study regenerative plasticity

The mammalian inner ear is a fascinating organ that endows us with the ability to hear sounds and to sense accelerated body movements. It consists of the vestibular and auditory compartments that house sensory epithelia containing mosaics of sensory HCs and non-sensory SCs (Fig. 4). In mammals, these cells remain mostly postmitotic after birth. The postmitotic state of these cells is an important underlying reason for sensory deficits, as lost HCs are not replaced by new ones following traumas. Recent data suggests there might be low-level natural turn-over of HCs in adult mammalian vestibular organs, but unfortunately traumas seem not to boost this activity (Bucks et al., 2017). Non-mammalian SCs possess a remarkable capacity to regenerate HCs following traumas, a discovery that has fueled intense research efforts aimed to trigger regeneration in the mammalian inner ear (see chapter 2.6 for details). Mammalian postmitotic HCs and SCs also serve as excellent models for studying the regenerative potential of highly differentiated, postmitotic cell types.

2.5.1 Structure and function of the mammalian inner ear

2.5.1.1 The auditory and vestibular system

The auditory sensory epithelium, termed the organ of Corti, is located inside the coiling cochlear duct and functions to detect sounds (Fig. 4). The vestibular system that mediates the sense of balance, consists of two macular organs, utricle and saccule, and three ampullae (lateral, anterior and posterior) and their associated semicircular canals (Fig. 4). Macular organs are covered by an otoconial membrane that consists of otoconial crystals nested in a protein matrix, and sense linear head acceleration and gravity. The sensory epithelia in the ampullae are located at the ends of semicircular canals and are covered with gelatinous cupula. Ampullae function to sense head rotations.
Figure 4. Mammalian inner ear and its sensory epithelia. Schematic figure demonstrates that the mammalian inner ear consists of the auditory organ, called the organ of Corti, and five vestibular organs, three ampullae (amp), utricle, and saccule (sac). Hematoxylin-stained transverse sections of postnatal day 7 utricle and cochlea show that all inner ear sensory epithelia consist of a mosaic of HCs and SCs. In the organ of Corti, two types of HCs, the outer HCs (OHCs) and inner HCs (IHCs), and various types of SCs, inner phalangeal cell (IPhC), inner pillar cell (IPC), outer pillar cell (OPC), Deiters’ cells (DCs), Hensen’s cells (HNCs), and Claudius’ cells (CCs) can be distinguished. Tunnel of Corti is indicated by (T). Utricle contains two types of HCs, termed Type I (HC1) and Type II (HC2), and a relatively homogenous population of SCs. HCs can be distinguished by using an antibody against myosin 6 (Myo6) (Hasson et al., 1997) and SCs with antibodies directed against transcription factors Sox2 and Sox9 (Loponen et al., 2011; Oesterle et al., 2008). Scale bar, 20 µm.

2.5.1.2 Hair cells

All HCs in the inner ear sensory epithelia are mechanosensory cells that contain apical actin-rich stereociliary bundles that deflect in response to sensory stimuli. Mechanotransduction channels located at the tips of HC stereociliary bundles open as a result of bundle deflection, leading to influx of potassium ions from the endolymphatic fluid that bathes HC apical surfaces. Potassium influx leads to HC
depolarization and neurotransmitter release at their basolateral membrane, triggering action potentials in the afferent fibers of the cochleovestibular nerve. The somas of cochlear nerve fibers are located in the spiral ganglion. The central processes of spiral ganglion neurons together with those of the vestibular ganglion neurons constitute the VIII cranial nerve whose fibers runs to the auditory and vestibular nuclei in the brainstem.

The auditory sensory organ, the organ of Corti, contains two types of sensory HCs: the inner HCs and outer HCs (Fig. 4). The organ of Corti lies on the basilar membrane that vibrates in response to sound-induced fluid movement in the cochlear duct. The basilar membrane vibrations trigger the opening of HC mechanotransduction channels. Inner HCs are considered the main sensory cells in the cochlea. They transmit the timing, frequency, and intensity of sound stimulus to the afferent fibers of Type I neurons of the auditory nerve (Appler and Goodrich, 2011). Outer HCs function mainly to amplify low-intensity sound waves by changing the length of their cell bodies in response to changes in membrane potential (termed electromotility) (Ashmore, 2008). Outer HC electromotility leads to acceleration of the sound-evoked movement of the basilar membrane and increased stimulation of afferent fibers. Outer HCs are mainly innervated by neurons of the efferent neural pathway that suppress their electromotility. Sound-evoked activation of efferent pathway thus creates a negative feedback loop, leading to reduced vibrations of the basilar membrane and decreased sound-sensitivity of afferent fibers of the auditory nerve. The functional significance of this efferent pathway is unclear, but it has been proposed to protect from acoustic injury (Reiter and Liberman, 1995).

In the cochlear duct, HCs are arranged along a tonotopic axis (Mann and Kelley, 2011). Tonotopy means that based on its location, each HC is sensitive to only a narrow range of sound frequencies. Accordingly, HC morphology, physiology, and gene expression change in a graded fashion along the apical-to-basal tonotopic axis of the cochlea. In addition, the properties of the basilar membrane change along the tonotopic axis. Tonotopy is also maintained in the afferent nerve fibers forming the auditory nerve, and in its target neurons in the central auditory system. The tonotopic organization of the auditory system allows discrimination between different sound frequencies, with the basal coil responding to high frequencies and the apical coil to low frequencies. The range of frequencies that the human ear can detect is 20-20 000 Hz, whereas the mouse ear detects 1000-100 000 Hz. Tonotopy is essential for speech sound perception in humans.

Vestibular sensory epithelia contain two subtypes of vestibular HCs, Type I and Type II, that are distinguished based on the synapses they make with the afferent and efferent fibers of the vestibular nerve (Fig. 4). In macular organs, the HC stereociliary bundles deflect when head movements cause the shifting of the otoconial membrane. The sensory epithelia in the ampullae are termed cristae. In cristae, the deflection of HC stereociliary bundles occurs concomitantly with the displacement of the gelatinous cupula due to fluid motion in semicircular canals that occurs in response to head rotations.
2.5.1.3 Supporting cells

As their name suggests, SCs fulfill numerous supportive functions in inner ear sensory epithelia and are indispensable for HC function and viability, and thus proper functioning of the inner ear (Wan et al., 2013).

The organ of Corti has five subtypes of non-sensory SCs: Claudius´ cells, Hensen´s cells, Deiters´ cells, pillar cells, and inner phalangeal cells (Fig. 4). Subtypes of SCs can be distinguished by their morphology and molecular characteristics. However, based on morphological and molecular characteristics, there are no distinct subpopulations of SCs in vestibular sensory epithelia.

SCs have rigid cytoskeletons (especially in the organ of Corti) allowing them to maintain structural integrity in inner ear epithelia during sensory stimulation. The apical surfaces of SCs interconnect with HC apical surfaces via tight junctions, forming a reticular lamina that protects sensitive basolateral surfaces of HCs and SCs from toxic potassium-rich endolymph. SCs are also necessary for maintaining the ionic composition of endolymph, an essential property for HC mechanotransduction. Excess levels of excitatory glutamate neurotransmitter that are released by HCs following sensory stimulation are toxic for afferent nerve fibers. SCs regulate the uptake of glutamate. SCs also generate components of the acellular membranes covering the inner ear sensory epithelia (otoconial membrane and cupula in vestibular epithelia, and tectorial membrane in cochlea) that are essential for HC mechanotransduction.

HCs are highly sensitive cells that die easily following traumas. In many cases, SCs survive the insults that kill HCs and attempt to restore the homeostasis in the epithelium. SCs maintain the homeostasis in the epithelium by extruding injured HCs from the epithelium. Alternatively, SCs can phagocytose dead HC debris. Importantly, in inner ear epithelia of non-mammalian species and vestibular epithelia of adult mammals, SCs are able to regenerate lost HCs.

2.5.2 Development of the mammalian inner ear

The mammalian inner ear is an organ of high complexity. It is remarkable that the whole inner ear arises from a simple ectodermal thickening. The following outline of the main steps in inner ear development is based on mouse inner ear development. E0.5 is defined as the morning when the vaginal plug denoting pregnancy was detected.

2.5.2.1 Embryonic development of the inner ear

Auditory and vestibular sensory epithelia and their associated sensory ganglia derive from the otic placode, a thickening of the surface ectoderm located next to the presumptive hindbrain, around E8 (Fig. 5). Subsequently, the placode grows and invaginates into underlying mesoderm, forming a fluid-filled otocyst by E9.5. Around that time, neuroblasts that will give rise to the cochleovestibular ganglion of the VIII
cranial nerve delaminate from the ventral region of the otocyst (D'Amico-Martel and Noden, 1983). The otocyst is patterned along anterior-posterior and dorsal-ventral axes giving rise to the mature inner ear, which consists of the ventral auditory compartment and dorsally located vestibular compartment (Groves and Fekete, 2012). The prosensory domains that contain progenitors of sensory HCs and non-sensory SCs form by E12.5 in both auditory and vestibular compartments. HCs and SCs are proposed to arise from common progenitors similar to their non-mammalian avian counterparts (Fekete et al., 1998). The morphogenesis of the inner ear is coordinated by a variety of signaling molecules, secreted by surrounding tissues (periotic mesenchyme, notochord, and neural tube) and involves various cellular processes, such as proliferation, programmed cell death, and cellular migration (Groves and Fekete, 2012).
Figure 5. Morphogenesis of the inner ear. At E8, otic placode forms next to hindbrain (HB) and notochord (NT), and folds into underlying mesoderm forming an otic pit around E8.5. By E9.5, the otic pit has fully invaginated and formed an otic vesicle from where neuroblasts (indicated by red color) are delaminating. Around E11.5, the cochlear duct starts to grow out from the ventral bulge of the otocyst. Around E12.5-E13.5, cells evaginating from the dorso-medial bulge of the otocyst form anterior, posterior and lateral semicircular canals. Prosensor patches (indicated by blue color) have also formed by that time. By E18.5, the cochlear duct has elongated into its full one and three-quarters length and tube-like semicircular canals have formed the vestibular compartment. These stages of the gross development of the inner ear have been described with the aid of paint-filling technique (Morsli et al., 1998).
2.5.2.2 Cell cycle exit and differentiation in the auditory and vestibular sensory epithelia

Proliferating cochlear HC and SC progenitors located in the prosensory domain in the dorsal half of the cochlear duct exit the cell cycle in an apical-to-basal gradient between E12.5 and E14.5, concomitantly with the expression of a negative cell cycle regulator CKI p27^kip1 (Chen and Segil, 1999) (Fig. 6). In vestibular epithelia, cell cycle exit of HC and SC progenitors occurs concomitantly with p27^kip1 expression in a central-to-peripheral gradient, starting around E13.5-E14.5 and lasting until the first postnatal week (Burns et al., 2012a; Ruben, 1967; Sans and Chat, 1982; Slowik and Bermingham-McDonogh, 2016) (Fig. 6).

**Figure 6. Cell cycle exit and differentiation in the embryonic inner ear sensory epithelia.** In the embryonic auditory organ, the cell cycle exit and differentiation of HC and SC progenitors are uncoupled and occur in opposing gradients. Cell cycle exit occurs in an apical-to-basal gradient and differentiation midbasal-to-apical/basal and medial-to-lateral gradients. The medial-to-lateral gradient of differentiation that occurs concomitantly with the leading edge of midbasal-to-apical/basal differentiation gradient directs that first HCs to differentiate are inner HCs and the first SCs to differentiate are inner pillar cells. In vestibular organs, both cell cycle exit and differentiation of HC and SC progenitors occur in a central-to-peripheral gradient.

After cell cycle exit, cell fate determination is regulated by Notch signaling-mediated lateral inhibition. During cell fate establishment in the cochlea, presumptive HCs start to express membrane-bound Notch ligands Jagged 2 (Jag2) and Delta-like 1 (Dll1), which bind and activate Notch1 receptor on neighboring cells, leading to suppression of their HC fate (Lanford et al., 1999; Zine et al., 2000). Activated Notch signaling
suppresses HC fate via suppression of the expression of pro-neural genes, such as HC master regulator Atonal homolog 1 (Atoh1), and directly instructs SC development (Campbell et al., 2016; Zheng et al., 2000). The differentiation of cochlear HCs and SCs follows two simultaneously occurring gradients: midbasal-to-apical/basal and medial-to-lateral (Retzius, 1884) (Fig. 6). Atoh1 transcription factor that initiates HC differentiation is first expressed in midbasal turn and extends apically and basally between E13.5 and E16.5 (Chen et al., 2002; Woods et al., 2004). As evidenced by the opposing gradients of cell cycle exit and differentiation, cell cycle exit is not coupled to differentiation in the auditory sensory epithelium. In contrast, in vestibular organs, HC differentiation starts shortly following cell cycle exit and seems to be coupled with cell cycle exit (Sans and Chat, 1982; Slowik and Bermingham-McDonogh, 2016) (Fig. 6). By E18.5 all cell types can be distinguished in vestibular and auditory sensory epithelia.

2.5.2.3 Early postnatal morphological maturation of the auditory sensory epithelium

By birth, all HC and SC subtypes have formed in the cochlea, but they remain morphologically immature. During the first weeks of postnatal development, drastic morphological changes take place in auditory sensory epithelium to prepare it for hearing function (Forge et al., 1997). In mice, hearing function is initiated around P8 and it reaches its full maturity by P14 (Alford and Ruben, 1963). In contrast, in humans, fully functional inner ear has developed already by pregnancy week 20. Hence, unlike mouse embryos, human fetuses have the capacity to hear.

During the postnatal morphological maturation, the height and width of the auditory epithelium increases, due to the elongation of SC bodies (termed phalangeal processes) and enlargement of pillar and Deiters’ cell heads (Fig. 7). The apical domains of auditory HCs and SCs are sealed together by reticular lamina that separates their sensitive basolateral surfaces from toxic potassium-rich endolymph. By P6-7 in mouse, the inner and outer pillar cell phalangeal processes separate, forming a fluid-filled tunnel of Corti and around OHCs the spaces of Nuel. Tunnel of Corti is important for the rigidity of the organ of Corti and plays an indispensable role in the auditory-stimulus-triggered vibrations of the basilar membrane, and thus in hearing function (Davis, 1958).
Figure 7. Early postnatal morphological maturation of the auditory sensory epithelium. Hematoxylin-stained transverse sections of P4 and P7 paraffin-embedded cochlea. At P4, the organ of Corti consists of a mass of columnar cells. By the end of the first postnatal week (P7), the height and width of the epithelium increases due to elongation of SC bodies. Fluid-filled tunnels, the tunnel of Corti (T) and Nuel’s space (*) also form in the organ of Corti. Abbreviations: DCs, Deiter’s cells; IHC, inner hair cell; IP, inner pillar cell; OHC, outer hair cells; OP, outer pillar cell. Scale bar, 10 µm.

The morphological maturation of SCs is mediated by the establishment and remodeling of actin and microtubule cytoskeletons, and intercellular junctions (Forge et al., 1997; Ito et al., 1995; Souter et al., 1997). It is suggested that SCs maturation is dependent on concomitant HC maturation, as no SCs develop when HCs do not differentiate (Erkman et al., 1996).

The high morphological complexity of the mammalian auditory and vestibular sensory epithelia comes at a cost. It is proposed that the reinforcement of apical junctions between SCs by filaments of circumferential actin that takes place during postnatal maturation, underlies the incapacity for mammalian HC regeneration (Burns et al., 2008; Burns and Corwin, 2014). Highly reinforced junctions between SCs limit their capacity to spread, a property that has been shown to be necessary for cell cycle re-entry in vestibular SCs (Meyers and Corwin, 2007). Consistently, in non-mammalian sensory epithelia where SCs proliferate following injury, the apical junctions between SCs remain thin throughout life (Burns et al., 2008). The junctional reinforcement in mammals occurs concomitantly with the sharp decline in SC regenerative plasticity at the end of the first postnatal week (Gu et al., 2007; Meyers and Corwin, 2007).
2.5.4 Mechanisms underlying sensorineural loss of hearing and loss of balance

Hearing loss is categorized either as conductive or sensorineural, depending on which component of the hearing system is affected. Conductive hearing loss is associated with blockage or damage to the outer and/or middle ear. Sensorineural hearing loss (SNHL) is attributed to dysfunction of the cochlea and/or auditory nerve, and associated central auditory pathways. SNHL is caused by a variety of factors (loud noise, aminoglycoside antibiotics, chemotherapy drugs such as cisplatin, and inflammation) that damage auditory HCs, stria vascularis (cochlear tissue that maintains the ionic composition of endolymph) and spiral ganglion neurons of the auditory nerve. Spiral ganglion neurons can also be secondarily lost following HC damage, as they depend on HCs for their trophic support (Schimmang et al., 2003). In addition, extensive amounts of excitatory glutamate neurotransmitter released by inner HCs during exposure to loud sound are toxic for afferent fibers of the auditory nerve (Puel et al., 1998). Loud noise also damages HCs mechanically, especially the apically-located delicate stereociliary bundles that act as mechanoelectrical transducers (Liberman, 1987).

One of the main metabolic events that has been proposed to underlie the ototoxicity of loud noise and aminoglycoside antibiotics is oxidative stress that leads to the accumulation of cytotoxic levels of ROS (Henderson et al., 2006). ROS is deleterious for cellular structures, such as DNA, proteins and membrane lipids. In the case of ototoxic drugs, cytotoxic levels of ROS arise due to mitochondrial dysfunction (Esterberg et al., 2016). Mitochondria are the primary generators of ROS during oxidative phosphorylation-mediated energy production. Aminoglycosides overstimulate mitochondrial oxidative phosphorylation leading to excess levels ROS that the endogenous antioxidant-defense system cannot handle. In the case of loud noise, the accumulation of cytotoxic levels of ROS occurs in HCs via overdrive of their mitochondria due to the increased energy demand (Thalmann et al., 1975).

ROS accumulation during oxidative stress has been shown to activate the apoptotic c-Jun N-terminal kinase (JNK) stress-signaling pathway (Son et al., 2013). Consistently, in auditory HCs, JNK signaling is activated following intense noise and exposure to aminoglycoside antibiotics and has been associated with the apoptotic phenotype (Pirvola et al., 2000; Ylikoski et al., 2002). Accordingly, pharmacological inhibition of the JNK pathway protects HCs against both noise and aminoglycoside-induced apoptosis (Pirvola et al., 2000; Ylikoski et al., 2002).

In addition to damage to cochlear HCs and neurons, aminoglycoside antibiotics also damage vestibular HCs (Forge and Li, 2000), causing vestibular hypofunction. Vestibular hypofunction is manifested by dizziness, nausea, and loss of gaze fixation during movement. Like for auditory HCs, aminoglycoside toxicity towards vestibular HCs has been shown to arise from ROS accumulation (Wu et al., 2001). Likewise, pharmacological inhibition of the JNK pathway protects vestibular HCs from aminoglycoside-induced apoptosis (Sugahara et al., 2006; Ylikoski et al., 2002). Fortunately, aminoglycoside-induced vestibular damage is often temporary. The underlying mechanism is unclear, but the regenerative plasticity of vestibular epithelia has been proposed to underlie the functional recovery (Berggren et al., 2003).
Altogether, based on the outlined mechanisms underlying the ototoxicity of noise and aminoglycoside antibiotics, possible therapeutic interventions would include increasing the antioxidant supplies to combat cytotoxic levels of ROS (Hight et al., 2003). In addition, as already mentioned, pharmacological suppression of signaling pathways associated with the apoptotic response, such as JNK, and components of the apoptotic cascade, seem to protect HCs from apoptosis following ototoxic insults (Pirvola et al., 2000; Wang et al., 2004).

2.5.5 Hair cell regeneration in non-mammalian species

Inner ear HCs are extremely sensitive to a wide variety of insults (such as loud noise and aminoglycoside antibiotics) and are also lost during aging. Mammalian inner ear sensory epithelia, especially the auditory epithelium, possess very limited capacity to regenerate HCs, leading to permanent hearing and balance deficits (Cox et al., 2014; Forge et al., 1993; Warchol et al., 1993). Remarkably, non-mammalian vertebrates such as fish, amphibians, and birds, have a life-long capacity to regenerate lost sensory HCs (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Furthermore, in addition to the regenerative response to HC loss, avian vestibular epithelia exhibit continuous HC turnover, whereby HCs that periodically die are continuously replaced (Jorgensen and Mathiesen, 1988). In contrast, the avian auditory epithelium is quiescent until HC trauma triggers a regenerative response of SCs (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). New auditory HCs are observed already 2-3 days following the injury and functional recovery takes place within 1-2 months after trauma (Bermingham-McDonogh and Rubel, 2003). SCs are the source of new HCs in all non-mammalian inner ear sensory epithelia during both normal turn-over and HC damage triggered regeneration (Balak et al., 1990; Raphael, 1992; Roberson et al., 1992). Non-mammalian HC regeneration has been most thoroughly studied in birds, thus avian HC regeneration is probably the best-understood example of HC regeneration.

Following HC loss, avian auditory SCs start re-expressing developmental genes important for HC development, such as the gene encoding for transcription factor Atoh1, and change their phenotype to HCs by direct transdifferentiation (Cafaro et al., 2007; Roberson et al., 2004) (Fig. 8). Thereafter, remaining SCs are activated to replenish lost HCs via mitotic regeneration, whereby cell cycle re-activation and proliferation is followed by asymmetrical or symmetrical differentiation into HCs and SCs (Raphael, 1992; Tsue et al., 1994) (Fig. 8). In addition to replenishment of lost HCs, mitotic regeneration serves to restore SC population, depleted in the process of SC-to-HC transdifferentiation. It has been suggested that transdifferentiation is used as a means to regenerate HCs initially or in case a small number of HCs are lost, whereas mitotic regeneration takes over at later stages to generate larger numbers of both HCs and SCs (Roberson et al., 2004).
Fig. 8. Non-mammalian hair cell regeneration. Non-mammalian SCs can regenerate lost HCs either by direct transdifferentiation or mitotic regeneration. During transdifferentiation, SCs gradually adopt the characteristics of HCs. During mitotic regeneration, SC nuclei travel from the basal layer to luminal surface, where they enter mitosis and divide (Tsue et al., 1994). The resulting SC progeny differentiates and depending on the cell fate, their nuclei assume a specific position (in case of SCs more basal and for HCs more luminal).

