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Summary

The transcription factor p53 has recognized roles in cell cycle arrest, apoptosis, DNA damage response and cellular redox regulation. In situ hybridization has demonstrated high expression level of p53 mRNA in the developing embryonic mouse brain. Nevertheless, though a role in DNA damage induced apoptosis has been attributed to p53, the conventional view is that p53, unlike the other p53 family member p73, is dispensable for telencephalic development.

In this work I have set out to explore explanations for p53 expression in the germinal zones of the embryonic telencephalon. My findings point to the importance of ROS in the regulation of embryonic neural progenitor cell homeostasis and imply a role for p53 in the fine-tuning of progenitor cell ROS levels and the concomitant DNA damage, and proliferation versus neurogenesis/oligodendrogenesis decisions. Indeed, elevation in ROS in embryonic p53-/- neural progenitor cells may provide a compensatory mechanism, which prevents overproliferation inducing instead neurogenesis. The study presents novel neurogenic genes, whose expression is responsive to cellular redox status and shows that in the absence of p53 PI3K-Akt signalling is elevated. P53 contributes to redox regulation at least in part by regulating expression of gene(s) with antioxidant properties and by counteracting PI3K-Akt signalling. Furthermore, elevation in ROS is not only a characteristic of embryonic p53-/- telencephalons, since the SVZ and hippocampus of the adult p53-/- mice, the neurogenic regions of the adult brain, exhibit increase in ROS compared to wild type animals.

Taken together, the findings in this study imply that p53 has a role in redox regulation in both embryonic and adult brain. Moreover, though in the embryonic brain elevation in ROS seems to compensate the absence of p53 and induce neurogenesis and impair oligodendrogenesis, in the adult brain an increase in ROS is a potential source of neurological damage, which would deserve investigations.

Abbreviations

7AAD	7-aminoactinomycin D
ADHD	attention deficit-hyperactivity disorder
AP	apical progenitor
BER	break excision repair
BP	basal progenitor
CGE	caudal ganglionic eminence
CM-DCFDA	chloromethyl 2’7’-dichlorodihydrofluorescein diacetate
CNS	central nervous system
COX	cyclooxygenase
DHE	dihydroethidine
DSB	double strand break
E11	embryonic day 11
EGF	epidermal growth factor
FGF	fibroblast growth factor
FACS	fluorescence assisted cell sorting
FoxO	forkhead transcription factor, sub-family O
GABA	gamma aminobutyric acid
GAD65/67	glutamic acid decarboxylase 65/67
GE	ganglionic eminence
GEF	guanine nucleotide exchange factor
Gpx	glutathione peroxidase
GSH	glutathione (reduced)
HRR	homologous recombination repair
HSC	hematopoietic stem cell
ICC	immunocytochemistry
IHC	immunohistochemistry
IPC	intermediate progenitor cell
ISH	in situ hybridization

LGE	lateral ganglionic eminence
LY294002	2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochlorid
MGE	medial ganglionic eminence
NAC	N-acetyl-cysteine
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
NOX	NADPH oxidase
eNPC	embryonic neural progenitor cell
NSC	neural stem cell
NTD	neural tube closure defect
8-OHdG	8-hydroxy-2'-deoxyguanosine
OPC	oligodendrocyte progenitor cell
P7	postnatal day 7
PDGF	platelet derived growth factor
PI3K	phosphatidyl inositide triphosphate kinase
RCN	reactive carbonyl species
RNS	reactive nitrogen species
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
qRT-PCR	quantitative real time PCR
SOD	superoxide dismutase
SSB	single strand break
SVZ	sub-ventricular zone
TCR	transcription coupled repair
UCP2	mitochondrial uncoupling protein
VZ	ventricular zone
Wnt	wingless
XO	xanthine oxidase

I Introduction

1. Overview of the generation of neural cells in the embryonic CNS

1.1. Neuroepithelial cells and radial glia are the source for all neural cells of the mature brain

All neural cells of the mature brain have ultimately the same embryonic origin, the dorsal ectoderm (Moreau and Leclerc, 2004). Neural fate is the default state of the dorsal ectoderm (Hemmati-Brivanlou and Melton, 1994). The ventral side of the embryonic ectoderm is the source of epidermal progenitors, while its dorsal side gives rise to nestin positive neuroepithelial cells. Initially, the neuroepithelium is only a single cell layer, which lines the cerebral ventricles and the lumen of the spinal cord. Before the onset of neurogenesis, from embryonic day 9 (E9) onwards in the mouse telencephalon, neuroepithelial cells undergo rapid expansion of the stem cell pool, after which at E12-E13 the majority of them transforms to radial glia cells, the cells from which the majority of cortical neurons are derived (Malatesta et al., 2003). Radial glia cells exhibit some of the characteristics of mature astrocytes, but also retain features of neuroepithelial cells (Noctor et al., 2002; Götz and Huttner, 2005).

From very early on the neuroepithelium, and the subsequent radial glia, is patterned having restricted potential to generate neuronal sub-types according to the anterior posterior and dorsal ventral axis (Malatesta et al., 2003). This is largely determined by the expression of a particular set of proneural transcription factors characteristic to each telencephalic subregion (Molyneaux et al., 2007). Thus, the dorsal i.e pallial cortical neuroepithelium produces glutamatergic pyramidal projection neurons, whereas the ventral i.e sub-pallial, striatal neuroepithelium generates gabaergic interneurons (Yozu et al., 2005) (Fig. 1.). In other words dorsal telencephalon is involved in the generation of excitatory neurons, while ventral telencephalon produces inhibitory neurons. However, for example in primates a

proportion of inhibitory interneurons is produced in the dorsal telencephalon (Letinic et al., 2002).

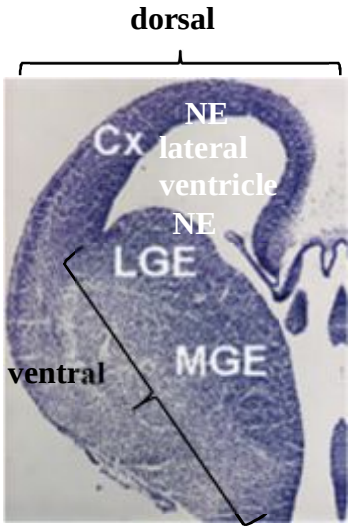


Figure 1. E14,5 mouse telencephalon. Neuroepithelium (NE), cortex (Cx), lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) (with modifications from Gulacsi and Anderson, 2008).

1.2.Neural progenitors undergo initially symmetric proliferative, later asymmetric and symmetric neurogenic cell divisions which terminate neurogenesis

Expansion of the founder progenitor cell pool, of so called apical progenitors (APs), produces two uncommitted mitotic daughter cells leading to the thickening of the germinal ventricular zone (VZ) (Haubensak et al., 2004; Noctor et al., 2004) (Fig. 2.). Interkinetic nuclear migration characterizes APs (Del Bene et al., 2008). Their nucleus resides during mitosis in the apical surface of the VZ, whereas during the S-phase it locates to a more basal position. With the onset of neurogenesis increasingly higher proportion of cell divisions becomes first asymmetric and later symmetric fate committed divisions (Noctor et al., 2004) (Fig. 3.). Asymmetric neurogenic cell divisions produce one uncommitted mitotic daughter cell and one doublecortin positive neuroblast whereas symmetric neurogenic divisions result in the generation of two committed daughters. The generation of a committed progenitor cell with restricted developmental potential and the regeneration of a stem cell in asymmetric cell

divisions stems from the polarization of the cell cortex, which targets cell fate determinants such as cell cycle regulators and proneural transcription factors unequally to daughter cells. Fate committed daughter cells, also called basal progenitors (BP) or alternatively intermediate progenitors (IPs), migrate from the VZ to the sub-ventricular zone (SVZ) where they can still undergo a few mitotic cycles before becoming permanently postmitotic (Arai et al., 2011). This leads to the thickening of the SVZ characteristic of the neurogenic period (Fig. 4.). Together the VZ, SVZ and ganglionic eminence (GE) form the germinal zones in telencephalic neurogenesis.

Symmetric committed cell divisions deplete the proliferative progenitor pool terminating neurogenesis. Hence, the timing of the switch from asymmetric to symmetric committed cell divisions, largely dictated by the timing of the fate determinant expression, is decisive as to the number of progenitors available and the number of neurons produced. Deviations from the correct timing can result either in macrocephaly (enlarged brain) or microcephaly (abnormally small brain) (Ishibashi et al., 1995; Regad et al., 2009).

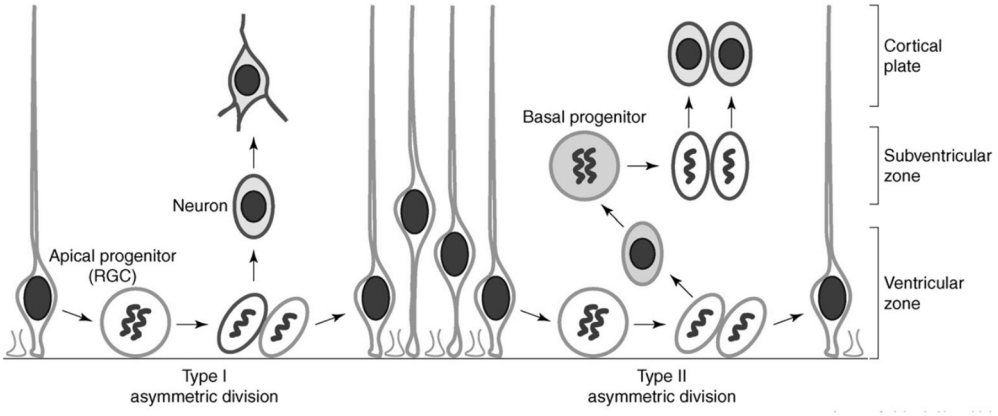


Figure 2. Apical progenitors (AP) and basal progenitors (BP) of the VZ and SVZ, respectively (from Zhong and Chia, 2008).

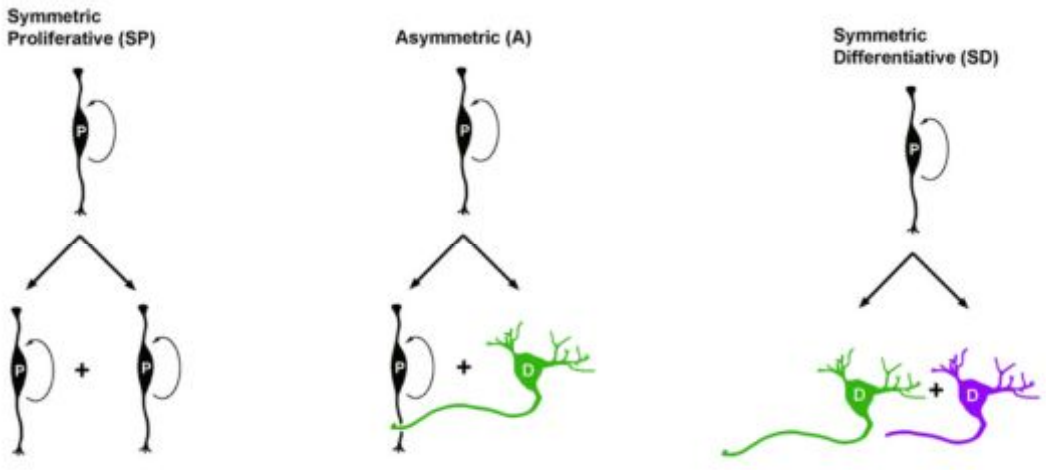


Figure 3. Modes of cell divisions during mammalian neurogenesis. Prolifearative cell (P), differentiating cell (D) (from Willardsen and Link, 2011).

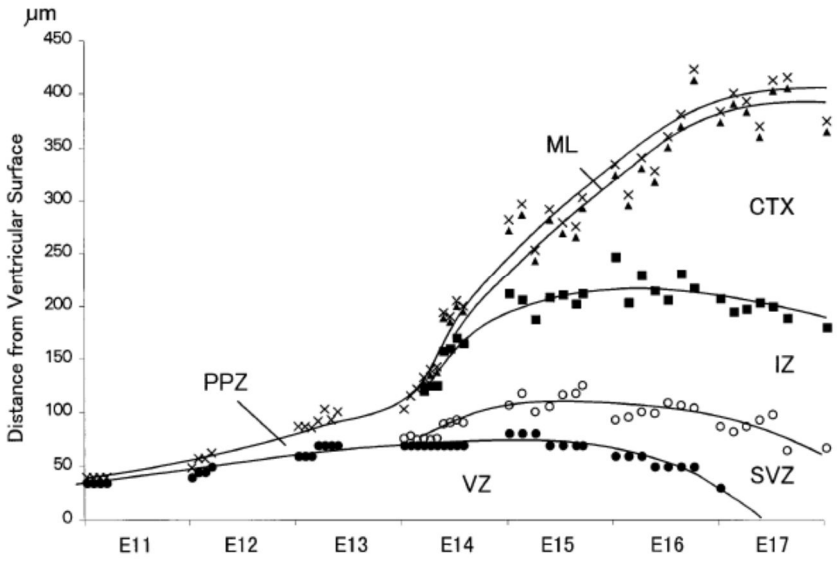


Figure 4. Growth of murine cerebral wall during neurorogenesis at E11-E17. Cortex (Ctx), intermediate zone (IZ), sub-ventricular zone (SVZ), ventricular zone (VZ) (from Takahashi et al., 1996).

1.3. A number of cell intrinsic and extrinsic factors regulate cell cycle and neurogenesis in NSCs

A number of factors have been implied in the regulation of cell cycle, the onset of neurogenesis and thus the number of neurons produced. Notch, FGF, Wnt and Sonic

Hedgehog (Shh) pathways coordinate progenitor cell proliferation and differentiation within the VZ-SVZ (Gaiano and Fishell, 2002; Lien et al., 2006; Borello et al., 2008; Gulacsi and Anderson, 2008; Kim et al., 2009). “The crowd control” model, for example, implies that the increase in cell density is sensed by an increase in the area occupied by adherens junctions per cell and this is translated into downregulation of Shh-signaling and a decrease in proliferation (Lien et al., 2006). GABA and glutamate released from newly generated neurons promote NPC proliferation in the VZ but decrease it in the SVZ (Haydar et al., 2000). Postmitotic neurons from the cortical plate execute negative feed-back control on NPC proliferation (Dicicco-Bloom et al., 1998), and early thalamic axons have an effect on cell cycle kinetics of cortical germinal zones (Dehay et al., 2001).

In addition, cellular energy status, potentially reactive oxygen species (ROS) etc. can have an impact on proliferation. Synthesis of proteins, nucleic acids and lipids requires energy. A cell is able to proceed from the G1 to S phase only when a certain level of macromolecules has been reached (Darzynkiewicz et al., 1980). Active metabolism and growth factor receptor stimulation, both characteristics of the expansion of the stem cell pool, are coupled with production of ROS (Le Belle et al., 2011; Mandal et al., 2011); persistent elevation in ROS can in various cell types increase cell cycle length and promote cell cycle exit (Burch and Heintz, 2005; Furukawa et al., 2005; Chua et al., 2009).

1.4. Cell cycle length hypothesis

Mouse neural progenitors undergo during neurogenic period a maximum of 11 cell cycles (Takahashi, et al., 1995; Takahashi et al., 1996). Cycles 1-8 in mice produce layer 6 and 5 neurons i.e. deep layer neurons of the cortex, whereas cycles 9-11 produce layer 4-2 neurons i.e. upper layer neurons (Takahashi, et al., 1995; Takahashi et al., 1996). There is a progressive increase in cell cycle length from 8.1h to 18.4h from E11 to E16 (Takahashi, et al., 1995) (Fig. 5.). The increase in the cell cycle length is due to the lengthening of the G1 phase of the cycle, which increases fourfold during neurogenesis. Cell cycle length hypothesis

postulates that lengthening of the G1 phase is a cause not a consequence of the induction of stem cell differentiation whether an embryonic, neural or hematopoietic stem cell (Calegari and Huttner, 2003; Lange and Calegari, 2010). An increase in the G1 phase would allow stem cells to be exposed to differentiation inducing determinants, such as proneural transcription factors, long enough so that differentiation can be launched.

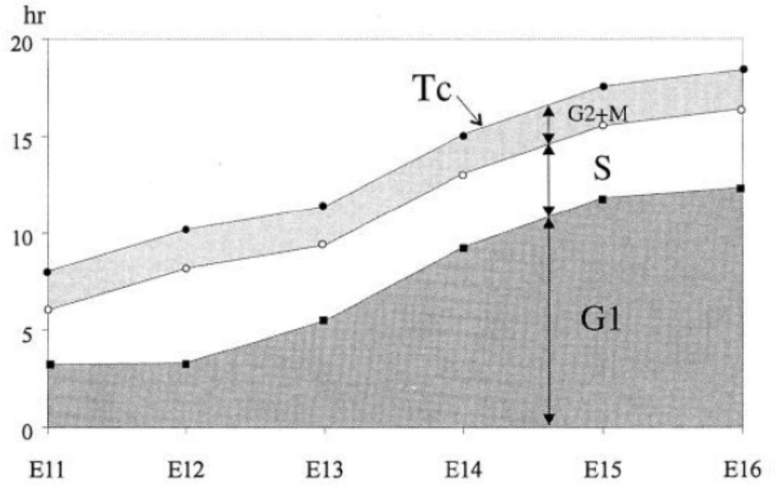


Figure 5. Lengthening of the G1 phase of the cell cycle during embryonic neurogenesis (from Takahashi et al., 1995).

1.5. Migration

Doublecortin positive young neurons have to migrate in some occasions long distances to their final destinations. While neuroblasts and young postmitotic neurons generated in the cortical germinal zone migrate radially in the cortical plate using radial glia as a scaffold, neurons born in the sub-pallial region of the ganglionic eminence (GE) migrate tangentially long distances to reach their targets in the cortical plate (neurons generated in the medial ganglionic eminence, MGE) but also to the more medial and caudal regions, such as hippocampus and thalamus (neurons born in caudal ganglionic eminence, CGE) (Kanatani et al., 2008). Radial glia cells and p73, a p53 family member, expressing Cajal Rezius cells of the marginal zone are thought to provide the migratory scaffold and signals to stop, respectively, during radial migration (Supèr et al. 2000; Meyer et al., 2002; Meyer et al,

2004). However, young migrating cells bear also intrinsic differences in their competence to respond to environmental cues as transplantation experiments demonstrate (Yozu et al., 2005; Kanatani et al., 2008). In mice neuronal migration is considered to be largely complete by P7 (Schuurmans et al., 2004).

1.6. The timing of neural cell production, neurons first then glia

The timing of neurogenesis and gliogenesis varies in different regions of the CNS. The common theme, however, is that neurons are generated first, which is followed by the production of oligodendrocytes and astrocytes (Morrow et al., 2001). This neuron-glia switch is governed by both intrinsic properties of progenitors and by extrinsic signals present in the environment leading to increased astrogenic and decreased neurogenic competence over the time. Consequently, neurogenesis in the mouse CNS begins on day E9 in caudal regions of the embryonic spinal cord and proceeds from there rostrally (Rowitch and Kriegstein, 2010). Neurogenesis peaks around day E10-E11 and continues till E13.5. While proliferative oligodendrocyte progenitors appear in the embryonic spinal cord around E12.5, the precise timing of astrogliogenesis is not clear, but it appears to occur like elsewhere in the CNS after neurogenesis is largely completed (Kessaris et al., 2007).

Telencephalic neurogenesis as well as that of the diencephalon and midbrain occurs later than that of the spinal cord and takes place between E10 to E17, the peak production of neurons being around the day E13-E14 (Götz and Huttner, 2005). The peak production of the deep layer neurons of the cortex in mice occurs around E11.5-E13.5 and that of the upper layer neurons at E15.5-E17.5 (Molyneaux et al., 2007). The onset of astrogliogenesis takes place once neurogenesis is largely completed, around day E17 in the mouse telencephalon, and continues postnatally (Freeman, 2010). However, the correct estimation of the onset of astrogliogenesis is hampered by the absence of reliable markers as for example GFAP and S100β, the markers for mature astrocytes, are expressed also by radial glia cells (Freeman, 2010). The first migratory oligodendrocyte progenitors (OPCs) expressing PDGFRα appear

in the MGE of the ventral telencephalon at E12 in mouse (Kessaris et al., 2006). These are followed by successive waves of OPCs from the LGE and the CGE during embryonic but also postnatal periods.

2. P53, NPCs and brain development

2.1. P53 is preferentially expressed in the germinal zones of the embryonic telencephalon though its role there in normal homeostatic conditions is largely unresolved

In situ hybridization (ISH) has demonstrated high expression level of p53 mRNA in all embryonic mouse tissues up to E10.5, after which p53 expression is prominent only in the developing brain being restricted there to the VZ and the GE (Schmid et al., 1991; Gottlieb et al., 1997; Komarova et al., 1997; van Lookeren Campagne and Gill, 1998). Later, p53 mRNA is also detected in the SVZ. When p53 expression was investigated using p53 responsive lacZ reporter, lacZ expression was detected in the developing E12.5 mouse forebrain (Gottlieb et al., 1997). A related report showed p53 responsive lacZ expression similarly preferentially in the developing nervous system both pre- and postnatally with profound decrease in the adult (Komarova et al., 1997). Yet, in spite of these findings, the conventional view is that p53, unlike the other two p53 family members p63 and p73, is dispensable during development (Donehower et al., 1992; Mills et al., 1999; Yang et al., 1999; Yang et al., 2000). Nevertheless, 16-24% of p53-/- female mouse embryos suffer from defective neural tube closure (NTD) in the hindbrain-midbrain boundary resulting in anencephaly and subsequent exencephaly clearly suggesting a role for p53 in brain development (Sah et al., 1995; Armstrong et al., 1995).

This raises the question on the role of p53 in the embryonic brain. Is p53 inactive in embryonic telencephalic germinal zones though obviously expressed as mRNA? Mdm2 and Mdm4 (Mdmx) null mouse embryos demonstrate that p53 is active in the developing embryonic brain. Mdms are ubiquitin-E ligases, which target p53 for degradation (Xiong et

al., 2006). In the absence of Mdm2 embryos develop hydranencephaly at E12.5, whereas the lack of Mdm4 leads to proencephaly by E17.5. Mutants are characterized by extensive apoptosis, which results in the formation of cysts or cavities in the cerebral hemispheres. Mdm4 null telencephalons also display extensive cell cycle arrest. P53 deletion rescues both conditions.

Additionally, early works implicated p53 in DNA damage induced apoptosis caused by γ-irridation (Gottlieb et al., 1997; Komarova et al., 1997). Yet, in normal homeostatic conditions p53 expression does prenatally not superimpose with apoptotic markers (van Lookeren Campagne and Gill, 1998). Nevertheless, a recent report connected p53 with postnatal developmental apoptosis in the CA3 region of the hippocampus (Murase et al., 2011). The CA3 region of p53-/- mice showed by P10 an increased number of neurons. This was linked to elevated Akt pathway activation, which promoted survival of maturing neurons.

2.2. P53 and NPC proliferation and differentiation

P53 has been implicated in the regulation of embryonic (Zheng et al., 2008; Armesilla-Diaz et al., 2009) and adult (Gil-Perotin et al., 2006; Meletis et al., 2006) NPC proliferation with p53 null NPCs exhibiting some increase in proliferation *in vitro*. In addition, enhanced NPC self-renewal in *in vivo* adult SVZ has been detected, a finding associated with glioma-like changes (Gil-Perotin et al., 2006; Zheng et al., 2008; Wang et al., 2009). Interestingly, these changes display an increase in P-Akt levels and a concomitant decrease in phosphatase and tensin homolog protein (PTEN) (Wang et al., 2009). However, evidence that embryonic p53-/- telencephalic germinal zones would exhibit an increase in NPC proliferation is missing.

P53 has been suggested to have a part in differentiation of neural progenitors (Zezula et al., 2001; Billon et al., 2004). OPC differentiation is impaired in p53-/- postnatal optic nerves (Billon et al., 2004). This may be due to the decreased expression of p21, a classical p53 target gene, which is required for oligodendrocyte differentiation independent of cell cycle withdrawal (Zezula et al., 2001). It has also been stated that p53-/- NPCs produce *in*

vitro more neurons compared to wild type NPCs, though molecular explanations of the phenomenon are missing (Gil-Perotin et al., 2006; Armesilla-Diaz et al., 2009).

In one report p53 null E13 embryos were found to exhibit higher basal DNA oxidation compared to wild type when analyzed for the 8-hydroxy-2'-deoxyguanosine (8-OHdG) oxidative modification of DNA (Chen et al., 2009). Yet, by E19 this difference had largely disappeared, though the brain still exhibited elevated levels. Moreover, p53 has been suggested to act as a suppressor, which protects the embryo from DNA-damaging chemicals and of importance from developmental oxidative stress (Nicol et al., 1995). The lack of p53 has been connected with cognitive impairments. P53-/- adult brains had apoptotic brain lesions, accompanied by learning defects, behavioural alterations and decreased levels of presenilin-1 (PS1), a protein associated with the early-onset of familial Alzheimer's disease (Amson et al., 2000).

Taken together, it might be that although no gross abnormalities except exencephaly have been described for p53 null embryonic brains, p53 may have a role in the regulation of the embryonic and adult brain homeostasis. During embryonic telencephalic development other compensatory mechanisms may ameliorate the lack of p53. Furthermore, the early lethality of p53-/- mice, development of tumours and death by the age of 4 to 6 months, has largely prevented studies on aging related neurodegeneration.

3. DNA damage in the developing CNS and its repair mechanisms

3.1. Functional DNA repair is essential for the correct telencephalic development

The hallmark of the embryonic brain development, as mentioned above, is the extensive expansion of the progenitor pool before and during the neurogenic period (Haubensak et al., 2004; Noctor et al., 2004). Rapid proliferation is associated with replication related DNA damage (Mandal et al., 2011). Moreover, nearly 10,000 abasic sites are spontaneously generated in a single eukaryotic cell each day due to oxidative and hydrolytic

events (Dahlmann et al., 2009). It is fundamental to all neural cells of the mature CNS that the genetic information is transmitted accurately to daughter cells. Hence, high demands are laid on the replication and repair machinery of NSCs. Defective DNA repair underlies many neurodegenerative diseases with the accompanying neuropathology reflecting the developmental stage at which the damage occurred (McKinnon, 2009) (Table 1.).

Homologous recombination repair (HRR) and non-homologous end-joining (NHEJ) recombination repair are the two main DNA repair mechanisms during embryonic brain development (Orii et al., 2006). HRR is important for replication related DNA repair of proliferating NSCs in the VZ, whereas NHEJ is active in the SVZ and cortical plate i.e. in proliferating and differentiating cells. Disruption of HRR sensitizes the CNS to apoptosis early in embryogenesis, in mice around E9-E10 (Orii et al., 2006). Impairments in NHEJ disturb embryonic neurogenesis later due to extensive apoptosis in early post-mitotic neurons in all parts of the CNS causing embryonic lethality by E16.5. Apoptosis occurs outside the VZ in the SVZ and in the cortical plate (Orii et al., 2006; Gao et al., 1998).

3.2. P53 in DNA damage response

Excessive apoptosis resulting from defective DNA damage repair is in most cases rescued by p53 knockout implying that one of the major functions of p53 in the developing CNS is the elimination of neural cells with DNA damage (Gao et al., 2000; Frappart et al., 2005). Yet, p53 has a role in DNA repair itself directly though this role during embryonic brain development remains to be explored. In other cellular systems p53 has been shown to protect cells from naturally occurring replication associated DNA double strand breaks (DSBs) (Kumari et al., 2004). P53 suppresses HRR by acting as a surveillance factor that interacts with stalled replication forks (Kumari et al., 2004). It inhibits Rad51 mediated strand exchange events and replication fork regression. Consequently, it has been proposed that p53 prevents restart of the replication before the lesion is repaired (Subramanian and Griffith, 2005). Moreover, p53 directly represses Rad51 expression (Arias-Lopez et al., 2006).

p53 is also involved in single strand break repair (SSBR), whose defects unlike those of DSBR deficiencies manifest themselves exclusively as neuropathological symptoms (McKinnon, 2009). Thus, spontaneous DNA base modifications, caused by endogenous oxidation, alkylation and deamination of DNA but also by exogenous insults are repaired in base excision repair (BER), the main repair mechanism for SSBs (Offer et al., 2001; Zhou et al., 2001). P53 interacts directly with DNA polymerase β (DNA pol β) and AP endonuclease stabilizing their interaction with DNA (Offer et al., 2001; Zhou et al., 2001). BER activity is considerably reduced in human and murine cells, which lack endogenous p53 and *in vivo* BER activity correlates with p53 levels. BER appears to be active *in vivo* in proliferative neural stem cells during the neurogenic period of the embryonic forebrain development after which BER activity declines (Hildrestrand et al., 2009). DNA pol β deficient mice show abnormalities exclusively in the nervous tissue and die immediately after birth indicating the importance of DNA pol β in nervous system development (Sugo et al., 2004).

p53 has been reported to contribute to nucleotide excision repair (NER) i.e. elimination of bulky adducts such as pyrimidine dimers typically caused by exogenous damaging agents such as UV radiation (Wang et al., 1995). Notably, neuropathology in defective NER is associated with impaired transcription coupled repair (TCR), a form of NER in which p53 has been implicated to play a decisive part (Wang et al., 1995; McKinnon, 2009).

Table 1. Neurodegenerative syndromes associated with DNA repair deficiency (from Rass et al., 2007).

