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Research Report

Dynamic expression of the p53 family members p63 and p73 in the mouse and human telencephalon during development and in adulthood

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ABSTRACT

p63 and p73, family members of the tumor suppressor p53, are critically involved in the life and death of mammalian cells. They display high homology and may act in concert. The p73 gene is relevant for brain development, and p73-deficient mice display important malformations of the telencephalon. In turn, p63 is essential for the development of stratified epithelia and may also play a part in neuronal survival and aging. We show here that p63 and p73 are dynamically expressed in the embryonic and adult mouse and human telencephalon. During embryonic stages, Cajal-Retzius cells derived from the cortical hem co-express p73 and p63. Comparison of the brain phenotypes of p63- and p73- deficient mice shows that only the loss of p73 function leads to the loss of Cajal-Retzius cells, whereas p63 is apparently not essential for brain development and Cajal-Retzius cell formation. In postnatal mice, p53, p63, and p73 are present in cells of the subventricular zone (SVZ) of the lateral ventricle, a site of continued neurogenesis. The neurogenetic niche is reduced in size in p73-deficient mice, and the numbers of young neurons near the ventricular wall, marked with doublecortin, Tbr1 and calretinin, are dramatically decreased, suggesting that p73 is important for SVZ proliferation. In contrast to their restricted expression during brain development, p73 and p63 are widely detected in pyramidal neurons of the adult human cortex and hippocampus at protein and mRNA levels, pointing to a role of both genes in neuronal maintenance in adulthood.

1. Introduction

The transcription factor family p53 has three members, p53, p63, and p73. Each has a distinct expression pattern and plays distinct roles, although the three proteins may work in concert (Bourdon, 2007; Levrero et al., 2000; McKeon and Melino, 2007). p53 is the classical tumor suppressor, mutated in more than 50% of human cancers (Murray-Zmijewski et al., 2006). Like

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p53, p63 and p73 have tumor-suppressor activities (Flores et al., 2002), but in addition, they are necessary for the development of specific tissues and organs. p63 is essential for the development and maintenance of stratified epithelial tissues; $p63^{-/-}$ mice are born without skin and limbs and die soon after birth (Mills et al., 1999; Yang et al., 1998). Mutations of the human *TP63* gene are associated with a variety of syndromes, such as ectodermal dysplasia, cleft lip/palate syndrome; split-hand/foot malformation (SHFM); limb–mammary syndrome; Rap-Hodgkin syndrome (RHS), and orofacial cleft (Cabiling et al., 2007; Chan et al., 2004; Chan et al., 2005; Huang et al., 2005; Julapalli et al., 2009; Rinne et al., 2007; van Bokhoven et al., 2001). In turn, the inactivation of the *p73* gene in mice gives rise to immunological, neurological, and pheromonal defects (Yang et al., 2000).

The p53 family members have a similar domain structure, displaying an N-terminal transactivation (TA) domain, a DNA-binding domain (DBD), and an oligomerization domain (OD). p73 and p63 share a sterile alpha motif domain (SAM) and an inhibitory domain (ID) at their C-termini and have a higher sequence homology as compared with p53. p53 family members have common target genes, e.g., p21 (Jost et al., 1997; Kaghad et al., 1997), as well as specific targets, such as CaN19 for p63 (Kirschner et al., 2008). The three proteins form a complex network, which may be of significance in cancer formation (Flores et al., 2002; Muller et al., 2006; Schilling et al., 2010). p63 and p73 use multiple promoters to generate an array of isoforms, including full-length TA isoforms, and amino-terminally truncated (ΔN) isoforms, which lack the TA domain. The TA isoforms of p63 and p73 can activate downstream target genes and induce apoptosis, whereas the ΔN isoforms act as dominant inhibitors of the full-length forms of p53, p63, and p73, thereby inhibiting transactivation of target genes and induction of apoptosis (Flores et al., 2002; Grob et al., 2001; Melino et al., 2004; Muller et al., 2005; Wu et al., 2003, 2005). Further complexity derives from differential splicing of the last four exons of p63 and p73. For p73, the combination between N-terminal (TA or ΔNp73) isoforms and C-terminal splice variants (p73α-η) can produce up to 14 p73 proteins (Murray-Zmijewski et al., 2006).

p73 is important for the development of the central nervous system (CNS), in particular, for hippocampal development and cortical regionalization (Meyer et al., 2004). A remarkable aspect of p73-deficient mice is their lack of Cajal-Retzius (CR) cells (Meyer et al., 2002; Yang et al., 2000), which in wild-type mice represent the most important source of Reelin, a secreted glycoprotein that controls radial migration into the cortex (D'Arcangelo et al., 1995). The most severe brain phenotype is obtained by complete inactivation of the p73 gene (Yang et al., 2000), whereas selective inactivations of TAp73 (Tomasini et al., 2008) and ΔNp73 (Ravni et al., 2010; Tissir et al., 2009; Wilhelm et al., 2010) give rise to much milder brain defects. In the adult human cerebral cortex, both the TAp73 and ΔNp73 isoforms are widely expressed (Cabrera-Socorro et al., 2006). Several studies have linked ΔNp73 with neuronal survival pathways in the central and peripheral nervous system (Lee et al., 2004; Pozniak et al., 2000, 2002; Tissir et al., 2009), and there is increasing evidence involving p73 in neurodegeneration, Alzheimer 's disease, and cortical ischemia (Bui et al., 2009; Wetzel et al., 2008; Wilson et al., 2004). On the other hand, also p63 may play a role in neuronal survival (Dugani et al., 2009; Jacobs et al., 2005). As yet, the contributions of p63 and p53 to brain development is not well known, although a subset of p53-deficient mouse embryos exhibit severe brain malformations such as exencephaly (Armstrong et al., 1995; Sah et al., 1995).

In order to better understand the possible activities of the p53 family in the nervous system, we analyze here the normal expression patterns of p73 and p63 in the telencephalon during development and in adulthood. We find that prenatally they are co-expressed in CR cells of the cerebral cortex and hippocampus. At early postnatal stages, we study their expression in the neurogenetic niche of the subventricular zone of the lateral ventricle. We also examine the brains of p73 and p63 knockout (ko) mice. In addition, by RT-PCR and immunohistochemistry using pan-p63 antibodies, we demonstrate the presence of p63 in the adult human cortex, where it is widely expressed, and colocalized with TAp73.