Despite decades of work, the identity of signals that trigger the regenerative response of SCs following HC loss remains unclear. An array of factors has been proposed to activate regenerative response in the avian auditory epithelium, including several growth factors, Notch, and Wnt (Hawkins et al., 2007; Oesterle and Hume, 1999). Notch signaling mediated cell-to-cell signaling seems to regulate the numbers of avian auditory SCs that transdifferentiate or whose progeny differentiates into HCs in response to HC loss, as suppression of Notch signaling leads to overproduction of HCs (Daudet et al., 2009).

Many additional unanswered questions remain. For example, it is still unclear whether all avian auditory SCs are equipotent in their capacity to regenerate HCs or whether there is a distinct subpopulation of cells designated for this purpose (Groves, 2010). Furthermore, little is known of how the regenerative response is toned down. The regenerative response needs to be able to orchestrate the restoration of a proper tissue architecture, as morphological abnormalities would make the proper functioning of the organ impossible.
2.6 Regenerative restrictions and attempts in the inner ear sensory epithelia

Mammalian cochlear and vestibular HCs and SCs remain postmitotic following cell cycle exit during mid-embryogenesis (between E12.5 and E14.5) and at early postnatal development, respectively (Chen and Segil, 1999; Ruben, 1967). Thereafter, HC loss leads to permanent functional deficits (hearing and balance), as mammalian inner ear sensory epithelia show only limited capacity to replace the lost HCs (Cox et al., 2014; Forge et al., 1993; Warchol et al., 1993). Like in non-mammalian species, non-sensory SCs are a source of limited numbers of new HCs generated following HC loss in mammals. Mammalian SCs can regenerate HCs both via direct transdifferentiation and mitotic regeneration. Mammalian vestibular sensory epithelia show limited capacity to replace lost HCs in adulthood, whereas in cochlea this capacity is restricted to neonatal stages (Cox et al., 2014; Golub et al., 2012; Warchol et al., 1993). Despite the extended regenerative capacity, mammalian vestibular SCs lose their capacity for mitotic regeneration by adulthood, being able to regenerate limited numbers of HCs only following severe manipulations that kill all HCs and by employing direct transdifferentiation that leads to depletion of SC population (Golub et al., 2012).

The manipulations to regenerate mammalian HCs are targeted mainly to postmitotic postnatal SCs, with the aim to trigger their cell cycle re-activation and/or SC-to-HC transdifferentiation. SCs are used as the main platform to generate new HCs, as postmitotic HCs seem to poorly tolerate forced cell cycle re-activation (Chen et al., 2003; Laine et al., 2007; Weber et al., 2008). Manipulations to trigger cell cycle re-activation target either the core cell cycle machinery or manipulate developmentally important signaling pathways Wnt and Notch, whose targets include core cell cycle regulators. The manipulations aiming for SC-to-HC transdifferentiation, target developmentally important HC fate regulators, such as Notch signaling and its downstream target HC master regulator Atoh1.

2.6.1 Stemness

Stem cells are undifferentiated cells that are defined by their ability to self-renew and to differentiate into specialized cells. Different types of stem cells exhibit different degrees of plasticity regarding the range of cell types they can give rise to. Embryonic stem cells are pluripotent and are able to generate all the cells of the body. Adult somatic tissue stem cells exhibit more limited plasticity and are considered multipotent, being restricted to generate cell types of their tissue of origin. Adult tissue stem cells are essential for homeostasis in the adult organism. The decline and abnormalities in adult stem cell populations are linked with aging and tumor formation.

Although mammalian SCs can proliferate and differentiate into HC-like cells upon isolation and culturing (Chai et al., 2012; Sinkkonen et al., 2011; White et al., 2006) and are able to regenerate limited numbers of HCs following traumas (Cox et al., 2014; Warchol et al., 1993), they are unlikely to function as tissue stem cells in the inner ear. Instead, mammalian SCs seem to represent a plastic cell population that can respond
to growth-promoting conditions and HC loss for a limited period of time during the early postnatal development. Thus, it seems that rather than fulfilling a role of tissue stem cells, the plasticity of mammalian SCs is a property associated with their developmental immaturity that progressively declines with maturation. Finally, a good indicator that mammalian inner ear is unlikely to contain tissue stem cells, is the fact that no tumors, except for benign vestibular schwannomas, have been described in the mammalian inner ear.

2.6.2 Cell cycle manipulations

From a therapeutic point of view, cell cycle re-activation in postmitotic SCs and surviving HCs is an inevitable step in restoring the numbers of lost HCs. SC proliferation is an important step for maintaining the essential SC population (Mellado Lagarde et al., 2013), especially if SC-to-HC transdifferentiation is used as a therapeutic strategy. Hence, in a variety of studies, the therapeutic potential of manipulating the core cell cycle regulators in auditory and vestibular HC and SC populations has been addressed.

Negative cell cycle regulator, CKI p27Kip1 binds cyclin-CDK complexes and inhibits their positive activity towards cell cycle progression. During development, upregulation of p27Kip1 is indispensable for the permanent cell cycle exit of HCs and SCs in auditory and vestibular sensory epithelia (Chen and Segil, 1999; Ruben, 1967). Consistently, in p27Kip1 knockout mice, auditory HCs and SCs continue to proliferate, in contrast to control mice where these cells exit the cell cycle at mid-embryogenesis (Chen and Segil, 1999; Lowenheim et al., 1999). p27Kip1 continues to maintain the postmitotic state in postnatal auditory SCs, as its ablation results in cell cycle re-activation (Lowenheim et al., 1999; Oesterle et al., 2011). Interestingly, in a recent study, where p27Kip1 was inducibly inactivated in neonatal auditory HCs, HCs also re-activated their cell cycles, although p27Kip1 has been shown to be downregulated during the course of HC differentiation (Chen and Segil, 1999; Walters et al., 2014). The authors attributed their results to the fact that although they were unable to detect p27Kip1 protein, p27Kip1 mRNA has been shown to be present in postnatal HCs (Laine et al., 2007). These newly generated auditory HCs developed to maturity and survived until adulthood, preserving the hearing function. Importantly, this study thus suggests that surviving HCs themselves could be manipulated to proliferate to restore the lost HC population.

Other CKIs besides p27Kip1, such as p19Ink4d and p21Cip1 play an important role in the maintenance of postmitotic state of mammalian auditory HCs (Chen et al., 2003; Laine et al., 2007). Consistently, both postnatal postmitotic p19Ink4d-deleted and p19Ink4d/p21Cip1-deleted auditory HCs re-enter cell cycle (Chen et al., 2003; Laine et al., 2007). However, the ectopic cell cycle re-activation is followed by their apoptosis, suggesting that cell cycle re-entry is lethal to postmitotic auditory HCs. In contrast, early postnatal vestibular HCs from p19Ink4d/p21Cip1 compound mutant mice are unable to re-enter the cell cycle (Laine et al., 2010). This inability is attributed to the lack of cD1 expression in these cells, as opposed to auditory HCs at a similar age (Laine et al., 2010). Cyclin D1 is the main cyclin responsible for cell cycle progression from G1-to-S phase in...
response to growth signals. Consistently, forced adenoviral vector-mediated cD1 overexpression in early postnatal utricular HCs from p19\textsuperscript{ Ink4d/p21\textsuperscript{Cip1} mutant mice leads to aberrant cell cycle re-entry (Laine et al., 2010). In wild-type utricular early postnatal and adult SCs, cD1 overexpression is sufficient to trigger cell cycle re-entry (Loponen et al., 2011). These results suggest that in vestibular SCs, cD1 overexpression can override cell cycle suppression by CKIs. As the survival of cell cycle re-activated postnatal utricular HCs and SCs has not been analyzed over the long-term, it is difficult to conclude whether these cells exhibit increased capacity for forced cell cycle re-activation compared to postmitotic auditory HCs. However, DNA damage and cell cycle arrest of these cell cycle re-activated utricular SCs, especially at adulthood (Loponen et al., 2011), suggests that the proliferative plasticity of vestibular postmitotic SCs is limited as well.

Tumor suppressor and a negative regulator of cell cycle, pRb, has also been shown to be an important regulator of postmitotic state in HCs and SCs of mammalian inner ear sensory epithelia. Like CKI p27\textsuperscript{Kip1}, pRb is indispensable for cell cycle exit of embryonic progenitors of cochlear and vestibular HCs and SCs, as its absence leads to their continued proliferation (Mantela et al., 2005; Sage et al., 2005). Proliferating Rb-deleted embryonic precursors of cochlear HCs and SCs never fully mature and succumb to apoptosis during early postnatal development, suggesting that pRb plays a role in their maturation (Sage et al., 2006). In contrast, Rb-deleted proliferating embryonic precursors of vestibular HCs are able to survive to adulthood, suggesting that these cells are able to mature independently of pRb (Sage et al., 2006). pRb continues to maintain the postmitotic state of cochlear HCs and SCs during early postnatal development, as its neonatal ablation in these cells leads to cell cycle re-activation, and in the case of SCs, also to successful cell cycle completion (Weber et al., 2008; Yu et al., 2010). However, both Rb-inactivated cochlear HCs with incomplete cell cycles and SCs that are able to complete cell cycle, succumb to apoptosis, suggesting that postnatal auditory HCs and SCs fail to tolerate ectopic cell cycle re-entry. Furthermore, these studies suggest that pRb exhibits additional roles besides maintenance of postmitotic state in these cells. In contrast, pRb does not maintain postmitotic state in adult cochlear and utricular SCs, as these cells are unable to re-activate their cell cycles following Rb depletion (Huang et al., 2011).

Altogether, these studies suggest that auditory HCs are unfavorable targets to induce cell cycle re-activation, as they easily succumb to apoptosis. SCs, especially the ones from vestibular sensory epithelia seem to exhibit higher proliferative plasticity. Thus, SCs seem to be more feasible targets for cell cycle manipulations aiming to trigger HC regeneration in the mammalian inner ear.

2.6.3 Manipulation of developmentally important regulators

Studies focusing on highly regenerative species (salamander) and mammalian organs that regenerate naturally and following injury (skin and liver) have demonstrated that signaling pathways important for development are re-employed to direct regeneration.
Thus, approaches to regenerate lost inner ear HCs have focused on signaling pathways and regulators regulating mammalian inner ear development, and HC regeneration in non-mammalian species. Two widely manipulated pathways are Wnt and Notch signaling pathways.

### 2.6.3.1 Wnt signaling

In mammalian inner ear, Wnt/β-catenin signaling (Fig. 9) regulates various aspects of development. It is important from the earliest stages of inner ear development, directing otic placode-epidermis fate decisions (Ohyama et al., 2006). At the otocyst stage, canonical Wnt signaling directs the development of the dorsal region of the otocyst into a vestibular fate (Brown et al., 2015; Noda et al., 2012; Riccomagno et al., 2005). Canonical Wnt signaling is important in the regulation of proliferation in the prosensory domain of the cochlear duct and HC differentiation (Jacques et al., 2012; Shi et al., 2014). Wnt signaling also regulates the finer aspects of HC differentiation by directing stereociliary bundle development of cochlear HCs (Dabdoub et al., 2003; Qian et al., 2007).

![Canonical Wnt signaling](image)

**Figure 9. Canonical Wnt signaling.** In β-catenin-dependent canonical Wnt signaling, binding of secreted Wnt ligand to Frizzled (Fzd) receptor and Low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptors activates Dishevelled (Dvl) that inhibits the β-catenin
destruction complex, which is composed of Axin, Adenomatous polyposis coli (APC), and Glycogen synthase kinase-3β (GSK3β). In the absence of Wnt ligand, this complex sequesters β-catenin to the cytosol and GSK3β phosphorylates it, targeting β-catenin for proteasomal degradation. Following Wnt ligand-mediated activation of Dvl, β-catenin is not degraded and it can translocate to the nucleus where it activates its target genes, such as positive cell cycle regulator cD1 (Shtutman et al., 1999) and Leucine-rich repeat-containing, G-protein-coupled receptor 5 (Lgr5), by forming a complex with TCF/LEF family of transcription factors.

Consistent with its role in stemness in other tissues (van Es et al., 2005), downregulation of Wnt signaling during the first postnatal week in mouse cochlea parallels the decline in regenerative potential (Geng et al., 2016). Lgr5 is a Wnt target gene and a receptor, the activation of which augments Wnt signaling (Fig. 9). It has been shown to be expressed in cells with regenerative potential, such as intestinal stem cells and hair follicle stem cells (Barker et al., 2007). Lgr5 is being used in the inner ear sensory epithelia to detect cells with active Wnt signaling that possess regenerative plasticity.

Under culture conditions, neonatal cochlear Lgr5-positive cells can clonally proliferate and form spheres, and they are able to differentiate into HC-like cells (Shi et al., 2012). Furthermore, also in vivo stimulation of Wnt signaling by stabilizing the transducer of Wnt signaling, β-catenin, triggers robust proliferation of neonatal auditory Lgr5-positive SCs (Chai et al., 2012; Shi et al., 2013). Importantly, these cell cycle re-activated Lgr5-positive SCs are able to differentiate into HC-like cells in vivo (Shi et al., 2013). The fact that Lgr5 marks cells with regenerative capacity is underlined by a study in the neonatal cochlea, showing that following HC loss, rare Lgr5-positive SCs are able to spontaneously transdifferentiate into HC (Bramhall et al., 2014). Another study links the activation of Wnt signaling with spontaneous HC regeneration in the neonatal cochlea, demonstrating that suppression of Wnt signaling suppresses the regenerative response of SCs (transdifferentiation and mitotic regeneration) following HC loss (Hu et al., 2016). However, the regenerative plasticity of Lgr5-positive SCs in response to in vivo Wnt signaling activation is restricted to neonatal stages, as similar manipulation fails to elicit any responses in a mature cochlea (Shi et al., 2013). Similar to cochlea, Wnt signaling plays an important role in mammalian vestibular HC regeneration. Following HC damage, Lgr5 is upregulated in utricular early postnatal SCs that are subsequently able to regenerate HCs both via direct transdifferentiation and mitotic regeneration (Wang et al., 2015).

Altogether, these studies demonstrate that Wnt signaling is active in mammalian auditory and vestibular SCs and that it boosts their regenerative response following HC damage. Unfortunately, Wnt-regulated regenerative capacity, like the regenerative capacity in mammalian inner ear sensory epithelia, is restricted to early postnatal stages, especially in the cochlea.
2.6.3.2 Notch signaling

During the development of the mammalian inner ear, Notch signaling (Fig. 10) is re-employed to regulate various processes. During early inner ear development, Notch signaling is required for cochlear prosensory specification (Daudet et al., 2007; Hayashi et al., 2008). At later stages, Notch signaling is required for patterning of the sensory epithelium by suppressing HC fate and inducing SC differentiation (Campbell et al., 2016; Lanford et al., 1999; Zine et al., 2000). Thereafter, active Notch signaling is required for the maintenance of SCs fate, at least up to postnatal day 6 (P6) in cochlea (Maass et al., 2015) and adulthood in utricle (Lin et al., 2011).

Figure 10. Notch signaling. In mammals, Notch signaling is mediated by a signaling cell expressing a membrane-bound Notch ligand, Jag1,2 or Dll1,3,4 and a receiving cell expressing transmembrane Notch receptor (Notch 1-4). The activation of Notch receptor by a transmembrane ligand on a neighboring cell results in a cleavage of the Notch extracellular domain by Tumor necrosis factor-α-converting enzyme (TACE) metalloprotease and a subsequent cleavage of its intracellular domain by γ-secretase. Notch intracellular domain (NICD), the product of γ-secretase cleavage, translocates to the nucleus and by interacting with Recombining binding protein suppressor of hairless (Rbpj) transcription factor and Mastermind-like (MAML) co-factors it activates its target genes (Bray, 2006). In the inner ear, Notch activation drives the expression of the basic helix-loop-helix transcriptional repressors of HES/HEY family whose targets include HC master regulator Atoh1 (Zheng et al., 2000).
In the auditory sensory epithelium of a mature chicken and lateral line neuromasts of zebrafish, where HC regeneration occurs spontaneously after trauma, Notch signaling appears to be active under homeostatic conditions (Daudet et al., 2009; Romero-Carvajal et al., 2015). Interestingly, suppression of Notch signaling in the absence of HC trauma has no effect on non-mammalian SCs, unlike in mammals where Notch signaling is required for the maintenance of SC fate (Daudet et al., 2009; Ma et al., 2008). Extra-HCs are produced from SCs following the suppression of Notch signaling in chicken auditory epithelium and zebrafish lateral line only when HCs are damaged (Daudet et al., 2009; Ma et al., 2008). Furthermore, during HC regeneration in the zebrafish lateral line, Notch signaling seems to regulate SC proliferation (Ma et al., 2008). These studies suggest that during non-mammalian HC regeneration, Notch signaling functions mainly to limit the number of SCs that differentiate into HCs and is not able to autonomously trigger transdifferentiation.

In contrast, in an intact undamaged, mammalian early postnatal cochlea, suppression of Notch signaling triggers the accumulation of extra-HCs, demonstrating that in mammals Notch signaling maintains SC fate (Mizutari et al., 2013; Yamamoto et al., 2006). However, the responsiveness of SCs to suppression of Notch signaling declines during maturation, so that very rare ectopic HCs are found in adult HC-damaged mammalian cochlea upon suppression of Notch signaling (Hori et al., 2007). It has been suggested that age-related decline in responsiveness to suppression of Notch signaling is due to downregulation of Notch signaling components in cochlea by the end of the first postnatal week (Maass et al., 2015). The expression of Notch signaling components in vestibular epithelia has not been extensively characterized. However, as SC-to-HC transdifferentiation occurs in aminoglycoside-treated adult utricle at low efficiencies suggests that vestibular epithelia retain higher levels of Notch signaling at adulthood compared to cochlea (Lin et al., 2011). In adult cochlea that is refractory to Notch inhibition, the components of Notch signaling remain very low even following noise trauma (Maass et al., 2015).

Altogether, these studies demonstrate that suppression of Notch signaling in mammalian auditory and vestibular SCs can be employed to transdifferentiate HCs from SCs. Furthermore, recent studies have demonstrated that Notch signaling limits the proliferation of mammalian auditory and vestibular SCs via suppression of Wnt signaling (Li et al., 2015; Wu et al., 2016). These studies thus suggest that in addition to transdifferentiation capacity, the proliferative capacity of mammalian SCs could also be boosted via suppression of Notch signaling.

One of the main negatively regulated targets of Notch signaling in the inner ear is HC master regulator Atoh1. Atoh1 is a basic helix-loop-helix transcription factor that is essential for HC differentiation in the inner ear (Bermingham et al., 1999; Chen et al., 2002). Atoh1 plays an important role also in HC regeneration in chicken auditory organ, where it is upregulated in SCs that give rise to new HCs upon HC damage (Cafaro et al., 2007). The potential of Atoh1 as the master regulator of mammalian HC fate is underlined by studies showing that its ectopic expression is sufficient to trigger the generation of HC-like cells from non-sensory cells in the auditory and vestibular sensory epithelia (Kawamoto et al., 2003; Kelly et al., 2012; Zheng and Gao, 2000). Furthermore,
the ectopic expression of Atoh1 can induce the formation of HC-like cells from cells outside the cochlear sensory epithelium, such as the cells in the greater epithelial ridge (GER) and non-neuronal cells in the spiral ganglion (Kelly et al., 2012; Zheng and Gao, 2000). The therapeutic potential of Atoh1 is further demonstrated by studies showing that Atoh1-overexpressing cells attract neurons and can trigger the formation of SC-like cells from surrounding cells (Kelly et al., 2012; Woods et al., 2004). The ability of cochlear cells to respond to Atoh1 overexpression declines concomitantly with the maturation of inner ear and the gradual disappearance of GER, so that by the end of the first two postnatal weeks, no ectopic HCs are generated upon Atoh1 overexpression (Kelly et al., 2012; Liu et al., 2012). The decline in the capacity to respond to Atoh1 overexpression is concomitant with the epigenetic silencing of its locus by the end of the first postnatal week in the mammalian cochlea (Stojanova et al., 2015). There are, however, some studies reporting the generation of rare HCs upon Atoh1 overexpression in mechanically injured adult guinea pig cochlea, suggesting that limited regenerative plasticity is retained in some contexts upon Atoh1 overexpression (Atkinson et al., 2014; Kawamoto et al., 2003). In contrast to auditory epithelia, the regenerative plasticity in vestibular epithelia following HC damage and in response to Atoh1 overexpression is extended to adult ages (Shou et al., 2003; Staecker et al., 2007).

Finally, both the ability of canonical Wnt signaling to trigger the formation of HC-like cells from Lgr5-positive SCs and the ability of SCs to transdifferentiate into HCs following suppression of Notch signaling are consistent with the fact that Atoh1 is targeted by both signaling pathways (Shi et al., 2010; Zheng et al., 2000). Altogether, these studies demonstrate that Atoh1 expression is indispensable for HC regeneration in the mammalian inner ear.
3. AIMS OF THE STUDY

Mammalian inner ear SCs demonstrate poor capacity to regenerate HCs following traumas, leading to permanent hearing and balance deficits. The responsiveness of postmitotic mammalian SCs to manipulations that aim to boost their regenerative capacity declines during postnatal maturation. To design a regenerative therapy to restore lost HCs, the understanding of the mechanisms underlying the regenerative restrictions of SCs are essential.

Aims of this thesis were:

1. To study the proliferative response and proliferation dynamics of SCs following forced cell cycle re-activation.

2. To study the role of DNA damage response and DNA repair in the age-dependent decline of the regenerative plasticity of SCs.

3. To study the role of p53 tumor suppressor in the regenerative plasticity of SCs.

4. To study the p53 sensitivity in different developmental cellular contexts (proliferation, differentiation, and homeostasis) during embryonic and postnatal inner ear development.
4. MATERIALS AND METHODS

The methods, mouse strains and antibodies used in this thesis are listed in tables below. Detailed information can be found in articles I and II, as indicated.