Table 1. DNA-Repair Deficiency and Neurodegeneration

Syndrome	Disease gene	Neurological implications	Other
Nucleotide excision repair			
Xeroderma pigmentosum (XP)	*XPA, XPB, XPD, XPF, XPG, XPV*	microcephaly, progressive neurodegeneration	neoplasm of the skin and eyes
Cockayne syndrome (CS)	*XPB, XPD, XPG, CSA, CSB*	microcephaly, progressive neurodegeneration	dwarfism
Trichothiodystrophy (TTD)	*XPB, XPD, TFB5/TTD-A*	microcephaly	brittle hair
DNA damage response/DSB repair			
Ataxia telangiectasia (A-T)	*ATM*	ataxia, progressive neurodegeneration	immunological implications, lymphoid malignancy
Ataxia telangiectasia-like disorder (ATLD)	*MRE11*	ataxia, progressive neurodegeneration	immunological implications, lymphoid malignancy
Nijmegen breakage syndrome (NBS)	*NBS1*	microcephaly	immunological implications, lymphoid malignancy, short stature
ATR-Seckel syndrome (ATR-Seckel)	*ATR*	microcephaly	dwarfism
Primary microcephaly 1 (MCPH1)	*MCPH1/BRIT1*	microcephaly	
LIG4 syndrome	*LIG4*	microcephaly	immunodeficiency, lymphoid malignancy, developmental and growth delay
Immunodeficiency with microcephaly	*Cernunnos/XLF*	microcephaly	immunodeficiency, lymphoid malignancy
SSB repair			
Ataxia with oculomotor apraxia 1 (AOA1)	*Aprataxin*	ataxia, progressive neurodegeneration, oculomotor apraxia	hypoalbuminemia, hypercholesterolemia
Spinocerebellar ataxia with axonal neuropathy (SCAN1)	*Tyrosyl-DNA phosphodiesterase 1*	ataxia, sensory loss	hypoalbuminemia, hypercholesterolemia

4. Oxygen tension and embryonic brain development

4.1. Early embryogenesis occurs at low oxygen tension, whereas differentiation and organic maturation takes places at higher oxygen tension

Normal embryonic developmental requires oxygenation that is appropriate to the gestational stage in concern (Fantel and Person, 2002). Embryonic and fetal development, early prenatal and later prenatal development, respectively, have profoundly different requirements for the availability of oxygen. Organogenesis i.e. formation of organs occurs at low oxygen concentration. The early post-implantation embryo is hypoxic its oxygen concentration being 2-8%, whereas the growth and the performance of more “adult-like” organic functions takes place at higher oxygen concentration (Wu et al., 2012). Placental maturation plays here an important part. In the early post-implantation mammalian embryo energy production relies on glycolysis (Fantel and Person, 2002). Later, around the time of neural tube closure and the onset of neurogenesis tricarboxylic acid cycle and oxidative

phosphorylation i.e. the use of mitochondria becomes increasingly important. Consequently, during early embryogenesis, when active proliferation and the related organogenesis are ongoing, embryonic environment is reducing (Hansen, 2006). When the organogenesis proceeds, the environment becomes more oxidizing shutting off proliferation and favoring differentiation (Fig. 6). Yet, too oxidative conditions activate cell death.

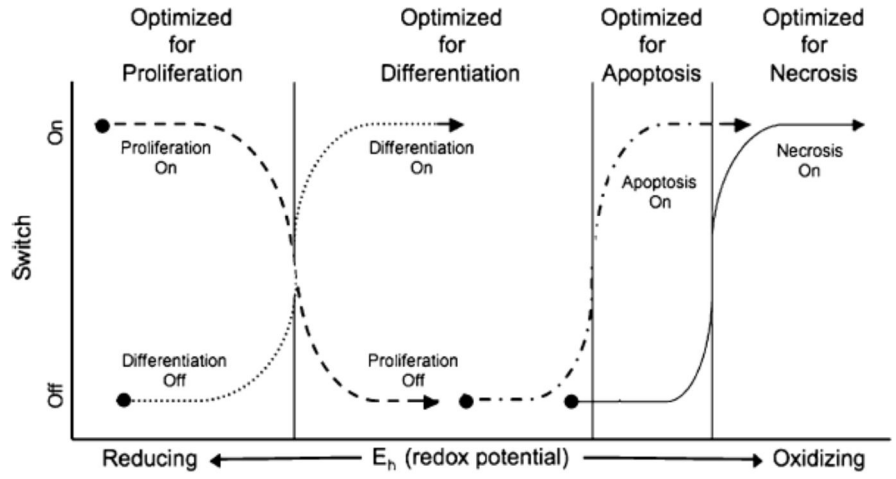


Figure 6. Redox switches during embryonic development (from Hansen, 2006).

4.2. Deviations from the correct oxygen tension can result in elevation of ROS and cause embryopathies

Factors that disturb normal oxygenation can have fatal consequences to the developing embryo causing embryopathies. The hazards include maternal diabetes and various teratogens such as ionizing radiation, thalidomide, metamphetamine and anticonvulsants valproic acid and phenytoin (Wells et al., 2010; Wu et al., 2012). In addition, hyperoxic *in vitro* culturing conditions are embryopathic. The adverse effects are largely due to initiation of the formation of ROS, which can disturb normal signal transduction and cause macromolecular damage. Neurulation in particular, which terminates in neural tube closure, is sensitive to oxidative stress (Fantel and Person, 2002).

4.3. Classical antioxidant systems appear to exhibit lower activity in the prenatal telencephalon compared to the postnatal telencephalon

When activities of the main antioxidant enzymes in the cerebrum and cerebellum were compared from E18 to P21 in mice, activity of glutathione peroxidase (Gpx) and catalase were found to increase, whereas that of superoxide dismutase (SOD), especially MnSOD/SOD2 in the mitochondria, but also Cu/ZnSOD/SOD1 in the cytosol decreased (Khan and Black, 2003). Glutathione (GSH) levels increased postnatally. In another study high expression of UCP2 (mitochondrial uncoupling protein) and Gpx were found in the adult SVZ and hippocampal subgranular zone compared to postmitotic regions (Madhavan et al., 2006). The conditions in the utero are hypoxic. Upon birth the newborn is exposed to about 4-fold elevation in oxygen concentration (hyperoxy) (Khan and Black, 2003). The increased activity of the most antioxidant enzymes upon birth is likely to reflect in part this. Hyperoxic conditions after birth also set high demands on redox regulation system in those parts of the brain, which are still actively undergoing proliferation and differentiation, like the cerebellum.

5. ROS dependent cellular signalling

5.1. ROS-rich signalling pockets

An emerging theme is that stem cells including neural, mesenchymal, embryonic and colon stem and progenitor cells employ ROS in the regulation of their normal cellular homeostasis and in self-renewal and differentiation decisions (Smith et al., 2000; Studer et al., 2000; Krabbe et al., 2009; Paik et al., 2009; Renault et al., 2009; Coant et al., 2010; Mazumdar et al., 2010; Yalcin et al., 2010; Le Belle et al., 2011). In fact, ROS and reactive nitrogen species (RNS) are utilized in normal signalling from early development on (D'Autréaux and Toledano, 2007). Cells use ROS and RNS they produce in normal physiological signalling to control processes such as cell proliferation, differentiation,

cytoskeletal reorganization, migration, protein trafficking, protein synthesis and degradation (Ushio-Fukai, 2006; Rhee et al., 2006; Giorgio et., 2007; D'Autréaux and Toledano 2007; Jones, 2008; Ducroc et al., 2010; Le Belle et al., 2011). Mitochondria and cellular oxidases, notably NADPH oxidases (NOX), xanthine oxidases (XO) and cyclo-oxygenases (COX) are the main sources of endogenous ROS in many cell types (Farrell et al., 2011; Le Belle et al., 2011). Binding of growth factors such as FGF, EGF, PDGF, VEGF or cytokines to their tyrosine kinase associated receptors on the plasmamembrane activates receptor associated oxidases and ROS mediated signaling (Meng et al., 2002; Kamata, 2009; Cattaneo et al., 2011; Le Belle et al., 2011). Two different cellular responses to changes in oxidative status have been proposed. In one of them, becoming slightly more oxidized is sufficient to inhibit proliferation and to induce differentiation. In the second one, similar changes drive proliferation (Noble et al., 2005). The outcome may depend on intrinsic cellular factors such as on the duration of the increase in ROS, developmental stage etc. In addition, other extracellular factors present may modulate the response (Noble et al., 2005; Le Belle et al., 2011).

ROS dependent signalling model postulates that cells have signalling pockets, which utilize ROS, RNS and reactive carbonyl species (RCS) as a part of their normal signalling (Halvey et al., 2005). Importantly, this kind of use of reactive species in signalling does not normally lead to generalized cellular oxidation, but is readily reversed by cellular antioxidant mechanisms (Halvey et al., 2005). Reversible oxidation-reduction reactions, especially those on thiols, function in receptor signalling, transcriptional regulation and apoptosis (Schlessinger, 2000; Dalton et al., 2004; Halvey et al., 2005; Ushio-Fukai, 2006; Farrell et al., 2011). Cysteinyl thiols are involved in binding interactions and in protein folding. Furthermore, they are important targets for various oxidative and nitrosative post-translational modifications. Oxidation of the active site Cys in enzymes and other signalling proteins is a common modification (Chiarugi et al., 2003; Sohn et al., 2003). Since these modifications

normally are reversible thiols can be thought to act as cellular redox sensors and signalling mediators (Forman, 2010).

5.2. Neural stem cells use ROS in the regulation of their normal cellular homeostasis

Studies on ROS levels of proliferative neural stem cells (NSCs) versus differentiating progenitors are somewhat conflicting. Adult proliferative NSCs have been reported to exhibit high ROS, which is needed to drive their proliferation (Limoli et al., 2004) on one hand and to promote both proliferation and neurogenesis on the other hand (Le Belle et al., 2011). In fact, adult NSCs have significantly elevated ROS levels compared to other primary cells and the elevation in ROS in them seems to depend on cell density (Limoli et al., 2004). Additionally, adult neurogenesis has been reported to generate transient oxidative stress typical for early postmitotic neurons (Walton et al., 2012). Nonetheless, low ROS levels appear to be typical to embryonic or early postnatal proliferating NSCs compared to newly-generated differentiating neurons (Madhavan et al., 2006; Tsatmali et al., 2005; Tsatmali et al., 2006). In fact, cultured NSCs have been reported to possess better antioxidant defense mechanisms than postmitotic neurons (Madhavan et al., 2006). Finally, in OPCs more reduced cellular environment promotes their proliferation, whereas an increase in the cellular oxidative status favors differentiation (Smith et al., 2000).

Oxygen tension regulates NPCs *in vivo* (Prozorovski et al., 2008; Mazumdar et al., 2010). This has been studied in the adult brain, where NSC niches next to the vasculature have adapted to less hypoxic conditions. Hypoxia promotes proliferation and inhibits precocious neuronal differentiation (Mazumdar et al., 2010). Oxygen tension also affects cellular metabolism with more hypoxic conditions shifting the balance towards glycolysis (Gustafsson et al., 2005; Shi et al., 2009).

Taken together, the available data may imply that proliferation of adult NSCs requires high ROS and that low ROS indicates quiescence (Limoli et al., 2004; Le Belle et al., 2011). However, proliferative embryonic and early postnatal NSCs appear to maintain relatively low

ROS levels. In fact, embryonic NSCs have been reported to possess enhanced steady-state "vigilance" of antioxidant defense (Madhavan et al., 2006). Finally, increase in ROS may promote neurogenesis (Tsatmali et al., 2005; Tsatmali et al., 2006; Le Belle et al., 2011).

6. PI3K-Akt-TOR signaling and embryonic brain development

The data available from various cell types would suggest that phosphatidylinositol 3,4,5 trisphosphate kinase (PI3K)-Akt pathway may be one of "the cellular redox rheostats", which orchestrates cellular responses to reactive oxygen species. Reactive lipids regulate the pathway at various levels (Wu et al., 2001; Lee et al.., 2002; Leslie et al., 2003). Moreover, extensive evidence shows that PI3K-Akt-dependent signaling by means of its downstream effectors mTOR, glycogen synthase kinase 3 (GSK3) and PTEN contributes to appropriate CNS development at multiple levels (Kwon et al., 2006; Kim et al., 2009a; Zhou et al., 2009; Fishwick 2010; Le Belle et al., 2011; Magri et al., 2011). Of relevance, PI3K-Akt-mTOR pathway is also linked to ROS production (Meng et al., 2002; Cattaneo et al., 2011; Le Belle et al., 2011). PI3K-Akt-mTOR dependent signaling is involved not only in the regulation of neural stem proliferation/differentiation decisions, but also in axon and dendrite outgrowth and arborization, and activity related neuronal plasticity. Various developmental abnormalities (macrocephaly, Tuberous Sclerosis Complex disease) and CNS disorders (autism, schizophrenia, seizures, malignant brain tumors e.g. Lhermitte-Duclos disease) have been linked to disturbed PI3K-Akt-PTEN-mTOR signaling. Hence, finely tuned activation of the pathway is developmentally important.

6.1. Conditional mouse knockouts demonstrate the importance of the PI3K-Akt-mTOR signalling for the correct homeostasis of eNSCs and for telencephalic development

Considerable evidence from conditional mouse knockouts generated for the components of the PI3K-Akt-mTOR pathway, has facilitated circumvention of the lethality associated with total knockouts before the onset of neurogenesis and has demonstrated the

importance of the PI3K-Akt-mTOR pathway for telencephalic development (Table 2.). Overactivation of the mTOR pathway by the loss of repressor PTEN leads to precocious neuronal differentiation (Bateman and McNeill, 2004; McNeill et al., 2008). In its active state PTEN lipid phosphatase dephosphorylates *in vivo* second messengers phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate produced by PI3K preventing translocation of Akt/PKB to the plasma membrane and thus its activation (Ushio-Fukai 1999; Lee et al., 2002). Likewise, overexpression of positive regulators of mTOR, such as PI3K, results in premature neuronal differentiation. Pharmacological inhibition of either PI3K or mTOR by LY294002 or rapamycin, respectively, attenuates neuron production (Fishwick et al., 2010). Mice with PDK1 deletion, the upstream activator of Akt, exhibit impaired neuronal differentiation in the neural tube and die by E11.5, whereas mice harboring targeted disruption of Akt3, the brain specific isoform of Akt, have considerable reduction in the brain size and weight due to reduced cell number and size (Tschopp et al., 2005; Fishwick et al., 2010). PI3K–Akt signalling also promotes survival of proliferating and differentiating embryonic NSCs as well as that of maturing neurons (Dudek et al., 1997; Chan et al., 2011).

6.2. PI3K-Akt-dependent signalling modifies neurite polarization, elongation, arborization and neuronal migration

PI3K/Akt/PTEN/GSK3β signalling has been associated with the regulation of neurite elongation, arborization and polarization. In embryonic phosphoinositide 3-kinase enhancer (PIKE)-/- cortical neurons, which naturally exhibit low PI3K/Akt activity dendritic arborisation, dendritic branch length and soma size is enhanced by forced activation of the PI3K/Akt pathway (Chan et al., 2011). Phosphorylated Akt has been implicated in the control of morphogenesis and dendritic development of postmitotic dentate granule cells of the adult hippocampus (Zhou et al., 2009). PI3K-Akt pathway also promotes embryonic axon outgrowth (Yoshimura et al., 2006; Bellon et al., 2010). Constitutively active GSK3β prevents

Table 2. Mouse mutants for the components of the PI3K-Akt pathway exhibit developmental brain defects.

Gene	Gene expressed during embryogenesis	Mode of knockout	Phenotype in mouse	Reference
PI3K catalytic subunit p110α	ubiquitously	systemic	embryonically lethal at E9.5-E10.5	Bi et al., 1999
Akt1 (PKBα)	ubiquitously	systemic	neonatally lethal, reduction in body weight and brain size	Chen et al., 2001
Akt2 (PKBβ)	ubiquitously, particularly high levels in insulin responsive tissues	systemic systemic	diabetes-like symptoms, insulin resistance	Cho et al.., 2001
Akt1(PKBα)-Akt2 (PKBβ)	ubiquitously	systemic	severe dwarfism, neonatally lethal	Peng et al., 2003
Akt3 (PKBγ)	brain	systemic	reduction in brain size and weight due to reduced cell number and size, neurons susceptible to glutamate toxicity	Tschopp et al., 2005
PDK1	ubiquitously	systemic	impaired neuronal differentiation, lethal at E11.5	Fishwick et al., 2010
PTEN	ubiquitously, from E15 onwards at high levels specifically in the brain	systemic	embryonically lethal at E9.5-E10.5	Di Cristofano et al., 1998
PTEN	ubiquitously, from E15 onwards at high levels specifically in the brain	PTEN $^{loxP/loxP}$ nestin-cre cells	enlarged brain with abnormal cytoarchitecture, increased NSC proliferation and size, decreased apoptosis, increase in synapse	Groszer et al., 2001, Groszer et al., 2006, Kwon et al., 2006,

			density, hypertrophic and ectopic dendrites and axonal tracts, abnormal social interaction, overactivation of the PI3K/Akt/ mTOR pathway	Zhou et al., 2009
Tsc1	ubiquitously	Emx1+ $^{loxP/loxP}$ nestin-cre cells	hyperproliferation, defects in cortical lamination, epileptic seizures, aberrantly expanded SVZ regions	Magri et al., 2011
Tsc2	ubiquitously	Tsc2 $^{flox/ko}$ GFAP-cre	postnatal megalencephaly, cortical lamination defects, abnormal myelination	Way et al., 2009
GSK3α, GSK3β	GSK3α ubiquitously GSK3β2 CNS specific	GSK3α-/- Gsk3β $^{loxP/loxP}$ nestin-cre cells	NPC hyperproliferation, generation of IPCs and postmitotic neurons greatly reduced	Kim et al., 2009b

axon formation, whereas pharmacological inhibition of GSK3β results in generation of several axons (Jiang et al., 2005; Yoshimura et al., 2005). Akt and PTEN are the upstream regulators of GSK3β Akt inhibiting constitutive GSK activity and PTEN promoting it. Indeed, lipid-rafts targeted Akt promotes axonal branching and growth cone expansion via mTOR and Rac1, respectively (Grider et al., 2009). Inactivation of PTEN in differentiating neurons *in vivo* correlates with increased P-Akt levels and concomitant phosphorylation and inactivation of GSK3β (Kwon et al., 2006). The outcome is elongated and thickened neuronal processes, increase in spine density and abnormal neuronal polarity. Disturbed activation of mTORC1 signalling contributes to the phenotype, while pharmacological inhibition of mTORC1 reverses neuronal hypertrophy and ameliorates behavioural symptoms (Zhou et al., 2009).

ROS produced by NADPH oxidases have been connected with cytoskeletal reorganization and migration, whereas PI3K signalling dependent activation of mTOR is linked to NOX activation and thus ROS production (Moldovan et al., 2006; Kim et al., 2009c). PI3K pathway and thus tangential migration from the GE to the cortical plate is activated upon BDNF or neurotrophin-4 (NT-4) binding to their cognate receptors, whereas inhibition of PI3K considerably attenuates it, for instance (Polleux et al., 2002). Differentiating PTEN+/- NPCs are more migratory and invasive and exhibit Akt hyperphosphorylation (Li et al., 2002). Akt mediates cell motility downstream of PDGFR/Rac/Cdc42 in some cellular systems, such as in fibroblasts (Higuchi et al., 2001; Moldovan et al., 2006).

6.3 Sestrins regulate cellular ROS levels, DNA damage response and autophagy

Sestrins are a family of three proteins (Sesn1/PA26, Sesn2/Hi95 and Sesn3), which have been reported to possess oxidoreductase activity towards oxidised peroxiredoxins ($Prxs_{ox}$) (Budanov et al., 2004). In fact, sestrins are along with sulfiredoxin1 (Srx1) the only enzymes stated to be able to reactivate i.e. reduce Prx_{ox}. Sestrins also inhibit mTORC1 signalling by activating AMP-responsive protein kinase (AMPK) with a concomitant activation of Tuberous sclerosis complex 1 (TSC1), a negative effector of mTORC1 (Budanov and Karin, 2008; Chen et al., 2010). Consequently, sestrins can regulate translation; sestrin2 has been reported for instance to prevent translation of c-Myc and Cyclin D1 mRNAs blocking thereby cell proliferation (Budanov & Karin, 2008).

Depletion of sestrins in cell cultures leads to accumulation of ROS, whereas their overexpression reduces ROS levels (Budanov et al, 2004; Kopnin et al., 2007; Liu et al., 2011). Silencing of Sesn2 has also been linked to increased expression of PDGFRβ, a ROS regulated gene (Liu et al., 2011). Moreover, up-regulation of Sesn2 and Srx1 expression in response to synaptic activity has been interpreted to contribute to a reduction of neuronal ROS in a peroxiredoxin dependent manner (Papadia et al., 2008). However, whether reactivation of

$Prxs_{ox}$ is directly performed by sestrins or is due to some assisting role is controversial (Budanov and Karin, 2008; Woo et al., 2009). Peroxiredoxins are enzymes able to reduce hydroperoxides generated in cellular signalling, as metabolic by-products and in cellular stress conditions (Wood et al., 2003). According to an alternative explanation the ability of sestrins to reduce cellular ROS might result from downregulation of mTOR signalling, which in its upstream part is linked to ROS production (Budanov and Karin, 2008).

Genotoxic stress induces expression of sestrin1 and 2 in a p53 dependent manner (Velasco-Miguel et al., 1999; Budanov et al., 2002). Thus, sestrins act as DNA damage indicators and have been implicated in the induction of cell cycle arrest and apoptosis upon DNA damage (Budanov et al., 2002; Budanov and Karin, 2008). Sestrin2 has been linked to apoptosis also in *in vivo* rat brains upon middle cerebral artery occlusion (MCA). On the other hand, sestrins can potentially inhibit DNA damage by enhancing cellular antioxidant response. Accordingly, down-regulation of Sesn1, 2 or 3 by using shRNA approach leads to a 2-fold increase in ROS and to a similar increase in the frequency of chromosome breaks in dividing cells (Kopnin et al., 2007). Though the induction of Sesn1 and 2 expression in response to genotoxic insult is p53 dependent, whether this is the case also in response to hypoxia and hyperoxia is controversial with reports for and against existing (Velasco-Miguel et al., 1999; Budanov et al., 2002; Sablina et al., 2005). Notably, the expression of Sesn3 appears to be p53 independent and is instead induced by Foxo transcription factors (Chen et al., 2010).

Active mTOR signalling, TORC1 in particular, inhibits autophagy (Cecconi et al, 2007). Sestrin2 enhances autophagy in response to nutrient depletion and rapamycin in p53 proficient cells, but not in cells depleted for p53 (Maiuri et al, 2009). It has been proposed that the decrease in ROS by Sesn2 overexpression might in fact be due to the elimination of damaged mitochondria by autophagy (Budanov and Karim, 2008). Autophagy is a catabolic process, which eliminates damaged organelles and macromolecules producing substrates for

glycolysis and oxidative phosphorylation. Impairments in autophagy have been connected with tumorigenesis and genomic instability (Maiuri et al., 2009). From the developmental point of view it is of interest that impaired autophagy has been suggested to play a part in NTDs (Cecconi et al., 2007). As already mentioned, a subset of p53 null female embryos suffers from defective neural tube closure (Sah et al., 1995; Armstrong et al., 1995). Whether this is related to impairments in autophagy has not been investigated.

6.4. Sestrins and telencephalic development

No evidence currently exists on the significance of sestrins during embryonic development. Sesn2 null mice, for instance, do apparently not have any obvious phenotype (Budanov and Karim, 2008). Nevertheless, sestrin2 is expressed in mouse E13.5 telencephalons with an increasing gradient from the VZ to the SVZ (Meechan et al., 2009). Notably, in LgDel mouse model of DiGeorge's syndrome, a developmental disorder in which cortical connectivity is disrupted, expression of Sesn2, CyclinD1 and E2f2, a proliferation related transcription factor, are considerably decreased, the proliferation of BPs but not APs is impaired and neurogenesis is disturbed (Meechan et al., 2009). Patients with DiGeoge´s syndrome are susceptible to ADHD, autism and schizophrenia (Campbell et al., 2006; Meechan et al., 2009). However, *Sesn2* is not localized to the chromosomal region affected in DiGeorge's syndrome. Hence, though the decrease in sestrin2 in the LgDel mutant mouse brain is a secondary event, this does not diminish the possibility that sestrin2 does have a developmental role, possibly in the regulation of BPs proliferation. It is noteworthy that no difference in apoptosis was reported between wild type and LgDel mouse telencephalons (Meechan et al., 2009).

The aim of the dissertation work

Comprehensive explanations for the high basal expression of p53 mRNA in the germinal zones of the developing telencephalon are missing. This dissertation work aims, in its part, to shed some light on the possible role(s) the p53 transcription factor might play in the germinal zones of the developing embryonic telencephalon.

Materials and Methods

Animals

B6.129S2-Trp53$^{tm1Tyj/J}$ and B6.129P2-Trp53^{tm1Brn}/J x B6.Cg-Tg(Nes-cre)$^{1Kln/J}$ and B129 S2 TRP53II mice were purchased from The Jackson Laboratory and maintained as heterozygous breeding pairs. Mice were kept and experiments were conducted according to the national and EU laboratory animal standards on Project License granted by local authorities.

Mouse neural progenitor cell culture

Neural progenitor cell cultures were prepared from heterozygous breedings. Embryos were genotyped using the following oligo pairs;

B129 S2 TRP53II:

oIMR0013 forward:CTTGGGTGGAGAGGCTATTC, neo cassette
oIMR0014 reverse: AGGTGAGATGACAGGAGATC, neo cassette
oIMR0036 forward: ATAGGTCGGCGGTTCAT, p53
oIMR0037 reverse: CCCGAGTATCTGGAAGACAG, p53

B6/129 P2-Trp53loxP-B6.Cg(SJL)-Tg (Nes-cre)1Kln:
oIMR8543 forward: GGTTAAACCCAGCTTGACCA, transgene
oIMR8544 reverse: GGAGGCAGAGACAGTTGGAG, transgene
oIMR1084 forward: GCGGTCTGGCAGTAAAAACTATC, transgene
oIMR1085 reverse: GTGAAACAGCATTGCTGTCACTT, transgene
oIMR7338 forward: CTAGGCCACAGAATTGAAAGATCT, internal positive control
oIMR7339 reverse: GTAGGTGGAAATTCTAGCATCATCC, internal positive control

Telencephalons of E13 or E16 embryos were dissected and dissociated as described previously (Garcion et al., 2004). Neural progenitor cells were maintained as floating neurosphere cultures in F12:DMEM (Invitrogen) 1:1, 2%B27 w/o vitamin A (Invitrogen),

20ng/ml bFGF-2 and EGF (both from Millipore), 5μg/ml heparin (Sigma) 1%PenStrep (Invitrogen), 2mM L-Gln (Invitrogen) in normoxic cell culture conditions (5% CO_2, 20% O_2, >95% humidity). Cells were passaged using accutase (Millipore) every 7 days.

In pretreatments neurospheres were exposed to 1μM H_2O_2, 10μM LY294002 (Cayman Chemical), 1mM N-acetyl cysteine (Sigma) or 100nM rapamycin (Alexis Biochemicals). Medium was changed back to normal differentiation medium after pretreatments to differentiate cells. In p53 overexpression experiments dissociated NPCs were transduced with Ad-GFP and Ad-GFP-p53 viruses, and progenitor cells overexpressed proteins for 18hrs before cells were processed for experiments. All experiments on *in vitro* neurospheres were performed before the 5^{th} passage and repeated 2-6 times.

Differentiation of neural progenitor cells

Neural progenitor cells were dissociated using accutase (Millipore) and plated on poly-D-Ornithine (15μg/ml, Sigma) and laminin (15μg/ml, Millipore) coated glass coverslips or wells in differentiation medium containing 1%FCS and 2%B27 supplement (Invitrogen), 1%PenStrep and 2mM L-Gln (Invitrogen), F12:DMEM (Invitrogen) 1:1. Cells were allowed to differentiate for 1-7 days before being lysed for RNA extraction or immunoblotting, or fixation for immunocytochemistry.

Immunoblotting

Telencephalons were lysed in Lysis buffer (20mM Hepes, pH 7,4, 2mM EGTA, 50mM β-glycerolphosphate, 50mM NaF, 1mM benzamidine, 1%Triton X-100, 1%SDS, 1mM DTT) in the presence of complete protease and phosphatase inhibitors (Roche). Samples were prepared according to standard immunoblot protocols. They were separated by SDS-Page and transferred to nitrocellulose membranes. 5% non-fat milk blocked membranes were incubated with primary antibodies o/n at $+4^oC$, washed three times and incubated with HRP-conjugated secondary antibodies for 1hr at RT, washed and detected using Enhanced Chemiluminesence

Detection Kit (Thermo Fisher Scientific) and Amersham Hyperfilm^{TM} ECL (GE Healthcare). *In vitro* cultured NPCs were lysed in SDS-Page sample buffer and immunoblotted as described above. The following primary antibodies were used: rabbit anti-phospho-S473Akt 1:1000, rabbit anti-leaved caspase3 1:400 from Cell Signaling, mouse anti-BIII-tubulin 1:1000 (Promega), mouse anti-nestin 1:1000, rabbit anti-NG2 1:500, rabbit anti-MAP2 1:1000, rabbit ant-GAD65&67 1:500, mouse anti-NeuN 1:1000 and rabbit anti-GFAP 1:1000 from Millipore, goat anti-DCX 1:1000 (Santa Cruz Biotechnology), mouse anti-β-actin 1:5000 (Sigma), mouse anti-PCNA 1:500 (Calbiochem) and rabbit anti-sestrin2 1:1000 (ProteinTech Group). ImageJ software was used to densitometrically quantify the films.

Microarray analysis

Total RNA from early passage E13 wild type and p53-/- neurospheres, three embryos per each genotype, was extracted with Trizol (Invitrogen) according to the manufacturer's protocol. Affymetrix, Mouse Genome 430 2.0 Array from triplicate samples was performed at the Microarray Genechip Facility at Universitäts Klinikum, Tübingen.

Quantitative real-time PCR

RNA from cell and tissue samples was isolated using Trizol reagent (Invitrogen). 1μg of total RNA was treated with Amplification Grade DNAse I (Invitrogen) as described by the manufacturer to eliminate possible genomic DNA contamination. RNA samples were reverse transcribed with SuperScript II Reverse Transcriptase and Oligo$(dT)_{12\text{-}18}$ primers (Invitrogen) after which cDNA was diluted 25-fold with nuclease free water and qRT-PCR reactions were prepared using Absolute qPCR SYBR Low Rox Mix (Thermo Scientific). qRT-PCR was run by 7500 Fast Real-Time PCR System (Applied Biosystems) with a standard Fast Mode and additional Melt Curve stage. A relative quantification analysis was applied using the ΔΔCt method with β-actin as an endogenous control. The following primer pairs were used:

Polκ forward: TCCAGCCTTGTACAGATGAGG

Polκ reverse: CTCGAGCTTTCTTTGGGTTG
Aen forward: CTGCTGCACAAGAAGATCCA
Aen reverse: TATCTGGGCCTCCATTTGTC
Ephx1 forward: TGAGGGGATGAAGGTCTTTG
Ephx1 reverse: CCCACGTTCCATGTAGGAAT
Sesn2 forward: TAGCCTGCAGCCTCACCTAT
Sesn2 reverse: GATTTTGAGGTTCCGTTCCA
Dcxr forward: CGTGCACTGACCAACCATAC
Dcxr reverse: TTCTCCACCTCAGCGAACTT
Redd2 forward: TGGTCCCTGAGAAACTGACC
Redd2 reverse: ACCACGCTAGCATCACACAC
p53 forward: ATAGGTCGGCGGTTCAT
p53 reverse: CCCGAGTATCTGGAAGACAG
p21 Cdkn1a forward: ACGACCTGGGAGGGGACAAG
p21 Cdkn1a reverse: TCCGTTTTCGGCCCTGAGAT
PdgfRα forward: TCCCTTGGTGGCACACCCTA
PdgfRα reverse: TGTCCCGGCAACAGGTTCTC
Ngn2 forward: GGTTGAATGCAAGCGTGGAAA
Ngn2 reverse: TGTGGCTGATCCTGGCAATG
Dcx forward: CCAAAGGCTTCCCCAACACC
Dcx reverse: TGTGCTTCCGCAGACTTCCA
Pax6 forward: ACCGCCCTCACCAACACGTA
Pax6 reverse: ACTCCGCCCATTCACTGACG
Otx1 forward: AGCGCCCATGCACTCTCATC
Otx1 reverse: ATTGAAGGCGAGCCCAGAGC
Nr2f2 forward: CCAACCAGCCAACACGGTTC
Nr2f2 reverse: TCCCGGATGAGGGTTTCGAT
Gabra2 forward: GGCTTGGGACGGGAAGAGTG
Gabra2 reverse: GTTCGGTTCTGGCGTCGTTG
Mbp var1 forward: GGGCAGAAGCCAGGATTTGG
Mbp var1 reverse: TCGCCATGGGAGATCCAG
Sox10 forward: CCCTGGTGTGGATGCCAAAG
Sox10 reverse: CTGGGAGGGCCCCATGTAAG
β-actin forward: ACCCAGGCATTGCTGACAGG
β-actin reverse: GGGGCCGGACTCATCGTACT

DNA damage assay

DNA damage assay Kit (BioVision) was applied according manufacture's protocol to quatify the level of apurinic/apyrimidinic sites in neural progenitor cell DNA.