2. Results

2.1. *p73 and p63 colocalize in Cajal-Retzius cells derived from the cortical hem*

In embryonic mice, p73 is highly expressed in the cortical hem, a transient signaling center at the interface of the cerebral cortex and the choroid plexus, where a substantial proportion of CR cells have their origin (Bielle et al., 2005; Meyer et al., 2002; Yoshida et al., 2006). Cajal-Retzius (CR) cells in the marginal zone of the developing cerebral cortex characteristically express Reelin and a variety of transcription factors, most notably p73. In mice at embryonic day (E) 12.5, p73 was expressed in virtually all CR cells in the prospective neocortex, closely matching the distribution of Reelin (Fig. 1A, C, E). Only at the level of the pallial/subpallial boundary (asterisk in Fig. 1A and C) a distinct triangular area was occupied by Reelin-immunoreactive (ir) neurons that were p73-negative. Two-color immunostaining showed an extensive colocalization of Reelin and p73 in the dorsal cortex (Fig. 1E), whereas in the ventral cortex, only a subset of CR cells expressed both proteins (Fig. 1F). Reelin-ir neurons were also abundant in the septum (Fig. 1C), which was mostly p73- negative (Fig. 1A). p73-ir CR cells were particularly abundant in medial limbic areas, such as the taenia tecta or rostral hippocampal rudiment (Fig. 1A).

A large proportion of the p73-positive CR cells were also positive for p63 (Fig. 1B), although on the whole, the p63 signal was less intense than the p73 signal (the skin epithelium, intensely ir for p63, served as a control). The expression of both p73 and p63 in CR cells persisted during the whole embryonic period and continued during the first postnatal week. By using confocal microscopy, we examined the colocalization of both proteins in CR cells at E15 (Fig. 1H–J). We found that intense positivity for p73 in a CR cell usually correlated with low levels of p63; conversely, cells that were more strongly stained for p63 usually showed a lower p73 signal. Some CR cells expressed only p73, but conversely, we did not observe p63-positive CR cells that were totally negative for p73. p53 immunoreactivity was not detected in CR cells.

The presence of p63 in Reelin-expressing CR cells was also observed in human embryos at 9 gestational weeks (Fig. 1G),

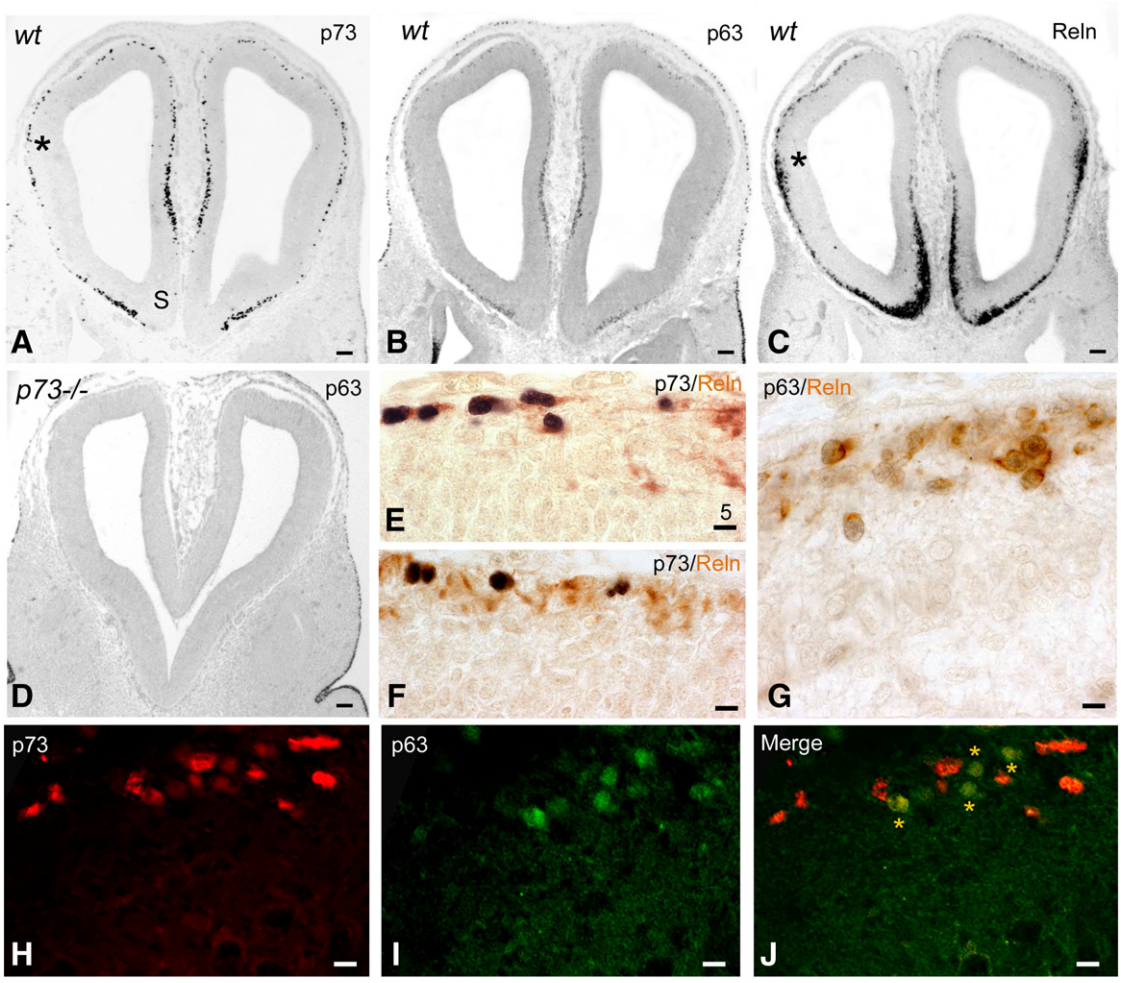


Fig. 1 – p73, p63, and Reelin in mouse and human Cajal-Retzius (CR) cells. A: distribution of p73-immunoreactive (ir) CR cells in E12.5 mouse. B: p63-ir CR cells in E12.5 mouse. C: Idem, Reelin. The asterisks in A and C point to the striato-pallial boundary where Reelin is increased, but p73 is not. S: septum. D: absence of p63-ir CR cells in a p73 ko mouse. Note the intense staining in the skin. E: colocalization of Reelin (brown) and p73 (black, in the nucleus) in a CR cells in the dorsal cortex at E12.5. F: E 12.5; at the striato-pallial boundary, Reelin-ir CR cells outnumber the p73-positive ones. G: human medial cortex, 8 gestational weeks. p63 gives a faint nuclear staining (grey) in Reelin-positive CR cells (brown). H–J: confocal microscopy images of p73 (H, red), p63 (I, green) and colocalization (J, yellow, asterisks). Scale bars: in A, for A–D: 40 μm; in E, for E–J: 5 μm.

where it was most prominent in the medial cortex adjacent to the cortical hem, and staining intensity gradually decreased toward more lateral areas. However, as in the mice, the p63 signal in CR cells was fainter than the p73 signal, and much less intense than the strong signal in the skin.