4.1 Methods

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4.1.1 Organotypic cultures

Organotypic cultures of the inner ear sensory epithelia were made by dissecting the epithelia from inside the otic capsule under sterile conditions, using fine forceps (Fig. 11). To allow better access to oxygen, a Trowell-type of organ culture system was used, where explants are cultured on the surface of the culture medium (Trowell, 1959). The dissected sensory epithelia were placed on top of pieces of Nucleopore filter membrane (Whatman) and transferred onto a metal mesh in a tissue culture plate with medium (DMEM/F-12 supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS) and 0.06% penicillin G sodium salt) (Fig. 12). The organotypic cultures were maintained inside an incubator with a humidified 5% CO₂ atmosphere at +37°C.
Figure 11. Inner ear and organotypic preparations of its sensory epithelia. (A) The cochlea and one of the vestibular end-organs, the utricle, are circled in an inner ear dissected from a newborn mouse (D, dorsal; V, ventral; P, posterior; A, anterior). (B, C) The culture preparations of microdissected cochlear and utricular sensory epithelia are shown. Scale bar, shown in A: A, 500 µm; B,C, 370 µm.
Figure 12. Organ culture system for inner ear sensory epithelia. (A) Schematic drawing showing that inner ear sensory explants are cultured at the interface of culture medium and air, on top of filter membranes placed on a metal mesh. (B,C) Cochlear and vestibular sensory epithelia from a newborn mouse that have been cultured for 1 day are shown. (D) Schematic drawing showing that for standing drop method, inner ear sensory epithelia on top of filter membranes are placed inside drops of virus-containing medium. Scale bar, shown in C: 500 µm.

4.1.2 Standing drop method for adenoviral infections of inner ear organotypic cultures

Serotype 5 adenoviral vectors were used as tools to deliver transgenes (β-galactosidase (βGal), green fluorescent protein (GFP), and cD1) under the control of cytomegalovirus (CMV) promoter to SCs in organotypic cultures prepared from postnatal auditory and vestibular epithelia (Fig. 11). Studies by Anna Kirjavainen (Kirjavainen et al., 2008) and Heidi Loponen (Laine et al., 2010; Loponen et al., 2011) have demonstrated that by postnatal development, the tropism of these viruses shifts from HC to SCs. Standing drop method to transduce inner ear sensory epithelia was developed by Anna Kirjavainen (Fig. 12).

Virus dilutions were prepared by adding viruses into culture medium containing 5% FBS, with a final concentration of 1x10⁷ plaque-forming units/mL. Prior to infection, the sensory epithelia were allowed to stabilize on filter membranes for at least 2 h. Thereafter, the filter membranes with sensory epithelia on top were placed inside 30 µl standing drops of the virus-containing medium. Cochlear explants were maintained in standing drops for 16 h and utricular explants for 7 h. Thereafter, the explants were transferred back onto the metal mesh in a tissue culture plate with 10% FBS-containing medium and cultured 2-14 days in vitro (DIV).
### 4.2 Mouse lines

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4.3 Primary antibodies

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5. RESULTS AND DISCUSSION

5.1 DNA damage signaling regulates proliferative capacity of postmitotic inner ear supporting cells

5.1.1 Serotype 5 adenoviruses transduce postnatal supporting cells (I)

Transgene delivery to the cells of inner ear sensory epithelia is inefficient and non-specific when non-viral gene transfer methods, such as electroporation and chemical-based transfection are used. Viral vectors enable the targeting of transgene expression to specific tissues and cell types by using different viral serotypes and promoters. In in vitro studies of inner ear, adenoviral vectors have often been selected for transgene delivery (Brandon et al., 2012; Burns et al., 2012b; Loponen et al., 2011), due to their high transduction efficiency, capability to transduce both proliferating and postmitotic cells, and capacity to carry large transgenes (up to 30 kb). The importance of adenoviral vectors as in vivo transgene-delivery tools in the inner ear is underlined by the fact that they have been chosen as viral vectors for a clinical trial, delivering Atoh1 to cochleas of patients with hearing loss (clinical trial no. NCT02132130).

Heidi Loponen demonstrated that serotype 5 adenoviral vectors are efficient tools to deliver transgenes to SCs in postnatal utricular explant cultures (Loponen et al., 2011). Thus, these viral vectors were employed for gene transfer in this study. The cell-type specificity and efficiency was studied in explants of juvenile (P6) utricles and cochleas, and adult (P50) utricles, using reporter-viruses carrying either βGal or GFP transgenes. After 3 DIV, 20-50% of SCs expressed the reporter transgene in P6 and P50 adenovirus-infected utricles. In P6 cochleas, the reporter transgene was restricted to a specific subtype of SCs, Deiters’ cells. These data (I, Fig. 1) suggest that serotype 5 adenoviruses, expressing transgenes under CMV promoter are efficient tools to ectopically express genes in postnatal SCs in vitro.

5.1.2 Utricular supporting cells show age-dependent decline in proliferative plasticity (I and unpublished)

In mammals, vestibular HCs and SCs remain postmitotic following their cell cycle exit between embryogenesis and the first days after birth (Burns et al., 2012a; Ruben, 1967). Postmitotic SCs show little proliferative plasticity following HC loss. In non-mammalian species, lost HCs are regenerated from surrounding SCs via direct transdifferentiation and mitotic regeneration (Stone and Cotanche, 2007). Although spontaneous HC regeneration does not occur in mammals, its feasibility in non-mammalian species places SCs into a main therapeutic focus as a potential source of new HCs. In response to cell cycle manipulations and growth factor treatments, early postnatal mammalian vestibular SCs exhibit limited capacity for proliferation that declines with maturation (Burns et al., 2012b; Gu et al., 2007; Loponen et al., 2011).
The mechanisms underlying this age-related decline of SCs to respond to regenerative manipulations are unclear.

In order to comparatively study the proliferative potential of postmitotic utricular SCs at the juvenile (P6) and adult (P50) ages, cD1, a positive cell cycle regulator, was ectopically expressed in these cells by adenoviral-mediated gene transfer to organotypic in vitro cultures. Cyclin D1 is a mediator for many proliferation-promoting signaling pathways, including the Wnt pathway (Shtutman et al., 1999). Analysis of incorporation of 5-ethynyl-2’-deoxyuridine (EdU) thymidine analog allowed to identify cells that had entered the cell cycle. By double-labeling with EdU and another thymidine analog, bromodeoxyuridine (BrdU), cells that had completed one cell cycle and had started DNA replication in the next cycle were identified. An average of 50% of EdU-positive SCs was counted in AdcD1-infected P6 utricles, whereas only 20% of SCs were found EdU-positive in AdcD1-infected P50 utricular cultures after 3 DIV (EdU pulse between 2 and 3 DIV). Non-infected and reporter-virus (AdGFP or AdβGal) infected P6 and P50 utricular explants lacked EdU-positive SCs, consistent with the postmitotic status of their cells (Burns et al., 2012a). After 7 DIV, the nuclei of EdU-positive cells in P6 AdcD1-infected utricles were organized as clumps of variable labeling intensity, suggesting that these cells had diluted the EdU label during DNA replication (I, Fig. 2). In contrast, most EdU-positive nuclei in P50 AdcD1-infected utricles were strongly labeled and fewer clumps of EdU-positive nuclei were found. These results suggest that complete cell cycles were less frequent in mature utricles (I, Fig. 2). EdU/BrdU double labeling confirmed this data, showing lower numbers of double-positive nuclei in adult compared to juvenile explants (unpublished, Fig. 13). In addition, a part of BrdU-positive SCs in adult utricles showed an apoptotic morphology, suggesting that unscheduled cell cycle re-activation is lethal for adult SCs (unpublished, Fig. 13). This data suggests that unlike juvenile SCs, most adult SCs are unable to progress through a complete cell cycle, arresting at G2/M transition and succumbing to apoptosis. Altogether, in accordance with previous studies (Burns et al., 2012b; Loponen et al., 2011), these results show that in utricular SCs, the efficiency of cell cycle re-activation and progression through the cell cycle decline during maturation. Of note, this decline is not associated with lower levels of viral transduction, as reporter-viruses transduced both P6 and P50 utricles at comparable efficiencies (I, Fig. 1).
Figure 13. The age-dependent proliferative response of utricular supporting cells to cyclin D1 overexpression. Adenovirus-infected utricular explants were pulsed with EdU and BrdU (EdU pulse between 2 and 3 DIV, and BrdU pulse between days 6 and 7). Double- and triple-labeled specimens were analyzed at 7 DIV. (A, A') AdcD1-infected P6 utricles accumulate EdU-positive (early pulse) SCs, some of which are also BrdU-positive (late pulse) (arrows in A'). Note the variable intensity of EdU labeling and EdU-positive cells arranged in small groups. (B) High magnification view of an AdcD1-infected P6 utricle shows EdU/BrdU double-positive nuclei (arrows). (C) AdβGal-infected P6 utricular sensory epithelium lacks replicating cells. (D, D') AdcD1-infected P50 utricles display lower numbers of EdU-positive and BrdU-positive cells. Rare EdU/BrdU double-positive nuclei (arrows in D') can be found. Note the apoptotic morphology of a part of BrdU-positive cells (arrowheads in D'). Scale bar, shown in C: A,A',C, 20 µm; B,D,D', 15 µm.
5.1.3 Cochlear supporting cells are more limited in their proliferative plasticity compared to utricular supporting cells (I and unpublished)

Mammalian auditory SCs are more limited in their proliferative plasticity compared to vestibular SCs (see Literature review for details). To assess the proliferative capacity of auditory SCs, explant cultures were prepared from P6 cochleas and infected with AdcD1. As evidenced by EdU-positive nuclei of Deiters’ cells at 3 DIV (EdU pulse between 2 and 3 DIV), cD1 overexpression led to cell cycle re-activation, similar to utricular SCs (I, Fig. 2 and 6). Thus, in both auditory and vestibular SCs, cD1 overexpression is sufficient to override the negative regulators that maintain the postmitotic state (I, (Loponen et al., 2011)). Non-infected and reporter-virus (AdGFP or AdβGal) infected explants lacked EdU-positive SCs and HCs, consistent with the postmitotic status of these cells.

Many of the EdU-positive Deiters’ cell nuclei in AdcD1-infected P6 cochleas appeared in doublets at 3 DIV and 7 DIV, suggesting that cytokinesis had been unsuccessful. To penetrate into cell cycle characteristics of AdcD1-infected Deiters’ cells, these cells were double-labeled for EdU and BrdU thymidine analogs (EdU pulse between 2 and 3 DIV, BrdU pulse between days 6 and 7) (unpublished). Rare EdU/BrdU-double positive Deiters’ cells were seen at 7 DIV, suggesting that few SCs that entered cell cycle following cD1 overexpression were able to complete the cell cycle (unpublished, data not shown). Furthermore, the morphological comparison between AdcD1-infected and control (non-infected and reporter virus-infected) cochleas showed no significant addition of extra cells (HCS and SCs) (I, Fig. 6). Thus, this data suggests that although the postmitotic Deiters’ cells exhibit the capacity to re-enter the cell cycle following cD1 overexpression, they are inefficient in progressing through the cell cycle. These results demonstrate that P6 auditory SCs show less proliferative plasticity compared to P6 utricular SCs.

There are several possible explanations as to why mammalian auditory SCs might show less proliferative plasticity compared to their vestibular counterparts. For example, the higher degree of morphological complexity of auditory SCs might restrict shape changes needed for proliferation. Indeed, it has been proposed that the ability for cell shape changes and remodeling of the components of the apical adherens junctions is required for the proliferative ability of SCs (Collado et al., 2011; Meyers and Corwin, 2007). Auditory and vestibular SCs are also different in terms of their maturation kinetics. Vestibular SCs are more immature as they exit the cell cycle later than auditory SCs, last SCs still proliferating on first days following birth, whereas auditory SCs exit the cell cycle during a restricted time-period around mid-embryogenesis (Burns et al., 2012a; Ruben, 1967).
5.1.4 Cell cycle re-activated supporting cells accumulate DNA double-strand breaks and activate the DNA damage response (I and unpublished)

It was hypothesized that inefficient cell cycle progression of SCs following cD1 overexpression is due to DNA damage and subsequent activation of DDR signaling. The basis for this hypothesis was the following: proliferation is an unnatural process for postmitotic cells; cD1 is a proto-oncogene and oncogenes are known to trigger DNA damage (Bartkova et al., 2006). As a marker for DNA DSBs, the serine 139 phosphorylated form of histone H2AX, termed γH2AX, was employed (Rogakou et al., 1998). Analysis of γH2AX expression in AdcD1-infected juvenile utricles and cochleas, and adult utricles showed that EdU-positive SCs accumulate γH2AX-positive foci (I, Fig. 3, 8). These results indicate that DSBs form in response to cell cycle re-activation. However, γH2AX can also accumulate at single-stranded DNA regions that form due to replication fork stalling (Ward and Chen, 2001). Therefore, in order to establish a true template of DSBs, ionizing radiation was applied to non-infected utricles, as only DSBs can cause γH2AX formation in nonproliferating cells after irradiation (de Feraudy et al., 2010). Comparative analysis of irradiated and AdcD1-infected utricles demonstrated that ectopic cell cycle re-entry of utricular SCs of both ages leads to DSB accumulation (I, Fig. 3). In addition, the presence of DSBs was confirmed by immunostaining 3 days cultured AdcD1-infected utricles for 53BP1, a component of DDR signaling that localizes to DSBs (Rappold et al., 2001). The fact that EdU-positive utricular SCs accumulated 53BP1-positive foci (I, Fig. 3), strengthened the view that γH2AX-positive foci indeed represent DSBs, and that DDR signaling is activated in these cells. These experiments were performed under culture conditions known to promote oxidative stress that might trigger DNA damage, and using adenoviral vectors that can activate DDR signaling and trigger γH2AX accumulation (Halliwell, 2003; Nichols et al., 2009). Thus, non-infected and reporter virus-infected utricles were analyzed for γH2AX expression. Nuclei in both non-infected and reporter virus-infected utricles of both ages contained 1 or 2 large γH2AX foci, suggesting that unlike adenoviral transduction, culture conditions appear to trigger low levels of DNA damage (I, Fig. 3).

These results raised the question of whether DSB-associated γH2AX accumulation is specifically associated with cD1 overexpression or whether it is a more general response to ectopic cell cycle re-activation. To answer this question, a conditionally inducible \( Rb^{loxP/loxP};Fgfr3-iCre-\text{ER}^{T2} \) mouse line was employed. In this mouse model, negative cell cycle regulator \( Rb \) was inactivated from P2 onwards in auditory SCs via tamoxifen administration. A subtype of auditory SCs, the pillar cells, re-entered the cell cycle and accumulated γH2AX foci, indicative of DSBs (I, Fig. 8). These results suggested that DSB-associated γH2AX accumulation is a general response to ectopic cell cycle re-activation.

It was also assessed whether DSBs accumulate during normal proliferation of embryonic HCs and SCs by using explant cultures prepared from E14 utricles that contain proliferating HCs and SCs. Explants were labeled for EdU for 24 h in culture before the analysis of γH2AX and 53BP1 expression (unpublished). Unlike SCs in AdcD1-infected and irradiated P6 utricles after 3 DIV, EdU-positive SCs in embryonic explants
lacked γH2AX-positive and 53BP1-positive foci (unpublished, data not shown). Thus, these results demonstrate that normal proliferation fails to trigger DSBs and DDR activation. Furthermore, these results demonstrate that DSB accumulation and activation of DDR signaling are specifically associated with ectopic cell cycle re-entry of postmitotic cells rather than with normal proliferation of progenitor cells.

In postmitotic, differentiated cells, such as postnatal SCs, most of the chromatin is condensed into heterochromatin. Forced cell cycle re-activation probably requires extensive structural changes to chromatin and re-expression of cell cycle-associated genes. Consistent with this notion, somatic cell reprogramming that also requires extensive chromatin modifications, has also been shown to trigger DNA damage (Gonzalez et al., 2013). Altogether, these results together with previously published data suggest that chromatin status imposes barriers for manipulations for proliferative regeneration.

5.1.5 DNA double-strand break repair in supporting cells (I)

DSBs are highly mutagenic DNA lesions that can lead to chromosomal rearrangements. Thus, it is imperative that the cell rapidly activates DNA repair mechanisms (NHEJ, or HRR in case of proliferating cells) or induces apoptosis when repair is unsuccessful. As DSBs were detected in both P6 and P50 utricular SCs following cell cycle re-activation, differences in DDR signaling/DNA repair kinetics between these ages might underlie the differences in the capacity for cell cycle progression.

The kinetics of DSB repair in SCs of AdcD1-infected juvenile and adult utricles was comparatively analyzed. γH2AX foci have been found to disappear concomitantly with DSB repair (Rothkamm and Lobrich, 2003), mainly due to γH2AX dephosphorylation by protein phosphatase 2A (PP2A) (Chowdhury et al., 2005). Therefore, the loss of these foci was considered to be an indication for successful repair. At 3 DIV, an average of 51% of EdU-positive SCs in P6 utricles contained γH2AX foci. By 7 DIV, this value had dropped to 18%, suggesting that a large fraction of these SCs (65% decline from 3 DIV to 7 DIV) were able to repair DSBs (I, Fig. 4). In contrast, at 3 DIV in P50 AdcD1-infected utricles, 83% of EdU-positive SCs contained γH2AX-positive foci. After 7 DIV, this value was 54%, suggesting that compared to P6 SCs a smaller number of EdU-positive SCs (35% decline from 3 DIV to 7 DIV) had been able to repair the DSBs in P50 utricles. By 14 DIV, the number of EdU-positive SCs with γH2AX-positive foci had dropped to 16%. This latter value (16%) resembles the percentage of γH2AX-positive SCs in P6 utricles at 7 DIV. Thus, γH2AX dephosphorylation is delayed in adult SCs. Alternatively, delay in γH2AX disappearance in adult SCs might also be associated with the lower activity of PP2A. It has been demonstrated that the disappearance of γH2AX foci can be used as a marker for successful DSB repair only in the case of low levels of DNA damage because γH2AX dephosphorylation is inefficient when DNA damage levels are high (Bouquet et al., 2006). Based on these considerations, it is possible that DSB repair kinetics is comparable between juvenile and adult SCs, but the amount of accumulated DSBs is
higher in the latter case. In order to confirm the assumption that the disappearance of γH2AX foci reflects DSB repair, the expression of Rad51, a component of HRR that functions in DSB repair during S and G2 phases of the cell cycle, was analyzed. At 3 DIV, Rad51-positive foci colocalized with EdU and γH2AX in SCs of both P6 and P50 AdcD1-infected utricles. Rad51 expression also disappeared concomitantly with γH2AX expression (I, Fig. 5). Reporter virus-infected and irradiated P6 utricles that contain postmitotic cells that cannot use HRR pathway to repair DSBs lacked Rad51-positive foci. Thus, this data links Rad51 expression exclusively with active HRR, characteristic for postmitotic, differentiated cells, has been shown to limit DNA damage repair (Goodarzi et al., 2008). Therefore, postnatal maturation and heterochromatinization likely create barriers for DNA repair in adult inner ear SCs.

In addition to the foci-like expression of γH2AX, AdcD1-infected P6 and P50 utricles contained EdU-positive SCs with a strong pan-nuclear γH2AX expression (I, Fig. 5). This expression pattern has been associated with apoptosis-related DNA fragmentation (de Feraudy et al., 2010; Rogakou et al., 2000). SCs with pan-nuclear γH2AX were also positive for cleaved caspase-3, a late apoptosis marker (I, Fig. 4). These results link the pan-nuclear γH2AX profile to apoptosis. The fact that these apoptotic SCs also lacked or showed low-level Rad51 expression are consistent with previous data showing that DDR/DNA repair components are downregulated in the course of apoptosis, concomitantly with apoptosis-related chromatin condensation (Ding et al., 2016). Comparative analysis revealed that the numbers of EdU-positive SCs with pan-nuclear γH2AX expression profiles were significantly higher in P50 compared to P6 AdcD1-infected utricles (I, Fig. 4). These findings link delayed DNA repair in adult SCs with apoptosis. Thus, hampered DNA repair due to prominent heterochromatin, combined with the attenuated DNA repair signaling (see Literature review for details) leads to a death-prone phenotype of cell cycle re-activated adult SCs. These results are consistent with previously published data showing that unscheduled proliferation of postmitotic cortical neurons leads to DNA damage and DDR activation, followed by cell cycle arrest and apoptosis (Ye and Blain, 2010).

5.2 DNA damage signaling is not linked with transdifferentiation plasticity in supporting cells

5.2.1 Transdifferentiation does not trigger DNA damage (I)

Direct transdifferentiation or lineage conversion of SCs to HCs is a naturally occurring process in non-mammalian species (see Literature review for details). In mammals, spontaneous transdifferentiation can be triggered in the neonatal cochlea and adult utricle only by severe manipulations that kill all HCs (Cox et al., 2014; Golub et al., 2012).

In mammalian inner ear sensory epithelia, Notch signaling maintains the SC fate via suppression of Atoh1 expression (see Literature review for details). Consequently,
transdifferentiation can be triggered by genetic and pharmacological manipulations that suppress Notch signaling (Yamamoto et al., 2006). However, the response of SCs to Notch signaling inhibition declines with maturation (Maass et al., 2015).

Somatic cell reprogramming that is known to involve extensive chromatin remodeling, has been shown to trigger DNA damage and activation of DDR signaling (Gonzalez et al., 2013). Thus, it was investigated here whether DNA damage is triggered in the postnatal inner ear by stimulated transdifferentiation, a process that also involves chromatin remodeling. In addition, it was studied whether DNA damage accumulation underlies the distinct age-related decline in transdifferentiation capacity. Explants were prepared from P2 and P6 cochleas, and P6 and P50 utricles, and treated for 3 days with the pharmacological inhibitor of Notch signaling, DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester). Ectopic HCs in these explants contained similar, low numbers of γH2AX foci as seen in normal HCs in the same explants, and in HCs in non-treated cultures (I, Fig. 9). These results suggest that stimulated transdifferentiation does not trigger DNA DSBs. Alternatively, it is possible that DNA damage accumulated during transdifferentiation, but it was rapidly repaired, before the analysis. This possibility is quite unlikely, because transdifferentiation is not a synchronized process. Thus, if there was any extensive DNA damage it should have been detected at least in some SCs or ectopic HCs. The lack of genomic abnormalities in transdifferentiating cells is surprising, considering the dramatic changes SC undergoes to become a HC. On the other hand, whereas somatic reprogramming is an unnatural process, SC-to-HC transdifferentiation occurs naturally in non-mammalian inner ears and at low levels in young mammalian inner ears.

Altogether, these results suggest that despite having lost the natural capacity for transdifferentiation, early postnatal mammalian SCs can be pharmacologically triggered to transdifferentiate into HCs without DNA damage. Furthermore, the age-related decline in transdifferentiation capacity in the mammalian inner ear appears to be unrelated to DNA damage accumulation.