Immunocytochemistry

Cells were fixed with 4%PFA in PBS at RT for 15 min and immunostained according to standard protocols. Primary antibodies used were GFAP (rabbit, 1:1000, Chemicon), βIII-tubulin (mouse, 1:1000, Promega), p53-CM5 (rabbit, 1:500, Novocastra), Ki67 (mouse, 1:100, BD Pharmingen), phospho-H3 (Ser10) (rabbit, 1:100, Millipore), doublecortin and phospho-S473Akt (both rabbit, 1:200, Cell Signaling). Alexa488 and Alexa568 (Molecular Probes) were applied 1:2000 as fluorescent secondary antibodies. Axiophot 2/Axioplan

microscope and AxioVision software 4.8. (Zeiss) were used in imaging. For quantifications 5 to 8 fields each containing approximately 20 neurospheres or 500-1000 differentiating cells were counted. Neurite length of tuj1+ differentiating neurons was measured using Neurolucida 8 software (MBF Bioscience) from 5-8 fields each contaning 500-1000 cells.

Immunohistochemistry

Brains were fixed in 4%PFA in PBS at +4^{o}C o/n, cryoprotected in 30% saccharose at +4^{o}C o/n after which they were cut to 18μm sections and stained according to the protocols described by the manufacturer specifically for each antibody. The primary antibodies used were p53-CM5 (rabbit, 1:500, Novocastra), Ki67 (mouse, 1:100, BD Pharmingen), phospho-H3 (Ser10) and nestin (rabbit and mouse respectively, 1:100, Millipore), phospho-S473Akt (rabbit, 1:100, Cell Signaling), BrdU (rat, 1:200, Novus Biologicals) and sestrin2 (rabbit, 1:100, ProteinTech Group). Fluorescent secondary antibodies applied were Alexa488 and Alexa568 at 1:2000 dilution (Molecular Probes). LSM 780 Laser scanning confocal microscope together with Zen 2011 software (Zeiss) and Axiophot 2/Axioplan microscope with AxioVision software 4.8. (Zeiss) were applied in imaging. 3-6 sections per animal, each separated by 40μm, and 3-6 animals per developmental stage and genotype were examined to quantitate phospho-H3 or BrdU positive cells.

Endogenous ROS measurement

ROS indicator chloromethyl 2'7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes) was used to assess endogenous cellular ROS levels in control and pretreated neurosphere cultures as well as in *ex vivo* ROS measurements. For CM-H2DCFDA staining cells were incubated with 5μM CM-H2DCFDA dye in Hibernate-E medium (Invitrogen) at 37^{o}C in the cell culture incubator for 20 min. After washing in PBS labeled cells were incubated in neural progenitor cell proliferation medium in the cell culture incubator for 30min to allow cellular esterases to hydrolyze the acetate groups and render the

dye responsive to oxidation. NPCs were then washed, dissociated with accutase to a single cell suspension and sorted by using CyAnADP FACS and Summit software (DakoCytomation). CM-H2DCFDA stained cells were also incubated with propidium iodide (PI) 4μg/ml for 30min in FACS buffer to detect late apoptotic and dead cells and side and forward scatter were set to eliminate dead and aggregated cells. Non-stained cells were run as a negative control.

Differentiating neural progenitor cells were stained with the ROS sensitive dye dihydroethidine (2μM, Invitrogen) for 30min in the cell culture incubator, washed with PBS and fixed for immunocytochemistry.

For E11 *ex vivo* ROS measurements the telencephalon was dissected, dissociated to single cells using papain (Worthington) and stained for CM-H2DCFDA (Molecular Probes) as described above. Stained cells were also incubated with propidium iodide 4μg/ml for 15min in FACS buffer to detect late apoptotic and dead cells and FACS was performed as above.

In vivo ROS levels of B6.129P2-Trp53^{tm1Brn}/J x B6.Cg-Tg(Nes-cre)$^{1Kln/J}$ embryonic telencephalons were examined using dihydroethidine dye (DHE, 5 mg/kg; Invitrogen, Kunz et al., 2007). DHE was administered intraperitonally 1 hour prior to sacrifice. Brains were dissected, fixed in 4%PFA in PBS and processed for cryocutting. Fluorescence intensity of 18μm coronal slices was analyzed using ImageJ software. 3-5 embryos per genotype were examined.

For embryonic or adult *ex vivo* ROS measurements the telencephalon or the SVZ and hippocampus from 4-5months old males was dissected, dissociated to single cells using papain (Worthington) and stained for CM-H2DCFDA (Molecular Probes) as described above. Stained cells were also incubated with propidium iodide (PI) 4μg/ml for 15min in FACS buffer to detect late apoptotic and dead cells and FACS was performed as above.

Flow cytometry

Immunostaining of NPCs for FACS was performed by using Fix and Perm Kit (Invitrogen). The following antibodies were used: phospho-AktS473 and doublecortin (both rabbit, 1:100, Cell Signaling), and sestrin2 (rabbit, 1:100, ProteinTech Group). Alexa488 and Alexa633 (Molecular Probes) were the fluorescent secondary antibodies and they were used at 1:1000 dilution. For double staining with CM-H2DCFDA NPCs were first stained for CM-H2DCFDA whereafter immunostaining was performed. CyAnADP FACS and Summit software (DakoCytomation) were used to FACS cells and to analyze the data. Sort gates were set by side and forward scatter to eliminate dead and aggregated cells. Non-stained cells were run as a negative control. Non-specific IgG/Alexa secondary fluorophore staining was applied to define background staining from positive cells.

For cell cycle FACS NPCs were stained with 7-aminoactinomycin D (7AAD) 10μg/ml at low pH in the presence of saponin according to the protocol described by Schmid et al., 2000.

***Ex vivo* electroporation and culture**

pEF1/V5-His and pEF1/V5-His-Sesn2 plasmids (Papadia et al., 2008) were used in *ex vivo* electroporation of embryonic telencephalons. DNA was purified with Endofree Plasmid Maxi Kit (Qiagen). 2μg (2-3μl) plasmid in saline with 0,05% Fast Green (Sigma) was microinjected into lateral ventricles of E13 mouse embryos. Five 3ms pulses, each 40V were applied to electroporate DNA into telencephalons. The hemisphere on the side of the positive electrode was used as a control in all experiments. Electroporation of pEF-empty vector was conducted as an additional control. After electroporation cortices were dissected and placed in growth medium F12:DMEM 1:1, 2%B27 (Invitrogen), 1% pen-strep, 2mM L-Gln in the cell culture incubator (5% CO_2, 20% O_2, >95% humidity). After 20 hours expression cortices were prepared for ROS measurements by FACS according to the procedure described above.

In vivo saline, N-acetyl-cysteine and BrdU injections

0.9% saline (control) or N-acetyl-cysteine 67mg/kg (Sigma) in 0.9% saline was injected intraperitonally from E10 onwards daily to pregnant dams. Embryos were sacrificed at E16 and the telencephalons were snap-frozen in liquid nitrogen for further experiments. Three embryos per treatment and genotype were analyzed.

Brdu 150mg/kg (Sigma) was injected 1hr before sacrifice intraperitonally to pregnant dams. Embryonic brains were dissected and prepared for BrdU immunohistochemistry as described above. Images were acquired using Axiophot 2/Axioplan microscope and AxioVision software 4.8. (Zeiss). 3-6 animals per group, 3 sections per animal were quantified. Successive sections were separated by 40μm.

BrdU proliferation assay

Proliferation of *in vitro* NPCs was assessed by BrdU Proliferation assay Kit, HTS01 (Calbiochem). NPCs seeded at the density of 10 000 or 100 000 cells per 96 well plate well were let to incorporate BrdU for 18hrs in proliferation medium and the assay was performed according to manufacturer´s protocol.

In silico promoter analysis

In silico promoter analysis for p53 binding sites was performed using MatInspector software (http://www.genomatix.de/).

Statistical analysis

Data are expressed as mean +/- sem, in some cases when indicated as mean +/- sd. Data were analyzed by unpaired two-tail Student t test to determine statistical significance of data sets. Statistical significance is expressed as *P value < 0.05, ** P value < 0.01 and *** P value < 0.001.

II RESULTS

1. P53 is preferentially expressed in proliferating progenitors both *in vivo* and *in vitro*

First, the expression pattern of the transcription factor p53 was characterized in the developing telencephalon the focus being on the germinal zones and on the cortical plate. Immunohistochemistry (IHC) on coronal sections of embryonic day 11 and 13 (E11 and E13 respectively) mouse telencephalons detected nuclear expression of p53, possibly suggesting a role in the regulation of gene expression, in germinal zones positive for the proliferative neural progenitor cell (NPC) marker nestin (Fig. 1.A,B,C). P53 was expressed equally in the pallial and sub-pallial proliferative germinal zone its expression decreasing towards the cortical plate. In line with the *in vivo* expression pattern, p53 was detected in the nucleus of a proportion of cultured E13 NPCs (Fig. 1.D). Co-staining for the pan-proliferative marker Ki67 demonstrated that the vast majority of p53+ NPCs was also Ki67+ that is cycling cells. Importantly, there was no staining for p53 in p53-/- NPCs, an indication of the specificity of the antibody. Quantification of p53 mRNA levels by quantitative real time PCR (qRT-PCR) showed that p53 mRNA is most abundant in the embryonic telencephalon between E13-E16 its expression decreasing thereafter considerably (Fig. 1.E). Similar quantification in *in vitro* cultured proliferating and differentiating NPCs confirmed that proliferating NPCs have the highest p53 expression level profound and progressive decrease in p53 expression occurring immediately upon induction of differentiation (Fig. 1.F).

1.1. The lack of p53 *in vivo* leads to a transient initial increase in proliferation in the sub-pallium

These findings inevitably raise the question on the role of p53 in eNPCs. As p53 has been connected with DNA damage induced prenatal apoptosis and postnatal developmental apoptosis (Komarova et al., 1997; Murase et al., 2011) the level of cell death on E13 and E16 telencephalons was investigated by tunnel and cleaved caspase-3 immunohistochemistry. However, the level of apoptosis that was detectable was very low even though DNAase I

treated telencephalic positive control slices displayed profound tunnel signal (data not shown).

Since p53 has been connected with cell cycle regulation in various cell types including adult SVZ and embryonic olfactory bulb derived NPCs (Gil-Perotin et al., 2006; Armesilla-Diaz et al., 2009), I set next out to explore whether embryonic telencephalic NPCs devoid of p53 exhibited impairments in proliferation control. Quantification of *in vivo* proliferation by immunoblotting of telencephalic protein extracts for the pan-proliferative marker PCNA showed that its amount was elevated in p53 null telencephalons at E13 (Fig. 1.G). However, by E16 that is by the end of the neurogenic period the amount of PCNA protein was normalized. No major deviations in the number of mitotic or S-phase cells could be detected between the genotypes by phospho-histone-3 (P-H3) or BrdU IHC, when the total number of P-H3+ and BrdU+ cells present in the pallial VZ and the SVZ of E13 and E15 telencephalons were counted (Fig. 1.H-J). Yet, E13 p53-/- telencephalons exhibited an increase in the number of sub-pallial basal mitoses though the number of apical mitoses did not differ. Consequently, the elevation in PCNA marker is likely to reflect increased proliferation in the basal regions of GE, which by E15 however is normalized (Fig. 1.I).

Proliferation capacity of cultured E13 NPCs was not markedly different between genotypes (Fig. 1.K-L). In fact, somewhat surprisingly cultured E13 and E16 NPCs derived either from the conventional p53 transgenic mice or nestin-cre/loxPp53 mice, in which p53 is deleted by cre recombination in nestin+ cells, displayed reduced proliferation at higher cell densities according to BrdU incorporation assays and P-H3 immunocytochemistry (ICC), while the total number of proliferative cells did not differ as Ki67 ICC demonstrated. This implies that at higher cell densities *in vitro* p53-/- NPCs exhibit lengthening of the cell cycle without however becoming postmitotic. Notably, proliferation between the genotypes did not diverge, when NPCs were seeded at low densities (Fig. 1.K-L). Finally, the *in vivo* expression

of the cell cycle inihitor p21 did not differ between genotypes prenatally, while it postnatally was downregulated in p53-/- cerebrums.

Consequently, these data imply some deviations in proliferation between wild type and p53-/- embryonic neural progenitors. Nonetheless, in contrast to the adult SVZ *in vivo* or the *in vitro* cultured NSCs no profound, persistent overproliferation can be detected in telencephalic p53-/- NPCs suggesting that if p53 has a role in proliferation control during telencephalic development other factors are able to compensate its absence.

2. P53-/- NPCs exhibit an increase in cellular ROS both *in vitro* and *in vivo*

ROS have been implicated in the regulation of stem cell homeostasis (Limoli et al., 2004; Le Belle et al., 2011). Elevation in cellular ROS is able to drive an initial increase in proliferation with a subsequent induction of cell cycle arrest and differentiation (Limoli et al., 2004; Chua et al., 2009; Le Belle et al., 2011). As p53 has been shown to possess an antioxidant function (Sablina et al., 2005), I reasoned that one explanation for p53 expression in NPCs might be its putative role in the regulation of NPC redox homeostasis. To determine whether endogenous ROS levels *in vitro* p53-/- NPCs differed from those of the wild type cells I used fluorescence-activated cell sorting (FACS) and a ROS sensitive dye DCFDA capable of detecting H_2O_2, $HO^{\cdot}$ and $ROO^{\cdot}$ species (Gomes et al., 2005). *In vitro* cultured p53-/- E13 or E16 NPCs derived from either nestin-cre/loxP-p53 or from the conventional p53 knockout mice displayed an increase in cellular ROS levels relative to wild type cells but no increase in cell death, when assessed by propidium iodide (PI) staining (Fig. 2.A,B). When a similar analysis was performed *ex vivo* on acutely dissociated E11 telencephalic neural stem cells (NSCs), p53-/- cells showed an increase in ROS but no increase in cellular death (Fig. 2.C,D). At this developmental stage, which delineates the beginning of neurogenesis, the great majority of telencephalic NSCs are still proliferative. Hence, it appears that the absence of p53 results in higher ROS levels in proliferative NPCs both *in vitro* and *in vivo*.

2.1. The putative role of p53 as “a guardian of the genome” in eNPCs

A classical read-out of cellular ROS is the occurrence of DNA damage (Yahata et al., 2011), and p53 has been implicated in DNA damage response including DNA repair (Wang et al., 1995; Offer et al., 2001; Seo and Jung, 2004). Labelling of apurinic/apyrimidinic (AP) sites in DNA with aldehyde reactive probe showed that while in the wild type the number of AP sites *in vitro* remained constant in successive passages in p53-/- NPCs DNA damage raised steadily indicating increased DNA damage with time in culture (Fig. 2.E). Importantly, overexpression of Adeno-GFP-p53 in proliferating *in vitro* p53-/- NPCs accumulated cells in the G1 phase of the cell cycle and decreased the number of AP sites compared to control transduction (Fig. 2.F,G). In addition, BrdU incorporation to DNA was increased upon Ad-GFP-transduction possibly indicating DNA repair (Fig. 2.H). qRT-PCR showed that p53 overexpression induced expression of p21 Cdkn1a, a classical p53 target, as well as Aen exonuclease, which has a putative role in DNA repair and replication (Shimada et al., 2010) and DNA polymerase κ (Polκ), a member of the Y family of DNA polymerases that permits continuity of the replication fork by allowing replication through DNA lesions (Fig. 2.I) (Jones et al., 2011). Notably, overexpression of p53 did not result in apoptosis like unaltered cleaved caspase-3 levels demonstrated (Fig. 2.J).

3. High ROS is a feature of young neurons

In order to further explore the generation of ROS *in vivo* dihydroethidine (DHE) was injected to pregnant dams 1 hour before sacrifice and densitometric intensity of hydroethidine stained cells emitting red fluorescence upon hydroethidine oxidation was measured on sectioned E16 nestin-cre/loxP-p53 brain slices. Similarly to dissociated p53-/- E11 telencephalons ROS levels were elevated in E16 embryonic brains (Fig. 3.A). In the absence of p53 both the E16 VZ-SVZ and the cortical plate displayed a significant increase in hydroethidine staining intensity. Furthermore, the DHE staining pattern suggested that high ROS is a feature of young neurons especially as the outmost layer of the cortical plate was

most intensively stained for DHE (Fig. 3.A). In line with this, staining of *in vitro* differentiating NPCs for DHE showed that the total number of DHE+ cells was increased in the absence of p53 (Fig. 3.B). Moreover, the great majority of young postmitotic neurons when double stained for DHE and Tuj1, an early postmitotic neuronal marker, were double positive, yet p53-/- cells displaying even higher percentage of DHE+/Tuj1+ neurons.

Finally, ROS were measured in the SVZ and hippocampus derived from 4-5 months old adult wild type and p53-/- males. *Ex vivo* FACS with DCFDA as a ROS indicator detected an elevation in ROS both in the SVZ and hippocampus of p53-/- males (Fig. 3.C,D). This demonstrates that not only embryonic telencephalic p53-/- prognitors but also adult p53-/- neurogenic regions exhibit an increase in ROS. Furthermore, although high ROS levels appear to belong to the physiology of young neurons both *in vivo* and *in vitro*, the absence of p53 contributes to even further elevation in ROS.

4. Deviations in neurogenesis and oligodendogenesis in p53-/- NPCs *in vivo* and *in vitro*

4.1. Elevation in neurogenesis in p53-/- NPCs

A recent work connected ROS and neurogenesis in adult NPCs (Le Belle et al., 2011). Moreover, p53-/- adult SVZ NPCs and embryonic olfactory bulb derived NPCs reportedly generate neurons at the expense of glia cells (Gil-Perotin et al., 2006; Armesilla-Diaz et al., 2009). Hence, I investigated neural lineage commitment and differentiation potential of *in vitro* cultured E13 and E16 wild type and p53-/- telencephalic neural progenitor cells. FACS analysis of DCFDA-doublecortin (DCX) double-stained progenitors demonstrated that the proportion of DCX+/DCFDA++ cells was enriched in p53-/- NPCs (Fig. 4.A). ICC confirmed the increase in the number of DCX positive NPCs in p53-/- neurospheres (Fig. 4.B). Furthermore, not only p53-/- neurospheres but also immature differentiating progenitors displayed elevated DCX expression compared to wild type (Fig. 4.C). Quantification of the total amount of doublecortin protein by immunoblotting from embryonic and early postnatal telencephalic protein extracts revealed that DCX expression was up-regulated in p53-/-

telencephalons during prenatal neurogenic period (Fig. 4.D,E). However, DCX amounts were normalized postnatally. The possibility that the increase in DCX+ cells/DCX protein levels was due to increased proliferation of DCX+ NPCs rather than to the generation of DCX+ cells *per se* was excluded in part by double-staining proliferating NPCs for P-H3, a marker for mitosis, and for DCX (Fig. 4.F). No difference in the number of DCX+/ P-H3+ double positive cells could be detected between genotypes. Importantly, transduction of p53-/- NPCs with Ad-GFP-p53 also decreased the number of DCX+ NPCs generated (Fig. 4.G).

In accordance with the elevation in the abundance of DCX+ neuroblasts, p53-/- E13 and E16 NPCs generated upon induction of differentiation more tuj1+ neurons and exhibited enhanced extension of neurites, while astrogliogenesis was not affected (Fig. 5.A). Neuronal maturation was not impaired in the absence of p53, like immunocytochemistry and immunoblotting for some neuronal maturation markers demonstrated (Fig. 5.B). In fact, for example gabaergic differentiation was accentuated.

I also explored neuronal differentiation on wild type and p53-/- telencephalons by immunoblotting protein extracts for some differentiation markers. In line with the *in vitro* results, differentiation and neuronal maturation did not seem to be grossly affected on p53 null telencephalons (Fig. 5.C). However, some differences in the timing of the expression of certain marker proteins were detected. Gabaergic interneuron marker GAD65/67 and NeuN, for instance, were more abundant prenatally and were also detectable in p53-/- telencephalons earlier than in the wild type (Fig. 5.C_1, C_2). Yet, by P7 these differences were lost. Likewise, expression of the gamma-aminobutyric acid receptor subunit alpha-2 mRNA was elevated in p53 null telencephalons implying increased gabaergic differentiation (Fig. 5.D). Altogether, these data demonstrate some deviations in the timing of neurogenesis in p53-/- telencephalons, which however postnatally are normalized.

4.2. Oligodendrogenesis is impaired in p53-/- NPCs *in vivo* and *in vitro*

In contrast, the expression of oligodendroblast/oligodendrocyte markers was downregulated in p53-/- telencephalons. Sox10 mRNA, expressed both by immature precursor cells and mature oligodendrocytes (Nishiyama et al., 2009), was present at lower levels in p53 null telencephalons as was Pdgfrα, a marker for oligodendroblasts, which is not expressed by premyelinating oligodendrocytes or by mature oligodendrocytes (Fig. 6.A). It is noteworthy that also the myelin basic protein variant chosen, Mbp var1, was significantly downregulated in p53 null P7 brains implying that myelination might be impaired or at least delayed in null brains. In line with the profiling of oligodendroblast marker transcripts NG2, a chondroitin sulphate proteoglycan core protein, expressed in oligodendrocyte precursor cells (OPCs) (Richardson et al., 2011; Zhu et al., 2011) was down-regulated in p53-/- telencephalic protein extracts (Fig. 6.B). OPCs are redox sensitive precursors, whose proliferation is promoted in reductive conditions, whereas an increase in cellular oxidation status drives their differentiation (Smith et al., 2000).

5. ROS drives initiation of neurogenesis

To cast some light on the reasons of increased neurogenesis a genome-wide Affymetrix gene expression analysis on *in vitro* cultured E13 wild type and p53-/- proliferating early passage NPCs was conducted. Altogether 300 genes were dysregulated, when the cut of limit for the fold change of mRNA expression in p53-/- versus wild type NPCs was set to 2 (Table 1., Fig. 7.A,B). Ingenuity functional pathway analysis of the array data for the transcripts involved in “physiological system development and function” identified genes related to nervous system development, but also to hematological system development as the biggest groups of deregulated genes. Similar analysis in the group “Molecular and cellular functions” according to Integuinity classification recognized genes involved in cellular development as the largest group. Interestingly, a number of genes having a role in cellular movement were likewise identified.

Microarray analysis proved that several genes related to neurogenesis were up-regulated in p53-/- NPCs. qRT-PCR validation of some of them confirmed that expression of *DCX*, *Pax6*, *Otx1*, *Nr2f2* (*CoupTFII*) and *Ngn2*, for instance, was increased in cultured p53-/- progenitors (Fig. 7.C,D). I next explored the possibility that increased expression of neurogenic genes might be related to cellular redox status. Indeed, treatment of NPCs with H_2O_2 or with the ROS scavenger N-acetyl cysteine (NAC) proved that the expression of all genes of interest, except that of *Ngn2* and *p21 Cdkn1a*, were responsive to redox balance manipulation (Fig. 7.E). Hence, the expression of *Pax6*, *Dcx*, *Otx1* and *Nr2f2*, for instance, was responsive to modulation of cellular redox status. The expression of *Dcx*, in particular, responded robustly to a more oxidative environment. In addition, expression of *Pdgfrα*, a classical ROS responsive gene (Ruef et al., 1998), was regulated in a redox status dependent manner. In support for these findings, pretreatment of proliferating NPCs with H_2O_2 promoted generation of tuj1+ neurons (Fig. 7.F). Conversely, pre-exposure of NPCs to NAC decreased the number of neurons produced upon induction of differentiation (Fig. 7.G).

These data indicated that more oxidative conditions can trigger expression of neuroblast/basal progenitor (BP) marker genes and drive neurogenesis. On the other hand, proliferation data had implied that though the total number of proliferative NPCs did not grossly differ between the genotypes (Fig. 1.K_1), p53 null NPCs exhibited some increase in cell cycle length (Fig. 1.K-L). Cell cycle length, lengthening of its G1 phase in particular, has been linked to the induction of neurogenesis (Calegari and Huttner, 2003). According to the cell cycle length hypothesis lengthening of the G1 phase allows NPCs to be exposed to growth and transcription factors long enough so that neurogenesis is launched (Salomoni and Calegari, 2010). I therefore investigated the possibility that elevated ROS might have an impact on cell cycle length promoting thereby neurogenesis. Proliferating NPCs were exposed to 1μM H_2O2 and cell cycle profile was examined by FACS using 7-aminoactinomycin D (7AAD) staining. Exposure to exogenous ROS led to an accumulation of progenitors in the

G0-G1a phase of the cell cycle and to a simultaneous decrease of NPCs in G2-M (Fig. 7.H). Cellular death was not increased like both the assessment of cell size and propidium iodide staining demonstrated (Fig. 7.I).

Finally, I investigated whether administration of the antioxidant NAC would normalize deviations in neurogenesis in p53-/- telencephalons also *in vivo*. NAC was injected daily to pregnant dams from E10 onwards. Animals were sacrificed at E16 and telencephalic protein lysates were immunoblotted for marker proteins. Importantly, NAC administration led to a reduction in DCX protein when compared to the vehicle group confirming that *Dcx* is a redox regulated gene also *in vivo* (Fig. 7.J).

6. P53 null telencephalons have lower levels of the putative redox regulator sestrin2

Taken together, these data imply that cellular redox status has a role in the initiation of neurogenesis. Moreover, the data suggest that deviations in neurogenesis in p53-/- NPCs presumably result, at least in part, from their more oxidative cellular environment. I continued by attempting to cast some light on the underlying reasons for these observations. Gene expression microarray analysis had revealed four dysregulated genes with a putative role in redox regulation in neural progenitors: sestrin2 (*Sesn2*), dicarbonyl/L-xylulose reductase (*Dcxr*), epoxide hydrolase-1 (*Ephx1*) and *Redd2/Ddit4l* (Table 1.). Sesn2 has been linked to a reduction of oxidized peroxiredoxins and to a negative regulation of mTOR pathway (Budanov et al., 2004; Budanov and Karin, 2008). Dcxr displays activity towards reactive carbonyl groups formed by lipid peroxidation (Son et al., 2011) and Ephx1 has been reported to decrease cellular ROS and enhance development of pre-implantation mouse embryos (Cheong et al., 2009). Redd2 is a close homolog of Redd1, which has been implicated in mTOR pathway and redox regulation and, interestingly, in the control of embryonic neurogenesis and migration (Corradetti et al., 2005; Malagelada et al., 2011). First microarray results were verified by performing qRT-PCR from unrelated samples and from Ad-GFP-p53 and control Ad-GFP transduced NPCs. Transient over-expression of p53 induced expression

of Sesn2, Dcxr, Redd2 and Ephx1 in p53 null neurospheres to the same level as in wild type cells implying that p53 positively regulates their expression (Fig. 8.A,B). P53-/- NPCs had lower levels of sestrin2 protein, which however was readily induced by Ad-GFP-p53 (Fig. 8.C). In support for this, *in silico* promoter analysis found consensus dipartite p53 binding sites on the Sesn2 promoter (Fig. 8.D). However, chromatin immunoprecipitation for p53 failed to demonstrate direct binding of p53 to Sesn2 promoter.

Since *Sesn2* is the best characterized molecule among the redox regulation related candidate genes emerged further work focused on it. IHC for sestrin2 showed that after the initial ubiquitous expression at E11 sestrin2 had by E16 become more restricted to the VZ-SVZ (Fig. 8.E,F). Importantly, according to immunoblotting sestrin2 protein levels decreased progressively in the course of neurogenesis with the highest protein abundance measured at E11 that is during the almost exclusively proliferative phase of the telencephalic development, and at E13 (Fig. 8.G). Sestrin2 was remarkably downregulated in p53 null telencephalic lysates at E13-E16 its expression becoming postnatally apparently p53 independent. Of importance, developmental downregulation of sestrin2 protein occurred in p53-/- null telencephalons earlier compared to wild type embryos. Quantification of telencephalic mRNA levels confirmed downregulation of Sesn2 in p53-/- embryonic brains (Fig. 8.H.) This characterization connects Sesn2 expression with the proliferative phase of the telencephalic development and implies a positive role for p53 in Sesn2 expression also *in vivo*.

6.1. Exogenous Sesn2 reduces ROS levels in embryonic telencephalons

To explore the function of sestrin2 in embryonic telencephalons *ex utero* model was applied. Either empty vector or a DNA construct encoding Sesn2 was injected into the lateral ventricle of E13 telencephalons. After 22hrs expression ROS were measured from the hemisphere which was injected with the Sesn2 expression plasmid or with the empty vector, and from the non-injected control hemisphere by FACS. Expression of exogenous Sesn2 decreased endogenous ROS levels compared to the control hemisphere according to the ROS

indicator DCFDA (Fig. 8.I). Nonetheless, no difference in DCFDA staining was detected between the empty plasmid injected hemisphere and the corresponding control hemisphere. Overexpression of Sesn2 was confirmed by FACS (Fig. 8.J.).

These data indicate that sestrin2 is able to fine-tune ROS levels in embryonic telencephalons. On the other hand, the expression of Sesn2 itself in proliferative NPCs was ROS responsive since exogenous ROS induced expression of Sesn2 mRNA (Fig. 8.K). Moreover, mTORC1 inhibitor rapamycin reduced the amount of Sesn2 mRNA in wild type NPCs. Together the data imply a reciprocal connection between sestrin2, cellular redox status and mTOR pathway in proliferative embryonic neural progenitor cells.