The p73 knockout mice lack CR cells (Yang et al., 2000, Meyer et al., 2002). To address the possibility that p63-positive CR cells were preserved in the absence of p73, we studied p73-deficient mice at E12.5. There were no p63-positive CR cells in the marginal zone of pallial and subpallial regions of the $p73^{-/-}$ mutant (Fig.1D), although p63 was strongly expressed in the skin. This finding indicates that p73 is the relevant protein for CR cell formation and that p63 cannot compensate the loss of p73 in this cell type.

Since the cortical hem is the source of a large proportion of CR cells, we analyzed the expression of p73, p63, and Reelin in CR cells at their place of origin. At E12.5, the cortical hem showed numerous p73-ir (Fig. 2A), p63-ir (Fig. 2B), and Reelin-ir (Fig. 2C) neurons in an almost identical position, continuous with a similar expression pattern in CR cells of the neighboring cortex. By contrast, immunostaining with a specific anti-TAp73 antibody showed positivity for the transactivation-competent TAp73 isoform only in the most medial segment of the cortical hem adjacent to the choroid plexus anlage (Fig. 2D), whereas TAp73 immunoreactivity was not observed in CR cells in the cortical marginal zone. This finding confirms that the dominant p73 isoform in CR cells is ΔNp73, in keeping with previous reports (Meyer et al., 2004; Tissir et al., 2009), whereas their birth place, the cortical hem, is also positive for TAp73.

p63 is expressed in most CR cells of the hippocampal fissure (compare Fig. 3C, p73, and D, p63), and thus we wanted to further assess its importance for brain development by examining p63-deficient mice (Yang et al., 1999) at E17 (they die at birth). We did not observe brain malformations in the

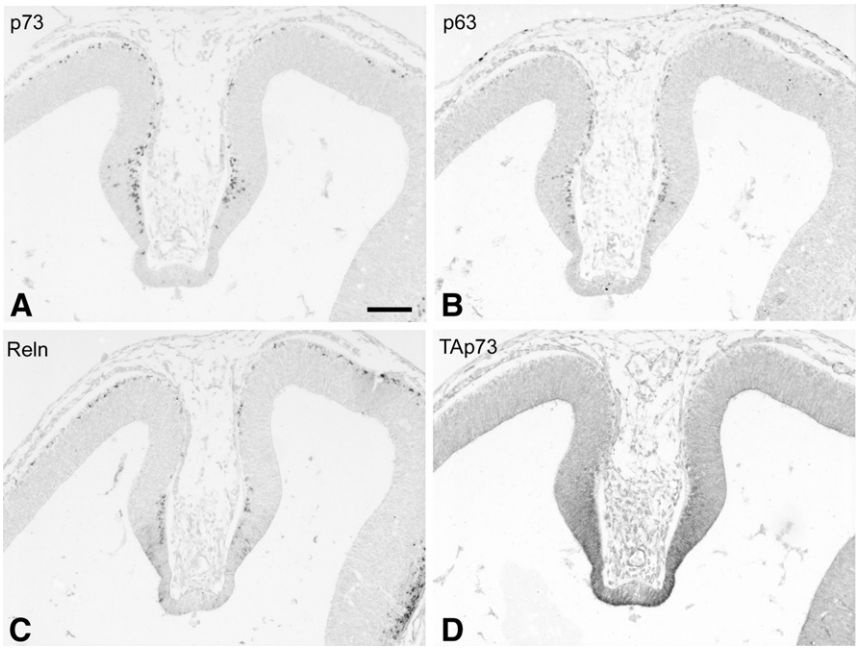


Fig. 2 – The cortical hem at E12.5. A: CR cells in the hem and the cortex are ir for p73α, B: for p63, and C: Reelin. However, immunoreactivity for the TAp73- isoform is only observed in the cortical hem, indicating that cortical CR cells express ΔNp73α. Scale bar in A, for A–D: 100 μm.

p63-mutant; cortical layering and telencephalic size were normal (Fig. 3A, B), CR cells in cortex and hippocampus were numerous and intensely immunoreactive for p73, and the hippocampal fissure was clearly present (Fig. 3E). This is in contrast to the $p73^{-/-}$ mouse, which displays a striking and characteristic hippocampal dysgenesis, absence of hippocampal fissure, and reduction of the cortical width (Meyer et al., 2004; Yang et al., 2000). Altogether, our results indicate that p63 is not necessary for CR cell genesis and cortex/hippocampus development.

2.2. *The p53 family in the neurogenetic niche of the lateral ventricle*

During embryonic life, we did not detect p53, p63, or p73 immunoreactivity in the proliferative zones of the telencephalon. However, during the first postnatal week, p53, p63, and p73 were expressed in cells of the subventricular zone (SVZ) of the lateral ventricle, specifically in its lateral angle, known as the neurogenetic niche (Fig. 4), where neurogenesis takes place into adult life, providing interneurons for the olfactory bulb through the rostral migratory stream (Lois and Alvarez-Buylla, 1993; Luskin, 1993). PCNA, a marker of proliferating cells, indicated the position and extent of the SVZ, which was very prominent during the first postnatal week (Fig. 4A). p73 was expressed in small cells scattered throughout the neurogenetic niche (Fig.4B) and in the ependymal cells that begin to line the ventricle in the early postnatal period. Double fluorescence immunohistochemistry revealed the co-expression of p73 and PCNA in the neurogenetic niche (Fig. 4E), indicating that p73 was expressed in cycling cells. A few small cells in the neurogenetic niche and the adjacent striatum were positive for p63 (Fig. 4C) and p53 (Fig. 4D); however, since all our commercial antibodies were raised in mice, double immunostaining with PCNA was not possible. The small size and the rather diffuse distribution of p53- and p63-positive cells within the SVZ did not allow us to identify them as belonging to the distinct cell types that have been described in the adult mouse SVZ (Lois and Alvarez-Buylla, 1993).