5.2.2 Cell cycle re-activation is not followed by transdifferentiation in supporting cells (I)

During HC regeneration in non-mammalian inner ears, SCs either directly transdifferentiate into HCs or first proliferate, and one or both daughter cells become HCs (see Literature review for details). It was studied whether forced cell cycle re-activation in P6 utricular explants through AdcD1 transduction is followed by spontaneous transdifferentiation into HCs. After 6 DIV, while most of the EdU-positive cells were Sox2-positive SCs, low numbers of EdU/Myo6-positive HCs were found in these explants (EdU pulse between days 2 and 3) (I, Fig. 9). These EdU/Myo6-positive cells might be AdcD1-transduced SCs that had transdifferentiated into HCs or they might represent a rare population of HCs that were transduced by adenoviruses (I, Fig. 1). Altogether, these results suggest that forced cell cycle re-activation seems not to be coupled with transdifferentiation, although further studies using lineage tracing are needed to conclusively determine this.
In the neonatal cochlea in vivo, β-catenin-mediated stimulation of Wnt signaling triggers proliferation of Lgr5-positive SCs (Shi et al., 2013). These cells are thereafter able to transdifferentiate into HC-like cells. It has also been shown in vivo that the Wnt target gene Lgr5 is rapidly upregulated in utricular SCs following HC damage at P1 and that these SCs are able to mitotically regenerate HCs (Wang et al., 2015). Under in vitro conditions, however, SCs do not proliferate following HC damage and HC regeneration occurs only via transdifferentiation. Thus, in accordance with these published data, the findings on the uncoupling of forced proliferation and transdifferentiation can at least partly be attributed to the insufficiency of organotypic culture conditions.

It was postulated that DSB accumulation and DDR activation might antagonize transdifferentiation, an important consideration to make if SC proliferation and transdifferentiation are employed as a combinatory strategy to regenerate HCs. To tackle this question, AdcD1-infected P6 utricular explants were treated with Notch signaling inhibitor DAPT for 3 days and the explants analyzed at 6 DIV. A three-fold increase in EdU-positive HCs (EdU pulse between days 2 and 3) was found in these explants, compared to utricles only infected with AdcD1 (I, Fig. 10). These results suggest that forced cell cycle re-activation does not block stimulated transdifferentiation. It can be further concluded that DNA damage and ongoing DNA repair (I, Fig. 5) seem not to hinder transdifferentiation.

5.3 p53 and the regenerative plasticity of the inner ear

5.3.1 p53 is upregulated in cell cycle re-activated auditory supporting cells (I)

p53 is an important regulator of cellular responses to stress. Its upregulation in proliferating cells is associated with cell cycle deregulation (see Literature review for details). As cell cycle re-activated auditory SCs showed defects in cell cycle progression, p53 expression was studied in these cells. After 7 DIV, p53 upregulation was found in EdU-positive nuclear doublets in AdcD1-infected P6 cochleas, but not in control cochleas (non-infected and reporter virus-infected) (I, Fig. 7). This data suggests that the observed defects in cytokinesis and cell cycle arrest of auditory SCs might be linked to p53 activation. Next, p53 expression levels were investigated in AdcD1-infected utricles of the same age, where SCs exhibited complete cell cycles. Compared to cochlear SCs, immunostainings revealed that p53 levels were much lower in EdU-positive utricular SCs (I, Fig. 7). These results suggest that utricular SCs tolerate ectopic cell cycle re-activation better than auditory SCs. This poses an interesting question: could the higher morphological complexity of auditory epithelium pose restrictions for cell shape changes necessary for cell division, leading to p53-mediated stress response? Alternatively, the threshold for p53 activation and subsequent cell cycle arrest might be higher in utricles, allowing cell cycle progression. In any case, these results suggest that p53 signaling likely poses restrictions on cell cycle progression and thus restricts regenerative plasticity in the postnatal inner ear.
5.3.2 p53 does not regulate the maintenance of the postmitotic state in the cochlea (II)

In addition to the data that links p53 upregulation with unscheduled proliferation in P6 cochlear SCs (I, Fig. 7), p53 has been shown to negatively affect regenerative plasticity in other cellular contexts (see Literature review for details). Thus, the role of p53 in the proliferative plasticity of cochlear SCs was investigated using p53\textsuperscript{fl/fl};Pax2-Cre mice (Jonkers et al., 2001; Ohyama and Groves, 2004). In these mice, p53 is inactivated in Pax2-positive inner ear progenitors (all inner ear epithelial cells and neurons of the cochlear and vestibular ganglia) from the otic placode stage onwards. By analyzing whole mount specimens and paraffin sections from P0 and P35 inner ears of p53\textsuperscript{fl/fl};Pax2-Cre mice, no abnormalities nor ectopically proliferating cells were found (data not shown, II, Fig. 8). This data suggests that p53 is dispensable for inner ear development and maintenance of the postmitotic state of its cells. These results are consistent with the normal development of most tissues in constitutive p53 knockout mice (Donehower et al., 1992).

Next, p53 inactivation was studied in the context of different growth-promoting conditions to identify whether p53 would regulate proliferative plasticity of auditory SCs in a combinatorial setting. First, p53-inactivated cochleas were studied as to whether growth-promoting culture conditions promote proliferative plasticity. Organotypic explants were made from p53-inactivated P6 cochleas and cultured for 3 days. EdU was added to the culture medium to detect proliferating cells. No EdU-positive cells were found in the auditory sensory epithelium, suggesting that growth-promoting culture conditions are insufficient to trigger cell cycle re-activation in p53-deficient cells.

Wnt signaling promotes SC proliferation in the mammalian cochlea, but this effect is restricted to neonatal stages (see Literature review for details). The combination of activated Wnt signaling and p53 inactivation was thus studied to investigate if it would extend SC proliferative plasticity beyond the neonatal age. Wnt signaling was activated in p53-depleted P6 cochlear explants by a small molecule compound 6-bromoindirubin-3'-oxime (BIO). These explants were cultured in the presence of EdU for 3 days to detect proliferating cells. No EdU-positive cells were found in BIO-treated nor p53-inactivated plus BIO-treated sensory epithelia (data not shown). These results are not entirely surprising, as Wnt signaling components have been shown to be downregulated in cochlea during the first postnatal week of development (Geng et al., 2016).

In non-mammalian species, auditory SCs proliferate upon HC loss, and HC loss has also been shown to trigger SC proliferation in neonatal mouse cochlea in vivo (Cox et al., 2014). To test whether the HC-trauma-induced proliferative response of neonatal SCs could be extended by p53 ablation, p53-ablated P6 organotypic explants were treated with aminoglycoside neomycin that kills HCs. Although a large proportion of HCs died in these cultures, EdU-positive SCs were not found after 3 days in culture, in both mutant and control explants (II, Fig. 8).

Altogether, this data suggests that p53 does not regulate the proliferative capacity of postnatal cochlear SCs, even in growth-promoting contexts. As p53 functions mainly to regulate stress responses and is not one of the core cell cycle regulators, these results are not entirely unexpected. Functional p53 was absent in the p53\textsuperscript{fl/fl};Pax2-Cre
mouse model throughout embryonic inner ear development. Thus, it is possible that compensatory mechanisms took over p53’s functions, masking its true role in the maintenance of postmitotic state.

5.3.3 p53 inactivation does not boost transdifferentiation efficiency (II)

Suppression of Notch signaling triggers SC-to-HC transdifferentiation in the neonatal cochlea. However, the efficiency of this manipulation dramatically declines around P6 in the mouse (Maass et al., 2015). p53 has been shown to suppress reprogramming of somatic cells to pluripotent cells (Kawamura et al., 2009) and restrict lineage conversion of fibroblasts to neurons (Jiang et al., 2015). Thus, it was investigated whether its ablation increases transdifferentiation efficiency in cochlea and extends the postnatal period when stimulated transdifferentiation can efficiently occur. Treatment of p53-inactivated (p53fl/fl;Pax2-Cre mouse line) and wild-type P1 and P6 cochleas with DAPT led to a comparable accumulation of extra HCs, evidenced after 3 DIV (II, Fig. 9). Together, this data suggests that p53 does not regulate transdifferentiation in the postnatal mouse cochlea. These results are also consistent with previous data, showing that transdifferentiation does not trigger DNA damage (I, Fig. 9).

5.4 Mdm2/p53 interaction is indispensable for embryonic and postnatal inner ear development

5.4.1 Mdm2 ablation-mediated p53 accumulation is lethal for embryonic inner ear progenitors (II)

During proliferation, p53 is especially important in coordinating cellular responses to DNA damage (see Literature review for details). In response to DNA damage, the interaction between p53 and its negative regulator, Mdm2, is disrupted, causing p53 stabilization. Stabilized p53 arrests the cell cycle and activates DNA repair signaling. In case DNA repair is unsuccessful, p53 directs cells to apoptosis. During proliferation, cells are especially sensitive to DNA damage and p53 upregulation, because there is a risk that their progeny will accumulate genomic abnormalities. Accordingly, p53 upregulation in proliferating cells in mouse models with Mdm2 ablation has been shown to lead to apoptosis (Grier et al., 2006; Zhang et al., 2014).

To study the p53 sensitivity of proliferating inner ear cells in vivo, the p53-binding domain of Mdm2 was genetically disrupted by generating Mdm2FM/FM;Pax2-Cre mutant mice (Grier et al., 2002; Ohyama and Groves, 2004). In these mice, Pax2 expression directs Cre recombinase-mediated Mdm2 ablation to E8.5 inner ear progenitor cells of the otic placode. In accordance with prior data on p53 sensitivity in other embryonic tissues containing proliferating cells (Francoz et al., 2006; Grier et al., 2006), p53 upregulation led to robust apoptosis of proliferating cells of the otic placode, as shown
by cleaved caspase-3 immunostaining (II, Fig. 1). Thus, the data presented here demonstrates that p53 upregulation is lethal for proliferating early otic progenitors.

5.4.2 p53 upregulation mediated loss of otic inner ear progenitors leads to impaired morphogenesis of the inner ear (II)

Despite strong upregulation of p53 and widespread apoptosis in the otic placodes of Mdm2<sup>FM/FM;Pax2-Cre</sup> mice, a portion of placodal cells survived and invaginated into small-sized otic vesicles by E9.0 (II, Fig. 2). Based on previously published data (Ohyama and Groves, 2004), it was proposed that incomplete recombination in otic progenitors led to maintained expression of functional Mdm2, allowing a portion of otic progenitors to circumvent death. Alternatively, as robust p53 upregulation was seen throughout the otic placode of mutant mice, it is possible that a subpopulation of otic progenitors are insensitive to p53 accumulation or might be able to downregulate p53 expression independently of Mdm2 and survive. This latter possibility is quite unlikely, as, in all tissues examined so far, Mdm2 is the main negative regulator of p53. Apoptotic cells have been shown to stimulate proliferation of neighboring cells (Li et al., 2010), offering a possibility that stimulated proliferation partially compensated for lost cells and allowed inner ear development to proceed. However, this possibility is also quite unlikely, as no differences in proliferative activity were evident between control and mutant otic placodes (data not shown). In any case, the survival of a portion of otic progenitor cells in Mdm2<sup>FM/FM;Pax2-Cre</sup> mice enabled the study of further development of the inner ear in the context of a dramatically reduced progenitor cell population.

Surprisingly, placodes of mutant mice developed into otic vesicles (by E9.0-9.5) and otocysts (by E10.5 and thereafter) (II, Fig. 2). In addition, delaminating neuroblasts were seen. However, developing mutant inner ears exhibited an overall reduction in size and patterning defects, manifested as an abnormal expression of patterning genes, such as Sox2 (II, Fig. 2). By the end of embryonic inner ear development (E17.5-E18.5), the morphology of inner ears derived from Mdm2<sup>FM/FM;Pax2-Cre</sup> mice was strongly altered (II, Fig. 3). This data suggests that reduced numbers of inner ear progenitors are unable to carry out the normal developmental program of the inner ear. Surprisingly, however, regions with a normal complement of auditory HCs, SCs, and islands of spiral ganglion neurons were found in some mutant inner ears (II, Fig. 3). These results suggest that, in some cases, the surviving progenitors are capable of executing inner ear development. It is possible that in these mutants with partially normal inner ear development, a distinct subpopulation of otic progenitors that exhibited enhanced capacity for inner ear development, had survived. Evidently, many unanswered questions remain and lineage-tracing experiments are warranted to be able to understand the mechanisms underlying aberrant inner ear development in this model. Altogether, the data presented here demonstrates that otic progenitors cannot compensate for the loss of fellow progenitor cells and are unable to carry out the normal development of the inner ear.
5.4.3 *Mdm2* ablation-mediated p53 upregulation is lethal for immature postmitotic auditory supporting cells and hair cells (II)

It is generally believed that postmitotic cells are insensitive to p53 upregulation. This general principle also underlies the design of cancer therapies. However, several studies have contradicted this belief, showing that p53 upregulation can be lethal also for postmitotic, differentiated cells (Boesten et al., 2006; Francoz et al., 2006; Zhang et al., 2014).

To study the p53 sensitivity of cochlear HC and SC progenitors, immediately following their cell cycle exit between E12.5 and E14.5, conditional, inducible *Mdm2* FM/FM;*Fgfr3*-iCre-ER<sup>T2</sup> mice were used (Grier et al., 2002; Young et al., 2010). Tamoxifen was administered at E13.5 and E14.5 to target iCre-mediated *Mdm2* ablation to Fgfr3-expressing differentiating HCs and SCs, and the cochleas were analyzed at E18.5. At E18.5, both *Mdm2*-ablated cochlear HCs and SCs showed upregulation of p53 and were dying by apoptosis (II, Fig. 6). This data is consistent with previously published data on postmitotic P1 auditory HCs that succumbed to apoptosis following pharmacological upregulation of p53 (Sulg et al., 2010). Altogether, this data demonstrates that differentiating, immature postmitotic HCs and SCs of the embryonic cochlea do not tolerate p53 upregulation.

5.4.4 p53 sensitivity decreases during postnatal maturation in auditory supporting cells (II)

The acquired data on p53-sensitivity of embryonic, postmitotic auditory SCs is quite surprising, as SCs are generally relatively resistant to stress stimuli compared to HCs. This has been shown, for example, in the context of ototoxic aminoglycosides where SC loss follows HC death (Oesterle and Campbell, 2009). However, SC sensitivity to insults has been difficult to assess, as HCs that are required for SC viability are usually abolished first. Hence, it is difficult to say whether SC loss occurred primarily due to their sensitivity to the insult or due to the loss of essential HCs.

Thus, the *Mdm2*<sup>Fm/Fm</sup>;*Fgfr3*-iCre-ER<sup>T2</sup> model was used to penetrate into p53-sensitivity of auditory SCs during postnatal maturation and in adulthood. The morphological maturation of mouse auditory SCs takes place during the first weeks of postnatal development. During this period, auditory SCs develop upright microtubule-rich cytoplasmic extensions called phalangeal processes (Ito et al., 1995). The phalangeal processes of a subtype of auditory SCs, the pillar cells, separate and form a fluid-filled tunnel of Corti.

During postnatal stages, conditional, inducible *Mdm2*<sup>Fm/Fm</sup>;*Fgfr3*-iCre-ER<sup>T2</sup> mouse line facilitates the targeting of iCre-recombinase mediated *Mdm2*-ablation and subsequent p53 upregulation to specific Fgfr3-positive subpopulations of auditory SCs, pillar and Deiters´ cells (Anttonen et al., 2012). To trigger *Mdm2*-ablation mediated p53
upregulation in auditory SCs at the neonatal stage, tamoxifen was administered to Mdm2<sup>Fm/Fm</sup>;Fgfr3-iCre-ER<sup>T2</sup> mice at P0 and P1, and the cochleas analyzed at P4 and P7. At P4, and more prominently at P7, the whole mount surface views of the mutant cochlea revealed a dramatic loss of auditory SCs (II, Fig. 4). HCs were viable in mutant cochleas, consistent with the fact that Fgfr3 that drives iCre recombinase expression, is not expressed in HCs during postnatal stages (except for apical and basal regions of the cochlea that were not included in this analysis). More thorough examination of paraffin sections of the mutant cochlea revealed that the tunnel of Corti was missing and SC phalangeal processes were disorganized (II, Fig. 4). The morphological maturation of SCs was most likely disrupted due to their ongoing apoptosis and degeneration, however, it is also possible that p53 upregulation directly affected morphological maturation. In several other contexts, for example in myoblasts (Porrello et al., 2000), p53 has been shown to orchestrate differentiation. It would be interesting to study, whether Notch, Fgf, and pRb signaling pathways that are implicated in SC differentiation (Campbell et al., 2016; Doetzlhofer et al., 2009; Yu et al., 2010), are dysregulated by p53 overexpression.

Next, Mdm2-ablation-mediated p53-upregulation was triggered in morphologically more mature auditory SCs, by injecting tamoxifen at P6-7 and analyzing the cochleas at P12 and P20. Whole mount views and paraffin sections of P12 mutant cochleas revealed that although the majority of SCs were still present in the organ of Corti, SC phalangeal processes were disorganized and the tunnel of Corti had collapsed (II, Fig. 5). By P20, a large part of the auditory SCs was lost in mutant cochleas and the organ of Corti had completely flattened (II, Fig. 5). This data reveals that in juvenile, P6 cochlea, p53 upregulation primarily disrupts the morphological maturation of auditory SCs and cell death seems to be a secondary consequence of disrupted morphological maturation. Thus, it is possible that morphological maturation defect, evident in P7 SCs following neonatal ablation of Mdm2, might also be a direct consequence of upregulated levels of p53 in addition to ongoing apoptosis and degeneration of SCs.

Comparative analysis of SC numbers 6-7 days following tamoxifen administration revealed that only 6.3% of auditory SCs were lost in P12 mutant cochleas (tamoxifen at P6-7), whereas 47.4% of SCs were lost in P7 cochleas (tamoxifen at P0-1). These results demonstrate that juvenile, P6 auditory SCs are less sensitive to p53 upregulation compared to their neonatal counterparts. Altogether, this data implicates that controlled levels of p53 are indispensible for proper SC differentiation and survival.

Interestingly, even though Mdm2 ablation and consequent p53 upregulation were targeted specifically to auditory SCs, scattered outer HC loss was evident already before significant SC loss in P12 mutant mice (tamoxifen P6-7) (II, Fig. 5). This data is consistent with a study where the loss of the same subtypes of auditory SCs led to a gradual HC loss that started by the end of the first postnatal week (Mellado Lagarde et al., 2013). Thus, HC loss also in this model seems to be unrelated to Mdm2-ablation-mediated p53-upregulation and appears rather to be a general consequence of the loss of essential tissue homeostasis maintaining SCs (Wan et al., 2013). Interestingly, no HC loss was seen when SCs were lost following neonatal ablation of Mdm2 (II, Fig. 4). As the gradual HC loss following Mdm2 ablation in juvenile P6 SCs coincided with the onset
of hearing function, it seems that HCs are especially sensitive to disruptions in homeostasis at the onset of their functional activity.

Finally, the p53 sensitivity of mature auditory SCs was investigated. Mdm2-ablation mediated p53 upregulation was triggered in mature auditory SCs by injecting tamoxifen to Mdm2\textsuperscript{Fm/Fm};Gfr3-iCre-\textit{ERT2} mice at P49-50. As analyzed at P55, despite strong p53 upregulation, the viability of auditory SCs was unaffected and the morphology of the organ of Corti was normal (II, Suppl. Fig. S3). This data demonstrates that p53 sensitivity declines with aging in auditory SCs. However, the possibility that longer-term accumulation of p53 would also affect the viability of mature SCs cannot be excluded. However, this could not be studied in this model, as the health of mutant mice was adversely affected by the latest time point of analysis (P55).

The p53-sensitivity of vestibular SCs could not be investigated in this model, as the expression of Gfr3 that drives iCre recombinase expression in Mdm2\textsuperscript{Fm/Fm};Gfr3-iCre-\textit{ERT2} mice is restricted to auditory SCs. Thus, to study p53 sensitivity in postmitotic mature vestibular SCs, P55 utricular organotypic explants were treated with nutlin3, a pharmacological compound that suppresses Mdm2/p53 interaction (Vassilev et al., 2004). After 7 days of continuous exposure to nutlin3, p53 was upregulated in utricular HCs and SCs (II, Fig. 7). Interestingly, as for mature cochlear SCs in vivo, pharmacological upregulation of p53 in utricular SCs and HCs in vitro yielded no significant cellular loss. This data suggests, that both mature auditory and vestibular SCs are resistant to p53 upregulation.

Altogether, this data demonstrates that p53 sensitivity decreases with maturation in postnatal auditory SCs. The p53 sensitivity seems to be associated with morphological maturation, as fully mature P55 SCs were resistant to upregulated levels of p53. Alternatively, p53 function has been shown to decline with aging, so that it’s efficiency to transactivate its target genes, required for the progression of apoptosis, is reduced (Feng et al., 2007). Maturation-associated heterochromatin accumulation has been suggested as the underlining reason for this decline. Indeed, in mouse kidney, it has been shown that the ability of p53 to transactivate the expression of proapoptotic \textit{Puma} is significantly decreased with aging (Zhang et al., 2014).
6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Postmitotic, differentiated cells are indispensable for proper tissue function and comprise most of the functional cell types in our body. In some tissues, multipotent stem cells renew these populations of cells throughout life. The mammalian inner ear seems to contain no true population of stem cells. However, a population of postmitotic cells, the SCs, maintains tissue homeostasis and shows limited regenerative plasticity in early postnatal life. SC regenerative plasticity seems to be linked with their developmental immaturity and it is not normally seen in the adult inner ear. However, in some studies regeneration in the adult inner ear has been achieved by genetic and pharmacological manipulations. In this thesis work, I studied the mechanisms behind restrictions of the regenerative plasticity of mammalian postnatal SCs. This topic has thus far remained largely unexplored.

This study revealed that DNA damage accumulation and its inefficient repair underlie age-dependent decline in the proliferative plasticity of postnatal SCs. Hence, one of the possible strategies that would allow more efficient cell cycle progression, would be to boost DNA damage repair. Indeed, the efficiency of somatic cell reprogramming that is also linked to the accumulation of DNA damage, is enhanced by the experimental augmentation of DNA damage repair (Lee et al., 2016). Postnatal maturation is associated with progressive accumulation of heterochromatin that creates obstacles for DNA repair (Goodarzi et al., 2008). It has also been indicated in the inner ear that epigenetic silencing associated with heterochromatin accumulation occurs concomitantly with early postnatal maturation (Stojanova et al., 2015). Still, future studies are needed to determine whether maturation-associated chromatin silencing underlies the age-dependent decrease in DNA repair efficiency in the inner ear.