7. PI3K-Akt signaling contributes to neurogenesis and neuronal maturation *in vivo* and *in vitro*

A number of studies have demonstrated that PI3K-Akt-mTOR pathway has a role in the regulation of NPC proliferation and differentiation (Kumar et al., 2005; Kwon et al., 2006; Kim et al., 2009a; Kim et al., 2009b; Mao et al., 2009; Magri et al., 2011). ROS production has been associated with PI3K-Akt pathway and with adult neurogenesis (Le Belle et al., 2011). Accordingly, I hypothesized that the increase in ROS, DCX and GAD65/67 in p53-/- *in vitro* and *in vivo* NPCs/differentiating neurons might be related to altered activation of the PI3K-Akt-mTOR pathway in p53 null cells.

I began by performing FACS for Akt-P-Ser473 and the ROS indicator DCFDA in *in vitro* cultured NPCs. P53-/- neural progenitors displayed an increase in the number of phospho-Akt++ cells, which correlated with DCFDA staining intensity (Fig. 9.A). Conversely, Ad-GFP-p53 transduction of p53-/- NPCs decreased the number of P-Akt++ cells (Fig. 9.B). When Akt P-Ser473 abundance was examined on telencephalic extracts E11-E13 p53-/- telencephalons were found to possess elevation in phospho-Akt (Fig. 9.C). This elevation was prominent at E11 in particular. However, by E16 the abundance of phospho-Akt was normalized to wild type levels. Interestingly, according to immunohistochemistry

Akt activation on E11 and E13 telencephalons was enriched to regions, where the newly generated postmitotic neurons reside that is to the cortical plate, but was detectable also on the germinal zones (Fig. 9.D,E). Quantification of the total level of the phosphorylated Akt had demonstrated that its amount increased from E11 to E16 staying thereafter relatively constant implying a connection with neurogenesis. Extension of this analysis to *in vitro* differentiating NPCs confirmed that Akt-P-Ser473 is most abundant in young maturing neurons, whereas other differentiating cells that is glia cells in eNPC cultures possessed lower amount of active Akt (Fig. 9.F). Consistent with the increased number of tuj1+ neurons produced the proportion of phospho-Akt+ cells was higher in differentiating p53-/- NPCs.

7.1. Inhibition of the PI3K-Akt pathway in proliferative p53-/- progenitors reduces the number of neuroblasts and neurons generated

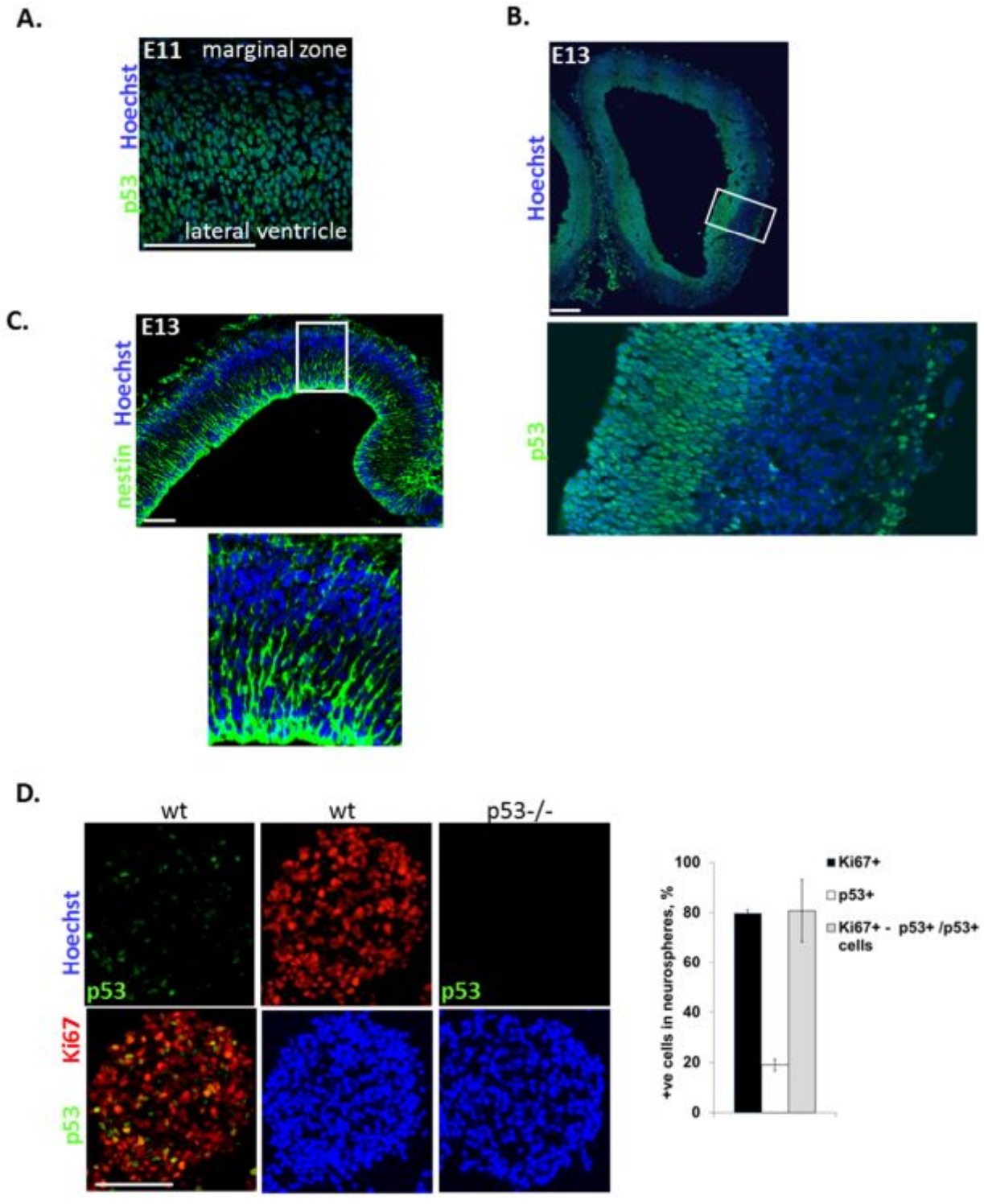
I continued by examining the effect of the pharmacological inhibition of the PI3K-Akt pathway on lineage commitment of NPCs. Pretreatment of p53-/- neurospheres in proliferation conditions with LY294002, a PI3K inhibitor, decreased the number of DCX++ cells suggesting that the increase in DCX expression in p53-/- NPCs was due to the elevated PI3K-Akt signalling (Fig. 10.A). Transient exposure of NPCs to LY294002 also decreased ROS as DCFDA FACS demonstrated (Fig. 10.B). Consistently, pretreatment reduced the number of tuj1+ neurons generated upon differentiation (Fig. 10.C). However, p53-/- progenitors displayed a more modest decrease in neuronal differentiation in response to LY pretreatment. This is reminiscent of the prepriming of proliferating p53-/- NPCs with H_2O_2, where they did not show similar increase in neuronal differentiation as the wild type cells (Fig. 7.F). Thus, it seems that the putatively priming responsive p53-/- NPCs were “already primed” to neuronal fate by their more oxidative cellular environment and higher P-Akt levels resulting in that a transient inhibition of the PI3K-Akt signalling was unable to turn them from neuronal fate. This may indicate long term changes in the signalling involving Akt in such a way that the proliferation phase largely determines subsequent signalling in post-mitotic

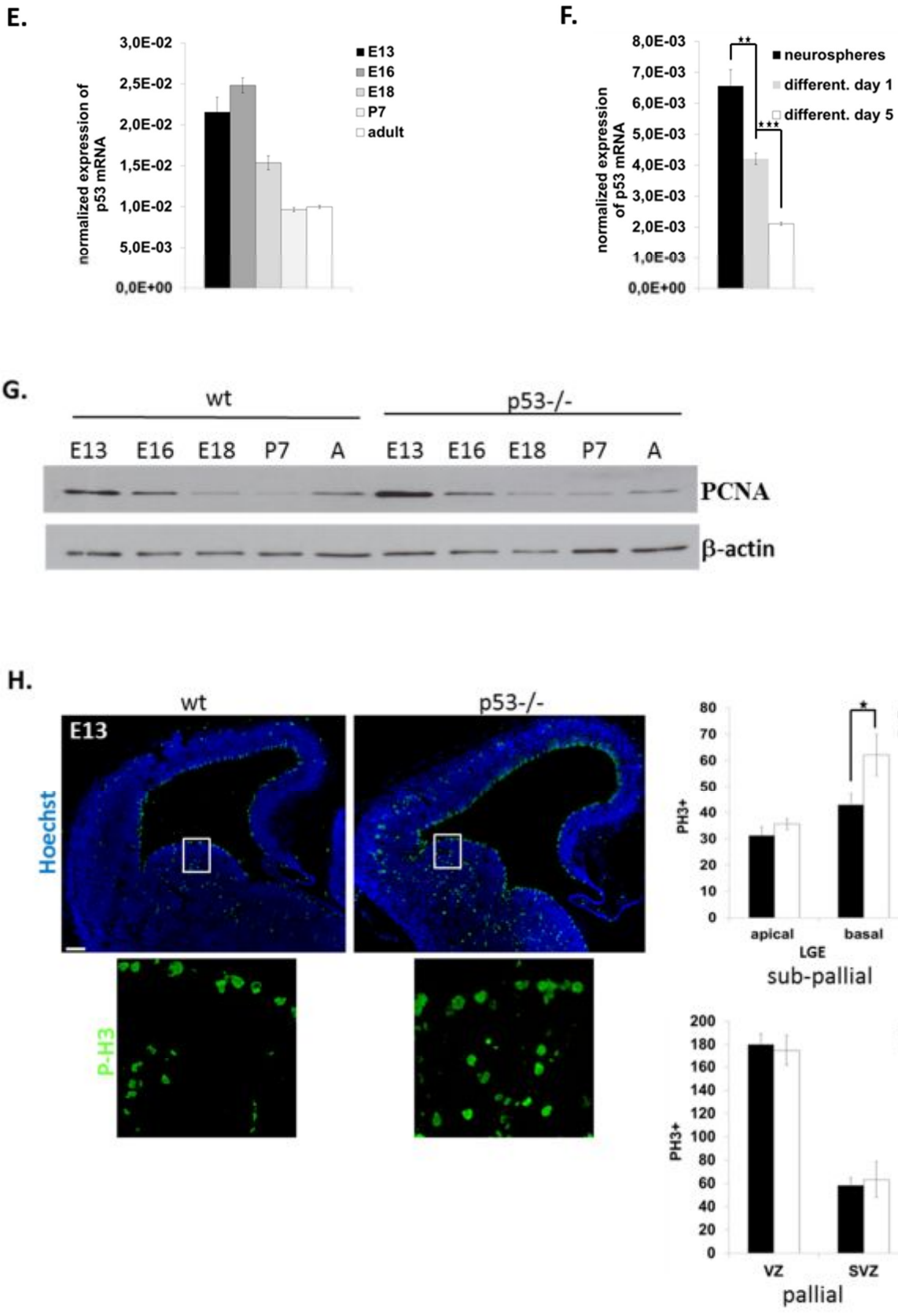
young neurons. In line with this, treatment of neurospheres in proliferation conditions with LY had a long lasting negative affect on the number of P-Akt+ cells produced upon differentiation. Conversely, prepriming of neurospheres during proliferation phase with H_2O_2 resulted in a higher abundance of P-Akt+ differentiating cells (Fig. 10.D). The importance of the PI3K-Akt signalling for neuronal differentiation is highlighted by the fact that young neurons were sensitive to modulation of this signalling route both by exogenous peroxide and by LY treatment. Both induced extensive cell death regardless of the genotype when applied to differentiating progenitors at the same concentration as to proliferative NPCs (data not shown).

Altogether, these data emphasize the importance of the PI3K-Akt signalling for neurogenesis/neuronal maturation and suggest that the increase in neurogenesis *in vitro* and the premature onset of neurogenesis as indicated by elevated DCX and GAD65/67 levels *in vivo* are at least in part due to a more oxidative environment and increased Akt dependent signalling in p53-/- neural progenitors. Furthermore, they also imply that the elevated ROS-PI3K-Akt signalling could provide a compensatory mechanism which prevents overproliferation by inducing neurogenesis in p53-/- embryos.

FIGURES AND FIGURE TEXTS

Figure 1.

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E13
E16
E18
P7
adult
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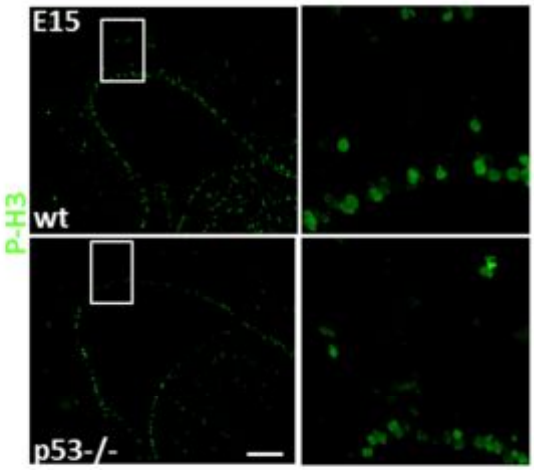
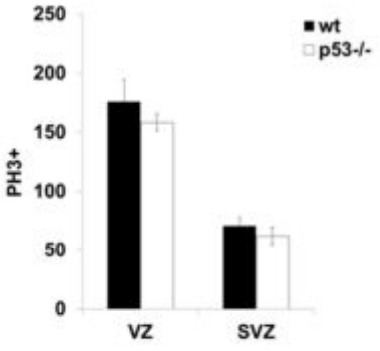
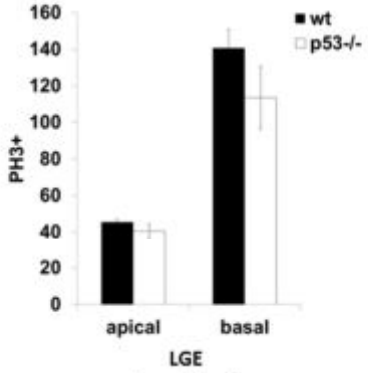
**
***
neurospheres
different. day 1
different. day 5
G.
wt
p53-/-
E13 E16 E18 P7 A E13 E16 E18 P7 A
PCNA
β-actin
H.
wt
p53-/-
E13
Hoechst
P-H3
PH3+
80
70
60
50
40
30
20
10
0
*
wt
p53-/-
apical
basal
LGE
sub-pallial
PH3+
200
180
160
140
120
100
80
60
40
20
0
wt
p53-/-
VZ
SVZ
pallial

I.



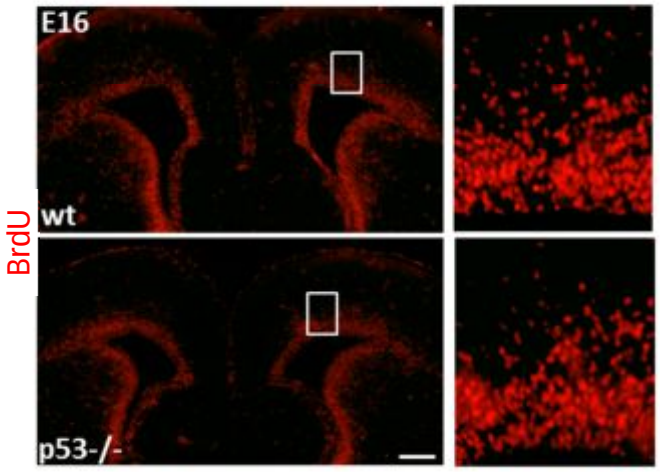


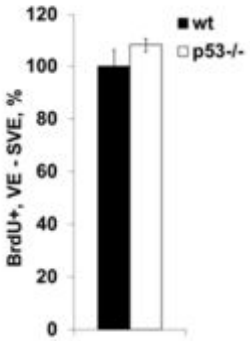
pallial



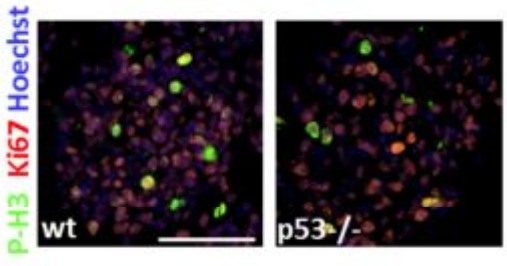
sub-pallial

J.

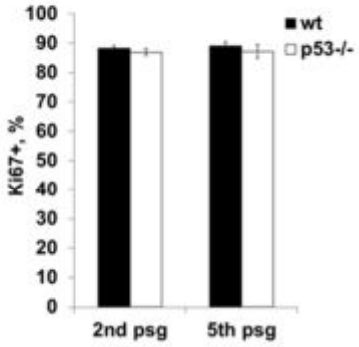




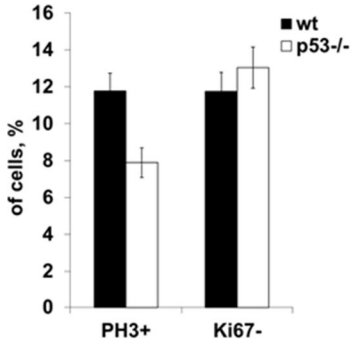
K.


P-H3 Ki67 Hoechst
wt
p53-/-

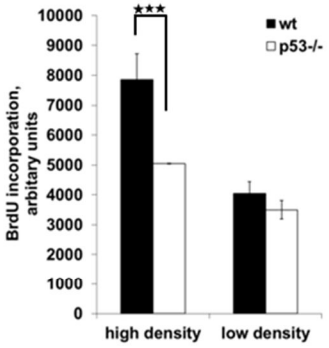
K_1.


wt
p53-/-
Ki67+, %
100
90
80
70
60
50
40
30
20
10
0
2nd psg
5th psg

K_2.


wt
p53-/-
of cells, %
16
14
12
10
8
6
4
2
0
PH3+
Ki67-

L.


***
wt
p53-/-
BrdU incorporation, arbitary units
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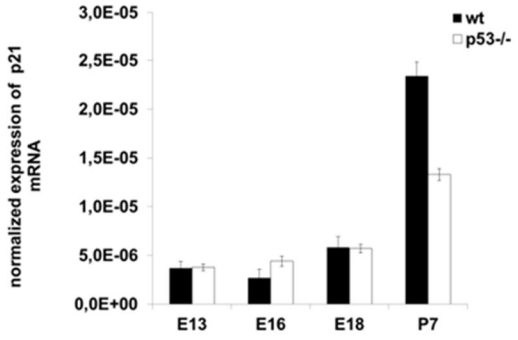
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
high density
low density

M.


wt
p53-/-
normalized expression of p21 mRNA
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1,0E-05
5,0E-06
0,0E+00
E13
E16
E18
P7

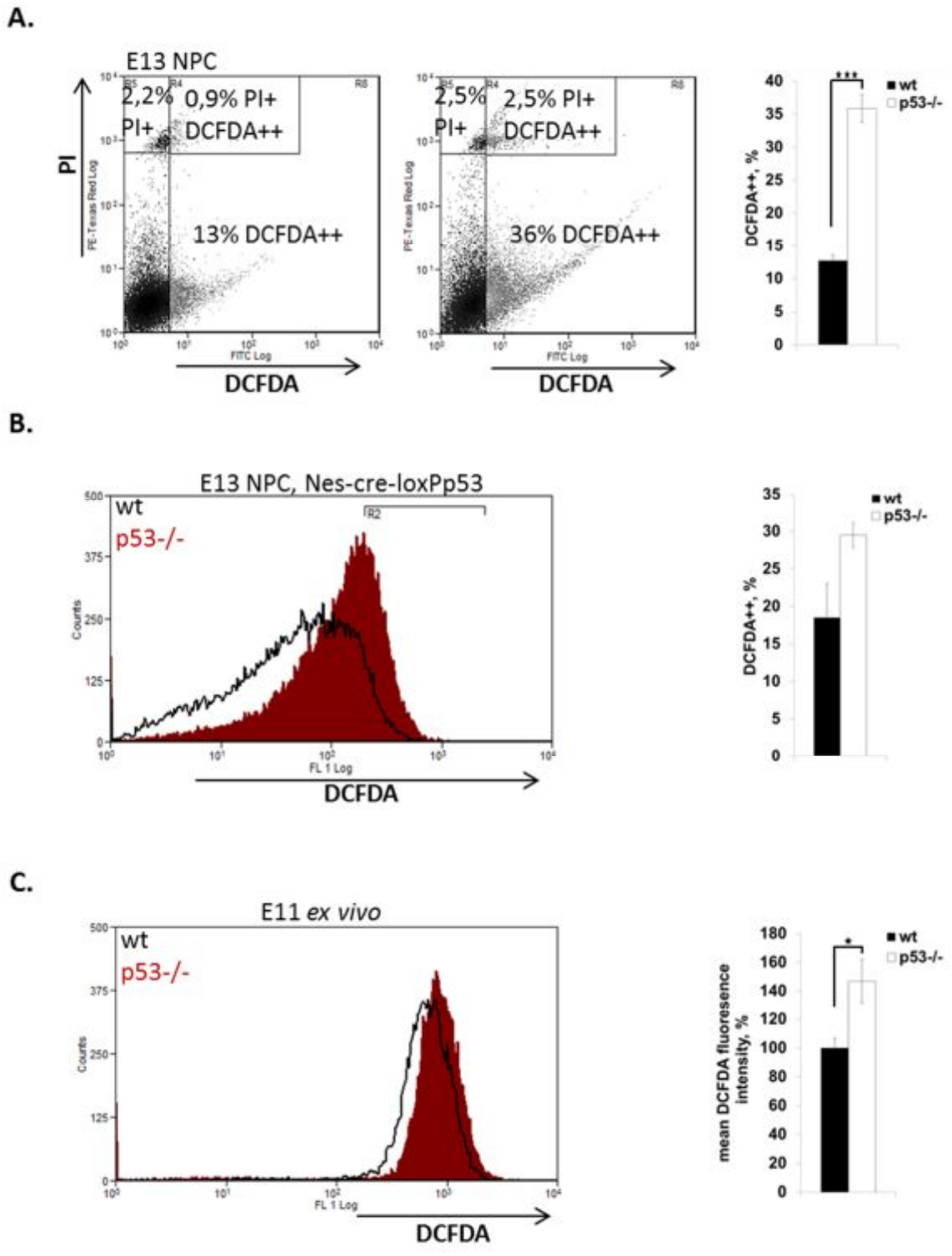
Figure 1. The lack of p53 *in vivo* leads to a transient initial increase in proliferation in the sub-pallium

A. Immunohistochemistry (IHC) for p53 (green) on E11 telencephalons. P53 immunoreactivity is ubiquitous at E11 telencephalons yet decreasing in the preplate that is at the outmost layer of the E11 telencephalon. Scale bar 50μm. **B.** P53 immunoreactivity is detectable in the germinal zones of E13 mouse telencephalons that is in regions where proliferative neural progenitors reside. Notably, the intensity of p53 signal decreases considerably towards the cortical plate. Scale bar 100μm. **C.** IHC for nestin, a NPC marker, on E13 telencephalons. The germinal regions, which are strongly positive for p53, are also immunoreactive for nestin. Scale bar 100μm. **D.** E13 neurospheres co-stained for the pan-proliferative marker Ki67 (red) and for p53 (green). The great majority of p53+ NPCs are also Ki67+ that is they are cycling cells. Importantly, there is no detectable p53 immunoreactivity in p53-/- neurospheres. In the graph the percent mean ± sem of Ki67+, p53+ and Ki67+/p53+ double positive cells in neurospheres from two unrelated experiments is shown. Scale bar 100μm. **E.** Quantification of p53 mRNA amounts across pre- and postnatal telencephalic development by qRT-PCR. P53 mRNA is most abundant at E13-E16 that is during the prenatal neurogenic period decreasing thereafter considerably. Mean p53 mRNA expression normalized to β-actin is shown. Three embryos per developmental stage and genotype were examined. Each experiment was conducted as triplicates. Error bars are ±sd. **F.** Reminiscent of the *in vivo* p53 staining pattern p53 *in vitro* mRNA levels decrease upon differentiation the highest amount of p53 mRNA being expressed in proliferative neurospheres when assessed by quantitative RT-PCR. Data represent mean p53 mRNA expression normalized to β-actin ±sd from two unrelated experiments each triplicates (** $p<0.01$, *** $p< 0.001$ by two-tail t test). **G.** Quantification of the abundance of the pan-proliferative marker PCNA in pre- and postnatal telencephalic samples. P53-/- telencephalons display a transient increase in PCNA protein at E13 compared to wild type. 2-3 telencephalons were examined per developmental stage and genotype. Representative immunoblot is shown. **H.** Quantification of the mitosis

marker phospho-histone-3+ (P-H3) (green) cells in the VZ and SVZ and in the lateral ganglionic eminence (LGE) on coronal sections of wild type and p53-/- E13 telencephalons. The number of basal mitoses is increased in the LGE on p53-/- telencephalons. Data show the mean number of P-H3+ cells ±sem for each germinal zone as indicated when counted from 3 embryos per genotype and 3 sections per each embryo. The distance of successive sections counted was 40μm. ($\star$ $p < 0.05$ by two-tail t test). Scale bar 100μm. **I.** Quantification of the mitosis marker phospho-histone-3 (P-H3)+ (green) cells in the VZ and SVZ and in the lateral ganglionic eminence (LGE) on E15 coronal sections of wild type and p53-/- telencephalons. The increase in P-H3+ cells detected in p53-/- E13 sub-pallial germinal zone is no longer present indicating normalization of sub-pallial proliferation. There is no difference in the number of P-H3+ cells in the pallial germinal zone (the VZ-SVZ) either. The data show the mean of P-H3+ cells ±sem for each germinal zone as indicated when counted from 3 embryos per genotype and 3 sections per each embryo. The distance of successive sections assessed was 40μm. Scale bar 100μm. **J.** Immunohistochemistry for BrdU (red) on E16 sectioned wild type and p53-/- telencephalons. Quantification of BrdU+ cells in the VZ-SVZ of E16 wild type and p53-/- telencephalons does not reveal a difference in the number of S-phase (BrdU+) cells between genotypes. Pregnant dams were sacrificed after 1hr BrdU pulse and embryonic brains were dissected for IHC. Data show percent mean of BrdU+ cells ±sem for the VZ-SVZ when counted from 3 embryos per genotype and 3 sections per each embryo. The distance of successive sections counted was 40μm. Scale bar 100μm. **K.** P53-/- NPCs do not exhibit increased proliferation compared to wild type according to P-H3/Ki67 **i**mmunocytochemistry. E13 neurospheres were stained for mitosis marker P-H3 (green) and for pan-proliferative marker Ki67 (red). Nucleus was stained with hoechst (blue). Scale bar 100μm. $\mathbf{K_1}$. There is no difference in the total number of proliferative cells between the genotypes across successive passages. $\mathbf{K_2}$. Yet, the number of PH3+ cells is lower in p53-/- neurospheres, an indication of the increase in cell cycle length. In $\mathbf{K_1}$ and $\mathbf{K_2}$ data

are presented as percent mean of Ki67+ or P-H3+ cells in neurospheres ±sd from three independent experiments. **L.** P53-/- neurospheres (nes-cre/loxPp53 embryos) display at higher cell densities reduced incorporation of BrdU to DNA compared to the wild type. However, at lower cell density there is no difference between the genotypes. Data show mean BrdU incorporation in arbitrary units ±sem from 3 independent experiments. (*** $p < 0.001$ by two-tail t test). **M.** P21 mRNA is expressed at lower level during prenatal development, yet up-regulated at P7. Moreover, no difference in p21 expression can be detected prenatally between genotypes, while postnatally p21 is downregulated in p53-/- telencephalons. qRT-PCR quantification data represent mean p21 mRNA expression normalized to β-actin ±sd from two unrelated experiments each triplicates.