We compared the neurogenetic niche of p73 knockout mice at postnatal day (P) 7 with that of their wild-type littermates (Fig. 5). On the whole, the size of the niche and the number of proliferating cells were clearly reduced in the mutant while the lateral ventricle was dilated (Fig. 5A, B). The choroid plexus was not visible at the level shown in Fig. 5B, because it is much smaller in the mutant compared to the wild type (Cabrera-Socorro et al., 2006). The numbers of p53 and p63-expressing cells in the p73-mutant SVZ niche were very low (not shown), perhaps due to the small size of this compartment. The size reduction of the mutant neurogenetic niche was not accompanied by an increase of apoptotic cells stained with activated caspase 3 (Fig. 5C, D), at least not during the period examined in our study.

GFAP stains astrocytes of the SVZ, considered progenitor cells of postnatal neuro- and gliogenesis (Doetsch et al., 1999). The morphology and distribution of GFAP-ir cells differed between wild-type and p73 knockout mice. In the former, GFAP-ir cells covered the entire ventricular surface and extended radial processes reminiscent of radial glia (Fig.5 E); in the latter, GFAP-ir cells were more irregularly distributed and the radial processes appeared truncated (Fig. 5F).

We then examined the expression of proteins that characterize young neurons in the SVZ. Tbr1 marks excitatory neurons of the cerebral cortex (Hevner et al., 2001) but is also present in the SVZ. In wild-type animals from P1 to P7, Tbr1 was expressed in the dorsal aspect of the lateral ventricle, possibly representing late-born neurons destined for the cortical gray or white matter

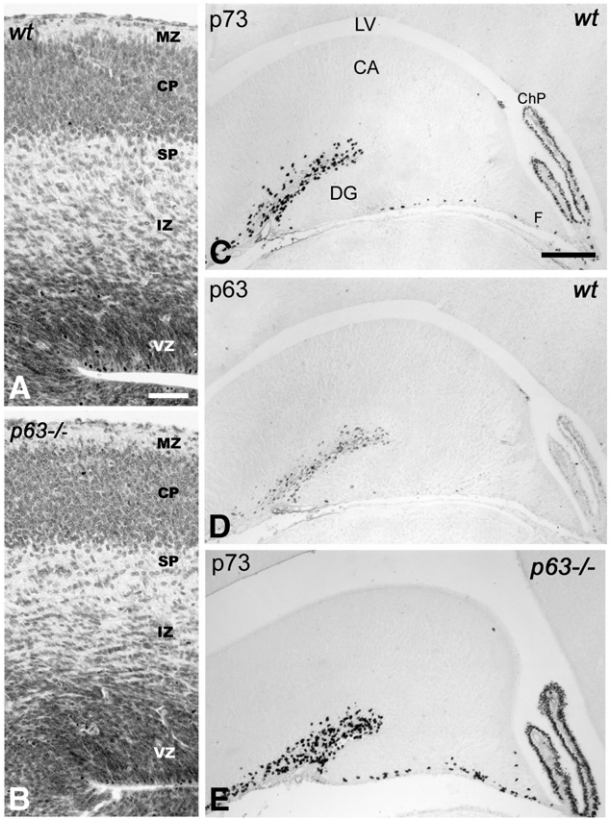


Fig. 3 – Cortical layers, hippocampus, and CR cells are normal in p63-deficient mice. Wild-type (wt) and p63-deficient mice were compared at E17.5. A: Wt cortical layers, B: mutant cortical layers. MZ: marginal zone, CR: cortical plate, SP: subplate, IZ: intermediate zone; VZ: ventricular zone. C: p73-ir CR cells in the hippocampal fissure of a wt mouse, E17.5. D: Idem, p63. E: p73 immunoreactivity in a p63 ko mouse shows the same distribution as in wt. CA: Cornu Ammonis, DG: dentate gyrus; LV: lateral ventricle. Scale bars in A, for A and B: 60 μm; in C, for C–E: 50 μm.

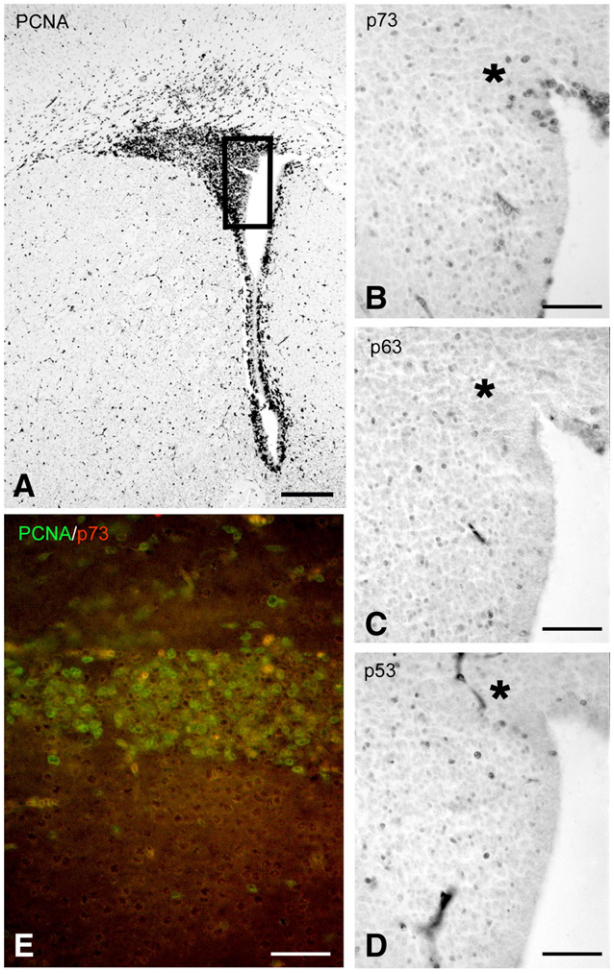


Fig. 4 – The p53 family in the postnatal neurogenetic niche. A: the neurogenetic niche of the lateral ventricle of a postnatal (P) 5 mouse, stained with PCNA. The boxed area is shown in B–D, stained for p73α (B), p63 (C), and p53 (D). Scattered small cells are observed with the 3 antibodies. E: double-staining fluorescence micrograph of PCNA (green) and p73α (red). A few cells (yellow) co-express both proteins, indicating that p73 is in cycling cells. Scale bars: A: 200 μm; in D, for B–E: 50 μm.

(Fig. 5G). In the mutant littermates, Tbr1-ir cells were virtually missing in the SVZ, although they were clearly stained in layer VI of the cortex (Fig. 5H). A similar result was obtained with calretinin, a marker of non-pyramidal neurons. In the early postnatal wild-type animals, calretinin-ir neurons lay scattered in the dorsal aspect of the ventricle (Fig. 5I) but were almost absent in the same location of the mutant mice (Fig. 5J). Notice, in all figures, the reduced size of the cortical white matter in the p73-mutant mice. Doublecortin (DCX) marks newly born neurons; in the neurogenetic niche of the wild-type mice, it was prominently expressed by numerous small neurons (Fig. 5K). In contrast, they were sparse in the small niche of the p73 mutant animals (Fig. 5L).