The data presented here implicates that strategies, like dedifferentiation that would relax the compact chromatin of mature SCs, would facilitate cell cycle progression. Dedifferentiation, like differentiation, is believed to be regulated at the epigenetic level. During dedifferentiation, a specialized type of cell loses the expression of lineage-specific genes, leading to structural and physiological changes. In other contexts, such as in the regenerating zebrafish heart, dedifferentiation of morphologically complex cardiomyocytes facilitates proliferation following amputation (Jopling et al., 2010). However, in the non-mammalian inner ear, where SCs can regenerate HCs, it is still unclear whether dedifferentiation is part of the regenerative response. Furthermore, in mature mammalian cochlea, SCs appear to be unable to spontaneously dedifferentiate following HC loss (Oesterle and Campbell, 2009). In any case, dedifferentiation of postmitotic SCs represents an attractive strategy to stimulate regenerative proliferation, especially since in many cases it is coupled to proliferation, such as in Schwann cells during peripheral axon regeneration (Chen et al., 2007). Thus, in a best-case scenario, experimentally triggered dedifferentiation would be naturally followed by proliferation and a separate unnatural forced cell cycle re-activation step.
could be avoided. Unfortunately, at this point experimentally induced dedifferentiation remains out of reach.

This study also showed that experimentally induced transdifferentiation, one of the mechanisms that SCs could use to replace lost HCs, does not trigger DNA damage. The results thus suggest that compared to forced proliferation, experimentally induced transdifferentiation is a more natural and feasible process for postnatal mammalian SCs. Despite the lack of DNA damage, the efficiency of experimentally induced SC-to-HC transdifferentiation declines during maturation. Notch signaling maintains the cochlear SC fate only until the end of the first postnatal week (Maass et al., 2015). Consequently, manipulation of Notch signaling becomes inefficient after this stage. To extend transdifferentiation plasticity to more mature stages, future studies are needed to determine the mechanisms that take over the maintenance of the cochlear SC identity following the early postnatal period.

Following HC loss in non-mammalian species, SCs spontaneously regenerate lost HCs via direct transdifferentiation or mitotic regeneration (proliferation followed by transdifferentiation). The results presented here revealed that forced cell cycle re-activation is not spontaneously followed by transdifferentiation in mammalian SCs. However, as pharmacological suppression of Notch signaling was sufficient to trigger transdifferentiation of cell cycle re-activated cells, the results suggest that stressed cells are nevertheless able to initiate lineage conversion. Thus, this study shows that even proliferating SCs with an ongoing DNA damage response signaling, can be induced to transdifferentiate into HCs.

The downregulation of p53 tumor suppressor has been shown to be necessary for regeneration in several contexts. However, the results presented here showed that the absence of p53 in the inner ear fails to lead to cell cycle re-activation during postnatal development, even in the context of various growth promoting settings (in vitro culture, activation of Wnt signaling, and HC loss). Thus, in inner ear sensory epithelia, p53 appears not to maintain the postmitotic state. In contrast to somatic cell reprogramming, where the suppression of p53 has an enhancing effect, experiments presented here showed that the absence of p53 has no effect on the efficiency of transdifferentiation. Unlike somatic cell reprogramming, pharmacologically triggered transdifferentiation does not trigger DNA damage, thus the lack of p53 involvement is no surprise. However, these results are inconclusive, as functional p53 was absent in this model throughout the embryonic inner ear development and compensatory mechanisms might have taken over the essential functions of p53. Thus, future studies using acute, preferably transient, inactivation of p53 are warranted, to conclusively determine the role of p53 in the maintenance of postmitotic and differentiated states of mammalian SCs and HCs.

The results on upregulated levels of p53 during different stages of inner ear development demonstrated that the sensitivity to p53-upregulation triggered apoptosis decreases with maturation in SCs. There are several possible implications of these results. For example, does the decreased sensitivity to p53 mean that mature SCs have a higher threshold for stress-induced apoptosis? Furthermore, is the decreased p53-sensitivity the underlying reason why SCs are more refractory to various ototoxic
insults compared to HCs? Finally, does this insensitivity represent a protective mechanism to spare essential SCs from death? In relation to these aspects, it would be very interesting to study HC sensitivity to p53 at different time points during their maturation. These studies could determine, whether age-associated p53 insensitivity is a SC-specific characteristic or whether it is a more general feature associated for example with heterochromatin-accumulation associated decline in p53 function during aging (Feng et al., 2007; Zhang et al., 2014).

As the regenerative plasticity of mammalian SCs seems to be a property associated with their immature developmental status, more efforts should be directed towards identifying signaling pathways and regulators that are prominent in these postnatal, maturing SCs. In this regard, more knowledge on epigenetic mechanisms that regulate differentiation and maintenance of the postmitotic state might be useful. During recent years, the role of epigenetic regulation of inner ear development and regenerative plasticity has started to unveil (Doetzlhofer and Avraham, 2016).

Altogether, it appears there are no shortcuts to HC regeneration therapy and there is still a long way to go before a therapeutic intervention becomes a reality. What complicates the design of regenerative intervention in addition to cell intrinsic restrictions, is a complex morphological architecture of the mammalian sensory epithelia that should be restored precisely to restore the functionality of the organ. Furthermore, the therapeutic intervention should consider the variability of degeneration of the sensory epithelium between individuals. The so-called flat epithelium that lacks any specialized cells that forms after strong trauma has very different properties from sensory epithelium where only a part of HCs is missing (Taylor et al., 2012).

In conclusion, this work implicates maturation-associated changes of SC chromatin in restricting the regenerative capacity of SCs and provides a stepping stone for future studies that characterize the epigenetic changes that occur during postnatal maturation.
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Maerja Laos
REFERENCES


Daudet, N., Ariza-McNaughton, L., and Lewis, J. (2007). Notch signalling is needed to maintain, but not to initiate, the formation of prosensory patches in the chick inner ear. Development 134, 2369-2378.


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ORIGINAL PUBLICATIONS
DNA damage signaling regulates age-dependent proliferative capacity of quiescent inner ear supporting cells

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Abstract: Supporting cells (SCs) of the cochlear (auditory) and vestibular (balance) organs hold promise as a platform for therapeutic regeneration of the sensory hair cells. Prior data have shown proliferative restrictions of adult SCs forced to re-enter the cell cycle. By comparing juvenile and adult SCs in explant cultures, we have here studied how proliferative restrictions are linked with DNA damage signaling. Cyclin D1 overexpression, used to stimulate cell cycle re-entry, triggered higher proliferative activity of juvenile SCs. Phosphorylated form of histone H2AX (γH2AX) and p53 binding protein 1 (53BP1) were induced in a foci-like pattern in SCs of both ages as an indication of DNA double-strand break formation and activated DNA damage response. Compared to juvenile SCs, γH2AX and the repair protein Rad51 were resolved with slower kinetics in adult SCs, accompanied by increased apoptosis. Consistent with the in vitro data, in a Rb mutant mouse model in vivo, cell cycle re-entry of SCs was associated with γH2AX foci induction. In contrast to cell cycle reactivation, pharmacological stimulation of SC-to-hair-cell transdifferentiation in vitro did not trigger γH2AX. Thus, DNA damage and its prolonged resolution are critical barriers in the efforts to stimulate proliferation of the adult inner ear SCs.

INTRODUCTION

Sensory hair cells and supporting cells (SCs) of the cochlear and vestibular organs are highly differentiated cells that do not proliferate at adulthood. Because mammalian hair cells do not regenerate, their loss following trauma leads to permanent functional (hearing and balance) deficits. SCs hold promise as a platform for therapeutic hair cell regeneration, analogous to natural regeneration in the non-mammalian inner ears in which SCs divide and transdifferentiate into hair cells [1]. In the field of regenerative medicine, stimulation of cellular reprogramming and expansion of quiescent cells have been shown to cause genetic abnormalities and cell death, for example in response to DNA damage triggered by ectopic oncogene expression [2, 3]. We have previously shown that, upon forced cell cycle re-entry, SCs of the adult utricle, one of the vestibular organs, accumulate DNA damage and arrest in the cell cycle. We hypothesized that the limited capacity of adult SCs to cope with DNA damage underlies the restrictions in completing normal cell cycles [4].

Oncogene overexpression and genotoxic agents cause several types of DNA lesions, including DNA double-strand breaks (DSBs) that represent the most lethal form of DNA damage. The activated DNA damage response (DDR) orchestrates damage detection and repair. DDR activates cell cycle checkpoints to guard against inappropriate cell cycle progression until DNA lesions are resolved. If DNA damage remains unresolved, the outcome is cell cycle arrest or death [5]. Activation of the proximal components of the DDR pathway, the members of a family of phosphoinositide-3-kinase-
related protein kinases (PIKKs), feeds to their downstream effectors, including the histone H2A variant H2AX. It is activated by PIKK phosphorylation on serine 139, termed as γH2AX. γH2AX accumulates in megabase regions surrounding DSBs, resulting in the formation of foci that act as docking sites for other DDR components [6, 7]. γH2AX foci can be detected by antibodies and used as indicators for the presence of DNA damage [6]. The rapid γH2AX activation after damage and the resolution of the foci after repair have suggested that γH2AX can be used as a marker for monitoring cellular DDR [8, 9].

Compared to proliferating cells, DNA damage signaling in postmitotic cells is poorly studied, especially in connection with cell cycle manipulation. Upon forced proliferation, γH2AX induction has been shown in neurons of the central nervous system and in the inner ear hair cells, and was found to lead to apoptosis of these highly differentiated cells [10, 11]. Upon DNA damage triggered by genotoxic agents, activation of other components of the DDR pathway, besides the PIKKs, feeds to their downstream effectors, including the histone H2A related protein kinases (PIKKs), feeds to their downstream effectors, including the histone H2A variant H2AX. It is activated by PIKK phosphorylation on serine 139, termed as γH2AX. γH2AX accumulates in megabase regions surrounding DSBs, resulting in the formation of foci that act as docking sites for other DDR components [6, 7]. γH2AX foci can be detected by antibodies and used as indicators for the presence of DNA damage [6]. The rapid γH2AX activation after damage and the resolution of the foci after repair have suggested that γH2AX can be used as a marker for monitoring cellular DDR [8, 9].

RESULTS

Adenoviral tropism to postnatal inner ear supporting cells

In the present study, the sensory epithelia from postnatal day 6 (P6) utricles and cochleas, and from P50 utricles were prepared for organotypic cultures, as previously described [4]. The P6 and P50 SCs were used to represent the juvenile and adult SCs, respectively. Explants were maintained for 2 to 14 days in vitro (DIV). The auditory sensory epithelium, the organ of Corti, was not studied at adulthood due to difficulties in preserving in vitro the normal cytoarchitecture of the mature organ and the survival of its hair cells. SCs were marked by antibodies against Sox9 and Sox2 [4, 17]. In postnatal utricles, Sox2 is expressed in both SCs and hair cells. However, the...
nuclei of two cell types are located at different heights in the sensory epithelium and have different morphology, allowing cell type-specific analysis in whole mount surface preparations (Fig. 1A,B). In some experiments, hair cell-specific markers, parvalbumin and myosin 6 (myo6), were used.

Our previous work has established optimal conditions for transduction by adenoviruses encoding cD1 (AdcD1) and β-galactosidase (AdβGal) in adult utricular explants [4]. In the present study, also AdGFP reporter viruses were used to investigate viral tropism, an important issue, because our model organ comprises different cell types and because we studied different ages. AdGFP viruses transduced P6 and P50 utricular SCs, as detected by the presence of GFP+/Sox2+ (Fig. 1C,D) and GFP+/Sox9+ cells (data not shown) at 3 DIV. Transduction efficiency varied between individual explants, ranging from 20 to 50%. Only occasional AdGFP-infected hair cells were found in adult utricles (data not shown). P6 utricles showed higher amount of infected hair cells, based on quantification of parvalbumin+/GFP+ cells. The average infection rate of hair cells was 10% (10.1 ± 0.7, n = 3, total number of hair cells counted = 843). Together, even though infected hair cells were present in juvenile utricles, their amount was clearly outnumbered by infected SCs (Fig. 1E,F) [18].

In AdGFP- or AdβGal-infected P6 cochleas analyzed at 3 DIV, transgenes expressions were concentrated to Deiters’ cells, a specific subtype of auditory SCs (Fig. 1F,F”). This expression was concentrated to the upper half of the cochlear duct, transduced Deiters’ cells being often arranged in small patches (Fig. 1F”,G). Hair cells were not transduced, based on the absence of GFP+/parvalbumin+ cells (data not shown). In the AdβGal-infected P6 cochlea shown in Fig. 1G, the boxed area represents the cochlear region analyzed in the present study. Taken together, under the experimental conditions used, the adenoviral serotype 5 vector (Ad5) with the CMV promoter preferentially transduces SCs in the juvenile and adult inner ear sensory epithelia, with an interesting Deiters’ cell-specific pattern in the cochlea.

Response of juvenile and adult utricular supporting cells to AdcD1 infection

We used ectopic cD1 expression as a tool to force SCs into the cell cycle, based on the fact that many proliferation-promoting signaling pathways target this core cell cycle component. Particularly, cD1 is a central mediator of the proliferative response following activation of the Wnt/β-catenin pathway. It has been shown in mutant mouse models that Wnt/β-catenin activation increases proliferative activity of neonatal inner ear SCs [19]. To study cell cycle activity of SCs transduced by AdcD1, we pulsed explants with EdU for 24 h between days 2 and 3, and analyzed at 3 and 7 DIV. (A,A’) AdcD1-infected P6 utricle displays high numbers of EdU+/Sox2+ SCs. (B,B’) AdcD1-infected P50 utricle shows lower numbers of EdU+ SCs. (C,C’) Non-infected utricular explant is devoid of proliferating SCs. (D,D’) The AdcD1-infected P6 utricle shows EdU+/cD1+ SCs (arrowheads). In addition, there are only EdU-positive and only cD1-positive SCs due to cell cycle dynamics and EdU pulsing (see Results). Abbreviations: AdcD1, adenovirus encoding cyclin D1; utr, utricle. Scale bar, shown in D’,A-C’, 20 μm; B’,D’, 8 μm.

Figure 2. Proliferative response of juvenile and adult utricular supporting cells to cyclin D1 overexpression. Explants were pulsed with EdU for 24 h between days 2 and 3, and analyzed at 3 and 7 DIV. (A,A’) AdcD1-infected P6 utricle displays high numbers of EdU+/Sox2+ SCs. (B,B’) AdcD1-infected P50 utricle shows lower numbers of EdU+ SCs. (C,C’) Non-infected utricular explant is devoid of proliferating SCs. (D,D’) The AdcD1-infected P6 utricle shows EdU+/cD1+ SCs (arrowheads). In addition, there are only EdU-positive and only cD1-positive SCs due to cell cycle dynamics and EdU pulsing (see Results). Abbreviations: AdcD1, adenovirus encoding cyclin D1; utr, utricle. Scale bar, shown in D’,A-C’, 20 μm; B’,D’, 8 μm.
robust cell cycle activity of this juvenile cell population. Both P6 and adult utricles showed EdU-labeled SCs with cD1 expression, linking ectopic proliferation with adenovirus-mediated cD1 overexpression (Fig. 2D,D’; data not shown). Of note, because cD1 expression is rapidly downregulated in cells that initiate replication and because we retrospectively analyzed EdU-pulsed cells, SCs did not always show colabeling of cD1 and EdU.

**AdcD1-infected utricular supporting cells show a DNA damage response**

Oncogene-induced aberrant proliferation stimulates replication stress that can lead to DSB formation and DDR activation [20, 21]. We hypothesized that this is the case also with SCs following ectopic expression of the cD1 proto-oncogene. Furthermore, unresolved DSBs or other type of DNA damage might explain the low-level cell cycle activity of AdcD1-infected adult SCs compared to juvenile SCs (Fig. 2) [4]. To find out whether DSBs are formed in SCs, we compared using confocal microscopy the expression pattern of γH2AX, a DNA damage marker, in SCs challenged with AdcD1 and ionizing radiation (IR). γH2AX marks various forms of DNA damage, but the characteristic foci-like expression pattern seen in non-replicating cells following IR exposure represents DSBs [22]. Therefore, we used the γH2AX expression pattern seen 1 h after 2 gray (Gy) dose of IR as an established template of DSB profiles. Comparative analysis after 3 DIV showed that, similar to IR-treated SCs, AdcD1-infected and EdU-labeled (pulse between culture days 2 and 3) SCs of both ages expressed γH2AX as abundant small nuclear foci (Fig. 3A-D). These findings suggest that DSBs are induced in juvenile and adult SCs upon cell cycle re-entry.

To determine whether culture conditions and adenoviral transduction itself trigger DNA damage, non-infected as well as AdGβGal- and AdGFP-infected utricles were analyzed after 3 DIV. One or two large γH2AX foci were found in SCs of both non-infected and reporter virus-infected utricles of both ages (Fig. 3E-G; data not shown). Thus, *in vitro* conditions induce low-level DNA damage, and viral transduction does not markedly alter this basal pattern. However, the DNA damage profile in these control explants is clearly different from the DSB-like profile with abundant small γH2AX foci seen following AdcD1-infection and IR. To confirm that the baseline γH2AX expression arising in *vitro* does not negatively affect proliferation, we prepared utricular cultures at P2, at the stage when a part of utricular SCs still naturally proliferates *in vivo* [16]. Both non-infected and AdGFP-infected P2 cultures showed EdU-labeled SCs after a 3-day-long pulsing period (data not shown), suggesting that the baseline γH2AX expression in explant cultures does not prevent natural SC proliferation.

**Figure 3.** Utricular supporting cells show γH2AX and 53BP1 foci upon forced cell cycle re-entry, as revealed by confocal imaging. (A,B) At 3 DIV, AdcD1-infected P6 and P50 utricles display EdU+ SCs with numerous, small γH2AX foci. (C,D) Similar γH2AX profiles are seen 1 h post-irradiation in Sox2+ utricular SCs of both ages. (E-G) At 3 DIV, Sox2+ SCs in both AdGβGal-infected P6 and P50 utricles, and in non-infected P6 utricle show one or two large γH2AX foci per nucleus. (H-J) At 3 DIV, Edu+ SCs in AdcD1-infected P50 utricle display 53BP1 foci. Edu+ SC nuclei containing 53BP1 foci are outlined (M). (I) Four hours post-irradiation, P50 utricular Sox2+ SCs show 53BP1 foci. (J) At 3 DIV, AdGFP-infected P50 utricle is devoid of 53BP1 foci. A Sox2+GFP+/53BP1-nucleus is outlined. Abbreviations: AdcD1, adenovirus encoding cyclin D1; AdGβGal, adenovirus encoding β-galactosidase; AdGFP, adenovirus encoding green fluorescent protein; Gy, gray; γH2AX, Ser 139 phosphorylated histone H2AX; utr, utricle. Scale bar, shown in J: A–I, 5 µm.
As an additional marker for activated DDR, we used p53 binding protein 1 (53BP1), a component of the DDR pathway that localizes to the area of DSBs [23]. Similar to IR-challenged SCs, EdU-labeled SCs in AdcD1-infected utricles of both ages displayed 53BP1 foci (3H-I; data not shown). These foci were not detected in Sox2+/GFP+ SCs in explants infected with reporter viruses (Fig. 3J). We conclude that DDR is activated in juvenile and adult SCs upon forced cell cycle re-entry, with DSBs as a major type of DNA lesion.

γH2AX foci resolution in AdcD1-infected utricular supporting cells

We next examined the temporal pattern of γH2AX expression in AdcD1-infected and EdU-labeled SCs (Fig. 4). In P6 utricles, the first replicating SCs were found at 2 DIV (EdU pulse between days 1 and 2). These cells showed γH2AX foci (data not shown), but the intensity of this expression was not as prominent as one day later when widespread γH2AX expression was found in the SC population, concomitantly with the high proliferative activity of these cells. Based on quantification at 3 DIV, 51% of EdU+ SCs (EdU pulse between culture days 2 and 3) showed the typical DSB pattern of γH2AX (SC numbers counted per explant: EdU+ 326.8 ± 27.8; γH2AX+/EdU+ 164 ± 12.2). This value was only 18% at 7 DIV (SC numbers counted per explant: EdU+ 274.8 ± 38.3; γH2AX+/EdU+ 48.6 ± 8.6) (Fig. 4A). The disappearance of SCs with γH2AX foci was not caused by cell death, based on the findings of only rare cleaved caspase-3-positive profiles in the juvenile explants analyzed at 7 DIV (data not shown). Of note, it is likely that the rather low percentage of EdU+ SCs with γH2AX foci at 3 DIV is an underestimate, because a part of the foci had already been resolved by this time point. These data on γH2AX resolution in AdcD1-infected juvenile SCs suggested successful DNA repair.

Next, using the same EdU pulsing regimen, the temporal pattern of γH2AX expression was analyzed in AdcD1-infected adult utricles (Fig. 4B). At 3 DIV, 83% of EdU+ SCs displayed γH2AX foci (SC numbers counted per explant: EdU+ 68.6 ± 4.1; γH2AX+/EdU+ 56.6 ± 2.4). This value was 54% at 7 DIV (SC numbers counted per explant: EdU+ 40.6 ± 10.8; γH2AX+/EdU+ 22.6 ± 6.5). By 14 DIV, the percentage of double-positive cells had decreased to 16% (SC numbers counted per explant: EdU+ 39.5 ± 3.7; γH2AX+/EdU+ 6 ± 0.4), a value comparable to that seen in juvenile utricles at 7 DIV (Fig. 4B). Compared to the juvenile specimens, these results showed that the γH2AX foci resolution is prolonged in adult utricles and, thus, suggested slower DNA repair.

At 7 DIV, in addition to EdU+ SCs with γH2AX foci, AdcD1-infected adult utricles displayed EdU+ SCs with intensive, pan-nuclear γH2AX staining (Figs. 3B, 4C). This γH2AX pattern has been previously associated with severe DNA damage and cell death [22]. Consistently, the majority of SCs with pan-nuclear γH2AX co-expressed cleaved caspase-3, a marker for apoptotic death (Fig. 4C,C’). Quantification at 7 DIV showed a highly significant difference (p < 0.005) between the relative amount of γH2AX+ SCs with intensive, pan-nuclear staining in P6 and P50 utricles (Fig. 4D). This γH2AX expression profile was absent in explants transduced by reporter viruses (data not shown). Together, these data suggest that prolonged DNA damage resolution in cell cycle reactivated adult SCs predisposes these cells to apoptosis.