Figure 2.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E13 NPC
2,2% PI+
0,9% PI+ DCFDA++
13% DCFDA++
PI
PE-Texas Red Log
FITC Log
DCFDA
2,5% PI+
2,5% PI+ DCFDA++
36% DCFDA++
DCFDA++, %
wt
p53-/-
***
B.
E13 NPC, Nes-cre-loxPp53
wt
p53-/-
Counts
FL 1 Log
DCFDA
C.
E11 ex vivo
mean DCFDA fluoresence intensity, %
*

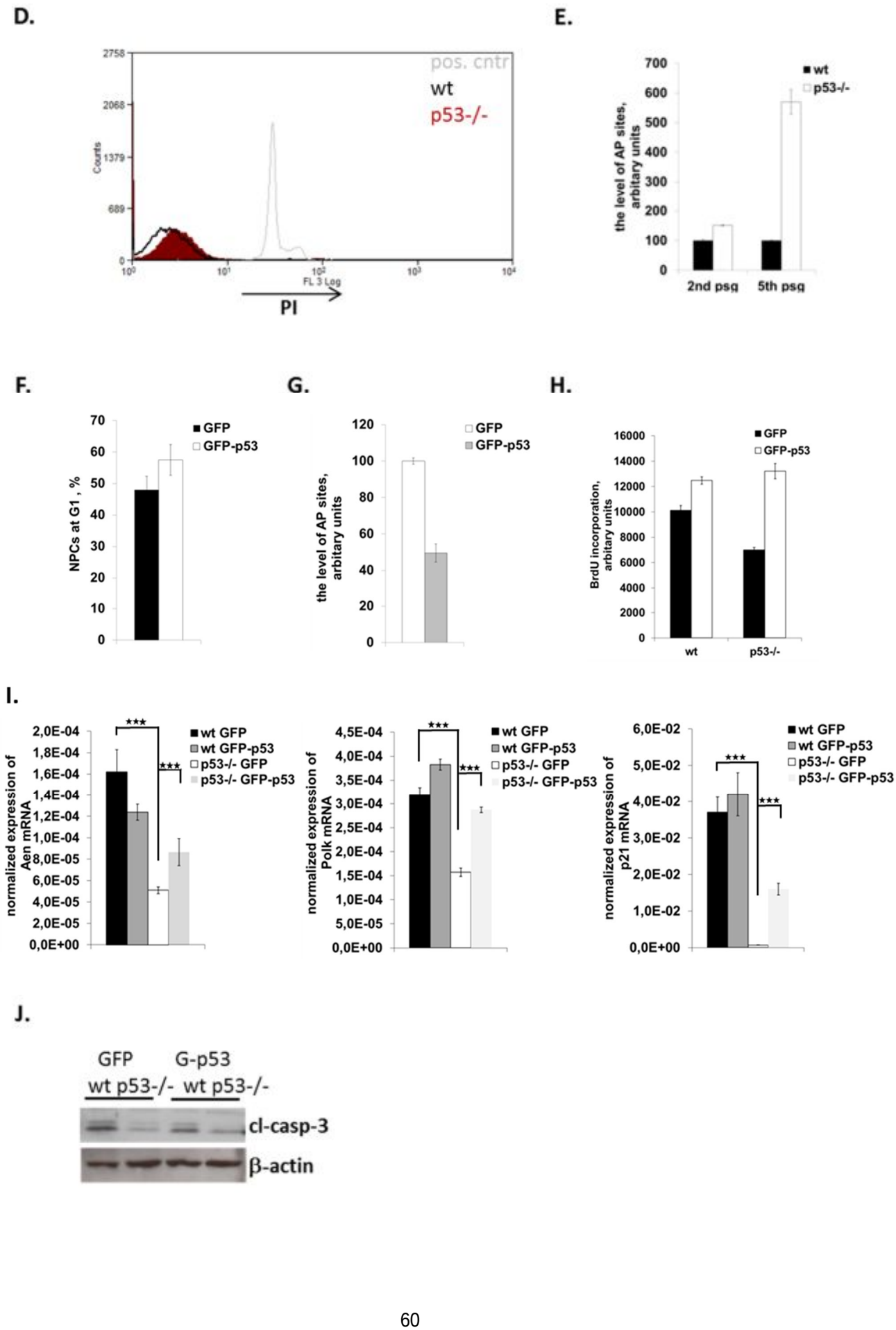
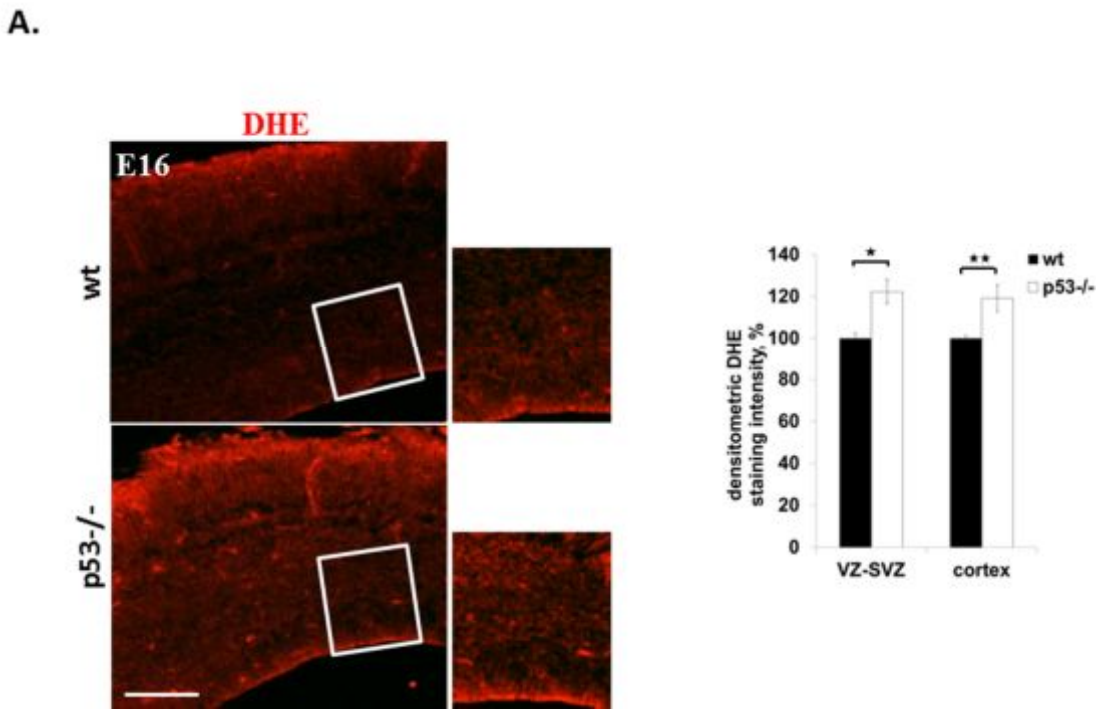

D.
pos. cntr
wt
p53-/-
Counts
FL 3 Log
PI
E.
wt
p53-/-
the level of AP sites, arbitary units
2nd psg
5th psg
F.
GFP
GFP-p53
NPCs at G1 , %
G.
GFP
GFP-p53
the level of AP sites, arbitary units
H.
GFP
GFP-p53
BrdU incorporation, arbitary units
wt
p53-/-
I.
wt GFP
wt GFP-p53
p53-/- GFP
p53-/- GFP-p53
normalized expression of Aen mRNA
normalized expression of Polk mRNA
normalized expression of p21 mRNA
***
J.
GFP
G-p53
wt p53-/- wt p53-/-
cl-casp-3
β-actin

Figure 2. P53-/- NPCs and differentiating young neurons exhibit an increase in cellular ROS both *in vitro* and *in vivo* and are prone to DNA damage

A. FACS for the ROS indicator chloromethyl 2'7'-dichlorodihydrofluorescein diacetate (CM-DCFDA) and for propidium iodide (PI detects dead cells) on cultured E13 NPCs. P53-/- NPCs exhibit an increase in DCFDA but not in PI staining intensity. Data are presented as mean ±sem, from 3 independent experiments. The graph shows the mean percentage of intensively DCFDA positive (DCFDA++) cells per genotype. Wt 13% DCFDA++ (sem ±0,9), p53-/- 36% DCFDA++ (sem ±2,1), Student´s two-tail t test *** P=0,001 . Wt DCFDA++/PI+ 0,9% (sem ±0,4), p53-/- DCFDA++/PI+ 2,5% (sem ±0,5), Wt PI+ 2,2% (sem ±0,3), p53-/- PI+ 2,5% (sem ±0,6). **B.** E13 *in vitro* p53-/- NPCs from nes-cre/loxPp53 embryos (p53 deleted only in nestin+ cells) exhibit increased DCFDA staining intensity compared to the wild type control when investigated by FACS. Data represent percent mean of intensively DCFDA positive (DCFDA++) cells ±sem from 3 independent experiments. **C.** E11 p53-/- telencephalons exhibit higher ROS ex v*ivo* when the mean DCFDA staining intensity is assessed by FACS. Data are mean ±sem, Wt N=3, p53-/- N=4. Student's two-tail t test * P<0,05. **D.** Propidium iodide (PI) staining of dying and dead NPCs in the experiment shown in C. No significant difference can be detected in the number of PI+ NPCs between the genotypes. Representative experiment is shown. N=3. **E.** The number of apurinic/apyrimidinic (AP) sites is elevated in E13 p53-/- neurospheres. Values are ±sd from two independent experiments, each performed as triplicates. **F.** Transduction of p53-/- NPCs with Ad-GFP-p53 accumulates p53-/- eNPCs to G1 phase of the cell cycle. FACS was performed on 7AAD stained (DNA dye), Ad-transduced p53-/- NPCs. Values are ±sd from two independent experiments. **G.** Transduction of p53-/- NPCs with Ad-GFP-p53 decreases the number of AP sites compared to Ad-GFP control transduction. Values are ±sd from two independent experiments, each performed as triplicates. **H.** Ad-GFP-p53 transduction of NPCs increases BrdU incorporation to DNA possibly indicating DNA repair related DNA

synthesis. Data represent mean ±sd from two independent experiments, each performed as triplicates **I.** Ad-GFP-p53 transduction of p53-/- NPCs induces expression of DNA repair/damage related genes and the classical p53 target p21/Cdkn1a. Data represent mean mRNA expression normalized to β-actin ±sd from two independent experiments, each performed as triplicates. (⋆⋆⋆ p< 0.001 by two-tail Student's t test). **J.** Transient exogenous p53 expression does not lead to the activation of the apoptotic pathway in Ad-GFP-p53 transduced NPCs compared to the control Ad-GFP transduced cells as immunoblotting for cleaved caspase-3 shows. Representative blot is shown. N=2.

Figure 3.



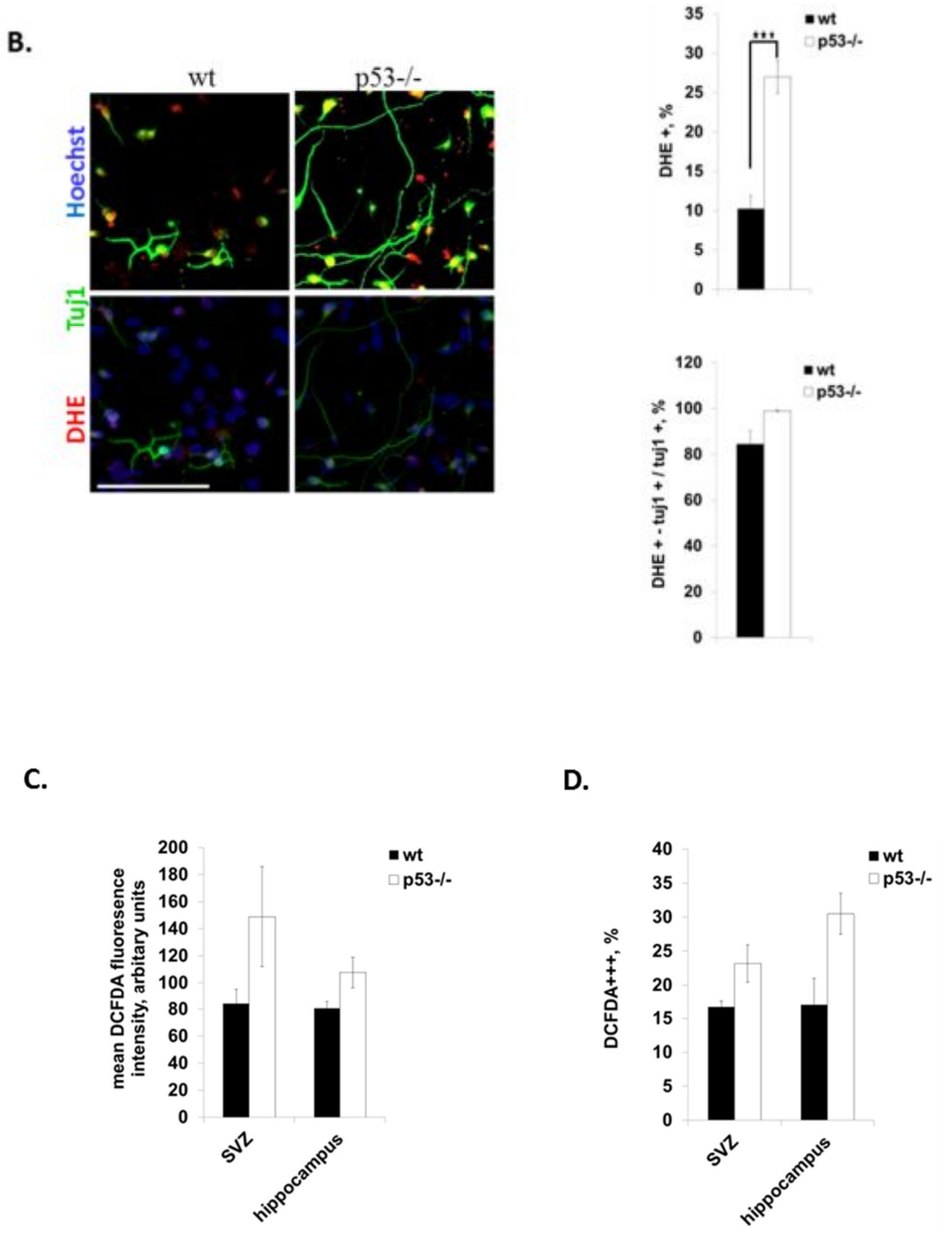
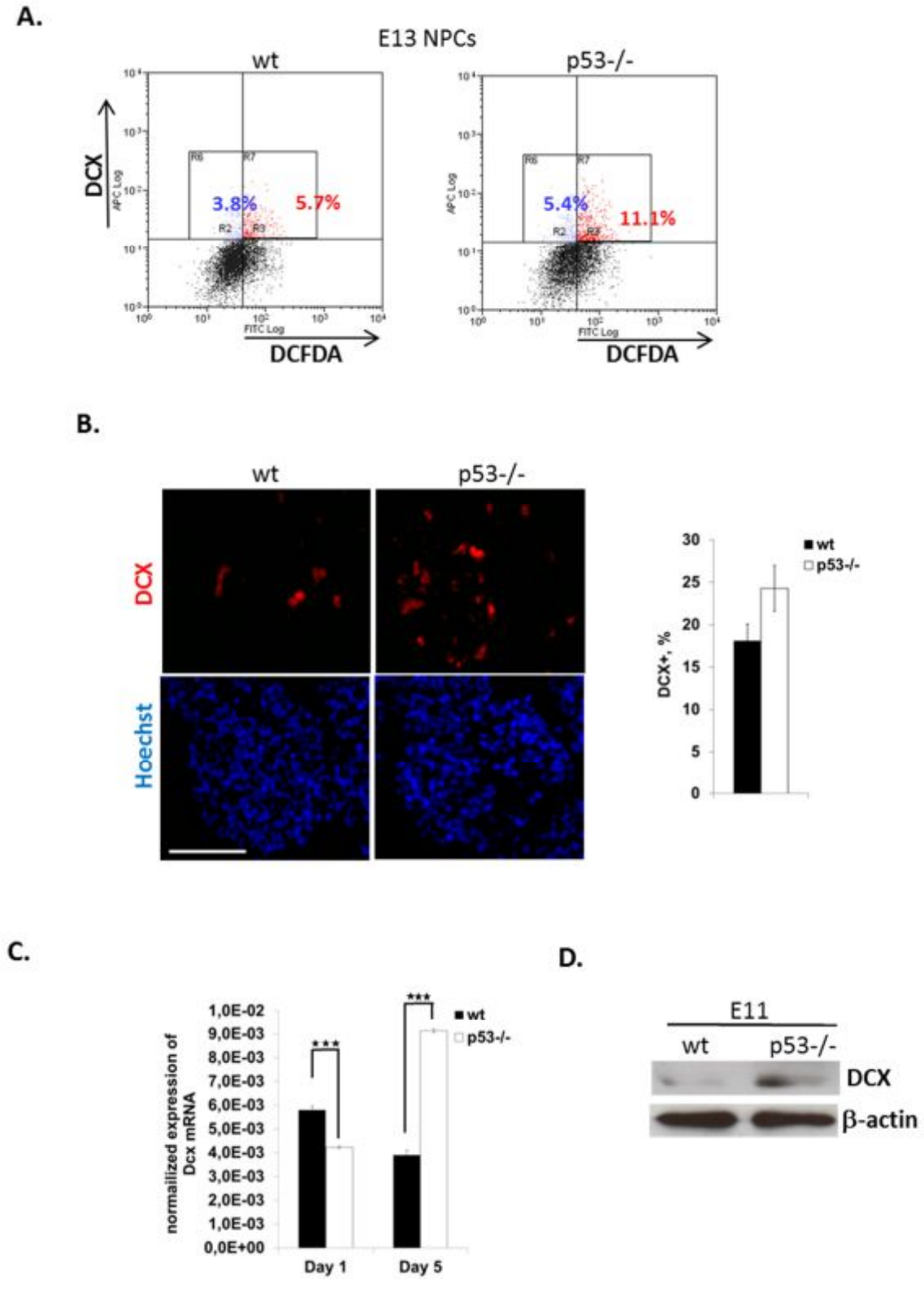


Figure 3. Young maturing neurons exhibit high ROS, yet p53-/- neurons display even further elevation in reactive oxygen species

A. One hour pulse of dihydroethidium (DHE) in *in vivo* E16 Nes-cre/loxPp53 embryos reveals that both the VZ and the cortical plate of p53-/- telencephalons have higher abundance of ROS compared to wild type telencephalons. Data show densitometric quantification of DHE signal intensity presented as percent mean ±sem. Wt N=4, p53-/- N=3. (Student's two-

tail t test VZ-SVZ * P<0.05, cortex ** P<0.01.) Scale bar 100μm. **B.** Staining of differentiating E13 NPCs for the neuron marker tuj1 (green), for the ROS indicator DHE (red) and the nuclear stain hoechst (blue) on day 5. The total number of DHE+ cells is higher in the p53-/- background, an indication of elevation in ROS also in differentiating p53-/- NPCs. Notably, the great majority of young tuj1+ neurons are also DHE+ demonstrating that ROS is characteristic of young neurons in particular. Values represent percent mean ±sd from three independent experiments. (*** p< 0.001 by two-tail Student's t test.) Scale bar 100μm. **C.** and **D.** *Ex vivo* ROS measurement of adult neurogenic zones of wild type and p53-/- mice. Both the SVZ and hippocampus of p53-/- 4-5 months old adult male mice exhibit an increase in ROS compared to the wild type mice of the same age. FACS was performed on dissociated tissue after *ex vivo* staining with DCFDA, a ROS indicator. **C.** Mean DCFDA staining intensity of the dissociated tissue in arbitrary units as indicated ±sem is shown. **D.** The percent mean of intensively DCFDA positive (DCFDA++) cells in the SVZ and hippocampus ±sem is presented. wt N=3, p53-/-N=6.

Figure 4.


A.
E13 NPCs
wt
p53-/-
DCX
APC Log
FITC Log
DCFDA
3.8%
5.7%
5.4%
11.1%
B.
wt
p53-/-
DCX
Hoechst
DCX+, %
C.
normailized expression of Dcx mRNA
1,0E-02
9,0E-03
8,0E-03
7,0E-03
6,0E-03
5,0E-03
4,0E-03
3,0E-03
2,0E-03
1,0E-03
0,0E+00
Day 1
Day 5
***
D.
E11
wt
p53-/-
DCX
β-actin

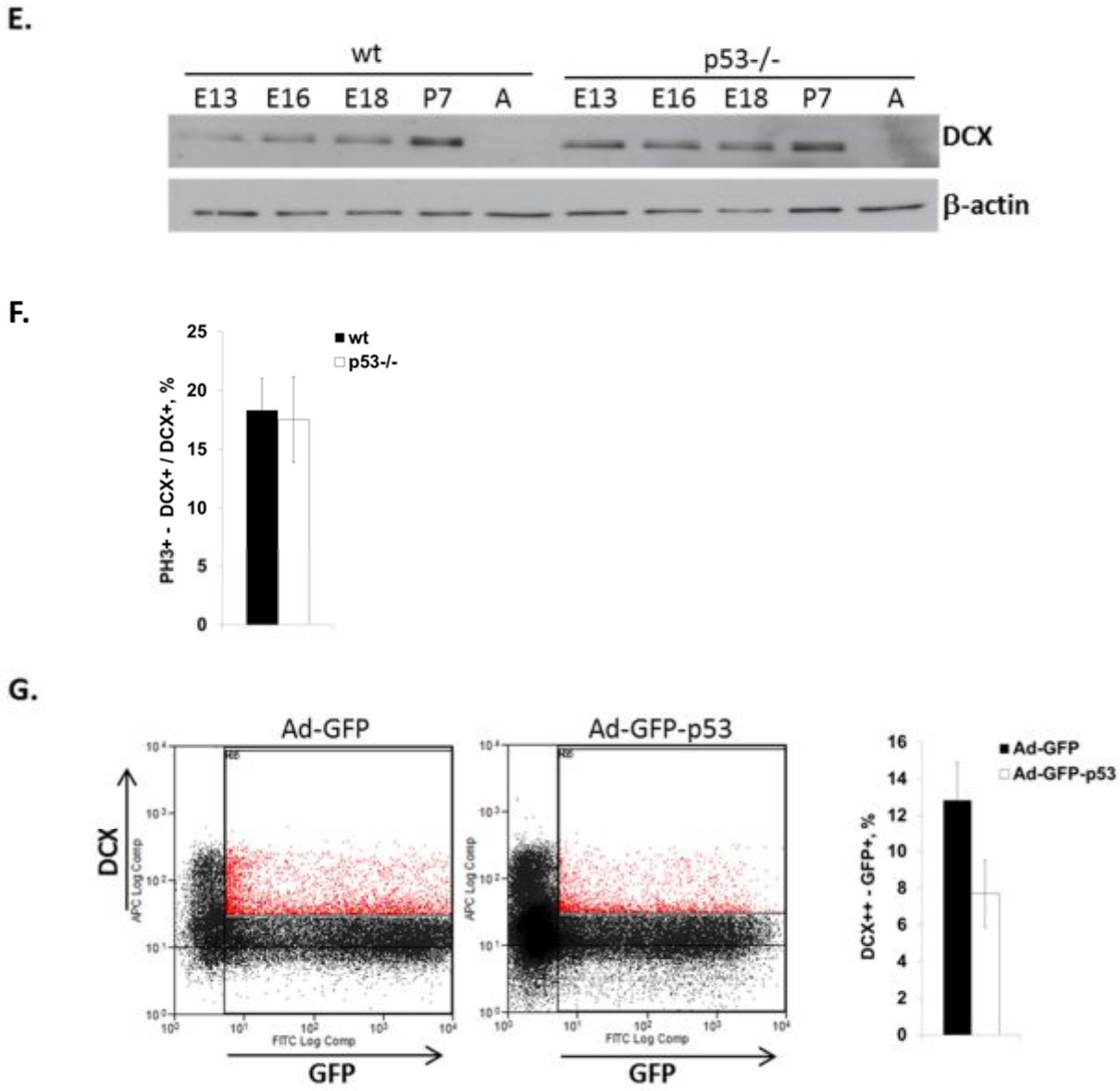
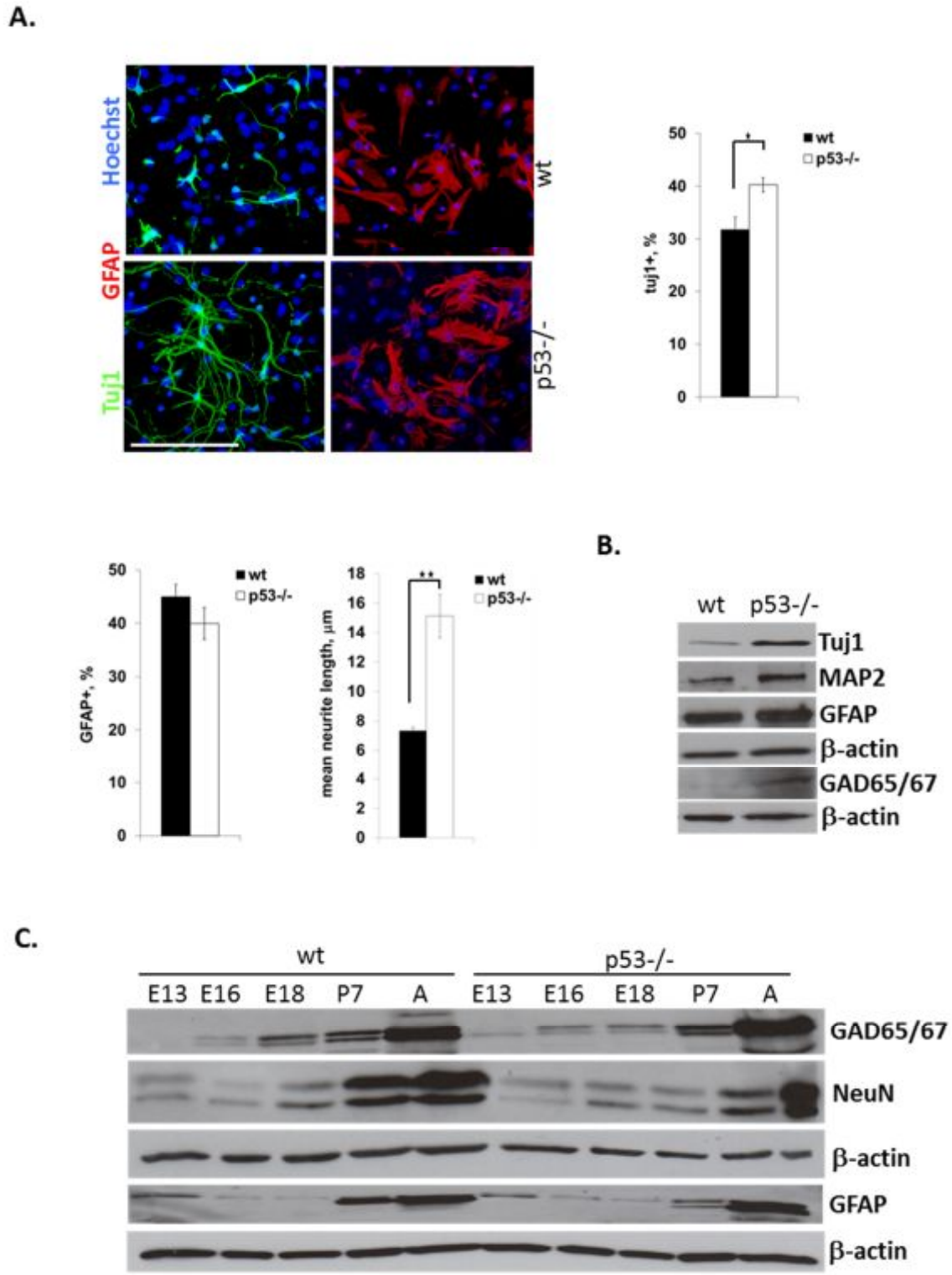


Figure 4. P53-/- telencephalons exhibit deviations in the timing of expression of neuroblast markers

A. FACS of DCFDA-DCX doublestained E13 neurospheres. P53-/- NPCs display an increase both in the total number of DCX+ cells and in the number of DCX-DCFDA double positive cells. Three independent experiments with similar results were conducted. Representative experiment is shown. **B.** Immunostaining of E13 neurospheres for neuroblast marker doublecortin (red). Nuclear staining hoechst in blue. A greater proportion of p53-/- NPCs are DCX+ compared to wild type. Data are presented as percent mean ±sd of DCX+ cells in

neurospheres in two unrelated experiments. Scale bar 100μm. **C.** Quantification of Dcx mRNA in differentiating NPCs by qRT-PCR. P53-/- NPCs exhibit *in vitro* significant elevation in Dcx mRNA also in differentiation conditions. Data represent mean mRNA expression normalized to β-actin ±sd, N=3, each in triplicates (⋆⋆⋆ $p < 0.001$ by two-tail Student's t test). **D.** E11 p53-/- telencephalons express higher amount of doublecortin protein according to immunoblotting. 2-3 telencephalons were examined per genotype and developmental stage. Representative experiment is shown. **E.** Immunoblotting of pre- and postnatal telencephalic lysates for doublecortin. The amount of DCX protein is elevated prenatally in p53-/- telencephalons. 2-3 telencephalons were used per developmental stage and genotype. Representative blot is shown. **F.** P-H3(green)/DCX(red) double staining of E13 neurospheres. The increase of DCX+ cells in p53-/- neurospheres does not result from increased proliferation of DCX+ p53-/- NPCs when compared to the wild type. Values represent percent mean ±sd, N=3. **G.** Ad-GFP-p53 transduction of proliferating p53-/- NPCs *in vitro* reduces the number of DCX+ NPCs according to FACS. Data are percent mean of DCX+/GFP+ double positive NPCs of all GFP+ cells ±sem, N=3.

Figure 5.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Hoechst
GFAP
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p53-/-
GFAP+, %
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p53-/-
Tuj1
MAP2
GFAP
β-actin
GAD65/67
β-actin
C.
wt
p53-/-
E13 E16 E18 P7 A
E13 E16 E18 P7 A
GAD65/67
NeuN
β-actin
GFAP
β-actin

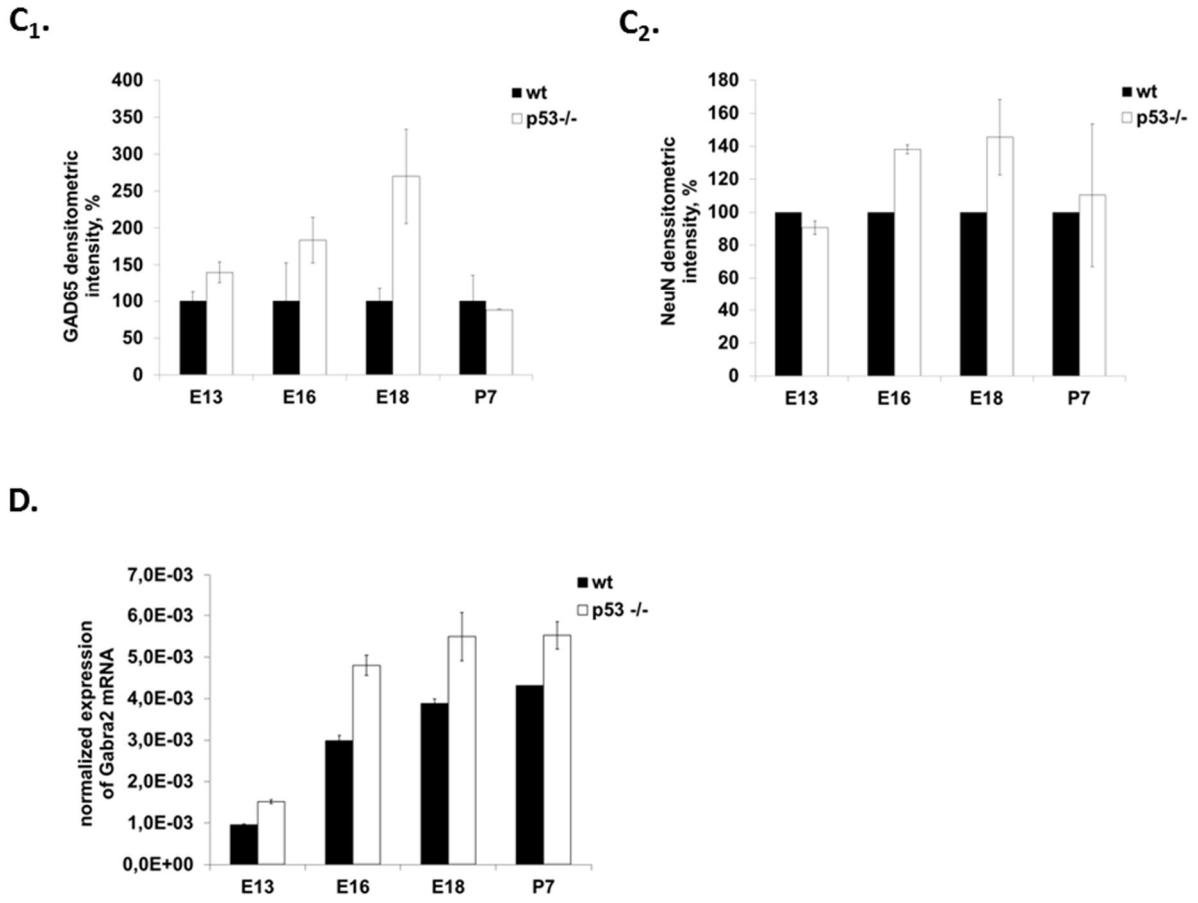


Figure 5. Neuronal differentiation is increased in p53-/- NPCs

A. *In vitro* differentiation of NPCs to main neural lineages. P53-/- NPCs do not have impairments in differentiation. They generate more tuj1+ young neurons and show also enhanced neurite extension. Differentiation to GFAP+ astrocytes is not impaired either. Differentiating cells were stained for neuron marker tuj1 (green) and astrocyte marker GFAP (red) and with nuclear stain hoechst (blue) on day 7. Values represent percent mean of tuj1+ neurons or GFAP+ astrocytes normalized to the total number of differentiating cells ± sd from four (tuj1) and 3 (GFAP) independent experiments. In neurite length measurements the mean neurite length measured from tuj1 stained differentiating NPCs cultures on day 7 from three experiments ±sem is shown. (* $p< 0.05$, ** $p< 0.01$ by two-tail Student's t test). Scale bar 100μm. **B.** Immunoblotting of *in vitro* differentiated NPCs (DIV7) for some differentiation markers as indicated. Neuronal differentiation is increased in p53-/- NPCs, whereas

astrogliogenesis is not affected. B-actin is used as a loading control. Similar results were obtained in independent experiments. Representative blots are shown. **C.** Immunoblotting of pre- and postnatal telencephalic lysates for marker proteins as indicated. 2-3 telencephalons were used per developmental stage and genotype. Representative blots are shown. **$C_{1.}$ and $C_{2.}$** Densitometric quantification of immunoblots for GAD65/67 and NeuN. Both GAD65/67 and NeuN are elevated in pre- but not in postnatal samples. Shown is percent densitometric signal intensity ±sem when normalized to β-actin. N=2-3. **D.** qRT-PCR quantification of mRNA for Gabra2, a GABA-receptor subunit. Gabra2 mRNA is elevated in p53-/- telencephalons pre- and postnatally. Data represent mean mRNA expression normalized to β-actin ±sd from two independent experiments, each performed as triplicates. 2-3 embryos per genotype and developmental stage were examined.