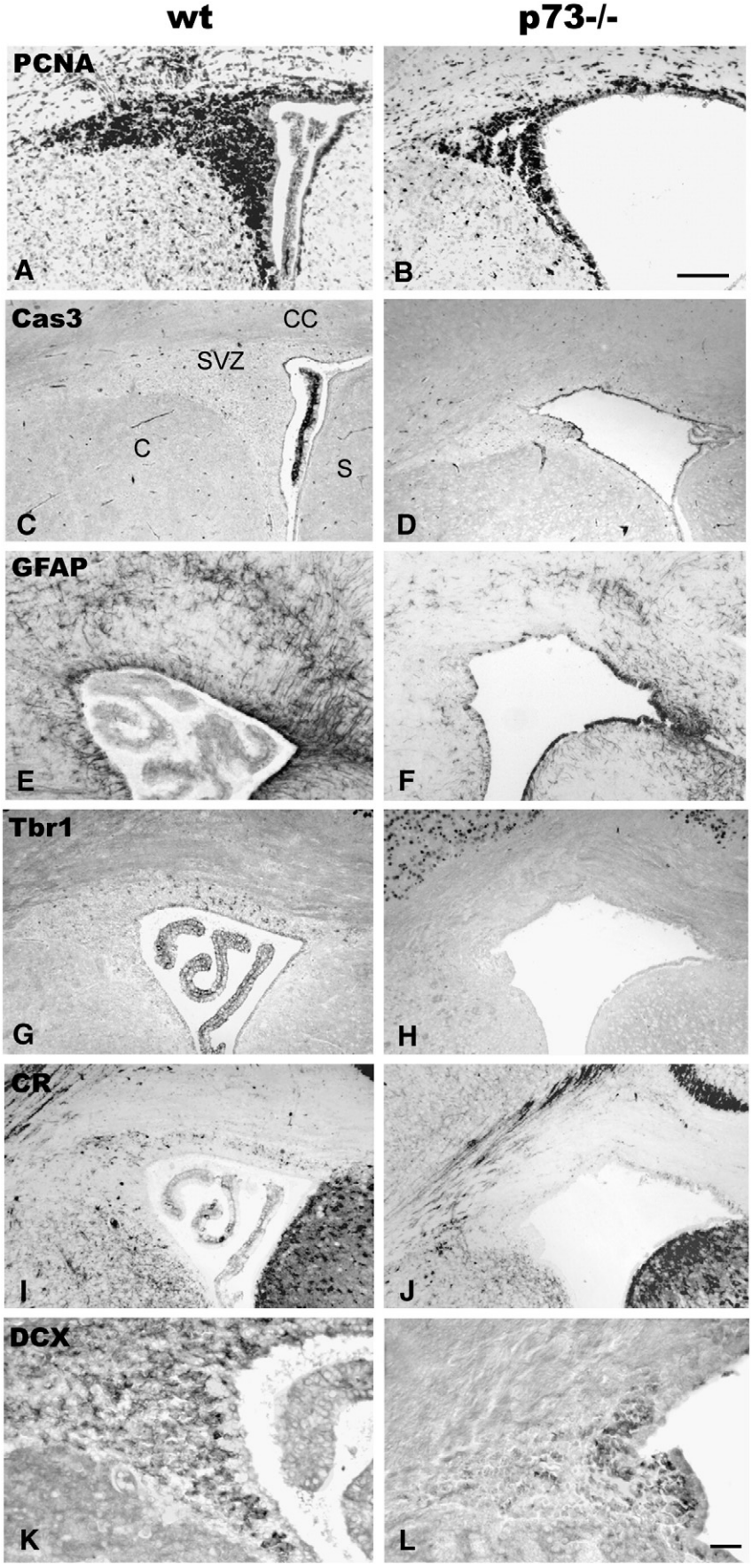
Altogether, our results indicate that loss of p73 negatively affects neurogenesis and gliogenesis in the postnatal SVZ.

2.3. *p63 and p73 in the adult human cortex and hippocampus*

We extracted the mRNA of p73 and p63 from adult human hippocampus and temporal cortex, using specific primers for the core region (coding for the DNA binding domain) of both genes. RT-PCR revealed the presence of the corresponding cDNAs, confirming that transcripts of both genes are expressed in the adult human cortex and hippocampus (Fig. 6A).

To further confirm the presence of both p63 and p73 in the human brain at protein levels, we performed Western blotting on extracts of adult human hippocampus and temporal cortex and obtained a band of the predicted weight of about 70 kDa for p63, and 3 bands of approximately 65, 70, and 80 kDa for p73, which may correspond to different isoforms of this protein (Fig. 6B).

In the embryonic and early postnatal mouse telencephalon and in the fetal human cortex, p63 and p73 immunoreactivity


wt
p73-/-
PCNA
A
B
Cas3
CC
SVZ
C
S
C
D
GFAP
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F
Tbr1
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CR
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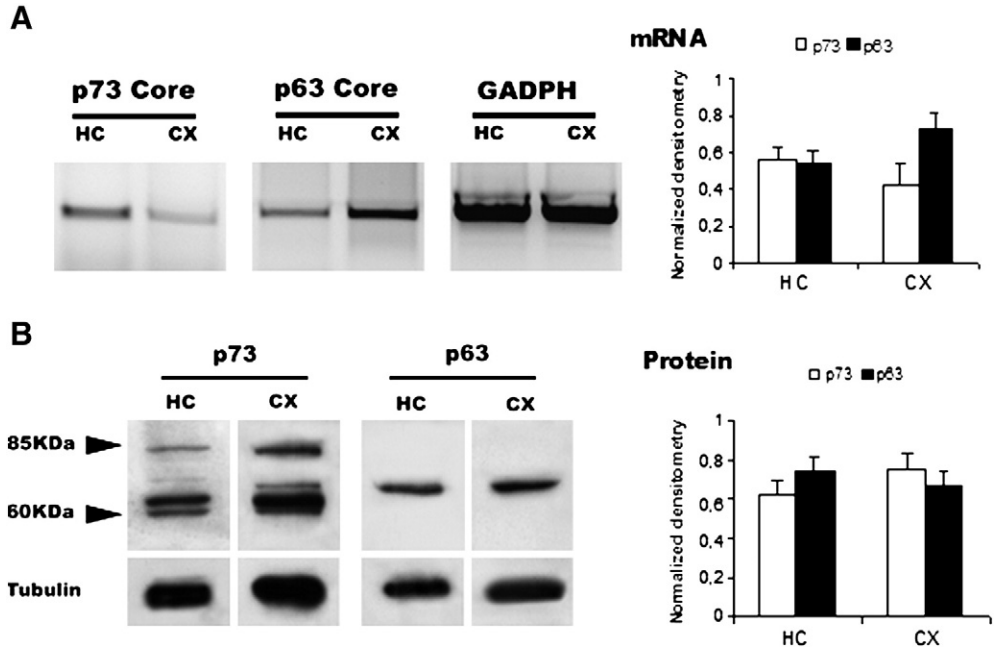