The repair protein Rad51 is dynamically expressed in AdcD1-infected utricular supporting cells

Rad51 is a DNA repair protein engaged in homologous recombination repair (HRR) and is detected in the respective repair foci. HRR is relevant in the context of cell cycle reactivated SCs investigated in the present work, because it predominates in S and G2 phases of the cell cycle [24]. We used Rad51 as a marker for HRR, and examined whether its expression correlate with the observed γH2AX foci resolution.

At 3 DIV, Rad51 was upregulated in AdcD1-infected P6 utricles, specifically in SCs with γH2AX foci (Fig. 5A,A’). This was readily seen under light microscopy and low magnification. The nuclear Rad51 expression appeared fuzzy at low magnification, because the expression was formed by numerous small foci, as revealed by confocal imaging at high magnification (Fig. 5B,B’). Interestingly, in juvenile SCs, Rad51 expression was downregulated by 7 DIV, paralleling the resolution of γH2AX foci (Fig. 5C,C’). AdG4al-infected explants did not show Rad51 expression (Fig. 5D,D’). As an additional control, IR-challenged, non-proliferating SCs lacked Rad51 expression (Fig. 5E,E’), consistent with the predominance of non-homologous end-joining rather than HRR in DSB repair in non-proliferating cells. Together, in juvenile SCs, HRR was induced upon ectopic proliferation and DDR activation, and was down-regulated concomitantly with successful DNA repair.

Also AdcD1-infected adult utricles showed Rad51 induction at 3 DIV, but as opposed to juvenile explants, downregulation was not observed before 14 DIV. This is consistent with the resolution of γH2AX foci by this late post-infection time point (Fig. 5F–H’). Similar to younger SCs, confocal microscopy revealed Rad51 expression as small nuclear foci in adult SCs (Fig.
Quantification showed that Rad51 expression closely paralleled that of γH2AX both in juvenile and adult SCs (Fig. 5K). As the presence of γH2AX foci matched with EdU labeling (Fig. 4A,B), we conclude that Rad51+ cells represent cell cycle reactivated and DNA damaged SCs. Interestingly, at 7 DIV, SCs with the strong, pan-nuclear γH2AX expression lacked or showed only very weak Rad51 expression (Fig. 5G,G’,J,J’). Because many of these cells were also positive for cleaved caspase-3 (Fig. 4C,C’), these data link delayed DNA repair to cell death.

AdcD1-triggered cell cycle re-entry of auditory supporting cells

We next studied cell cycle activity in cochlear SCs transduced by AdcD1 at P6. Consistent with the transduction pattern revealed by reporter viruses (Fig. 1F’,G), the upper half of AdcD1-infected cochlear explants showed EdU labeling exclusively in Sox2+ Deiters’ cells (EdU pulse between days 2 and 3). These double-positive cells were found both at 3 and 7 DIV (Fig. 6A-B’). Non-infected and reporter virus-infected cochleas were devoid of EdU+ cells (Fig.6C; data not shown). Following AdcD1 infection, similar to utricles, cochleas showed EdU-labeled Deiters’ cells with cD1 upregulation (Fig. 6D,D’).

Several EdU+ doublets of Deiters’ cells were found in AdcD1-infected P6 cochleas. In many cases, the nuclei of these doublets appeared to be firmly connected with each other, suggesting for incomplete cytokinesis (Fig. 6A-B’). Consistently, the Deiters’ cell population did not show clear signs of expansion, based on morphological examination and comparison to the control (non-infected and reporter virus-infected) cochleas at 7 DIV (Fig. 6A-C). An underlying reason might be the induction of p53, an anti-proliferative transcription factor often activated upon unscheduled proliferation [25]. Indeed, we found p53 upregulation in Deiters’ cells of AdcD1-infected cochleas, particularly in cell doublets, but also in cells with large nuclei, typical to replicating cells (Fig. 7A,A’). Interestingly, SCs of age-matched, AdcD1-infected utricles did not show p53 upregulation (Fig. 7B,B’).
consistent with their high proliferative activity (Fig. 2A, A’). p53 expression was undetectable in control explants of both the cochlea and utricle (Fig. 7C, D).

Thus, these data suggested an inverse correlation between the proliferative activity and p53 response in the cochlear Deiters’ cells.

**Figure 5.** Dynamics of the DNA repair protein Rad51 in cell cycle reactivated supporting cells. Adenovirus-infected utricular explants were maintained for 3 to 14 DIV and double-labeled for γH2AX and Rad51. Imaging by conventional (A, A’, C–H’) and confocal microscopy (B, B’, I–J’). (A, A’) At 3 DIV, AdcD1-infected P6 utricle shows Rad51 upregulation in SCs with γH2AX foci. (B, B’) High magnification view of an AdcD1-infected P6 utricle showing Rad51 expression as small foci that partially colocalize with the γH2AX foci. (C, C’) By 7 DIV, the reduction in Rad51 levels is paralleled by γH2AX resolution. (D, D’) AdβGal-infected P6 utricle lacks Rad51 expression. (E, E’) P6 utricle exposed to ionizing radiation and analyzed 1 h post-irradiation contains SCs with DSB-like γH2AX foci. These cells lack Rad51 expression. (F, F’) At 3 DIV, also the AdcD1-infected P50 utricle shows Rad51 induction in γH2AX+ SCs. (G, G’) Rad51 expression is maintained at 7 DIV. Note that adult SCs with bright, pan-nuclear γH2AX staining lack or show only weak Rad51 expression (arrowheads). (H, H’) Rad51 levels are reduced in the adult utricle by 14 DIV, paralleling γH2AX resolution. (I, I’) High magnification view shows that adult SCs with pan-nuclear γH2AX staining express only low levels of Rad51. (K) Quantification shows that, at all timepoints studied and both in P6 and P50 utricles, Rad51 expression closely parallels that of γH2AX. Only 3–5% of Rad51+ SCs were negative for γH2AX. Mean ± SEM and the number of explants (n) are shown. Abbreviations: AdcD1, adenovirus encoding cyclin D1; AdβGal, adenovirus encoding β-galactosidase; γH2AX, Ser 139 phosphorylated histone H2AX; utr, utricle. Scale bar, shown in H’: A, A’, C–H’, 20 µm; B, B’, I–J’, 5 µm.

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γH2AX foci accumulate in cell cycle reactivated auditory supporting cells in vitro and in vivo

We next studied the dynamics of γH2AX expression in AdcD1-infected Deiters’ cells. At 3 DIV, P6 cochlear explants showed EdU+/Sox2+ Deiters’ cells with γH2AX foci, typical to DSBs (Fig. 8A,A’). At 7 DIV, most of these cells, including most of the cell doublets, lacked γH2AX foci (Fig. 8B,B’). Combining these results to the utricular data, we conclude that the dynamics of DSB resolution in the inner ear SCs is age-dependent.

To reveal whether the accumulation of γH2AX foci is a specific response to cD1 overexpression or whether it is a general response to cell cycle re-entry, the retinoblastoma (Rb) gene was inactivated in vivo in auditory SCs. We generated Rb<sup>loxP/loxP</sup>;Fgf3-iCre-ER<sup>T2</sup> mutant mice and injected the pups with tamoxifen in order to inactivate Rb in auditory SCs from P2 onward. The Fgf3-iCre-ER<sup>T2</sup> transgenic mouse line mediates efficient and cell type-specific recombination in the cochlea, as previously shown [26]. Cross-sections were prepared from the cochleas of mutant animals and littermate controls at P7, and stained for γH2AX and Ki-
67. Pillar cells, a subtype of auditory SCs that similar to Deiters’ cells do not proliferate postnatally, showed unscheduled proliferation in the mutant mice during the first two postnatal weeks (Fig. 8C). We focused our analysis on pillar cells, because only a few Deiters’ cells of the Rb\textsuperscript{loxP/loxP}, Fgfr3-iCre-ER\textsuperscript{12} mice showed cell cycle reactivation, consistent with prior data [27]. In contrast to control specimens, pillar cells of mutant animals showed induction of γH2AX foci (Fig. 8D,E). Thus, these data show that both ectopic cD1 in vitro and genetic inactivation of a negative cell cycle regulator in vivo can trigger cell cycle re-entry of auditory SCs and that this event is linked with DSB accumulation.

**Figure 8.** Unscheduled proliferation is a specific trigger for the DNA damage response in auditory SCs in vitro and in vivo. (A,A’) AdcD1-infected P6 cochleas pulsed with EdU (for 24 h between days 2 and 3) and maintained for 3 DIV show accumulation of γH2AX foci in cell cycle reactivated EdU+ Deiters’ cells. (B,B’) Most EdU+ Deiters’ cells show γH2AX downregulation by 7 DIV. (C-E) Cross-sections through the cochlea of a Rb\textsuperscript{loxP/loxP}, Fgfr3-iCre-ER\textsuperscript{12} mutant mouse at P7 show Ki-67-stained pillar cells (arrowhead in C). γH2AX foci can be seen in pillar cells (arrowhead in D) of mutant, but not control mice. Abbreviations: AdcD1, adenovirus encoding cyclin D1; γH2AX, Ser 139 phosphorylated histone H2AX; co, cochlea; DCs, Deiters’ cells; IHC, inner hair cell; OHCs, outer hair cells; IP, inner pillar cell; OP, outer pillar cell; Rb mut, Rb\textsuperscript{loxP/loxP}, Fgfr3-iCre-ER\textsuperscript{12}. Scale bar, shown in E: A–E, 20 µm.

**Figure 9.** Transdifferentiation does not trigger DNA damage. (A–C’) In both P2 and P6 cochlear explants, DAPT treatment for 3 DIV triggers production of ectopic, myo6-labeled outer hair cells. The extent of γH2AX expression is similar as in non-treated cochleas. (D–E’) Similarly, in P6 utricular explants, DAPT treatment for 3 DIV triggers production of ectopic, myo6-labeled hair cells. New hair cells (outlined in D–D’) are in direct contact with each other and lack mature stereociliary bundles, based on myo6 and phalloidin labelings. The extent of γH2AX expression in ectopic hair cells is similar to normally developed hair cells and to hair cells in vehicle-treated control utricles. Abbreviations: co, cochlea; γH2AX, Ser 139 phosphorylated histone H2AX; Myo6, myosin 6; Phall, phalloidin; utr, utricle. Scale bar, shown in E’: A–C’, 20 µm; D–E’, 13 µm.
Supporting cell-to-hair-cell transdifferentiation does not trigger DNA damage

Having found that DSBs are formed in cell cycle reactivated SCs and that DNA damage resolution directs proliferative activity, we investigated whether SC-to-hair-cell transdifferentiation, the other main step in the regeneration process, triggers DNA damage as well. Transdifferentiation can be triggered by the γ-secretase inhibitor DAPT that inactivates Notch signaling [28, 29]. In addition to P6 explants, we treated P2 cochlear explants with DAPT, because SC’s response to DAPT is more pronounced at this earlier age. Cochleas were analyzed after a 3-day-long DAPT incubation. It is known that DAPT stimulates new hair cell formation during this whole culturing period [30]. Unlike control specimens (treated with DMSO), DAPT-treated cochleas showed supernumerary, myo6-positive hair cells, based on surface views where the normal mosaic-like organization of the sensory epithelium (Fig. 1B) was in places lost. In these areas, hair cells were in contact with each other, unlike in control explants where hair cells were contacted by SCs. Notably, these ectopic hair cells did not show γH2AX induction above the level seen in normal hair cells of the same explants or of control cultures (Fig. 9A-C’). Similar results were obtained in P6 and adult utricles after a 3-day-long DAPT treatment. Regions were found in these specimens where myo6-positive hair cells lacked mature stereociliary bundles, based on phalloidin staining that marks filamentous actin-rich stereocilia, suggesting that these cells had been generated through transdifferentiation (Fig. 9D-E’; data not shown). Thus, as opposed to forced cell cycle re-entry, stimulation of transdifferentiation of SCs does not trigger DNA damage.

Uncoupling between forced proliferation and transdifferentiation of utricular supporting cells

Next we wanted to investigate whether forced cell cycle re-entry is coupled to transdifferentiation, because natural regeneration in non-mammalian inner ears often involves SC divisions, followed by transdifferentiation of a part of the progeny into hair cells [1]. We used AdcD1-infected P6 utricles for these experiments, applying a 24-h-long EdU pulse between days 2 and 3. We took into account the fact that, in addition to SCs, a small part of hair cells of juvenile utricles are infected by adenoviruses (Fig. 1E,E’). At 6 DIV, the amount of EdU+/myo6+ hair cells was less than 10% in AdcD1-infected utricles, and this value corresponded to the amount of hair cells in the explants infected with reporter viruses (Figs. 1E,E’ and 10A,A’). These results argue against coupling between forced proliferation and transdifferentiation of postmitotic SCs.

We then asked whether forced proliferation can be combined with DAPT-induced transdifferentiation. AdcD1-infected P6 utricles were cultured for 3 days (EdU pulse between days 2 and 3) and then treated with DAPT for another 3 days. After 6 DIV, a robust, threefold increase in EdU+/myo6+ hair cells was found in these specimens compared to only AdcD1-infected explants (AdcD1: number of myo6+ cells counted 768, number of EdU+/myo6+ cells 22, n = 3 explants; AdcD1 plus DAPT: corresponding values 1096 and 109, n = 3) (Fig. 10A-B’). Thus, preceding cell cycle reactivation does not prevent pharmacological stimulation of SCs to transdifferentiate into hair cells.

Figure 10. Uncoupling between forced proliferation and transdifferentiation. (A, A’) AdcD1-infected P6 utricles show rare EdU+/myo6+ hair cells at 6 DIV (arrow) (B, B’) AdcD1-infected and DAPT-treated P6 utricles contain high numbers of EdU+/myo6+ hair cells (arrows) at 6 DIV (regimen of the treatments explained in Results). Abbreviations: AdcD1, adenovirus encoding cyclin D1; Myo6, myosin 6; utr, utricle. Scale bar, shown in B’: A-B’, 20 μm.
DISCUSSION

Current research puts efforts on understanding the mechanisms that restrict hair cell regeneration in the mammalian inner ear and how these restrictions could be overcome, the aim being the design of therapeutic strategies to replace lost hair cells by new ones. SCs serve as a potential platform for these regenerative interventions. We have studied SC’s response to forced cell cycle re-entry and taken into account prior data on the decline of proliferative potential of these cells during the early postnatal maturation period [15]. Juvenile SCs are proliferation-proficient; they are mostly postmitotic [16], but their proliferation can be stimulated in vitro by exogenous mitogens, manipulation of cell cycle regulators and conditions that promote stemness [31]. These stimuli have only little effect on cell cycle activity of adult SCs. Although hair cell re-growth might be achieved through SC-to-hair-cell transdifferentiation, SC depletion at the expense of new hair cells may not be a viable therapeutic approach [32]. Unscheduled cell cycle re-entry and DNA damage have been linked to neurodegenerative diseases [33] and to restrictions in stem cell reprogramming [2]. Based on these considerations, understanding of DNA damage signaling in adult SCs is important for the development of safe and efficient strategies of proliferative regeneration, an event that could potentially be followed by transdifferentiation into hair cells.

Present results demonstrate that Ad5 viruses with the CMV promoter efficiently target postnatal SCs in explant cultures. In the juvenile cochlea containing different types of SCs, the transduction was specific to Deiters’ cells. This SC subtype-specific infection and the fact that auditory hair cells were largely non-infected, make Ad5 vectors excellent tools in targeted manipulations of a specific population of the auditory sensory epithelium. Besides possessing regenerative potential, Deiters’ cells are the key cellular players in the pathophysio logically important wound healing in the auditory sensory epithelium [34]. Thus, Ad5-mediated manipulations could be useful in revealing molecular regulation underlying these events. Prior studies have shown that adenoviral tropism changes rapidly during development: mainly hair cells are transduced at late-embryogenesis, while both hair cells and SCs are transduced around birth. Thereafter, during the early postnatal maturation period, the tropism gradually shifts towards SCs [4, 18, 35].

We found that γH2AX was expressed, in addition to reporter virus-transduced SCs, in SCs of non-infected explants. These results suggest that, rather than viral transduction, stressors such as oxidative stress known to be associated with in vitro conditions might cause γH2AX induction [36]. However, the intensity and pattern of this basal γH2AX expression was clearly different compared to the expression seen in AdcD1-infected SCs. Nuclei of these cells showed numerous small γH2AX and 53BP1 foci that characterize DSB damage. Thus, we conclude that AdcD1-induced DSBs are caused by the transgene, a core cell cycle component that promotes G1/S transition, rather than by adenoviral transduction itself. These data are supported by our in vivo results in mice with the conditional, inducible inactivation of Rb, a downstream target of the D-type cyclin/cyclin-dependent kinase 4/6 complexes. In these mutant animals, abnormal cell cycle re-entry of juvenile auditory supporting cells, mostly pillar cells, was coupled with the accumulation of γH2AX foci. Similarly, γH2AX induction has been demonstrated in Rb-depleted, cell cycle reactivated adult forebrain neurons in vivo [37]. Furthermore, our results showing that stimulation of SC-to-hair cell transdifferentiation by the γ-secretase inhibitor DAPT does not elicit DSB-like γH2AX foci speak for the cell cycle re-entry being a specific trigger for this DNA damage.

One of the key findings of the current study was the differences in the DDR and DNA repair dynamics between AdcD1-infected juvenile and adult SCs. The results showing rapid resolution of γH2AX foci in juvenile SCs indicated that successful DNA repair was accomplished. This was also suggested by the foci formation and subsequent resolution of the core HRR component Rad51, indicating the engagement of HRR. In contrast, the slower resolution of γH2AX and Rad51 foci in adult SCs implies a delayed DNA repair. Further, the findings of adult SCs with intense, pan-nuclear γH2AX that were positive for cleaved caspase-3 and showed only negligible Rad51 expression suggested that incomplete DNA repair could lead to apoptosis. These results are consistent with prior data showing signs of apoptosis of cell cycle reactivated SCs of the adult utricle upon c-Myc overexpression [38]. What might be the mechanisms underlying slower kinetics of DNA damage signaling in adult SCs? Cellular differentiation is accompanied by an increase in heterochromatinization that is known to affect DNA repair [39]. DSBs occurring near or within heterochromatin, the tightly packed and transcriptionally inactive form of DNA, are repaired more slowly due to the chromatin challenge [40]. Thus, an increase in heterochromatin accumulation and condensation along maturation might delay DNA repair and impair survival and cell cycle progression of adult SCs.

In response to AdcD1 transduction, in addition to dif-
ferences between juvenile and adult utricular SCs, we found differences in proliferative activity between age-matched utricular and cochlear SCs, at the juvenile stage. At this stage, SCs of both organs are postmitotic, but are undergoing structural maturation. Production of ectopic Deiters’ cells of the cochlea was less evident than that of utricular SCs. Specifically, the numerous doublets of EdU-labeled Deiters’ cells and the signs of impaired separation of the nuclei of the doublets suggested that the cell cycles were incomplete. Interestingly, this has also been found in developing hair cells after cell cycle reactivation [11, 41]. We found that p53, a mediator of cycle arrest and apoptosis, was upregulated in Deiters’ cells of AdcD1-infected cochleas, in most cases in the cell doublets. p53 induction was not found in SCs of AdcD1-infected, juvenile utricles that showed robust cell cycle activity. Although these results link p53 upregulation with incomplete cell cycles of Deiters’ cells, direct evidence of the role of p53 in these cells remains to be shown, similar to the understanding of the mechanisms behind p53 upregulation.

AdcD1-triggered proliferation of P6 utricular SCs was not followed by transdifferentiation into hair cells. Thus, upon forced proliferation of postmitotic SCs, the two regenerative events seem to be uncoupled. Recently, a pulse-labeling study with thymidine analogs in the mouse utricle in vivo showed that, at birth and a few days thereafter, a part of SCs or SC-like-cells still naturally proliferate and the progeny produced from those divisions differentiate into hair cells [16]. In another in vivo study in which hair cells were killed form the neonatal mouse utricle, SC’s proliferative activity increased, resulting in mitotic hair cell replacement [42]. Combined with our results with slightly older SCs, the acquired postmitotic status, maintained by negative cell cycle regulators [31] and epigenetic mechanisms [43], is likely to underlie the observed uncoupling of forced cell cycle re-entry and transdifferentiation. Also, our findings showing that, in the postmitotic SCs, transdifferentiation can be pharmacologically stimulated after forced cell cycle re-entry, speak for independent molecular regulation of the two events.

To conclude, we have here used the inner ear SCs as models of postmitotic cells to understand the limitations in DNA damage signaling in the context of cell cycle reactivation. Several other types of quiescent cells, such as tissue-resident quiescent stem cells, are likely to pose similar barriers in the attempts to stimulate proliferative activity. Most studies on DNA damage signaling have been performed with cultured single cells. Our explant culture model retains many aspects of in vivo tissue integrity, yet it allows manipulation and analysis at the cellular level. It appears that the capacity of adult SCs to proliferate is a staggering step in the efforts to stimulate hair cell re-growth. Therefore, dedifferentiation of mature SCs towards immature-like cells may be required for successful proliferation. In addition to being a component of DDR signaling, histone H2A variant H2AX is an epigenetic regulator of proliferation, as recently shown in quiescent adult stem cells [44]. Thus, the link between DDR and epigenetic signaling might be an important factor in creating barriers against proliferative regeneration also in the inner ear SCs. Similar to the findings made in β-cells of the adult human pancreas [45], our results show that restrictions in DNA damage signaling form an important barrier of proliferation of adult SCs.

METHODS

Mice. Explant cultures of the utricular and cochlear sensory epithelia were prepared from NMRI mice, and histological sections from the inner ears of RhloxPlox;Fgfr3-iCre-ER mice. Conditional, inducible inactivation of Rh in auditory SCs of these mutant mice was induced by tamoxifen at P2 and P3, as described previously [26]. RhloxPlox and Fgfr3-iCre-ER mice were genotyped by PCR as described in the original publications [46, 47]. The day of birth was considered as P0. All animal work has been conducted according to relevant national and international guidelines.