Figure 6.

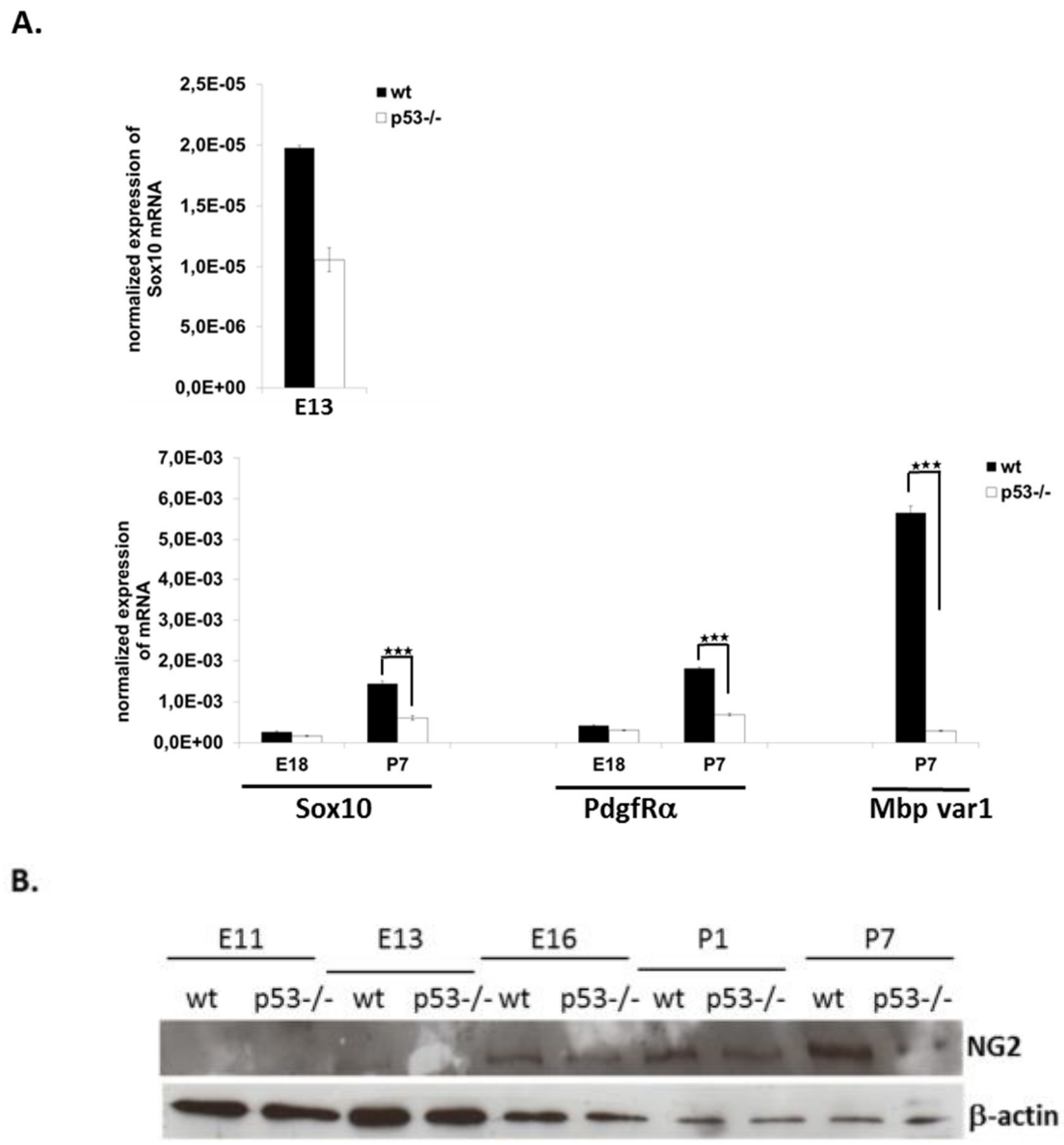
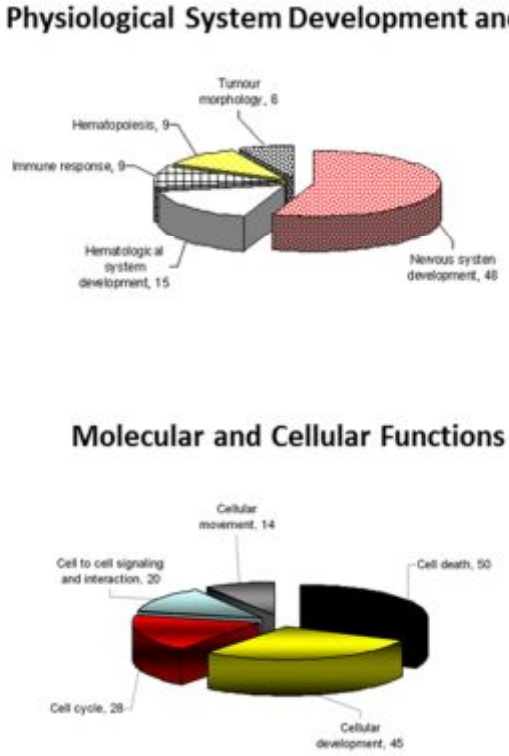


Figure 6. Decreased expression of OPC marker genes in p53-/- telencephalons

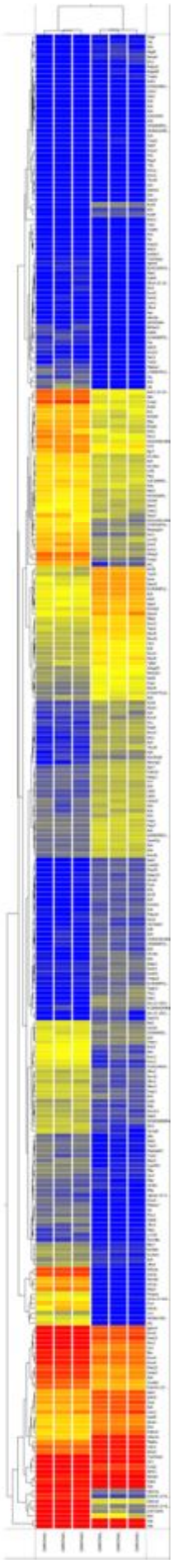
A. qRT-PCR quantification of the expression of some OPC and maturing oligodendrocyte marker genes. P53-/- telencephalons exhibit across the development reduced expression of genes related to oligodendrocyte lineage. Data represent mean mRNA expression normalized to β-actin ±sd from two unrelated experiments each conducted in triplicates. 2-3 embryos per developmental stage and genotype were examined (*** $p < 0.001$ by two-tail t test.) **B.** The expression of NG2, a chondroitin sulphate proteoglycan core protein, expressed by OPCs is reduced in p53-/- telencephalons. N=2-3 per developmental stage and genotype.

Figure 7.

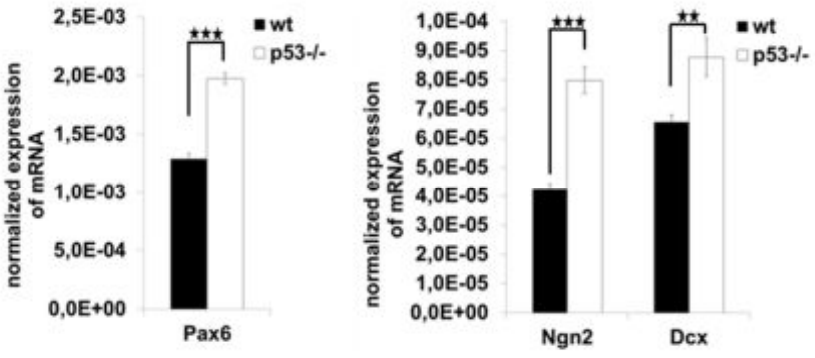
A.


Physiological System Development and Function
Tumour morphology, 6
Hematopoiesis, 9
Immune response, 9
Hematological system development, 15
Nervous system development, 48
Molecular and Cellular Functions
Cellular movement, 14
Cell to cell signaling and interaction, 20
Cell death, 50
Cell cycle, 28
Cellular development, 45

B.



C.


normalized expression of mRNA
2,5E-03
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1,5E-03
1,0E-03
5,0E-04
0,0E+00
***
wt
p53-/-
Pax6
normalized expression of mRNA
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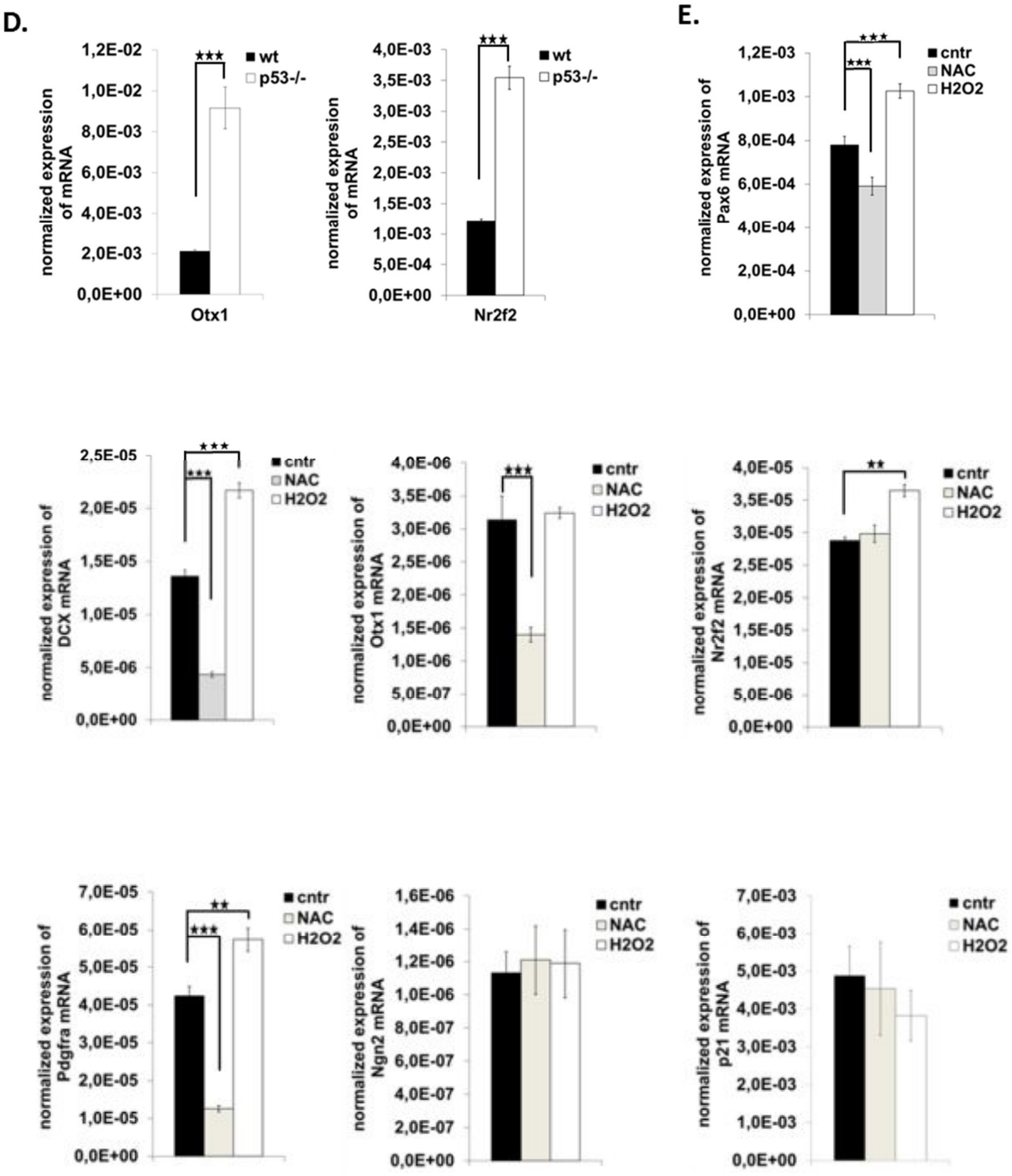
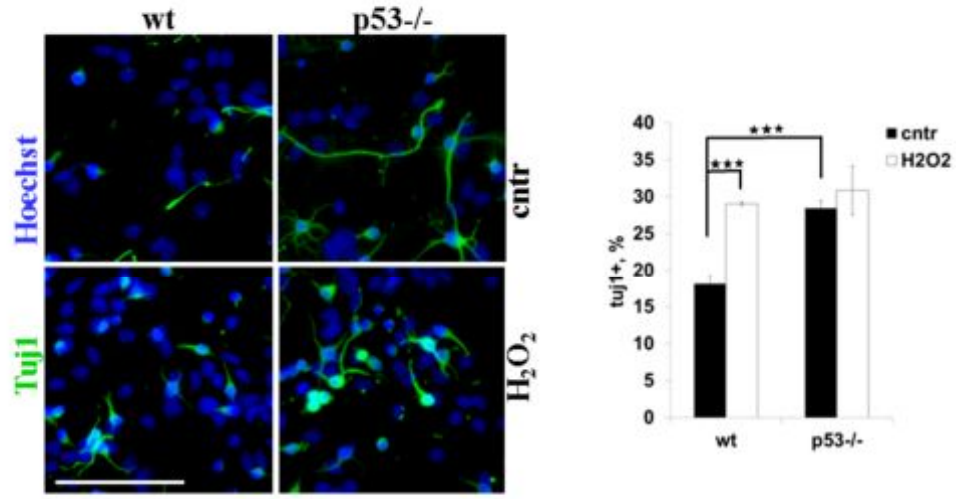
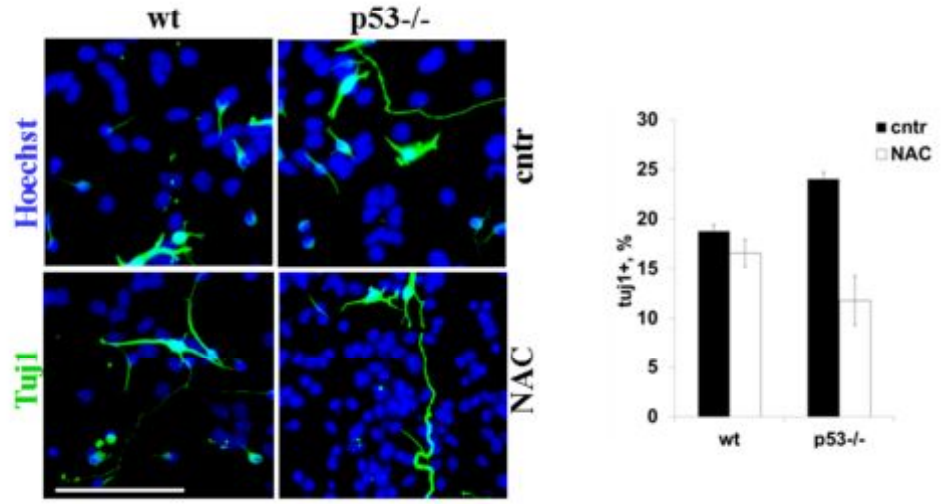
9,0E-05
8,0E-05
7,0E-05
6,0E-05
5,0E-05
4,0E-05
3,0E-05
2,0E-05
1,0E-05
0,0E+00
***
**
wt
p53-/-
Ngn2
Dcx


D.
E.
normalized expression of mRNA
wt
p53-/-
Otx1
Nr2f2
normalized expression of Pax6 mRNA
cntr
NAC
H2O2
normalized expression of DCX mRNA
normalized expression of Otx1 mRNA
normalized expression of Nr2f2 mRNA
normalized expression of Pdgfra mRNA
normalized expression of Ngn2 mRNA
normalized expression of p21 mRNA

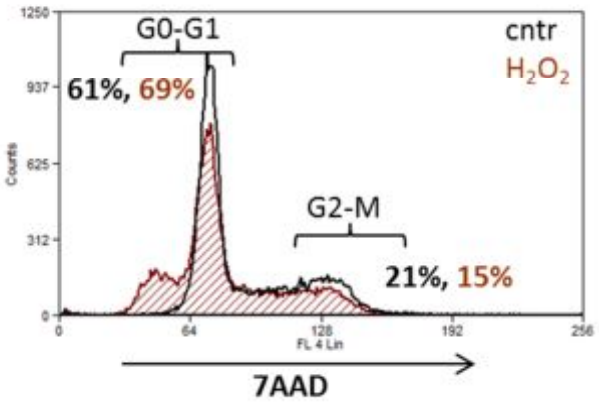
F.

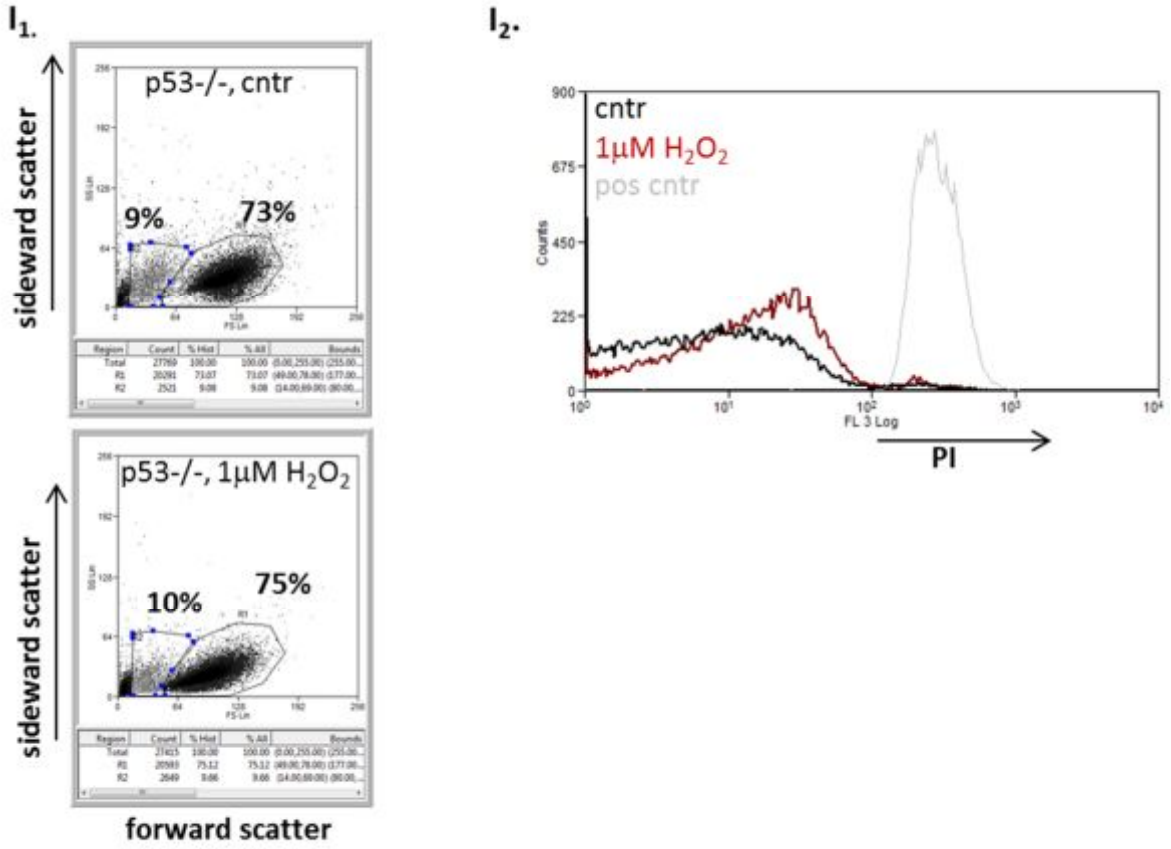

wt
p53-/-
Hoechst
Tuj1
cntr
H_2O_2
***
***
cntr
H2O2
tuj1+, %
40
35
30
25
20
15
10
5
0
wt
p53-/-

G.


wt
p53-/-
Hoechst
Tuj1
cntr
NAC
cntr
NAC
tuj1+, %
30
25
20
15
10
5
0
wt
p53-/-

H.


G0-G1
cntr
H_2O_2
61%, 69%
G2-M
21%, 15%
Counts
1250
937
625
312
0
0
64
128
192
256
FL 4 Lin
7AAD



J.

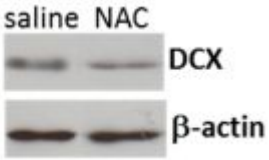


Figure 7. An increase in ROS drives neurogenesis

A. Ingenuity functional pathway analysis of the Affymetrix whole genome microarray gene expression data for genes involved in "physiological system development and function" identified genes related to nervous system development but also to hematological system development as the biggest groups of deregulated genes. Similar analysis for deregulated genes in the group "Molecular and cellular functions" according to Integuinity classification

recognized genes involved in cellular development as the largest group. Interestingly, a number of genes having a role in cellular movement were also deregulated according to Integuinity. **B.** Heat map of the deregulated genes according to Affymetrix analysis. **C.** Pax6, Ngn2 and Dcx mRNA are elevated in proliferating p53-/- NPCs according to qRT-PCR quantification. **D.** Otx1 and Nr2f2 mRNA expression is considerably higher in p53-/- differentiating NPCs on day 5 compared to wild type when assessed by qRT-PCR. In **C.** and **D**. data represent mean mRNA expression normalized to β-actin ±sd from three unrelated experiments, each in triplicates. (** $p< 0.01$, *** $p< 0.001$ by two-tail t test). **E.** Expression of genes which promote neurogenesis is responsive to redox regulation. mRNA quantification of a selection of neurogenic genes as indicated and of PdgfRα in control, antioxidant N-acetyl cysteine (NAC) and H_2O_2 treated neurospheres by qRT-PCR. NAC treatment decreases while H_2O_2 increases expression of the neurogenic genes selected. PdgfRα control gene expression is stimulated by H_2O_2 but reduced by NAC. Neither Ngn2 nor p21 expression is responsive to redox status manipulation. Data represent mean mRNA expression normalized to β-actin ±sem from three unrelated experiments, each in triplicates. (*** $p< 0.001$, ** $p< 0.01$ by two-tail Student's t test.) **F.** Pretreatment of neurospheres with H_2O_2 increases neurogenic differentiation in wild type but not in p53 null. Immunocytochemisry of H_2O_2 preprimed wild type and p53-/- differentiating neural progenitor cells for neuron marker tuj1 (green) and nuclear stain hoechst (blue) on day 5. Data represent percent mean of tuj1+ cells normalized to the total number of differentiating cells ±sem from three independent experiments. (*** $p< 0.001$ by two-tail t test.) Scale bar 100μm. **G.** Pretreatment of proliferative NPCs with antioxidant N-acetyl-cysteine (NAC) decreases neurogenic differentiation in p53-/- NPCs. Immunocytochemisry of NAC pretreated wild type and p53-/- differentiating neural progenitor cells for neuron marker tuj1 (green) and nuclear stain hoechst (blue) on day 5. Data presentation as in F. Scale bar 100μm. **H.** Cell cycle FACS (7AAD staining) of H_2O_2 treated

neurospheres. Exposure to 1μM H_2O_2 leads to the accumulation of NPCs in G1/G0 indicating lengthening of the cell cycle. Representative experiment is shown. N=3. **I.** Assessment of cell death in control and H_2O_2 treated NPCs. **I_1.** Exposure to 1μM H_2O_2 for 18hrs does not elicit any remarkable cellular deterioration according to cell size measurement by FACS. **I_2.** Exposure to 1μM H_2O_2 does not increase the number of dead and dying cells compared to control NPCs when analysed by propidium iodide (PI) staining. Permeabilized NPCs were used as a positive control. Representative experiment is shown. N=2. **J.** Immunoblotting of E16 p53-/- saline and NAC pretreated telencephalons for neuroblast/basal progenitor (BP) marker DCX. Pregnant dams were injected daily from E10 onwards either with saline or the antioxidant NAC. Embryos were sacrificed at E16. Antioxidant treatment decreases the amount of DCX protein. B-actin as a loading control. N=3.

Table 1. A selection of genes which according to Affymetrix gene expression analysis were dysregulated in p53-/- NPCs

Neurogenesis

Gabaergic	
Gad1	2.4 up
Gabra3	2.2 up
Glutamateric	
Grik2 (kainateR)	2.4 up
Grin3a (NR3a)	2.0 up
Grin2c (NR2c)	3.0 up
Gria3 (AMPAR)	4.4 down
Camk2a	2.5 up
Nrxn3	2.3 up
Otx1	3.4 up
Ngn2	4.3 up
Cholinergic	
Chrnb1	2.7 up
Dopaminergic	
Moxd1	2.2 up
Potassium channels	
Kcnj12	2.7 down
Kcnc1	4.8 down
Kctd4	4.7 up
Dpp10	2.0 up
Calcium channels	
Cacng5 (VGCC)	4.7 down

Redox regulation

Dcxr	24.7	down
Sesn2	3.1	down
Ephx1	2.1	down
Redd2/Ddit4l	5.2	down
Gpx7	2.3	up

DNA repair/Proliferation/Survival

Trp53inp1	3.1	down
Cdkn1a	26.5	down
Polk	2.1	down
Aen	4.7	down

Proliferation

Sox1	5.9	down
Lix1	10.1	down
Zic1	3.5	down
Zfhx3	3.1	down
Twist1	2.1	up
Glipr2	3.1	down
Cdkn2a	5.3	up
Wwox	2.1	up
Anxa6	2.1	up
Cdkn1a	26.5	down
Cdk6	2.3	up

Neurite extension/migration

Elavl4 (HuD)	2.8 up
E130309F12Rik (Prg3)	2.4 up
Dcx	2.8 up
Tagln3 (NP25)	3.1 up
Nr2f2	4.0 up
Gdap1l1	2.1 up
Gdap1	2.2 up

GEFs

Sipa1l1 (SPAR)	2.0 up
Rabif	2.4 up
Arhgef3	2.4 up
Cdc42ep3	2.3 up
Dock3 (PBP/ MOCA)	2.0 up
Gbp2	2.8 down

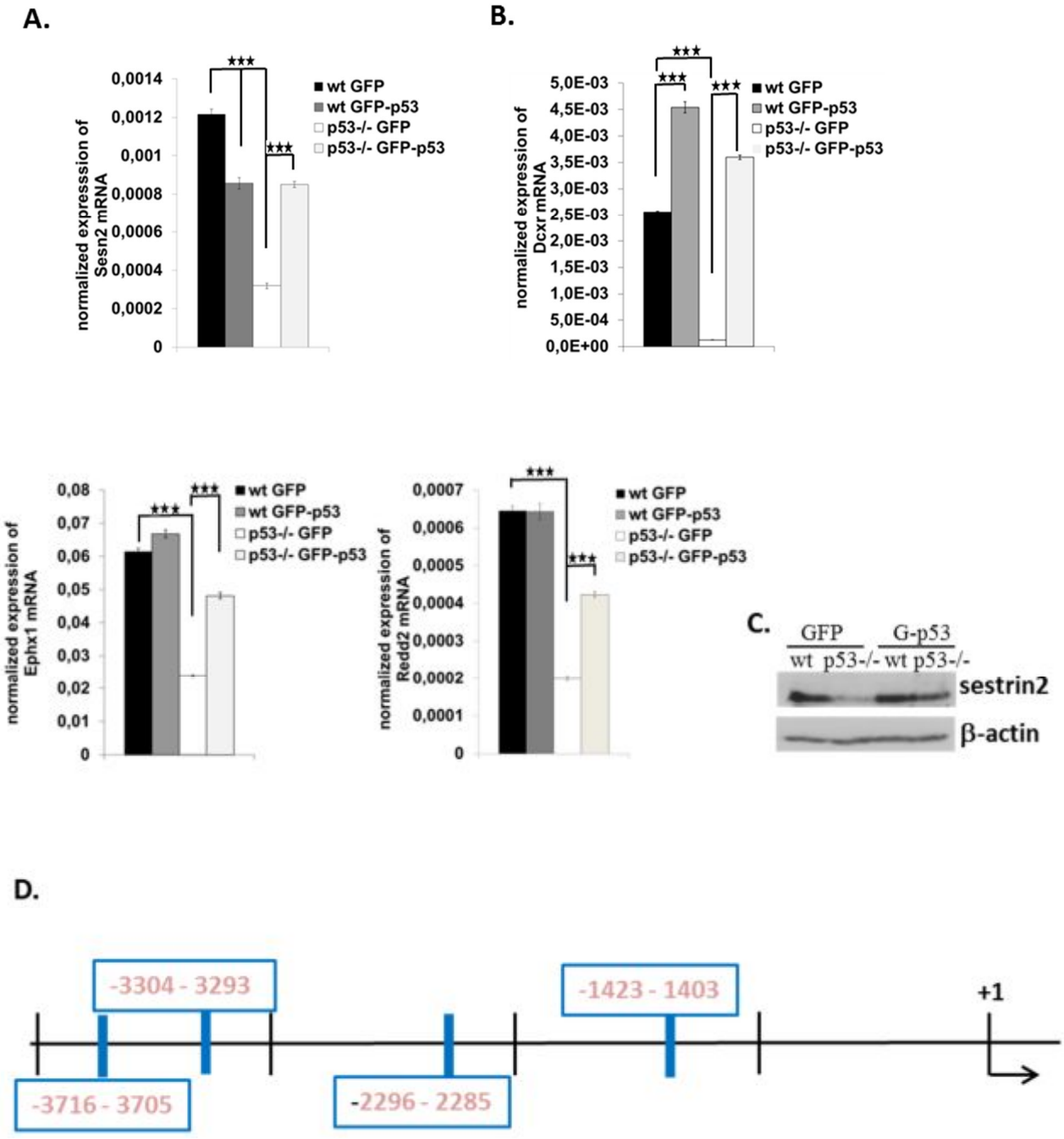
Oligodendrogenesis

Pdgfra	2.8 up
Mbp	2.3 down
Sox10	2.5 down
Plp1	2.0 down

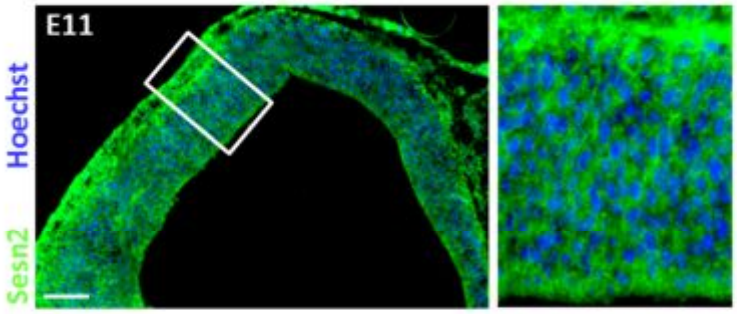
Apoptosis

Pmaip1	11.2 down
Bax	2.3 down
Bbc3 (PUMA)	2.8 down
Fas	3.7 down

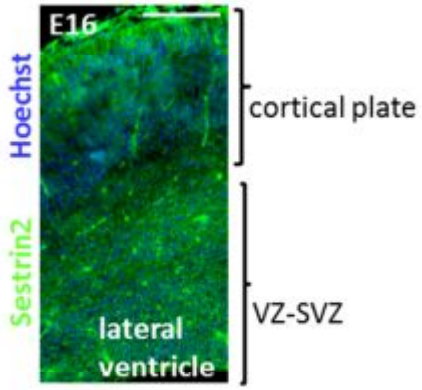
Figure 8.