Fig. 6 – p63 and p73 cDNAs and proteins in adult human brain. A: RT-PCR, performed from mRNA of p73 and p63 from adult human hippocampus and temporal cortex using primers specific for the core domain of both genes, reveals the presence of the corresponding cDNAs. Transcripts of both genes are expressed in the adult hippocampus (HC) and temporal cortex (CX). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as control of the PCR reaction. The bar graph shows relative values of p73 and p63 cDNAs normalized to GAPDH. B: Western blotting of hippocampus and temporal cortex extracts for p63 and p73 was performed by using a monoclonal antibody against p63 and a polyclonal antibody against TAp73 (aa 1–15). The monoclonal antibody anti-p63 recognized one band of approximately 70 kDa, whereas the polyclonal antibody anti-TAp73 gave three bands at approximately 65, 70, and 80 kDa, possibly corresponding to different isoforms. The graph represents the relative density of p73 and p63 bands normalized to α-tubulin. The data are mean values from 4 cases. Error bars indicate + SD.

was restricted to the CR cells and few cells in the SVZ. In particular, the CR cells were only visualized with C-terminal specific antibodies against p73α, but not with N-terminal specific antibodies against TAp73, consistent with the expression of Np73α. This situation changed during postnatal development, when the expression levels of TAp73 and p63 increased. In adult mouse (not shown) and human brains, both proteins were almost ubiquitously expressed. Since we previously reported the expression of various p73 isoforms in the adult human brain (Cabrera-Socorro et al., 2006), we focus here on the presence of p63. p63 immunoreactivity was prominent in neurons of all neocortical layers, with the most intense cytoplasmic staining in pyramidal cells (Fig. 7A). In the hippocampal formation, the granule cells of the dentate gyrus were almost p63 negative, whereas the neurons in the hilus area were strongly positive (Fig. 7B). High expression of p63 was also noted in pyramidal cells of Ammon's horn (Fig. 7C). The p63-staining pattern closely resembled that of TAp73 reported previously, and in fact, confocal microscopy demonstrated extensive colocalization of p63 and TAp73 in the cytoplasm of pyramidal cells of Ammon's horn (Fig. 7D–F) and temporal cortex.

In addition, the low expression of p63 in the dentate gyrus suggests that the amount of each protein as well as the ratio p63/p73 may vary among brain regions. Further mapping of p63 and p73 expression in the human brain may indicate areas of particularly high activity, perhaps related to a differential susceptibility for neurodegeneration.

3. Discussion

3.1. *p73 and p63 are co-expressed in Cajal-Retzius cells derived from the cortical hem*

CR cells are important for cortical development because they express high levels of the extracellular matrix protein Reelin,

Fig. 5 – The neurogenetic niche in wt and p73-deficient mice at P5. A: PCNA in wt, B in p73 ko mice. Notice the size reduction of the niche in this and the following panels. C, D: activated caspase 3 (casp3) is not changed in the p73 ko. C: caudate nucleus; CC: corpus callosum; S: septum; SVZ: subventricular zone. E, F: glial fibrillary acidic protein-ir (GFAP) radial glia is altered in the ko mice (F), with truncated processes and irregular distribution. G, H: Tbr1, marker of pallial neurons, is present in the subventricular zone (SVZ) of the dorsal aspect of the wt ventricle (G), but absent in the ko (H). I, J: the same is valid for the interneuron marker calretinin (CR), almost absent in the ko SVZ (J). K, L: doublecortin (DCX) stains newborn neurons in the SVZ and is dramatically reduced in the p73 ko (L). Scale bars in B, for A–J: 100 μm, in L, for K and L: 100 μm.

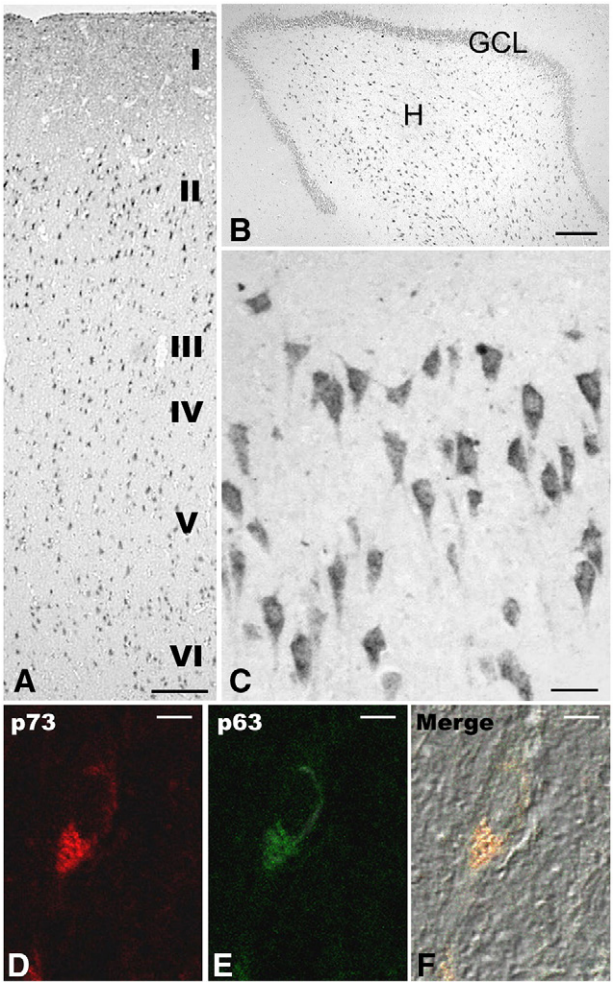

Fig. 7 – p63 in the adult human cortex and hippocampus. A: expression of p63 in virtually all neurons of the temporal cortex. B: intense p63 immunoreactivity in neurons of the dentate hilus (H), with faint positivity in dentate granule cells (GCL = granule cell layer). C: high expression in field CA1 of Ammon's horn. D–F: confocal images of p73, p63, and their co-expression in pyramidal cells of CA1. Scale bars: in A, B: 200 μm, C: 40 μm, D–F: 5 μm.