Explant cultures and viral infections. Explant cultures were established at P2 and P6 from the cochlea, and at P2, P6 and P50 from the utricle. Explants were maintained on pieces of Nuclepore filter membrane (Whatman) placed on a metal grid in Dulbecco’s modified Eagle’s DMEM/F-12 medium supplied with 2 mM L-glutamine and penicillin (100 U/mL) (Gibco/Invitrogen) and 10% fetal bovine serum (FBS) (HyClone/Thermo Scientific). Incubations were done in a humidified 5% CO2 atmosphere at 37°C. Ad5 vectors harbouring the βGal, GFP or cDI transgenes and CMV promoter were used. Cloning and propagation of the viruses have been described previously [48]. Cochlear and utricular explants were infected for 16 and 7 h, respectively, with Ad/βGal (4.2 x 107 pfu/ml), AdGFP (3.6 x 107 pfu/ml) or AdcDI (2.9 x 107 pfu/ml). Infections were done in 30 µl drops of medium containing 5% FBS. Thereafter, explants were maintained for 2 to 14 DIV in medium containing 10% FBS. Medium was changed every other day.

Whole mount specimens. Explants were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 10 min and permeabilized in PBS containing
Histological sections. For histological cross-sections as well as wholemount surface preparations were analyzed under Axio Imager.M2 microscope (Zeiss) using brightfield and epifluorescence optics. Images were acquired through CCD colour camera (AxioCam HRC, Zeiss) and ZEN software (Zeiss), and processed using Adobe Photoshop CS6 (Adobe Systems). Confocal images were acquired using a Leica TCS SP5 microscope with Plan Apochromat 63×/1.3 NA objective. The acquisition software was Leica LAS AF. Z-projections were processed with Imaris 7 (Bitplane Scientific Software). Blind 3D deconvolution was made for Z-projections with AutoQuant X2 (Media Cybernetics).

Quantification. To quantify the amount of adenovirus-infected utricular hair cells, parvalbumin+/GFP+ hair cells were counted from one random 40x microscopic field per AdGFP-infected P6 utricular explant at 3 DIV, each field covering striolar and non-striolar areas. Data are shown as the percentage of parvalbumin+/GFP+ hair cells (mean ± SEM).

To quantify the amount of proliferating cells following AdcD1 infection, Sox2+ and EdU+/Sox2+ SCs were counted from one random 40x microscopic field per infected P6 and P50 utricular explant at 3 DIV, each field covering striolar and non-striolar areas. Data are shown as the percentage of EdU+/Sox2+ SCs out of Sox2+ SCs (mean ± SEM).

To quantify the resolution of γH2AX+ foci, EdU+ and EdU+/γH2AX foci+ SCs were counted from one random 40x microscopic field per AdcD1-infected P6
and P50 utricular explant at 3, 7 and 14 DIV, each field covering striolar and non-striolar areas. Data are shown as the average percentage of EdU+/γH2AX+ cells out of EdU+/ γH2AX+ cells (mean ± SEM).

To determine the relative proportion of SCs with pan-nuclear γH2AX profiles, Sox2+/total γH2AX+ (foci plus pan-γH2AX) and Sox2+/pan-γH2AX+ SCs were counted from one random 40x microscopic field per P6 and P50 utricle at 7 DIV, each field covering striolar and non-striolar areas. Data are shown as the relative numbers of Sox2+/pan-γH2AX+ SCs out of Sox2+/total γH2AX+ SCs (mean ± SEM).

To determine the relative proportion of γH2AX+/Rad51- SCs, γH2AX+/Rad51+ and γH2AX+/Rad51- cells were counted from one random 40x microscopic field per P6 and P50 AdcD1-infected utricle at 3, 7 and 14 DIV, each field covering striolar and non-striolar areas. Data are shown as the percentage of Rad51+/γH2AX+ SCs out of Rad51+/γH2AX+ SCs (mean ± SEM).

To quantify the amount of EdU+ hair cells following AdcD1-infection alone and in combination with DAPT treatment, EdU+/myo6+ cells were counted from one random 40x microscopic field per P6 AdcD1-infected and, AdcD1-infected and DAPT-treated utricle at 6 DIV, each field covering striolar and non-striolar areas. Data are shown as the total number of myo6+ and EdU+/myo6+ cells.

Three or five individual explants (n) were used for each quantification. Two-tailed Student’s t test and one-way ANOVA were used for statistical analysis. A P-value ≤0.005 was considered highly significant.

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Author contributions. ML and UP designed research; ML performed research; ML and UP analyzed data and wrote the paper; TH helped with irradiation experiments; TA, AK and MLaiho helped drafting the manuscript; all the authors reviewed the manuscript.

Conflict of interest statement

The authors of this manuscript declare no conflict of interests.

REFERENCES

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Indispensable role of Mdm2/p53 interaction during the embryonic and postnatal inner ear development


p53 is a key component of a signaling network that protects cells against various stresses. As excess p53 is detrimental to cells, its levels are tightly controlled by several mechanisms. The E3 ubiquitin ligase Mdm2 is a major negative regulator of p53. The significance of balanced p53 levels in normal tissues, at different stages of lifetime, is poorly understood. We have studied in vivo how the disruption of Mdm2/p53 interaction affects the early-embryonic otic progenitor cells and their descendants, the auditory supporting cells and hair cells. We found that p53 accumulation, as a consequence of Mdm2 abrogation, is lethal to both proliferative progenitors and non-proliferating, differentiating cells. The sensitivity of postmitotic supporting cells to excess p53 decreases along maturation, suggesting that maturation-related mechanisms limit p53's transcriptional activity towards pro-apoptotic factors. We have also investigated in vitro whether p53 restricts supporting cell's regenerative capacity. Unlike in several other regenerative cellular models, p53 inactivation did not alter supporting cell's proliferative quiescence nor transdifferentiation capacity. Altogether, the postmitotic status of developing hair cells and supporting cells does not confer protection against the detrimental effects of p53 upregulation. These findings might be linked to auditory disturbances observed in developmental syndromes with inappropriate p53 upregulation.

p53 is well known for its role in guarding genomic integrity upon DNA damage and oncogene activation. Its tumor suppressor function is exerted through promotion of cell cycle arrest and apoptosis. p53 null mice are born alive and several of their tissues show an unaltered phenotype. Whether other p53 family members play a compensatory role remains an open question. p53 is a short-lived protein. The key regulator maintaining its low levels in normal tissues is Mdm2. Mdm2 is an E3 ubiquitin ligase that promotes p53 degradation by the proteasome, but it also suppresses p53's transcriptional activity. Genetic disruption of Mdm2/p53 interaction leads to widespread induction of apoptosis and spontaneous lethality of early embryos. Studies employing mouse models with conditional Mdm2 ablation have shown that proper Mdm2/p53 interaction is needed for morphogenesis during mid- and late-embryogenesis as well. Proper Mdm2/p53 interaction is also required in adult tissues, particularly in those containing high numbers of proliferating cells, shown both by inducible Mdm2 ablation and by inducible, direct p53 upregulation.

Except for global examination of various tissues, only a few studies exist where the response of a given cell type to increased p53 stability has been comparatively studied in a continuum from development to adulthood, with the aim to disentangle the importance of balanced p53 levels for cellular survival, differentiation and homeostasis. We have here addressed these questions in the proliferating progenitor cells of the embryonic inner ear and in one lineage of their descendants, the postmitotic epithelial supporting cells (SCs) of the auditory organ, at different stages of life. We have employed a conditional Mdm2 mutant mouse model to disrupt Mdm2/p53 interaction in vivo. We have also pharmacologically disrupted Mdm2/p53 interaction in vitro.

In regeneration research, auditory SCs serve as a potential platform for the replacement of lost auditory sensory cells, the hair cells, by new ones. Many attempts have been made to trigger mammalian SCs to re-enter the cell cycle, to stimulate their transdifferentiation into hair cells, and to increase their stemness. These studies have shown that while some degree of regenerative plasticity is retained in neonatal SCs, plasticity becomes very limited after this young age. Understanding the p53 response of auditory SCs with potential regenerative capacity is relevant, as p53 is known to antagonize regenerative interventions in various cellular contexts, best-known in restricting...
reprogramming of somatic cells into induced pluripotent stem cells. Therefore, in addition to gain-of-function approaches, we have here studied whether p53 inactivation confers regenerative plasticity to auditory SCs.

**Results**

**Disruption of Mdm2/p53 interaction is detrimental to embryonic otic progenitor cells.** By generating $Mdm2^{F906L/F906L}$; $Pax2-Cre$ mice, we first studied the response of otic placodal cells to the disruption of the p53 binding domain of Mdm2$^{12}$. The ectodermal otic placode contains progenitor cells that give rise to the epithelial and neuronal compartments of the inner ear. $Pax2$ is one of the earliest markers of the otic lineage and it is broadly expressed in the otic placode$^{14}$. In the $Pax2-Cre$ mouse line, Cre-mediated recombination occurs in the early otic placode. Thereafter Cre mRNA expression is quickly downregulated, already at the late placodal stage$^{16}$.

At embryonic day 8.5 (E8.5), otic placodes had formed in $Mdm2^{F906L/F906L}$; $Pax2-Cre$ embryos, but they were abnormally small and filled with apoptotic cells, detected by cleaved caspase-3 immunostaining (Fig. 1A–F). The concomitant strong p53 upregulation suggested that the cell death was a consequence of p53 accumulation (Fig. 1G,H). However, a portion of placodal progenitors - apparently those spared from recombination and consequent p53-mediated cell death - invaginated and formed a vesicle at E9.0. These mutant vesicles were clearly smaller than those of littermate controls (Fig. 2A,B). Consistent with our suggestion that the otic vesicles of mutant mice were formed by non-recombined cells, the extent of apoptosis in E9.5 vesicles was comparable to...
Neonatal auditory supporting cells are highly sensitive to the disruption of Mdm2/p53 interaction. We next focused on postnatal auditory SCs. Mdm2/p53 interaction was selectively abrogated in SCs by generating Mdm2<sup>Fgfr3-iCre<sup>ER<sup>T2</sup></sup>-<sup>18</sup>;Pax2-Cre<sup>+</sup> mice. Prior studies have shown efficient Fgfr3-iCre<sup>ER<sup>T2</sup></sup>-mediated recombination in two types of auditory SCs, the pillar and Deiters’ cells, during the early postnatal life<sup>17</sup>. The first postnatal week is characterized by active differentiation of the cells of the organ of Corti. During the second postnatal week, signaling cascades regulating cell fates and differentiation are downregulated<sup>18</sup>. At these stages, SCs, located between the hair cell rows, mature and develop prominent actin and microtubule-based cytoskeletal structures.Concomitantly, fluid-filled spaces open within the organ of Corti, of which the tunnel of Corti is the most prominent<sup>18</sup>. After tamoxifen treatment at postnatal day 0 (P0) and P1, cochleae from Mdm2<sup>Fgfr3-iCre<sup>ER<sup>T2</sup></sup> mice were analyzed at P4 and P7. A part of mutant mice had to be killed during this analysis period due to their worsened general health. At P4 (data not shown) and more prominently at P7 (Fig. 4A,B), mutant animals displayed an organ of Corti with reduced width (lateral-to-medial) and height (basal to luminal surface), as assessed in whole mount surface specimens. SC loss was evident in these specimens. Quantitative analysis revealed that a significant part of SCs were rapidly lost following recombination and that this loss had progressed by P7 (P4: 38.0% ± 1.4 vs. P7: 47.4% ± 1.4). All hair cells, detected by myosin 6, were preserved in Mdm2<sup>Fgfr3-iCre<sup>ER<sup>T2</sup></sup> mice (Fig. 4A,B). These data are consistent with prior data showing that Fgfr3-iCre<sup>ER<sup>T2</sup></sup>-mediated recombination does not target hair cells, with the exception of the most apical and basal parts of the cochlea that were not analyzed in the present study<sup>17</sup>. As shown in paraffin sections at P7, global cochlear morphology was comparable between mutant and control mice (Fig. 4C,D). However, the morphology of the organ of Corti of mutant mice was abnormal due to the loss of Sox2-positive SCs (Fig. 4D,I). SC loss was seen throughout the cochlear duct (data not shown). The presence of ApopTag-positive profiles in the organ of Corti, detected in adjacent sections, pointed to apoptotic cell death (Fig. 4E,F). p53 accumulation in SC nuclei indicated that apoptosis was mediated by p53 (Fig. 4E,K). γH2AX was not upregulated in SCs of mutant cochleae, excluding the involvement of DNA damage in cellular death (data not shown). β-tubulin immunostaining revealed impaired morphological differentiation of SCs, likely associated with ongoing degeneration of these cells (Fig. 4G,L). These sections also revealed that hair cells were unaffected in mutant mice, in line with the results obtained with whole mount specimens (Fig. 4A,B). Together, our data demonstrates that high p53 sensitivity is maintained in the early postnatal, postmitotic progeny of the embryonic otic progenitors.

Juvenile auditory supporting cells show increased resistance to the disruption of Mdm2/p53 interaction. To investigate whether p53 sensitivity is maintained in juvenile SCs undergoing morphological maturation, tamoxifen was administered to Mdm2<sup>Fgfr3-iCre<sup>ER<sup>T2</sup></sup> mice at P6 and P7, and their cochleae were analyzed at P12 and P20. Only a small part of SCs were lost at P12 (Fig. 5A,B), but longer-term p53 accumulation resulted in the loss of the majority of SCs, as evidenced at P20 (Fig. 5C,D). When SC loss in juvenile (P6/P7) and neonatal (P0/P1) cochleae was quantitatively compared 6 days after recombination, juvenile SCs showed a highly malformed cochlea.

that seen in controls (Fig. 2C,D). Comparable mitotic activity in E10.5 mutant and control otocysts, detected by phospho-histone 3 staining, pointed to our conclusion as well, as high levels of p53 would be expected to antagonize cell cycle activity (Fig. 2E,F). Importantly, mutant otocysts did not anymore show p53 upregulation (Fig. 2E,F; insets). Sox2 staining revealed that although neuroblasts delaminated from the otocyst epithelium at E10.5, the neuroblast population and otocyst size were reduced compared to controls (Fig. 5G,H). Also, even though Sox2 expression was regionalized in mutant otocysts, this domain was abnormally broad (Fig. 2G,H). E10.5, the neuroblast population and otocyst size were reduced compared to controls (Fig. 2G,H). Also, even though Sox2 expression was regionalized in mutant otocysts, this domain was abnormally broad (Fig. 2G,H). E10.5, the neuroblast population and otocyst size were reduced compared to controls (Fig. 2G,H). Also, even though Sox2 expression was regionalized in mutant otocysts, this domain was abnormally broad (Fig. 2G,H). E10.5, the neuroblast population and otocyst size were reduced compared to controls (Fig. 2G,H). Also, even though Sox2 expression was regionalized in mutant otocysts, this domain was abnormally broad (Fig. 2G,H). E10.5, the neuroblast population and otocyst size were reduced compared to controls (Fig. 2G,H). Also, even though Sox2 expression was regionalized in mutant otocysts, this domain was abnormally broad (Fig. 2G,H). E10.5, the neuroblast population and otocyst size were reduced compared to controls (Fig. 2G,H). Also, even though Sox2 expression was regionalized in mutant otocysts, this domain was abnormally broad (Fig. 2G,H). E10.5, the neuroblast population and otocyst size were reduced compared to controls (Fig. 2G,H). Also, even though Sox2 expression was regionalized in mutant otocysts, this domain was abnormally broad (Fig. 2G,H).
Figure 2. Morphogenesis is impaired in the early-developing inner ear of Mdm2<sup>−/−</sup>;Pax2-Cre mice. (A,B) At E9.0, otic vesicles of mutant embryos are smaller than those of controls. (C,D) At E9.5, the amount of cleaved caspase-3-positive cells (arrow) is comparable in mutant and control vesicles. (E,F) At E10.5, phosphorylated histone-3 staining shows mitotic activity at the lumenal surface of both control and mutant otocysts. Insets show that p53 is not anymore upregulated at the otocyst stage in mutants. (G,H) The small-sized E10.5 otocysts of mutants show expanded Sox2-positive domain and limited neuroblast delamination. A rudimentary cochleovestibular ganglion is seen. (I,J) Pax2-positive domain is prominently present in both mutant and control otocysts. (E,G,I and F,H,J) are from adjacent sections. Abbreviations: CVG, cochleovestibular ganglion; D, dorsal; HE, hematoxylin; M, medial; ov, otic vesicle; PH3, phosphorylated histone-3. Scale bar, shown in J: (A–D), 35 μm; (E–J), 85 μm, insets in (E,F), 165 μm.
Taken together, p53 accumulation arrests maturation of juvenile SCs, ultimately leading to their death. It appears that outer hair cell death is a secondary event, caused by degeneration of adjacent, recombined SCs. Adult auditory supporting cells are resistant to the disruption of Mdm2/p53 interaction.

To study the p53 response of mature auditory SCs, tamoxifen-mediated recombination was induced in Mdm2<sup>Fm/Fm</sup>;Fgfr3-iCre-ER<sup>T2</sup> mice at P49 and P50. The efficiency of recombination at adulthood was confirmed by generating Ai14 (tdTomato);Fgfr3-iCre-ER<sup>T2</sup> mice. Based on red-fluorescent-protein (RFP) immunohistochemistry on paraffin sections, the whole population of adult pillar and Deiters’ cells was recombined (Supplementary Fig. S2), consistent with previous studies using the same reporter mice at a younger age<sup>17</sup>.

In contrast to the early postnatal SCs, SCs of adult Mdm2<sup>Fm/Fm</sup>;Fgfr3-iCre-ER<sup>T2</sup> mice survived and failed to show morphological defects, as assessed 6 days after the induction of recombination, at P55 (Supplementary Fig. S3). Despite this unaltered morphology, p53 was strongly upregulated in the adult SCs (Supplementary Fig. S3C,D; insets). These findings point to increased p53 resistance of adult compared to early postnatal SCs. Due to the worsened general health of these mutant animals, the long-term fate of adult SCs with p53 accumulation could not be followed.

p53 upregulation is detrimental to hair cells and supporting cells of the late-embryonic cochlea.

In order to study p53 sensitivity of the cells of the late-embryonic organ of Corti, Mdm2<sup>Fm/Fm</sup>;Fgfr3-iCre-ER<sup>T2</sup> mice received tamoxifen at E13.5 and E14.5, and their cochleas were analyzed at E18.5. This mid-embryonic recombination paradigm enabled us to study the consequences of Mdm2 ablation in both hair cells and SCs, as described previously<sup>21</sup>. As cell cycle exit of precursor cells occurs at the time of recombination, these experiments focused on the response of postmitotic, early-differentiating cells to Mdm2 ablation. Consistent with the known sensitivity of developing auditory hair cells to pharmacological p53 accumulation in vitro<sup>22</sup>, hair cells

Figure 3. Strongly altered cochlear morphology of Mdm2<sup>Fm/Fm</sup>;Pax2-Cre mice at birth. (A–C) At E18.5, hematoxylin-stained sections show two examples of mutant cochleas with strong dysmorphogenesis. Boxed areas correspond to the higher magnification views shown in insets. Insets show varying degrees of cell organization in mutant organ of Corti. Arrowheads in insets denote Deiters’ cells. (D,E) Adjacent sections show organized pattern of hair cells, marked by myosin 6, and supporting cells (arrows). A rudimentary spiral ganglion is seen as well. Abbreviations: d, Deiters’ cell; HE, hematoxylin; hs, hair cells; ih, inner hair cell; ip, inner pillar cell; myo6, myosin 6; ohs, outer hair cells; op, outer pillar cell; SG, spiral ganglion. Scale bar, shown in E: (A–C), 200 μm; insets in (A–C), 25 μm; (D,E), 55 μm.
Figure 4. Disruption of Mdm2/p53 interaction triggers rapid death of neonatal auditory supporting cells, revealed in Mdm2<sup>ff/ff</sup>Fgfr3-iCre-ER<sup>T2</sup> mice. Tamoxifen-mediated recombination was induced at P0 and P1, and analysis performed at P7. Images are from the medial part of control and mutant cochleas. The rows of the different supporting cell (A) and hair cell types (A’) are marked. (A, A’) The mutant whole mount specimen shows the loss of Sox2-positive supporting cells, but maintained population of myosin 6-positive hair cells. The width of the supporting cell region is decreased (compare the bars spanning the width of this region in A and B). (C–G) A hematoxylin-stained cross-section through the control cochlea (C). The boxed area corresponds to the higher magnification views of the organ of Corti seen in adjacent sections (D–G). Sox9 marks supporting cells (D). ApopTag staining marks cell death that is seen in the greater epithelial ridge during the first postnatal week (arrowhead in E). Control specimen lacks nuclear p53 staining (F). β-tubulin antibody marks the prominent microtubule bundles in supporting cells (arrowheads in G). (H–L) Hematoxylin-staining shows unaltered global morphology of the mutant cochlea (H). The boxed area corresponds to the higher magnification views of the organ of Corti seen in adjacent sections (I–L). Sox9 staining reveals partial loss of supporting cells (I). ApopTag staining shows fragments of dying cells in the supporting cell area as well as in the greater epithelial ridge (arrow and arrowhead, respectively, in J). p53 is strongly expressed in the supporting cell nuclei (K). β-tubulin immunostaining shows the organ of Corti with perturbed cytoarchitecture (L). Asterisks mark hair cell nuclei. Abbreviations: Apop, ApopTag staining; β-tub, β-tubulin; d, Deiters’ cell; HE, hematoxylin; h, Hensen’s cell; ih, inner hair cell; ip, inner pillar cell; myo6, myosin 6; ohs, outer hair cells; op, outer pillar cell; t, tunnel of Corti. Scale bar shown in L: (A, B*), 50 μm; (C, H), 200 μm; (D–G, I–L), 15 μm.
from Mdm2<sup>FM/FM</sup>; Fgfr3-iCre-ER<sup>T2</sup> mice showed a strong death-prone phenotype (Fig. 6A,B,E,F). Also SCs were lost in these cochleas (Fig. 6A,E,F), similar as in neonatal cochleas (Fig. 4B). Cell death was confirmed by cleaved caspase-3 staining (Fig. 6G,H). p53 upregulation in adjacent sections suggests that SC death is caused by p53 induction (Fig. 6G,H; insets). Altogether, neither SCs nor hair cells tolerate p53 upregulation during their differentiation.
Pharmacological p53 upregulation abrogates supporting cell survival in an age-dependent manner. The small molecule compound nutlin3 blocks Mdm2/p53 interaction, triggering p53 upregulation. By preparing explants of the neonatal auditory sensory epithelium and maintaining them for 4 days in vitro (DIV) together with nutlin3, we first confirmed p53 upregulation in the explants (Fig. 7A, B'). A large part of SCs were lost after 4 DIV and all of them had disappeared by 7 DIV (Fig. 7A, B, data not shown). There was a total absence of hair cells already at 4 DIV (Fig. 7A, B). The nutlin3 response of mature auditory cells could not be studied due to the difficulties in maintaining the adult organ of Corti in culture conditions. Therefore, we prepared explants of the adult utricle, one of the balance organs that tolerates culture conditions. The utricle contains hair cells and SCs that resemble those of the cochlea. P53 utricular explants from wildtype mice were maintained for 7 DIV with continuous nutlin3 exposure. Despite p53 induction, SCs in these adult cultures were viable (Fig. 7C, D'). Interestingly, also hair cells were present in amounts comparable to those seen in vehicle-treated, age-matched specimens (Fig. 7C, D). Altogether, these data are consistent with our in vivo data demonstrating age-related decrease in the sensitivity of SCs to the disruption of Mdm2/p53 interaction.

p53 inactivation is dispensable for cell cycle re-entry of auditory supporting cells. To study the effects of p53 inactivation, we generated p53<sup>fl/fl</sup>;Pax2-Cre mice where p53 is conditionally inactivated in the Pax2-positive otic progenitor cells from the placodal stage onwards. These mice were viable and lacked phenotypic changes during development and adulthood, as assessed at birth and at P35 (data not shown; Fig. 8A).