A.
B.
normalized expresssion of Sesn2 mRNA
normalized expression of Dcxr mRNA
normalized expression of Ephx1 mRNA
normalized expression of Redd2 mRNA
wt GFP
wt GFP-p53
p53-/- GFP
p53-/- GFP-p53
***
C.
GFP
G-p53
wt p53-/- wt p53-/-
sestrin2
β-actin
D.
-3304 - 3293
-3716 - 3705
-2296 - 2285
-1423 - 1403
+1

E.


E11
Hoechst
Sesn2

F.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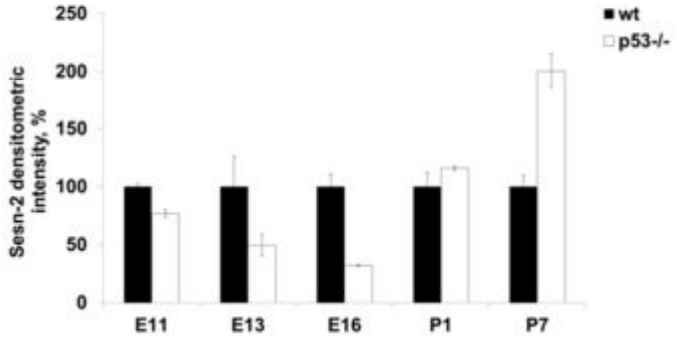
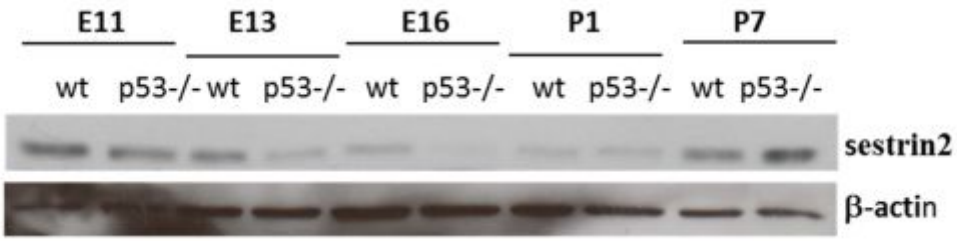
Hoechst
Sestrin2
cortical plate
VZ-SVZ
lateral
ventricle

G_1.


Sesn-2 densitometric intensity, %
250
200
150
100
50
0
E11
E13
E16
P1
P7
wt
p53-/-

G.


E11
E13
E16
P1
P7
wt
p53-/-
wt
p53-/-
wt
p53-/-
wt
p53-/-
wt
p53-/-
sestrin2
β-actin

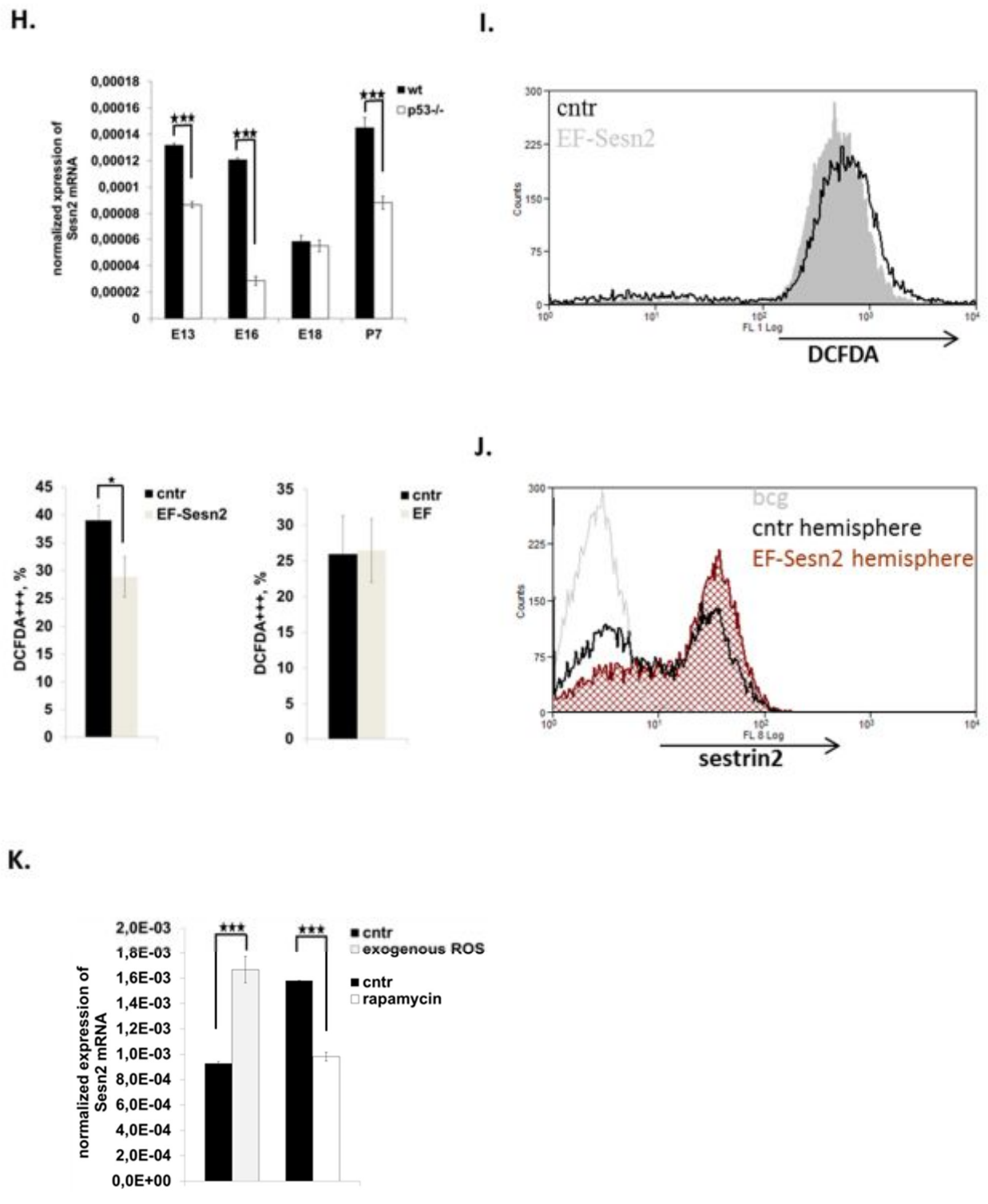


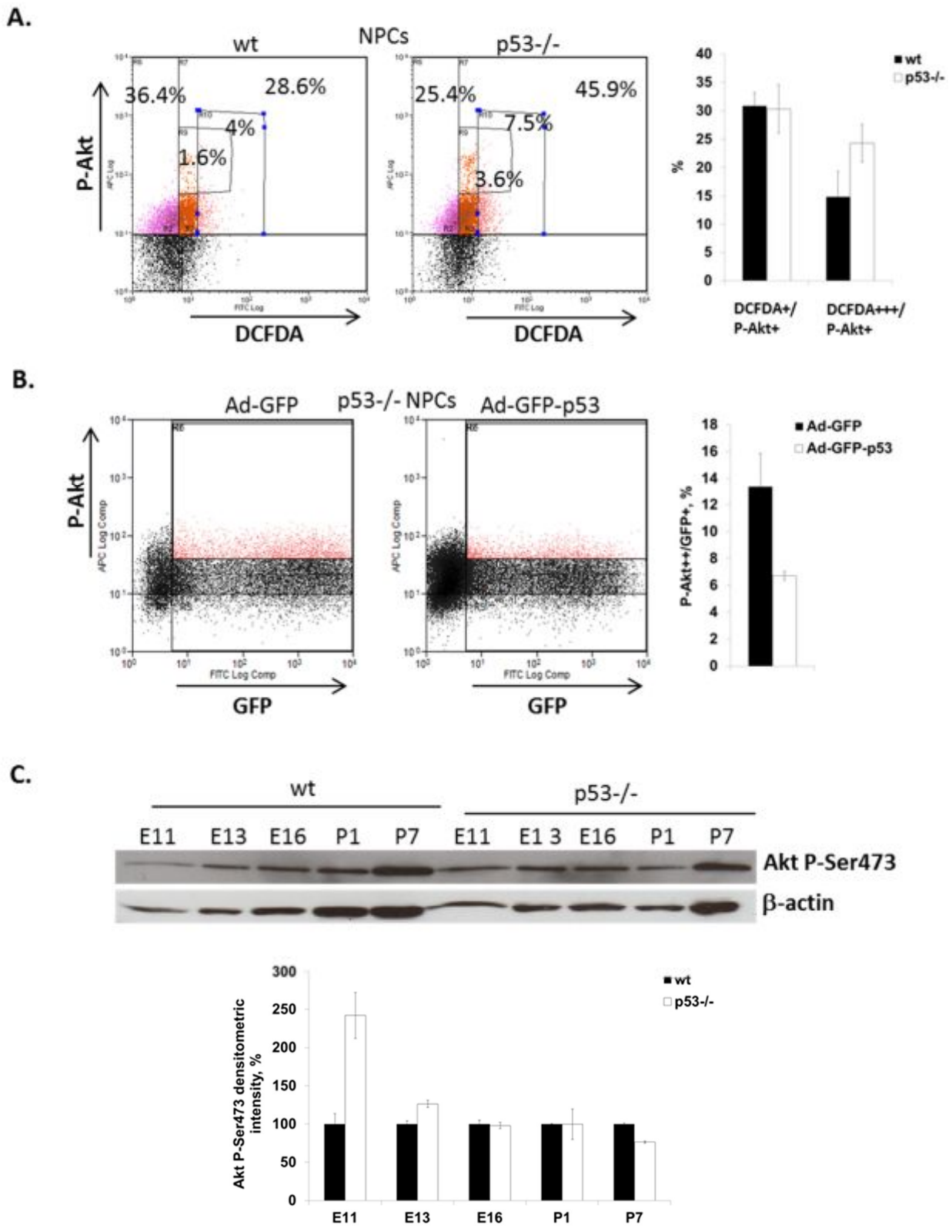
Figure 8. Sesn2, a p53 and mTOR pathway target gene, contributes to fine-tuning of redox homeostasis in embryonic telencephalons

A. Expression of Sesn2 mRNA is induced by exogenous p53 expression in p53-/- NPCs. Ad-GFP and Ad-GFP-p53 transduction of proliferative wild type and p53-/- NPCs. Mean mRNA expression normalized to β-actin ±sd is shown. Independent experiments were conducted as

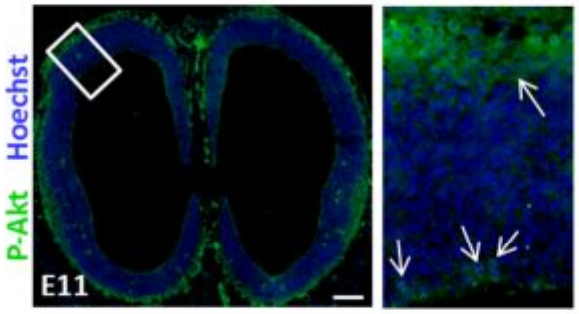
triplicates. (*** $p < 0.001$ by two-tail Student's t test.) **B.** Expression of the putative redox regulators Dcxr, Ephx1 and Redd2 mRNA upon Ad-GFP or Ad-GFP-p53 transduction of wild type and p53-/- proliferating NPCs. Exogenous p53 induces their expression in p53-/- NPCs to wild type levels when assessed by qRT-PCR. Mean mRNA expression normalized to β-actin ±sd is shown. Independent experiments were conducted as triplicates. (*** $p < 0.001$ by two-tail Student's t test). **C.** Immunoblotting of Ad-GFP and Ad-GFP-p53 transduced proliferative NPCs. Ad-GFP-p53 induces expression of sestrin2 protein in p53-/- NPCs to the same level as in the wild type. Experiment was performed twice from independent samples with similar results. Representative immunoblot is shown. **D.** Mouse Sesn2 promoter bears several consensus binding sites for p53, indicated by blue boxes, according to MatInspector *in silico* promoter analysis. **E**. and **F.** Immunohistochemistry for sestrin2 (green) on coronal sections of E11 and E16 telencephalons. At E11 sestrin2 is expressed ubiquitously in the telencephalon, whereas at E16 sestrin2 is present in the VZ and SVZ its level decreasing towards the cortical plate. Scale bar 100μm. **G.** Immunoblotting of pre- and postnatal telencephalic samples for sestrin2. Sestrin2 is prenatally downregulated in p53-/- telencephalons. 2-3 embryos per genotype and developmental stage were examined. Representative immunoblot is shown. $\mathbf{G_1}$. Densitometric quantification of sestrin2 blots when normalized to β-actin. According to the densitometric quantification sestrin2 is downregulated in prenatal but not in postnatal p53-/- telencephalons. Error bars are ±sd. N=2. **H.** Expression of Sesn2 mRNA across the pre- and postnatal telencephalic development by real-time PCR quantification. Sesn2 mRNA is downregulated in p53-/- telencephalons when compared to the wild type. Data represent mean Sesn2 mRNA expression normalized to β-actin ±sd. Three embryos per developmental stage and genotype were examined. Each sample as triplicates. (* ** $p < 0.001$ by two-tail Student's t test). **I.** Expression of exogenous Sesn2 decreases ROS in *ex vivo* E13 telencephalons, whereas control empty vector does not have an effect. ROS were

measured from either EF-empty vector or EF-Sesn2 *ex vivo* electroporated E13 telencephalons by FACS. Plasmid was injected to the lateral ventricle and after 20h expression the cortices were dissociated to single cells and stained for the ROS indicator DCFDA and FACS was performed. Non-injected hemispheres were used as controls. Data are presented as mean percent of intensively DCFDA positive (DCFDA++) cells of all cells ±sem. Two embryos were used for EF-empty vector and five for EF-Sesn2 electroporation. (* $p< 0.05$ by two-tail t test.) **J.** FACS quantification of sestrin2 protein of EF-Sesn2 electroporated and non-electroporated control E13 hemispheres. EF-Sesn2 electroporated hemispheres have higher amount of sestrin2 protein compared to control hemispheres. Representative experiment is shown. N=2 EF-empty vector, N=5 EF-Sesn2. **K.** The expression of Sesn2 mRNA is regulated in a ROS and mTOR pathway dependent manner. Neurospheres were treated either with 1μM H_2O_2 or with 100nM rapamycin. H_2O_2 induced whereas rapamycin, an mTOR inhibitor, decreased Sesn2 mRNA abundance compared to the control when assessed by qRT-PCR. Data represent mean mRNA expression normalized to β-actin ±sd from three unrelated experiments, each in triplicates. (*** $p< 0.001$ by two-tail t test.)

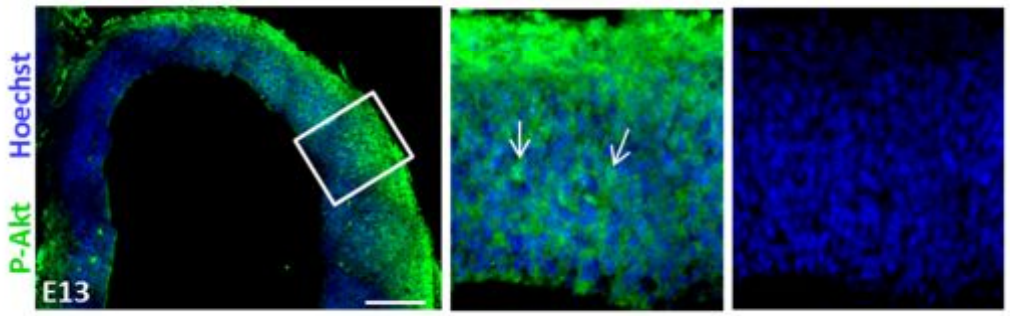
Figure 9.


A.
wt
NPCs
p53-/-
36.4%
28.6%
4%
1.6%
25.4%
45.9%
7.5%
3.6%
P-Akt
DCFDA
DCFDA
wt
p53-/-
%
DCFDA+/ P-Akt+
DCFDA+++/ P-Akt+
B.
Ad-GFP
p53-/- NPCs
Ad-GFP-p53
P-Akt
GFP
GFP
Ad-GFP
Ad-GFP-p53
P-Akt++/GFP+, %
C.
wt
p53-/-
E11 E13 E16 P1 P7 E11 E1 3 E16 P1 P7
Akt P-Ser473
β-actin
Akt P-Ser473 densitometric intensity, %
wt
p53-/-
E11
E13
E16
P1
P7

D.


Hoechst
P-Akt
E11

E.


Hoechst
P-Akt
E13

F.

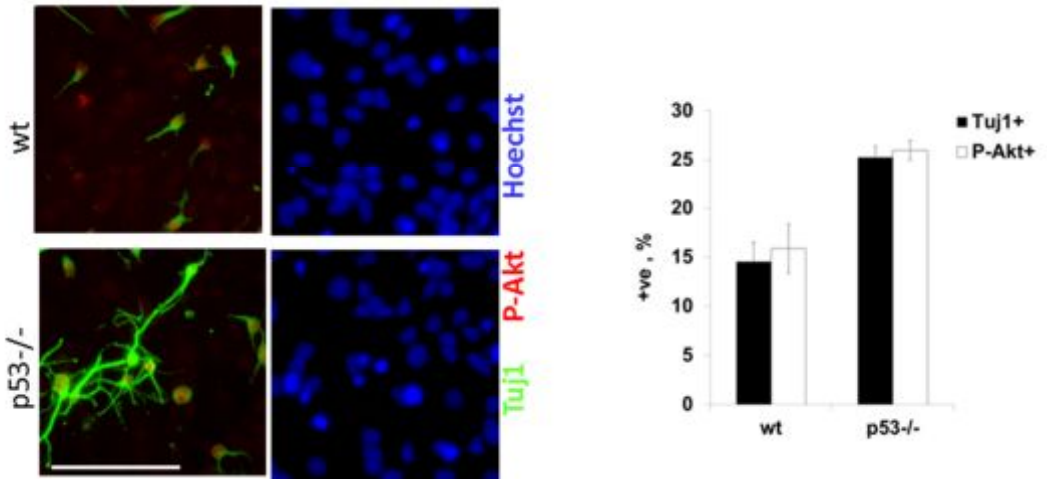

wt
p53-/-
Hoechst
P-Akt
Tuj1
+ve , %
30
25
20
15
10
5
0
Tuj1+
P-Akt+
wt
p53-/-

Figure 9. Precocious neurogenesis in p53-/- NPCs is due to the hyperactive PI3K-Akt pathway

A. ROS indicator DCFDA/P-Akt double staining of E13 *in vitro* NPCs. P53-/- NPCs exhibit an increase in P-Akt/DCFDA double positive cells as well as an increase in the total number of P-Akt+ NPCs. Data represent percent mean of double positive cells ±sem from four independent experiments. **B.** Ad-GFP-p53 transduction of P53-/- NPCs reduces the number of P-Akt++ NPCs. Proliferative NPCs were transduced either with Ad-GFP or with Ad-GFP-p53 and after 18hrs expression the proportion of Akt-P-Ser473++ cells was assessed by FACS. Values represent percent mean of intensively GFP+/P-Akt+ double positive cells ± sem from three independent experiments. **C.** Akt phospho-Ser473 immunoblotting of pre- and postnatal telencephalic samples. According to densitometric quantification p53-/- telencephalons have higher abundance of phospho-Akt protein compared to wild type at E11 and at E13 when normalized to β-actin. 2-3 embryos per developmental stage and genotype were examined. Error bars are ±sd. **D.** Akt phospho-S473 immunohistochemistry on E11 coronal telencephalic sections. Immunoreactivity for P-Akt (green) is localized to the preplate, the layer where the first postmitotic neurons reside, though some phospho-Akt+ cells can also be detected in the germinal region lining the lateral ventricle. Scale bar 100μm. **E.** Immunohistochemistry for Akt phospho-Ser473 on coronally sectioned E13 telencephalons. Phospho-Akt immunoreactivity is most intensive in the outer cortical plate, where the newly generated neurons reside, and in the marginal zone. However, there are P-Akt+ cells also in germinal zones. Scale bar 100μm. **F.** Double immunocytochemistry for tuj1 (green)/Akt phospho-S473 (red) on differentiating wild type and p53-/- NPCs on day 5. Nuclear hoechst staining in blue. P53-/- NPCs display an increase in the number of both P-Akt and tuj1 positive cells. Data represent percent mean of positive cells ±sd from three independent experiments. (*** $p < 0.001$ by two-tail Student's t test). Scale bar 100μm.

Figure 10.

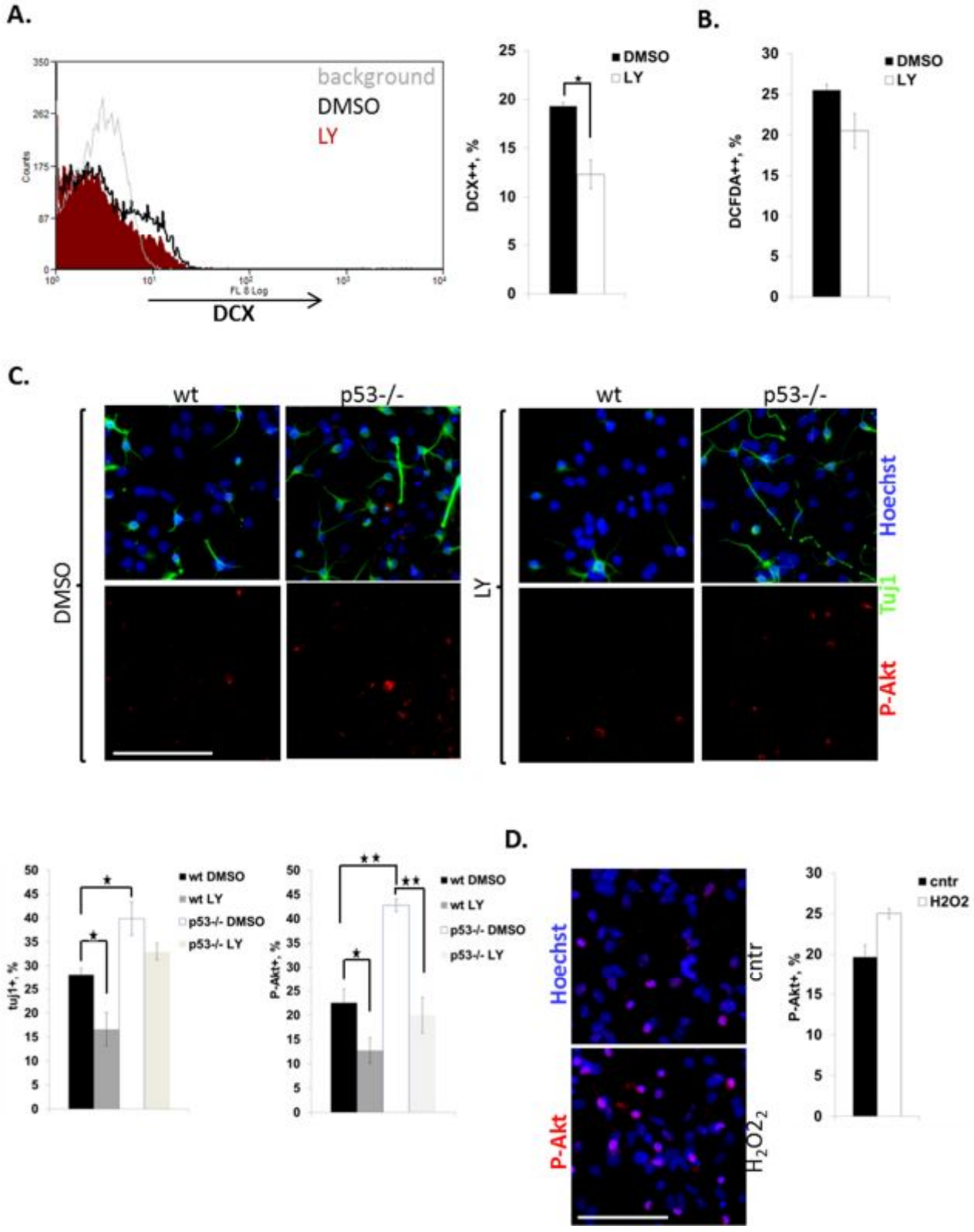

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Figure 10. Pharmacological inhibition of the PI3K-Akt pathway normalizes elevation in neuroblast formation in p53-/- NPCs

A. 10μM LY294002, a PI3K inhibitor, treatment of proliferative p53-/- NPCs reduces the number of DCX+ NPCs generated according to FACS. Data represent percent mean of DCX++ NPCs ± sem from three independent experiments, (* $p < 0.05$ by two-tail t test). **B.** Transient exposure of NPCs to the PI3K inhibitor LY294002 (10μM) decreases ROS as FACS for DCFDA demonstrates. Shown is the percent mean of intensively DCFDA positive (DCFDA++) cells ±sem from 3 independent experiments. **C.** Immunocytochemistry for tuj1 (green) and phospho-Akt (red) on differentiating wild type and p53-/- neural progenitors after 10μM LY294002 prepriming. Proliferative NPCs were pretreated either with DMSO or 10μM LY294002 whereafter they were plated for differentiation without LY for 5 days. LY294002 pretreatment decreases the number of tuj1+ and phospho-Akt+ neurons generated upon differentiation. Percent mean of tuj1+ neurons normalized to the total number of cells ±sem from three independent experiments is shown. (* $p < 0.05$, ** $p < 0.01$ by two-tail t test). Error bar is 100μm. **D.** Prepriming of proliferative NPCs with H_2O_2 drives an increase in the number Akt phospho-S473+ (red) cells in differentiation conditions. Nuclear staining (hoechst) in blue. Mean percent of P-Akt+ cells of all cells ±sd is shown, N=2. Scale bar 100μm.

III Discussion

The consensus view is that p53 is dispensable for embryonic brain development. The brain of p53-/- embryos develops normally without gross structural abnormalities (Donehower et al., 1995). However, 16-23% of female p53 knockout embryos suffer from neural tube closure defects, which lead to anencephaly and subsequent exencephaly (Armstrong et al., 1995; Sah et al., 1995). Both conditions are incompatible with life and occur also in humans with the incidence of 0.5-2/1000 established pregnancies (Copp and Greene, 2010). In addition, a reduction in the number of p53-/- female progeny from p53+/- x p53+/- matings has been reported, though normal Mendelian ratios have also been stated (Donehower et al., 1992; Armstrong et al., 1995).

Mdm2 and Mdm4 (Mdmx) null mouse embryos have demonstrated that p53 is active in the germinal zones of the developing telencephalon (Xiong et al., 2006). Mdms act as negative regulators of p53 and the absence of either of them causes embryonic lethality. The loss of Mdm2 leads to p53 dependent apoptosis, while that of Mdm4 results in cell cycle arrest accompanied by cell death. Additionally, studies from γ-irridated animals have suggested a role for p53 in DNA damage induced apoptosis (Gottlieb et al., 1997; Komarova et al., 1997), yet in normal physiological conditions p53 does not co-localize in the embryonic telencephalon in cells which undergo developmental cell death (van Lookeren Campagne and Gill, 1998). Nevertheless, p53 appears to have a central role in postnatal developmental apoptosis in the hippocampus (Murase et al., 2011). Evidence that p53 is involved in developmental prenatal cell death of the nervous system concerns only the peripheral nervous system (Kaplan and Miller, 2000). Thus, comprehensive explanations for the high basal expression of p53 in the germinal zones of the developing telencephalon are missing.

This study shows by immunostaining and by mRNA quantification that p53 is most abundant in proliferative NPCs and in the proliferative germinal zones of the developing

mouse telencephalon its expression decreasing profoundly upon differentiation and once the neurogenic period is over. This raises the question on the role of p53 in proliferative neural progenitors. One possible interpretation is that p53 may indeed play a role in the developing telencephalon but in its absence other factors, such as the other p53 family members, p63 and p73, compensate it. This could in principal be the case as both of them are expressed in embryonic NPCs (Dugani et al., 2009; Talos et al., 2010). Redundancy is a common theme in the regulation of eNPC homeostasis like for example the forkhead transcription factor (FoxO) null mice have demonstrated (Renault et al., 2009; Paik et al., 2009). Yet, the data in this study point to another possibility. The lack of p53 leads to an increase in cellular ROS and to the activation of the PI3K-Akt signaling thereby launching neurogenic program and preventing excessive proliferation (Fig. 11).