necessary for radial migration and laminar positioning of neurons destined for the cortical plate (D'Arcangelo et al., 1995). Transduction of the Reelin signal involves the Reelin receptors ApoER2 and VLDLR and leads to tyrosine phosphorylation of the adapter protein disabled 1 (Dab1) (Hiesberger et al., 1999; Rice and Curran, 1999). Disruption of the Reelin-Dab1 signaling pathway gives rise to a "reeler-like" phenotype, characterized by a roughly inverted cortical plate and neuronal malposition (Tissir and Goffinet, 2003). We describe here that a large proportion of Reelin-expressing CR cells are also positive for the p53 family members p73 and p63. What may be the role of p73 and p63 in CR cells? Importantly, the phenotype of the full p73 ko mouse shows that p73 is the critical protein for the formation of CR cells in the cortical hem, because it has no p63-positive CR cells that might compensate the loss of p73. Furthermore, specific inactivation of Np73 leads to the premature death of hem-derived CR cells (Tissir et al., 2009). By contrast, in the absence of p63, CR cells are present, express high levels of p73, and carry out their function with apparent normality.

The dominant p73 isoform expressed in CR cells is the anti-apoptotic Np73 (Meyer et al., 2004; Tissir et al., 2009; Yang et al., 2000), even though they undergo programmed developmental cell death during early postnatal life (del Rio et al., 1995; Derer and Derer, 1990; Meyer et al., 2002). In mouse embryos, the transcriptionally active TAp73 is expressed only in the cortical hem where it is involved in the development of the hippocampus (Meyer et al., 2004; Tomasini et al., 2008). We previously implicated an E2F1–TAp73 pathway in the development of hippocampus and caudal telencephalon (Meyer et al., 2004).

The possible activity of p63 in CR cells is less clear. TAp63 has been reported to be the dominant isoform expressed in neurons (Jacobs et al., 2005), whereas experimental evidence supports a prosurvival activity of Np63 in neural precursors (Dugani et al., 2009). Since the commercial antibodies used in our study do not discriminate between pro-apoptotic TAp63 and anti-apoptotic Np63, we are unable to solve the important question of which p63 isoform is expressed in CR cells. Our confocal microscope observations show that high levels of p73 in CR cells are usually accompanied by low levels of p63 and vice versa, which suggests that the two proteins complement each other, and CR cell death may be the result of a disturbed balance between various pro-survival and pro-apoptotic forms of p73 and p63.

In the context of the cortical hem, a putative signaling center and the main birth place of CR cells, it is of particular interest that the regulation of some BMP (bone morphogenetic protein) genes is mediated by p63 and p73 (Laurikkala et al., 2006; Yan and Chen, 2007). The BMPs, together with the Wnt/B-catenin pathway, form part of signaling pathways that are crucial for the development of the neocortex and highly expressed in the cortical hem. In particular, TAp73 expressed in the cortical hem may regulate the BMP pathway at this level. The phenotype of the p63 knockout mouse – presence of CR cells, absence of brain malformations, and normal expression of p73 – suggests that p73 is necessary and sufficient for the formation of CR cells and signaling in the cortical hem, whereas p63 expression seems to be a rather accessory feature of this cell type, and not relevant for brain development in general.

3.2. *The p53 family in the postnatal SVZ*

p53, p63, and p73 regulate cell fate, and thus their presence in the postnatal SVZ may influence life and death of cell populations in this compartment. The decrease in size of the neurogenetic SVZ niche in the p73 ko mice is particularly striking, and the scarcity of doublecortin-positive young neurons emerging from this source illustrates a dramatic loss of neurogenetic capacity in these animals. The postnatal and adult SVZ of the lateral ventricle harbors neural stem cells able to provide a life-long supply of new neurons and glia for the olfactory bulb, which migrate in the rostral migratory stream (Lois et al., 1996; Luskin, 1993). Our results in the postnatal SVZ point to an additional destination of cells deriving from the dorsal wall of the lateral ventricle. Tbr1 is a marker of excitatory neurons of telencephalic origin (Hevner et al., 2001), and Tbr1- ir

neurons born in the lateral ventricle stream dorsally toward the cortex even after the formation of the corpus callosum. Similarly, calretinin-ir neurons appear to migrate dorsally as well and may represent late-born interneurons destined for the cortex. Interestingly, both cell classes, late Tbr1 and calretinin-positive neurons, are severely reduced in numbers in the p73 ko mice, which suggests that loss of p73 function gives rise to a generalized reduction of postnatal neurogenesis, rather than affecting specific subsets of progenitor cells.

The neural stem cells of the neurogenetic niche may express the intermediate filament glial fibrillary acidic protein (GFAP) and exhibit the phenotypical and ultrastructural features of astroglia (Alvarez-Buylla et al., 2001; Doetsch et al., 1999; Garcia et al., 2004). They derive from the embryonic radial glia that fulfill a double function: they serve as a guidance substrate for radially migrating neurons (Rakic, 1972) and also function as neural progenitor cells (Malatesta et al., 2003; Noctor et al., 2001). In postnatal life, radial glia derivatives remain close to the ventricular surface, conserve their neurogenic potential, and persist as local multipotent astrocytic stem cells (Laywell et al., 2000; Merkle et al., 2004). For the function of the neural stem cell niche, interactions between ependyma and SVZ seem to be important. Ependymal cells express the BMP-binding protein Noggin, which appears to support neurogenesis by blocking endogeneous BMP signals that would induce gliogenesis (Lim et al., 2000). Another important regulatory mechanism of neural stem cell fate is through the Notch signaling pathway. Notch1 influences the developmental path of undifferentiated cells and determines the neuronal or glial identity during embryogenesis (Louvi and Artavanis-Tsakonas, 2006). Maintenance of the pool of neural stem cells within this area depends on cell–cell communication, and most of the expression of Notch1 in the adult SVZ occurs in polysialylated neural cell adhesion molecule (PSA-NCAM)-positive neural precursors and in GFAP-positive astrocytes (Givogri et al., 2006). Our finding of the presence of p63 and p73 in the SVZ of the postnatal brains and the partial colocalization of p73 with the proliferative marker PCNA point to potentially important activities of these genes in the maintenance of the adult neuronal stem cell environment (Dugani et al., 2009). The severe morphological abnormalities of GFAP radial glia in the p73 ko mice may reflect the damage of the signaling pathways that influence postnatal neurogenesis.