Next, we studied whether the loss of p53 facilitates cell cycle re-entry of the normally quiescent SCs. Cochlear explants were prepared from p53<sup>fl/fl</sup>;Pax2-Cre mutant and control mice at P6, and maintained for 3 DIV. Neither mutant nor control explants supplemented with the replication marker EdU showed SC proliferation (Fig. 8C, D, data not shown). To test whether p53 inactivation facilitates β-catenin-mediated proliferative plasticity, the glycogen synthase kinase-3α/β (GSK-3α/β) inhibitor BIO was added to P6 cultures to activate β-catenin. However, BIO treatment did not abrogate SC quiescence, neither in control nor mutant explants, based on the lack of EdU labeling (data not shown). We conclude that the absence of p53 does not confer proliferative plasticity to immature SCs.

Figure 6. Disruption of Mdm2/p53 interaction is lethal to late-embryonic auditory hair cells and supporting cells, revealed Mdm<sup>FM/FM</sup>;Fgfr3<i>iCre-ER</i> mice. Tamoxifen-mediated recombination was induced at E13.5 and E14.5, and analysis performed at E18.5. (A, B') Whole mount specimens show the loss of both myosin 6-positive hair cells and Sox2-positive supporting cells. Width of the organ of Corti is decreased in the mutant specimen (bracket in B). (C–F) Immunohistochemistry on paraffin sections shows degeneration of hair cells and supporting cells in the mutant organ of Corti. (G, H) The apoptotic marker cleaved caspase-3 is seen in the mutant, but not control organ of Corti. Insets show that p53 is upregulated only in the mutant organ of Corti. Abbreviations: casp, cleaved caspase-3; d, Deiters’ cell; ih, inner hair cell; ip, inner pillar cell; myo6, myosin 6; ohs, outer hair cells; op, outer pillar cell. Scale bar shown in H: (A–B'), 50 μm; (C–H), 20 μm; insets in (G, H), 55 μm.
Hair cell loss has been shown to stimulate cell cycle re-entry of neonatal SCs. To test whether p53 loss extends this proliferative response to the juvenile stage, P6 explants were prepared from p53<sup>fl/fl</sup>;Pax2-Cre mutant and control mice, and the explants were exposed to neomycin, an aminoglycoside antibiotic that kills hair cells. After neomycin challenge for 24 h, explants were maintained for additional 48 h in EdU-supplemented medium. Even though most hair cells were lost in these cultures, no EdU-positive SCs were detected, neither in control nor mutant explants (Fig. 8C,D'). Furthermore, although previous studies have reported that p53 inactivation confers hair cell protection against ototoxic agents<sup>29</sup>,<sup>30</sup>, we could not see a difference in the extent of hair cell loss between neomycin-exposed mutant and control explants (data not shown).

p53 inactivation does not boost supporting-cell-to-hair cell transdifferentiation. Neonatal SCs can transdifferentiate into hair cells in response to Notch inhibition<sup>31</sup>. This transdifferentiation capacity is prominent neonatally, but abruptly declines at around P6<sup>18</sup>. As p53 is known to limit somatic cell reprogramming, we next studied whether p53 inactivation increases transdifferentiation efficiency and whether it extends the period of reprogramming plasticity beyond the neonatal stage. Transdifferentiation was triggered in P1 and P6 cochlear explants from p53<sup>fl/fl</sup>;Pax2-Cre mutant and control mice. The β-secretase inhibitor DAPT, an inhibitor of Notch signaling, was used to trigger transdifferentiation. DAPT-treated explants from both genotypes showed comparable amounts of supernumerary hair cells (Fig. 9A–E, data not shown). In line with these findings, p53 was not upregulated in DAPT-treated control explants (Fig. 9F, G'). In these experiments, nutlin3-treated explants were used as positive controls for p53 upregulation (Fig. 9E, F'). Thus, the absence of p53 appears not to affect the molecular remodeling events that set the limits to the capacity of SCs to transdifferentiate into hair cells.

Discussion

Cells have a complex machinery that maintains low levels of p53 under normal conditions. p53 is most commonly upregulated following abnormal proliferation and ensuing DNA damage, when it orchestrates the DNA damage...
response and DNA repair. If DNA damage remains unrepaired, p53 mediates cellular senescence or death. Also, certain developmental syndromes lead to p53 stabilization and activation. In addition, p53 is induced in adult tissues upon a wide spectrum of stressful conditions and, thus, can also affect non-proliferating cells. The postmitotic status of a cell is considered to increase resistance to elevated p53 levels. An interesting question is whether the cells that have exited the cell cycle, but are still undergoing differentiation or maturation respond to elevated p53 levels more like proliferating progenitor cells or like mature cells. These immature cells often show plasticity towards regenerative manipulations, such as stimulated cell cycle re-entry or cell fate conversion. It has been suggested that p53 upregulation limits the extent of these regenerative events and sets barriers to the regenerative capacity of adult cells. Based on these considerations and the fact that auditory SCs serve as an attractive cell model in regeneration studies aiming to restore hearing loss, we found it important to study the response of these cells to increased and, on the other hand, suppressed p53 expression.

As an E3 ubiquitin ligase, Mdm2 mediates ubiquitination and proteasome-dependent degradation of p53. We found that the inner ear cells respond to Mdm2 abrogation by p53 upregulation and by phenotypic changes. Therefore, it can be concluded that these cells express p53 under normal conditions and that p53 levels are actively suppressed by Mdm2. We detected endogenous p53 in the cochlea by Western blotting assays, but not by immunohistochemistry. These results point to low levels of p53 in this tissue, consistent with the low abundance of p53 found in other organs. These data reflect the key role of Mdm2 in preventing p53 accumulation under normal conditions.

In Mdm2<sup>−/−</sup>;Pax2-Cre mice, Mdm2 was abrogated in the inner ear anlagen, the otic placode. Its progenitor cells upregulated p53, leading to rapid and extensive apoptosis. In the Pax2-Cre mouse line, Cre mRNA is induced in the early otic placode, but it is quickly downregulated. Thereby, by using Mdm2<sup>−/−</sup>;Pax2-Cre mice, we were able to study what happens when the earliest otic progenitors are lost. It has been shown that a portion of placodal progenitors remain unrecombined in Pax2-Cre mice. Most probably these unrecombined cells, that escaped Mdm2 inactivation, gave rise to the small otic vesicles in Mdm2<sup>−/−</sup>;Pax2-Cre mice. The ultimate outcome of the extensive loss of early progenitors was a highly dysplastic inner ear, as assessed at birth. From the perspective of...
inner ear development it is interesting that an otic placode with strongly reduced numbers of progenitor cells can generate a vesicle. However, the vesicle was abnormally small, indicating that the early-occurring, extensive apoptosis cannot be later compensated by enhanced proliferation. These growth defects were linked with abnormalities in the expression of patterning molecules in the otocyst and, further, with impaired cochlear morphogenesis. Our results are in line with previous results on other embryonic tissues, showing that Mdm2/p53 abrogation and consequent p53 upregulation trigger severe pathologies. Importantly, prior studies have shown that cell death, defects in organogenesis and embryonic lethality can be rescued by concomitant p53 inactivation, indicating that p53 is a major target of Mdm2.

The present results on Mdm2FM/Fm;Fgfr3-iCre-ERmice show that Mdm2 continues to be critical in regulating p53 levels in postmitotic, differentiating SCs. Following birth, Mdm2/p53 abrogation was induced exclusively in SCs in the cochlea. We found that the effects of p53 upregulation were distinctly age-dependent. During the first postnatal week, differentiating SCs were hypersensitive to p53, evidenced by their acute death upon p53 induction. During the second postnatal week, SCs undergoing structural maturation died only after a prolonged exposure to p53. Also adult SCs tolerated p53 accumulation surprisingly well. These age-dependent differences in p53 sensitivity were not due to differential recombination patterns, as both the early postnatal and adult SCs showed nearly 100% recombination efficiency, based on the analysis of Ai14(tdTomato);Fgfr3-iCre-ERT2 reporter mice (present study). Consistent with our findings, late-embryonic neurons of the central nervous system die rapidly and in large amounts upon conditional Mdm2 ablation. In another study, the effects of inducible, global Mdm2 inactivation were studied in various adult tissues. In line with our results, that study showed that tissues comprising postmitotic cells (“radio-insensitive tissues”) are more sensitive to p53 upregulation during the young adult compared to older, more mature stage.

What confers increased p53 resistance to juvenile (second postnatal week in our study) and adult SCs compared to neonatal (first postnatal week) SCs? In Mdm2FM/Fm;Fgfr3-iCre-ERT2 mice, cell death was not associated with activation of the DNA damage response, based on the absence of γH2AX induction. This result is not unexpected considering the postmitotic status of SCs. In adult cells in general, a large portion of chromatin is condensed and silenced. Accessibility of the p53 transcription factor to pro-apoptotic target genes, particularly to Puma, Bax, and Noxa, becomes limited along aging, resulting in weakening of its apoptosis-promoting function. In the mouse kidney, binding of p53 to Puma is significantly decreased in mature compared to young cells. In auditory SCs of the mouse, signaling cascades regulating differentiation are downregulated by the end of

Figure 9. Supporting cell transdifferentiation into hair cells is not enhanced in cochlear explants from p53fl/fl;Pax2-Cre mice. (A–D) DAPT-treated explants from P1 mutant and control cochleas show accumulation of myosin 6-positive outer hair cells, as opposed to the organized rows of hair cells in DMSO-treated explants, assessed after 3 DIV. (E) Quantification reveals no significant difference in the amount of extra hair cells between the genotypes. (FG) p53 is not upregulated in the DAPT-treated control explant, as opposed to the nutlin3-treated control explant, assessed after 4 DIV. Sox2 marks supporting cell nuclei and DAPI cell nuclei. Note that a part of cells is lost in the nutlin3-treated specimen. Abbreviations: ih, inner hair cell; myo6, myosin 6; oh, outer hair cell. Scale bar shown in (G'), 20μm.
the first postnatal week, shown, for example, in the case of Notch signaling. Epigenetic mechanisms might be involved in limiting transcriptional activation of differentiation promoting genes by Notch signaling. It has also been suggested that the proenoral gene Atoh1 is epigenetically silenced in auditory SCs as the first postnatal week. In line with these data, epigenetic mechanisms that promote heterochromatinization at the loci of pro-apoptotic genes might slow down their transcriptional activation by p53 and thereby cause delayed rather than accelerated cell death. Juvenile SCs in Mdm2\(^{+/+}\)fgf3-icre-ER\(^{T2}\) mice displayed delayed cell death, indicating that it took longer time for the upregulated p53 to execute cell death. Also, p53 was acutely and strongly upregulated in SCs in response to Mdm2 abrogation, despite delayed death of these cells. This implies that the age-dependent decline in p53 responsiveness is due to mechanisms downstream of p53 expression.

Inner ears of p53 inactivated mice (p53\(^{-/-}\);Pax2-Cre) failed to show gross developmental abnormalities, consistent with the unaltered phenotype of several p53 inactivated tissues thus far studied. However, p53 suppression has been shown to increase regeneration efficiency. For example, p53 silencing enhanced fibroblast conversion into neurons and it was suggested that p53 is a major gatekeeper in the maintenance of the existing transcription network of the cell. With the reprogramming intervention in the cochlea, transdifferentiation of SCs into outer hair cells, can be induced by Notch inhibition, mainly during the first postnatal week. However, we did not see enhanced transdifferentiation in DAPT-treated cochlear explants from p53\(^{-/-}\);Pax2-Cre mice. Correspondingly, DAPT-treated wildtype explants did not show p53 accumulation. This result was not actually surprising, since SC-to-hair cell transdifferentiation triggered by Notch inhibition is not associated with cell death or cell cycle re-entry. If this would be the case, DNA damage signaling would be activated, leading to p53 induction.

Could endogenous p53 restrict proliferative plasticity of quiescent auditory SCs? Critical regulators of cell cycle exit and the maintenance of the postmitotic state are the cyclin-dependent kinase inhibitors (CKIs) of the core cell cycle machinery. CKI inactivation has been shown to trigger ectopic replication of SCs during the early postnatal life. As p53 is not directly involved in the maintenance of the postmitotic state, it was not surprising that p53-depleted SCs failed to show unscheduled proliferation in vivo and in vitro. p53 inactivation has, nevertheless, been shown to potentiate cell cycle re-entry of quiescent cells when applied in combination with exogenous mitogens, shown in the case of Muller glia in retinal cultures. We did not see this effect in the early postnatal cochlear explants supplemented with the GSK-3\(^{β}\) inhibitor BIO, neither in explants from p53\(^{-/-}\); Pax2-Cre nor control mice. BIO activates the p21 checkpoint, a pathway whose activation has been shown to stimulate cell cycle re-entry of neonatal SCs. As also aminoacids-induced hair cell death failed to activate SC proliferation in explants from p53\(^{-/-}\);Pax2-Cre mice, we conclude that p53 loss does not facilitate the capacity of SCs to cell cycle re-entry or transdifferentiation.

Our results on nutlin3-treated explants support the data obtained with Mdm2\(^{+/+}\)fgf3-icre-ER\(^{T2}\) mice, showing the death-prone phenotype of developing SCs following p53 upregulation. The Fgfr3-icre-ER\(^{T2}\) mouse line enabled us to abrogate Mdm2 during late-embryogenesis in auditory hair cells as well. We found that disruption of Mdm2/p53 interaction in vivo is detrimental to differentiating hair cells, consistent with prior studies showing the sensitivity of developing auditory hair cells to nutlin3 in explant cultures. Certain human developmental syndromes cause unrestrained p53 activity, with a less severe phenotype and with delayed lethality compared to Mdm2 ablation, apparently due to less dramatic increase in p53 levels. Interestingly, some of these syndromes have been shown to include inner ear malformations or hearing loss, particularly the CHARGE syndrome. This syndrome has been modeled in mice and the animals show defects in the morphogenesis of the vestibular labyrinth. Based on our results, impaired survival of hair cells and SCs of the cochlea might be included in the spectrum of CHARGE phenotypes and perhaps also in some other developmental syndromes with unrestrained p53 activity. In conclusion, our results demonstrate an important role for the Mdm2/p53 interaction in ensuring survival of both proliferating progenitor cells and differentiating cells of the auditory organ.

**Methods.**

- **Mice.** Mdm2\(^{+/+}\);Pax2-Cre mice were generated by crossing floxed Mdm2 mice (Mdm2\(^{fl/fl}\)) with Pax2-Cre mice. Mdm2\(^{fl/fl}\) mice were used as littermate controls. At least 4 mutant and 4 control embryos were analyzed per age (E8.5, E9.0, E9.5, E10.5, E17.5, E18.5). Both ears of each animal were analyzed.
- **Mice.** Mdm2\(^{+/+}\);Pax2-Cre mice were generated by crossing Mdm2\(^{+/+}\) mice with Fgfr3-icre-ER\(^{T2}\) mice. Fgfr3-icre-ER\(^{T2}\) mice were obtained by crossing floxed Fgfr3-iCre-ER\(^{T2}\) mice, showing the death-prone phenotype of developing SCs following p53 upregulation. The Fgfr3-icre-ER\(^{T2}\) mouse line enabled us to abrogate Mdm2 during late-embryogenesis in auditory hair cells as well. We found that disruption of Mdm2/p53 interaction in vivo is detrimental to differentiating hair cells, consistent with prior studies showing the sensitivity of developing auditory hair cells to nutlin3 in explant cultures. Certain human developmental syndromes cause unrestrained p53 activity, with a less severe phenotype and with delayed lethality compared to Mdm2 ablation, apparently due to less dramatic increase in p53 levels. Interestingly, some of these syndromes have been shown to include inner ear malformations or hearing loss, particularly the CHARGE syndrome. This syndrome has been modeled in mice and the animals show defects in the morphogenesis of the vestibular labyrinth. Based on our results, impaired survival of hair cells and SCs of the cochlea might be included in the spectrum of CHARGE phenotypes and perhaps also in some other developmental syndromes with unrestrained p53 activity. In conclusion, our results demonstrate an important role for the Mdm2/p53 interaction in ensuring survival of both proliferating progenitor cells and differentiating cells of the auditory organ.
All mouse lines were maintained in a mixed background and both females and males were used in the analysis. Timed pregnancies were established by the detection of vaginal plug, taking the morning of plug observation as E0.5. The day of birth was considered as P0. Genotyping was performed as previously described. All animal work has been conducted according to relevant national and international guidelines. Approval for animal experiments has been obtained from the local Laboratory Animal Centre of University of Helsinki (permission number KEK14-001) and the National Animal Experiment Board (permission number ESAVI/4606/04.10.07/2016).

Induction of ifcre-mediated recombination. Mdm2<sup>fl/fl</sup>;Fgfr3-iCre-ER<sup>T2</sup> and control littermate mice were intraperitoneally injected with tamoxifen (Sigma-Aldrich) at 2 consecutive days: at E13.5 and E14.5 (3 mg per pregnant mother), at P0 and P1 (50 μg/g body weight), at P6 and P7 (50 μg/g body weight) and at P49 and P50 (200 μg/g body weight). This latter paradigm was also used for Ai14/TdTomato;Fgfr3-iCre-ER<sup>T2</sup> mice.

Histological sections. Between E8.5 and E10.5, Mdm2<sup>fl/fl</sup>;Pax2-Cre mutant and control mice were fixed with 4% paraformaldehyde (PFA) for 6 h. At E17.5 and E18.5, dissected inner ears were immersed in PFA overnight. Postnatal cochleas were perilymphatically fixed and immersed in PFA overnight, and decalcified in 0.5 M EDTA, pH 7.5. Specimens were embedded in paraffin and cut to 5-μm-thick sections. Epitopes were unmasked by microwave boiling (900 W) in 10 mM citrate buffer, pH 6.0, for 10 min. Sections were incubated with primary antibodies in PBS containing 0.25% Triton-X-100 (PBS-T) and 10% normal serum for 4 h at +4°C. The following primary antibodies were used: rabbit polyclonal Pax2 (1:2000, Zymed/Thermo Fisher Scientific), rabbit polyclonal p53 (1:250, Cell Signaling Technology), rabbit monoclonal cleaved caspase-3 (1:250, Cell Signaling Technology), rabbit polyclonal β-actin (1:1000, Abcam), rabbit polyclonal RPP (1:500, Rockland Immunochemicals), rabbit polyclonal Sox9 (1:3000, Millipore), mouse monoclonal β-H2AX (1:500, Millipore), rabbit polyclonal myosin 6 (1:3000, Proteus Biosciences), goat polyclonal Sox2 (1:3000, Santa Cruz Biotechnology) and mouse monoclonal PH3 (1:250, Cell Signaling Technology). Detection was done using the Vector Laboratories). Apoptag Peroxidase Vectastain Elite ABC and Mouse-on-Mouse Elite Peroxidase kits and the DAB Peroxidase Substrate kit (all from Vector Laboratories). ApopTag Peroxidase In Situ Apoprtosis Detection Kit (Millipore) was used to detect DNA fragmentation. Sections were counterstained with methyl green and mounted in Permount (Fisher Scientific). A part of the consecutive sections was stained with hematoxylin (Shandon Instant Hematoxylin, Thermo Fisher Scientific). Paraffin sections were imaged with the BX61 microscope equipped with UPlanApo 4x, 10x, 20x and 60x objectives. Images were acquired through the DP73 CCD color camera and CellSens software (all from Olympus).
Olympus). Whole mount specimens were imaged under epifluorescence illumination using the Axios Imager.M2 microscope equipped with PlanApo 20x and 40x objectives and with Apotome 2 structured illumination slider (all from Zeiss). Images were acquired with the black and white CMOS camera (Hamamatsu ORCA Flash 4.0 V2) and ZEN 2 software (Zeiss). Images were processed using Adobe Photoshop CS6 (Adobe Systems).

Statistical analysis. To determine the extent of cellular loss in the cochleas of Mdm2<sup>+/−</sup>p53fl/fl<sup>Ccgr-ER<sup>22</sup></sup> mice, Sox2-positive SCs and myosin 6-positive outer hair cells were counted from a 20-inner-hair-cell-width area from the medial cochlear coil. Data are shown as the average with s.e.m. of the percentage of extra outer hair cells. Three to five explants were used for each quantification. Two-tailed Student’s t test was used for statistical analysis. Values in the medial coil. Data are shown as the average with s.e.m. of the percentage of lost SCs and outer hair cells. To determine the amount of supernumerary outer hair cells in DAPT-treated cochlear explants from p53<sup>−/−</sup>pax2-Cre and control mice, myosin 6-positive cells were counted from a 20-inner-hair-cell-width area in the medial coil. Data are shown as the average with s.e.m. of the percentage of extra outer hair cells. Three to five explants were used for each quantification. Two-tailed Student’s t test was used for statistical analysis. Values were regarded as highly significant at p < 0.005.

References

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Author Contributions
M.L. and U.P. designed research; M.L. performed research; M.S. characterized Mdm2<sup>F<sub>M</sub></sup>FM; Pax2-Cre embryonic inner ear phenotype; M.L. and U.P. analyzed the data and wrote the paper; T.A., A.H. and M.S. helped drafting the manuscript; all the authors reviewed the manuscript.

Additional Information
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