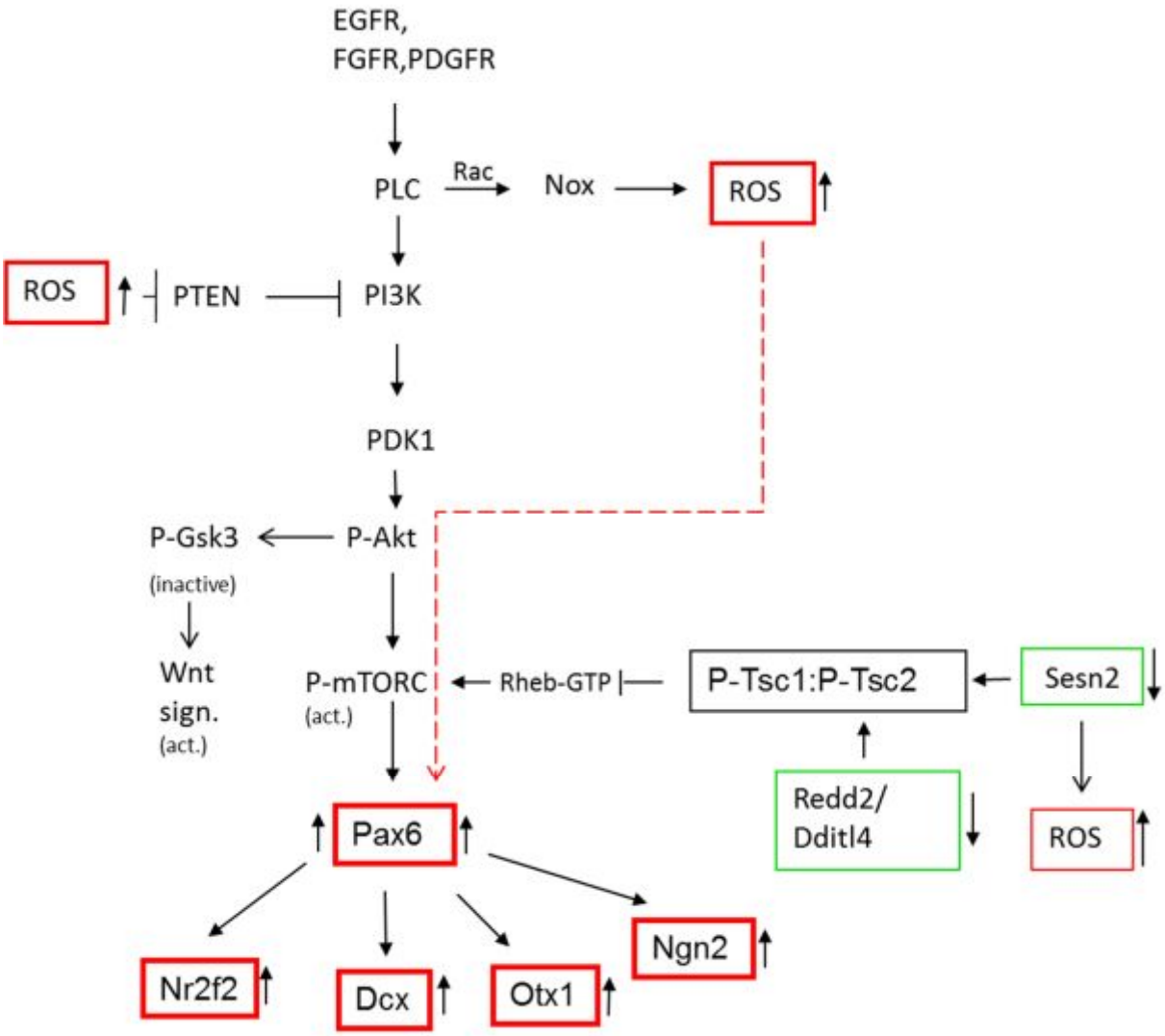

Figure 11. The putative pathway involved in launching an increase in ROS and thus neurogenesis in p53-/- eNPCs.

1. Elevation in ROS in embryonic p53-/- NPCs and neurons

That p53-/- cells and tissues exhibit increased ROS levels have been previously reported (Sablina et al., 2005). An increase in ROS in p53 null mice is connected with tumorigenesis and concomitant genetic instability, while administration of antioxidants protects against tumors such as lymphomas (Sablina et al., 2005). However, there are no published studies on ROS in p53-/- neural progenitor cells, neither embryonic nor adult. In

this study both the conventional p53 knock-out mice and the nestin-cre/loxPp53 NPCs and telencephalons *in vivo* and *in vitro* were found to exhibit elevation in ROS excluding the possibility that the increase in ROS would not be a cell autonomous property of p53-/- NPCs.

The report from Chen and co-workers is to my knowledge to date the only work exploring the impact of the absence of p53 on the embryonic forebrain in the context of macromolecular oxidation, yet not on ROS directly (Chen et al., 2009). Interestingly, DNA oxidation, when assessed by mass spectrometry for the presence of 8-oxoG lesions, was higher in E19 p53-/- mouse thymus and brain, whereas no difference was detected in the skin, for example. The amount of ROS itself was not investigated. The data in this study show that already proliferative p53-/- progenitors, both *in vitro* and *in vivo*, display an increase in ROS and that DNA damage *in vitro* is elevated in p53-/- NPCs. The level of DNA damage in *in vivo* telencephalons in the current study remains open, yet in the light of the report mentioned seems plausible.

Neural stem cells use endogenous ROS in the regulation of their normal cellular physiology (Le Belle et al., 2011). Two different cellular responses to changes in oxidative status have been proposed. In one of them, becoming slightly more oxidized is sufficient to inhibit proliferation and to induce differentiation (Smith et al., 2000; Noble et al., 2005). In the second one, similar changes drive proliferation (Limoli et al., 2004). The outcome has been suggested to depend on intrinsic cellular factors such as the duration of the increase in ROS, developmental stage etc. In addition, extracellular factors present may modulate the response (Noble et al., 2005).

Mitochondria and cellular oxidases, notably NADPH oxidases (NOX), xanthine oxidases (XO) and cyclooxygenases (COX) are the main sources of endogenous ROS generation in stem cells (Kamata et al., 2009; Coant et al., 2010, Le Belle et al., 2011). Stimulation of cells with growth factors or with cytokines activates receptor associated oxidases and ROS mediated signaling. Notably, growth factor receptor stimulation is also

coupled with the activation of the PI3K-Akt pathway. Complex feedback regulation takes place between ROS and receptor tyrosine kinase (RTK)-PI3K-Akt signaling. ROS can for example further augment RTK-PI3K-Akt signalling by inactivating protein and lipid phosphatases which negatively regulate the pathway (Leslie et al., 2003). The source of ROS in this study is not defined. Yet, the data point to the RTK-PI3K-Akt pathway, since the treatment of p53-/- NPCs with the PI3K inhibitor LY294002 ameliorated elevation in ROS. How this precisely is linked to the downregulation of ROS production by NOXs is unclear, but it has been reported (Le Belle et al., 2011). It also shows that RTK associated signaling is a central source of ROS in eNPCs. In spite of the fact that LY294002 decreased ROS the possibility that dysfunctional mitochondria, another potential source of ROS, would contribute to an increase in ROS in p53-/- NPCs, cannot be excluded. The importance of respiration rises with the onset of neurogenesis and continues to be of central significance in mature neurons, and p53 has been demonstrated to modulate respiration (Matoba et al., 2006; Hu et al., 2010).

2. Elevation in ROS is typical for tumor suppressor mutant cerebrums

The phenotype of several tumor supressor null NSCs has been linked to a deregulation of antioxidant genes and genes involved in the control of proliferation. FoxO triple knockout and Prdm16 null NSCs exhibit elevation in ROS and an initial increase in proliferation and neurogenesis followed by a decline in NSC pool (Paik et al., 2009; Chuikov et al., 2010). Global gene expression profiling of the VZ of new born mice brought hepatocyte growth factor (*Hgf*) as a candidate, whose downregulation would drive elevation of ROS in the Prdm16 null VZ. Similar analysis on Foxo triple null early passage adult neurospheres pointed to a deregulation of proliferation regulators, such as *Aspm,* and to Wnt signaling (Paik et al, 2009). It is noteworthy that the classical antioxidant genes, including *MnSOD* and catalase found to be positively regulated by FoxOs in some other cell types, did not show up in the array from Foxo triple null neurospheres (Tothova et al, 2007; Paik et al, 2009). Thus,

as the authors pointed out, FoxOs have clearly distinctive set of transcriptional targets in NSCs and HSCs, both of which however display the same phenotype; an increase in ROS and exhaustion of stem cell pool (Paik et al, 2009).

2.1. Sestrin2 has multiple possible ways to reduce ROS in embryonic telencephalons

The current work detected *Sesn2* as a candidate gene, whose deregulation seems to contribute to elevation in ROS in p53-/- NPCs. Sesn1 and 2 have been reported to be positively regulated by p53 in some cell types upon DNA damage and oxidative stress (Budanov et al., 2002). Yet, sestrins can be expressed at high levels also in the absence of stress and be still induced by p53 (Lee et al., 2010a, and this work). Sestrin2 has been implicated in the maintenance of normal cellular redox balance in various cells types and tissues, for example in antioxidant response of cortical neurons to NMDA receptor challenge (Sablina et al., 2005; Papadia et al., 2008). Sestrin1 and Sestrin2 are together with Sestrin3 peroxiredoxin oxidoreductases, though whether oxidorectase activity is the main way sestrins utilize to downregulate ROS is controversial (Budanov et., 2002; Woo et al., 2009; Lee et al., 2010a). In fact, sestrins may use various means in different cell types to fine-tune ROS. Sestrin2 or dSesn, the only sestrin *Drosophila* expresses, regulate for instance negatively mTOR signalling by sequestering Rheb, which inactivates mTOR shunting thereby down also ROS production (Budanov and Karin, 2008, Lee et al., 2010b). dSesn has been stated to influence negatively PI3K-Akt signaling (Lee et al., 2010b). This could potentially generate a negative feed-back loop to ROS production by NOXs, whose activation is linked to RTK activation. On the other hand, sestrin3 appears to influence ROS levels in neuronal cells by means of its effect on mitochondrial function (Hagenbuchner et al., 2012). Furthermore, a role in promoting autophagy of dysfunctional mitochondria has been attributed to sestrins (Lee et al., 2010b). Interestingly, tumor suppressor Pml also promotes autophagy by inhibiting Akt-mTOR pathway (Huang et al., 2011). Moreover, defective autophagy has been implicated in

NTDs (Cecconi et al., 2007), an intriguing possible explanation for the incidence of NTDs in p53-/- embryos. Taken together, which of the above mentioned possibilities, if any, is responsible for the downregulation of ROS in sestrin2 overexpressing p53-/- E13 telencephalons is open. However, considerable evidence has accumulated that shows that sestrins affect ROS levels both *in vitro* and *in vivo*.

In addition, this study identified three additional downregulated genes putatively involved in redox regulation: *Dcxr*, *Ephx1* and *Dditl4* /*Redd2*. Whether they are contributing to the redox dysbalance observed remains to be proven. Dditl4 is also a negative effector of mTOR (Corradetti et al., 2005). The highly homologous Redd1 has been associated with cellular redox response, and of interest with telencephalic neurogenesis and migration (Corradetti et al., 2005; Malagelada et al., 2011). Akin to p53, the amount of Redd1 was high in proliferative NPCs decreasing in young differentiating neurons. Furthermore, knockdown of Redd1 *in vitro* or *in vivo* accelerated neuronal differentiation and disrupted eNPC migration. It would be interesting to explore whether Dditl4/Redd2 has similar effects.

3. P53 as "a guardian of the genome" in eNPCs

P53 dependent cell cycle arrest takes place in G1 in response to DNA damage (Attardi et al., 2004). This allows DNA repair to occur before the onset of the S phase and transmission of mutations to be prevented to daughter cells. The data in this study would imply that a role as "a guardian of the genome" is indeed one of the major roles p53 has in embryonic neural progenitor cells (Lane, 1992). Overexpression of p53 both in wild type and p53-/- NPCs induced expression of p21, and caused accumulation of cells to G1 phase. In spite of this, BrdU incorporation increased in p53-/- progenitors to the same level as in the wild type. Simultaneously, DNA damage, when measured on the basis of apurinic/apyrimidinic (AP) sites present in DNA, decreased. Taken together, this is likely to be an indication of DNA damage repair, as DNA repair requires synthesis of new DNA.

AP sites in DNA molecules in all cells are generated spontaneously in vast numbers every day during spontaneous chemical processes like hydrolysis and via metabolic processes, which produce ROS. Rapidly proliferating cells, such as NPCs, are prone to replication induced DNA damage. AP sites unless repaired, can impair DNA replication and potentially retard cell cycle. P53 has been reported to interact directly with DNA polymerase β (DNA pol β) and AP endonuclease, both of which are important for the initiation of base excision repair (BER) (Offer et al., 2001; Zhou et al., 2001). BER activity correlates with p53 levels. Hence, the current study extends the possible functions attributed to p53 in the developing CNS apart from being a molecule involved in the elimination of neural cells with DNA damage (Gao et al., 1998; Frappart et al., 2005). However, the putative role p53 may have in DNA repair in eNPCs needs further examinations.

3.1. A putative increase in neurodegeneration in the absence of p53 in adult cerebrums

Oxidative macromolecular damage tends to accelerate itself in the absence of proper defense and repair mechanisms. Moreover, DNA damage can result in elevation of ROS and impair NPC proliferation like ATM-/- mice demonstrate (Kim and Wong, 2009). Mitochondrial DNA is due to the lack of histones more vulnerable than nuclear DNA to disruption of cellular redox homeostasis (LeDoux et al., 2007). This can potentially lead to a chain reaction in which an increase in ROS drives a further increase in reactive oxygen species due to impairments in mitochondrial respiratory chain. Of importance, this kind of sequence of events has been implicated in the initial stages of age related degenerative neurological diseases such as Alzheimer's and Parkinson's diseases (Taupin, 2010). In this study not only embryonic telencephalons but also the adult SVZ and hippocampus exhibited an increase in ROS. This may have implications in terms of neurodegeneration. In fact, adult female p53-/- mice have been reported to display impairments in learning, exhibit behavioural changes and their brains to have signs of neurodegeneration (Amson et al., 2000). Whether

the launching factors might include preceding impairments in redox balance regulation would be an interesting issue to examine.

4. A decrease in oligodendrogenesis is likely to be due to an increase in ROS in p53-/- NPCs

A decrease in the expression of oligodendroblast marker genes and proteins was detected in p53-/- telencephalons. These findings are consistent with the existing knowledge on the responsiveness of oligodendrocyte progenitor cells (OPCs) to cellular redox status modulation (Back et al., 1998; Smith et al., 2000; Noble et al., 2005) and are thus in line with the notion that p53-/- telencephalons and neural progenitors exhibit higher ROS. Proliferation of OPCs is remarkably sensitive to disturbances of cellular redox balance. More reduced cellular environment promotes their proliferation, whereas increased oxidization of signaling molecules drives their differentiation. In this study a continuous decrease in NG2, a marker for OPC progenitors, also called polydendrocytes (Nishiyama et al., 2009), was detected from its first appearance at E13 onwards. Simultaneously, platelet derived growth factor alpha (PDGFRα) another OPC marker, which is not expressed by maturing OPCs, was first precociously upregulated but afterwards downregulated in p53 null telencephalons compared to the wild type. Moreover, Sox10, an OPC marker that is expressed both by proliferating and maturing OPCs/oligodendrocytes was downregulated in p53-/- telencephalons as well as in *in vitro* cultured E13 NPCs as the genome wide microarray of E13 NPCs demonstrated. Unfortunately, maturation of OPCs in p53-/- cerebrums at protein level could not be investigated due to the lack of antibodies available in the lab. However, quantification of myelin basic protein (Mbp) mRNA levels demonstrated a dramatic decrease in Mbp transcript variant 1, the Mbp variant picked up, in p53-/- telencephalons at P7. Myelination of axons begins around P7-10 in the CNS (Chanderkar et al., 1986). This may imply that not only generation of oligodendrocyte precursors is impaired but also the concomitant myelination performed by mature oligodendocytes is affected. In this context it is interesting to note that

Prdm16 null mice, whose NSCs exhibit increase in ROS, also show akinesia of corpus callosum, the bundle of axons which connects the hemispheres (Chuikov et al., 2010). No molecular explanations for this observation were provided. Knowing the sensitivity of OPCs and myelination to ROS, elevation in ROS in Prm16 null mice could well be a contributing factor. It also implies that corpus callosum might be an interesting anatomical structure to compare between the wild type and p53-/- mice.

Consequently, the data propose a role for p53 in the fine-tuning of oligodendrogenesis, a role which has been attributed to p53 previously (Eizenberg et al., 1995; Zezula et al, 2001; Billon et al., 2004). P53 has been suggested to contribute positively to *in vitro* maturation of oligodendrocytes and to participate in developmental death of oligodendrocytes *in vitro* (Eizenberg et al., 1996; Billon et al., 2004). The data here expand putative roles of p53 in oligodendrogenesis to proliferative NPCs and to the *in vivo* field. Moreover, they propose that alterations in oligodendrogenesis identified in p53-/- telencephalons might be at least partially due to the disturbances in redox regulation. Yet, that this is really the case remains to be shown, as for example the effect of the antioxidant N-acetyl-cysteine treatment on the expression of OPC markers *in vivo* was not explored. Likewise, the effect of the Ad-p53 transduction of proliferative NPCs on *in vitro* OPC marker gene expression remains to be seen.

Postnatally another player may have a part. P21, a canonical p53 target gene, has been implicated in the maturation of oligodendrocytes to myelinating cells (Zezula et al., 2001). In p21 null brains myelination of axons is delayed. However, this is not related to impaired cell cycle exit of proliferating p21-/- OPCs but instead to impairments in the establishment of the differentiation program following cell cycle exit. The fact that p21 is expressed at low level during embryonic telencephalic development and without differences between the wild type and p53-/-, yet is early postnatally remarkably upregulated with p53-/- cerebrums displaying profound downregulation of p21 mRNA when compared to the wild type, suggests that p21 is

most likely a p53 target gene in postnatal cerebrums. It may also imply that lowered amount of Mbp1 var1 mRNA detected in p53-/- forebrains at P7 might in part be connected with a decrease in p21 abundance. Together, these findings clearly propose a part for p53 in OPC/oligodendrocyte biology *in vivo*, the elucidation of which however requires further work.

5. Elevation in ROS drives generation of neuroblasts

Enhanced neurogenesis in particular *in vitro* but also *in vivo* in p53-/- genetic background can be thought to represent another outcome of the elevation of intracellular ROS in p53-/- NPCs and telencephalons. In support for this, exposure of proliferative NPCs to H_2O_2 induced expression of neurogenic genes, among them novel genes whose expression has not previously been demonstrated to be cellular redox status responsive and resulted in increased neuronal differentiation, while N-acetyl-cysteine treatment shunted down neurogenic gene expression in proliferative NPCs, subsequent neuronal differentiation in differentiation conditions and the abundance of doublecortin protein *in vivo*. Ad-GFP-p53 transduction of proliferative NPCs decreased the number DCX+ neuroblasts generated *in vitro*. It also induced the expression of sestrin2 which, when electroporated to *ex vivo* telencephalons, dampened telencephalic ROS levels.

An increase in neuroblast formation has been previously reported in the adult p53-/- SVZ both *in vitro* and *in vivo* (Gil-Perotin et al., 2006) and increased proliferation *in vitro* (Gil-Perotin et al., 2006; Meletis et al., 2009). Likewise, embryonic olfactory bulb derived p53 null NPCs generated *in vitro* more neurons (Armesilla-Diaz et al., 2009). NPCs located in the olfactory bulb represent at the developmental stage Armesilla and colleagues focused on a neurogenic stem cell resource *per se*, since the SVZ derived NPCs have not yet migrated to the olfactory bulb in E13 mouse embryos (Armesilla-Diaz et al., 2009).

5.1. Elevated *in vitro* proliferation of p53-/- eNPCs may be an *in vitro* artifact

On the other hand, telencephalic p53-/- NPCs have also been stated to exhibit *in vitro* some increase in proliferation but no deviations in neuronal differentiation (Zheng et al., 2008). The increase in *in vitro* proliferation has been associated with downregulation of p21 cell cycle regulator in p53-/- *in vitro* NPCs/NSCs (Meletis et al., 2006), though direct evidence for the involvement of p21 has not been provided. Moreover, p21 may not be central in the regulation of eNPC proliferation (Kippin et al., 2005; this work). P53-/- telencephalons are not enlarged indicating that the increase in proliferation reported for *in vitro* eNPCs does not occur *in vivo*, and it may in fact represent an *in vitro* artifact. Consequently, some of the obvious discrepancies between studies may be due to different developmental stages investigated, embryo versus adult, due to mouse strain used etc. Furthermore, *in vitro* culturing conditions can contribute to different outcomes.

5.2. Genetic control of neurogenesis; sequential expression of Sox1-Pax6-Ngn2

Though the work from Le Belle and colleagues implied that an increase in ROS drove initial elevation in NPC proliferation, the expression of the classical markers of proliferative NPCs, such as nestin and Sox2, was not responsive to redox status manipulation (Le Belle et al., 2011). On the other hand, expression of *Dcx*, a gene expressed in neuroblasts and early postmitotic neurons, was found to be responsive to cellular redox status modulation. In this work Dcx was robustly induced by H_2O_2 , while NAC decreased its expression. Additionally, the current work identifies proneural transcription factors (TFs) *Pax6*, *Otx1* and *Nr2f2* as novel genes, which are regulated in a redox status dependent manner. Of interest, Ngn2 expression remained unresponsive to redox status manipulation.

A causal relationship between Sox1-Pax6-Ngn2-Dcx gene expression has been reported (Suter et al., 2009). In this work Sox1 was downregulated, while the other mentioned TFs were according to Affymetrix microarray upregulated in p53-/- NPCs. Sox1 and Sox2 maintain neuroepithelial cells in their uncommitted stage, whereas Pax6 drives them to

neurogenic pathway. Yet, not all future neurons are Pax6+. Only glutamatergic progenitors are positive for it, while gabaergic NPCs have their own characteristic marker genes (Suter et al., 2009). To add another level of complexity to their regulation, persistent high expression of Sox1 and Pax6 has been reported to drive neurogenic fate (Sox1) and cell cycle exit (Pax6) in NPCs (Sansom et al., 2009; Suter et al., 2009).

It appears that the level and duration of expression of particular transcription factors relative to other opposing effect having TFs determines whether a cell continues as an uncommitted stem cell, commits to a neurogenic proliferative fate or exits the cell cycle (Kan et al., 2007; Sansom et al., 2009; Suter et al., 2010). During neurogenesis a chain of successive waves of TFs induces expression of the next wave of TFs. Pax6 binds to and induces expression of Nr2f2 and Ngn2, for instance (Sansom et al., 2009). It also regulates positively Otx1 expression (Sansom et al., 2009). That Ngn2 was not induced by H_2O_2 might be related to the duration of the treatment. Pax6 was upregulated by H_2O_2. Nonetheless, since the duration of the exposure to the fate determining factors is crucial, Pax6 failed to elicit Ngn2 expression. It can be that ROS related signaling is in its part involved in the regulation not only of neurogenic fate but also the committed neuron progenitor sub-type generated. This is of course on the basis of the current data just speculative but possible, and would deserve investigation. Finally, Pax6 intriguingly binds also to the Sesn1 promoter (Samson et al., 2009). In the light of the current work it might be assuming that sestrin1 has an antioxidant function that it contributes to the regulation of cellular redox status and thus the expression of Pax6 and its downstream targets.

5.3. PI3K-Akt signalling drives elevation in ROS and neurogenesis

This study brings up PI3K-Akt pathway as a putative candidate whose dysregulation contributes to an increase in ROS and neurogenesis in p53-/- NPCs. It appears that a causal link between upstream activation of Akt signaling, ROS generation and neuroblast generation/neurogenesis exists in such a way that activation of PI3K-Akt signaling is

connected to ROS production, which in turn drives, most likely after reaching a certain threshold, expression of neurogenic genes inducing thus neuronal differentiation.

These results are consistent with the recent report stating that PI3K-Akt signaling regulates self-renewal and neurogenesis of adult NPCs *in vitro* and *in vivo* (Le Belle et al., 2011). High ROS drove NPC proliferation and subsequently induced generation of doublecortin+ neuroblasts/BPs, whereas production of astrocytes was not affected. Similarly to the current work, the PI3K inhibitor LY294002 decreased ROS and the number of DCX+ neuroblasts. Furthermore, overexpression of p53 in p53-/- NPCs decreased both Akt activation and DCX abundance in this study.

Mice devoid of PDK1, the upstream activator of Akt, exhibit impaired neuronal differentiation in the neural tube, and Akt activation drives positively for example gabaergic differentiation (Oishi et al., 2009; Fishwick et al., 2010). In line with this, GAD1 was upregulated in p53-/- neurospheres, while in *in vitro* differentiating p53-/- neurons GAD65/67 was more abundant compared to the wild type both *in vivo* and *in vitro*. In addition, PI3K–Akt signalling promotes survival of proliferating and differentiating embryonic NSCs as well as that of maturing neurons (Dudek et al., 1997; Chan et al., 2011). In this work Akt-P473 immunoreactivity was found to localize on E11 and E13 telencephalons in particular to regions, where new maturing neurons reside and which in separate ROS stainings exhibited high ROS connecting the two also in *in vivo* neurons. To my knowledge this kind of characterization of Akt-P473 immunoreactivity in embryonic telencephalons has not been reported in literature.

6. Multiple tumor supressors are expressed in the germinal zones of the developing telencephalon, the absence of which however does not inevitably result in overproliferation of eNPCs

At least four other tumor suppressors besides p53 have been found to play a part in the maintenance of stem cell, also neural stem cell, homeostasis: Prdmx6, promyelocytic

leukemia protein (Pml), the O-family of forkhead transcription factors (FoxOs) and PTEN (Paik et al., 2009; Renault et al., 2009; Regad et al, 2009; Chuikov et al., 2010). Like p53 null mice, mice null for Pml, Prdmx, Foxos and PTEN are prone to generate lymphomas (Sablina et al., 2005; Paik et al., 2007; Regad et al, 2009; Lee et al., 2010a). More importantly, they all are expressed in the germinal zones of the developing telencephalon; a phenotype in the developing CNS has been described for Pml, PTEN and Prdm16 mice. The lack of Pml results in overproliferation of NPCs and somewhat unexpectedly in the decrease in the brain size (Regad et al, 2009). Deletion of PTEN in nestin+ progenitors in the developing CNS leads to NPC overproliferation and to the increase in the brain size (Groszer et al., 2001). Ablation of Prdm16 is associated with reduced cortical thickness and agenesis of corpus callosum (Chuikov et al, 2010).

Akin to p53-/- mice, mice null for a single FoxO transcription factor do not show developmental abnormalities in the brain (Renault et al., 2009; Paik et al., 2009). However, in the early adulthood the SVZ of FoxO single null mice begins to exhibit increased proliferation followed by a decline in NPC pool and neurogenesis. Whether the increase in proliferation correlated with an increase in ROS was not investigated. Similar to single FoxO null mouse telencephalons, evidence that p53 deficiency would lead to uncontrolled proliferation in the embryonic VZ-SVZ *in vivo* has not been presented. Hence, exciting parallels exist between single null FoxO and p53 null mice (Gil-Perotin et al., 2006; Meletis et al., 2006; Renault et al., 2009; Paik et al., 2009).

The absence of Prdm16 or triple FoxO null leads to an increase in ROS in NPCs and in HSCs (Tothova et al., 2007; Paik et al., 2009; Chuikov et al., 2010, Yalcin et al., 2010). Consequently, Prdm16 and FoxOs have been implicated in the regulation of cellular redox status. They directly activate expression of some genes involved in cellular antioxidant response. The absence of Prdm16 leads to a progressive decrease in NPC proliferation derived from newborn mice finally depleting them, while in postnatal FoxO triple null mice

enlargement of ventricles and thinning of the neocortex was detected (Paik et al., 2009; Chuikov et al., 2010). Of interest, adult female p53-/- mouse brains were reported to exhibit enlargement of ventricles and thinning of the neocortex accompanied by neurodegeneration, impairments in learning and behavioral changes (Amson et al., 2000).

7. Environmental oxygen tension and neural stem cell cultures

Some of the differences in the properties of *in vitro* cultured versus *in vivo* NPCs can be expected to result from elevated environmental oxygen levels *in vitro*. Cultured NPCs are typically exposed to 20% environmental oxygen. Yet, the oxygen level of both embryonic and adult NSCs is in their *in vivo* niche 1-5% (Studer et al., 2000). Surrounding oxygen tension can have an effect on cellular redox status and consequently on proliferation versus differentiation decisions (Smith et al., 2000; Gustafsson et al., 2005; Noble et al., 2005; Krabbe et al., 2009; Renault et al., 2009; Mazumdar et al., 2010). Thus, proliferative capacity is enhanced and premature expression of differentiation markers is decreased, when embryonic mesencephalic NPCs are cultured in physiological oxygen levels (3%) (Studer et al., 2000). Moreover, there is a marked increase in the yield of dopaminergic but also serotonergic neurons upon induction of differentiation. However, the production of gabaergic and glutamatergic neurons was decreased in hypoxic conditions. Normoxy during differentiation did not have an effect on cellular subtype produced. *In vitro* propagated E13 cortical NPCs exhibited at 20% oxygen impaired expansion, and reduced generation of oligodendrocytes and astrocytes upon differentiation (Chen et al., 2007). The great majority of differentiated cells were neurons. Hence, these studies imply that oxygen tension to which precursors are exposed during their proliferation phase has a profound effect on the progeny produced upon differentiation (Smith et al., 2000; Morrison et al., 2000; Chen 2007). They also suggest that gaba and glutamaergic differentiation is favored at higher oxygen tension, while dopaminergic neurogenesis and oligodendrogenesis are impaired.

These reports are of importance when interpreting the results of this study, in particular its *in vitro* part, but also the previous reports stating elevated neurogenesis in p53-/- NSCs and in the p53-/- adult SVZ (Gil-Perotin et al., 2006; Armesilla-Diaz et al., 2009). The considerable increase in neurogenesis *in vitro,* which however *in vivo* appears to be more modest, possibly reflects the sensitivity of p53-/- NPCs to elevated oxygen tension. Nonetheless, although a profound increase in neurogenesis *in vitro* in p53-/- is apparently an artifact, the data of this study suggest that *in vivo* both the embryonic and adult p53-/- NPCs and brains exhibit impaired redox regulation. This is likely to be of importance during telencephalic development as the current study implies, it contributes to the initiation of neurogenesis and prevents overproliferation, but also suggests a putative involvement of p53 in neurodegeneration, an implication which would deserve investigation.

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