Previous studies have shown that the Notch ligands Jagged (JAG) 1 and 2 are controlled by both p73 and p63, although it is not clear whether this is done by TA or N isoforms (Sasaki et al., 2002). TAp73 may inhibit Notch signaling in some cells and contribute to increase Notch activity in the neighboring ones (Hooper et al., 2006; Sasaki et al., 2002). In the context of SVZ neurogenesis in the postnatal stages, p73 and p63 may contribute to the differentiation of newly born neurons through the regulation of the Notch signaling pathway. Notch has been shown to be active in astroglia-like neural stem cells but not in transit-amplifying progenitors of the subependymal zone (Andreu-Agullo et al., 2009). However, the absence of a cortical phenotype in the p63 ko mice suggests that p63 may not be involved in this molecular mechanism in the context of brain development, or that its absence may be compensated by other molecular mechanisms.

3.3. *p73 and p63 are co-expressed in the adult human cortex and hippocampus and may be involved in neuronal survival and degeneration*

We show here that p73 and p63 are widely co-expressed in the adult human telencephalon, with high protein levels in centers related to memory and cognition, in particular the hippocampus. We reported previously that TAp73 and ΔNp73 colocalize in adult cortical and hippocampal neurons (Cabrera-Socorro et al., 2006), suggesting that a precise balance between pro- and anti- apoptotic isoforms is needed to determine the fate of an adult cortical neuron. Our present observations complicate this picture, showing that p63 is an additional player in the game of death and life decisions in the adult cerebral cortex. Studies in sympathetic ganglion cells already provided evidence that p63 plays an essential role in neuronal survival or degeneration during development (Jacobs et al., 2005). In cortical neurons, the role of ΔNp63 promoting the survival of embryonic neural precursor cells has been demonstrated recently (Dugani et al., 2009). However, it is not clear yet which p63 isoform – TAp63 or ΔNp63 – is the dominant one in the adult human brain, because the available commercial antibodies do not discriminate between TAp63 and ΔNp63. Regardless of the p63 isoform involved, our data point to a complex interrelationship between pro-apoptotic and anti-apoptotic isoforms of p73 and p63 in adult neurons. On the whole, the p63 gene does not seem to play a significant role in brain development, since we did not detect any apparent brain malformation in the p63 ko mice. This is in keeping with the observations of Dugani et al. (2009) that p63 is dispensable for proliferation of cortical precursor cells *in vitro* and *in vivo*. On the other hand, since the p63 ko mice studied here do not survive into postnatal life, we cannot exclude that p63 may have important functions in the adult brain.

So far, the presence p63 in the adult human cerebral cortex has not been reported. However, there is evidence that links p63 and p73 with neurodegenerative disorders. p73 has been associated with molecular mechanisms involved in Alzheimer disease (Cancino et al., 2008; Li et al., 2004; Wetzel et al., 2008; Wilson et al., 2004), and ΔNp73-deficient mice display neurodegeneration (Wilhelm et al., 2010). p63, in turn, has been related to neuronal survival after cortical ischemia (Bui et al., 2009). Taken together, our results suggest that the p53 family members p63 and p73 may work in concert contributing to the survival/maintenance of cortical and hippocampal neurons of the adult human brain.

The mouse is the most widely used animal model in experimental genetics; however, regarding the clinically relevant questions as to whether p63 and p73 are equally important for neuronal survival and/or involved in neurodegenerative disorders, their expression has to be explored in the normal and pathological human brain. Importantly, a variety of mutations of the human *p63* gene have been described as giving rise to a complex spectrum of limb and skin malformations (Chan et al., 2004; Rinne et al., 2007), whereas brain malformations have not been reported. Our present study of p63 ko mice supports the view that despite its specific expression in CR cells of the developing cortex, p63 is dispensable for brain development. Altogether, p73 emerges as a key protein for the development of the nervous system,

whereas p63 may be more relevant for the adult or ageing brain.

4. Experimental procedures

4.1. *Mouse brains*

We examined p73 (Yang et al., 2000) and p63 (Yang et al., 1999) mutant and wild-type littermates. Of p73-deficient mice, a total of 42 C57BL/6 animals from heterozygous intercrosses were studied at embryonic days E12, E15, E16, and E18, and from postnatal day P4 to P10. Genotyping was performed using PCR as described (Yang et al., 1999, 2000). Prior to sacrifice, animals were deeply anesthetized with ketamine. Prenatal mouse brains were fixed by immersion, postnatal brains by perfusion with Bouin's fixative, and embedded in paraffin according to standard procedures. A total of 8 p63-deficient mice (Balb/c background) were collected at E17.5, since they die around birth. Sections were cut serially at 10 μm and processed for immunohistochemistry.

The $p73^{+/-}$ mice of our colony were a gift from Dr. Frank McKeon (Harvard University, Cambridge, MA) (Yang et al., 2000).

4.2. *Human tissues*

Tissue samples from human cerebral cortex and hippocampus were dissected from necropsies of four cognitively normal subjects (aged between 32 and 54 years) shortly after death (less than 10 h post mortem), with informed consent and in accordance with the guidelines of the Ethics committees of our institutions and following the Code of Ethics of the World Medical Association (Declaration of Helsinki). The causes of death were myocardial infarctation, pulmonary embolism, pancreatic tumor, and heart failure. For immunohistochemistry (IHC), the human tissue was fixed in Bouin and processed for paraffin sectioning (10 μm) following standard procedures.

4.3. *Immunohistochemistry*

Paraffin sections were deparaffinized, hydrated following standard protocols, boiled in 10 mM citrate buffer (pH6) at 100 °C for 20 min for antigen retrieval, rinsed in tris-buffered saline (TBS, pH7.6, 0.05 M), and incubated in the primary antibodies overnight in a humid chamber. After rinsing, they were incubated in the corresponding biotinylated secondary antibodies (rabbit anti-mouse IgG or goat anti-rabbit IgG; Dako, Glostrup, Denmark), diluted at 1:200 in TBS, washed, and incubated in a Strept ABComplexHRP Duet kit (Dako). Bound peroxidase was revealed using 0.04% 3,3-diaminobenzidine (Sigma, St. Louis, MO, USA), 0.05% ammonium nickel (II) sulphate, and 0.03% hydrogen peroxide in TBS, pH 7.6. The sections were dehydrated, cleared, and covered with Eukitt (O. Kindler, Freiburg, Germany). Double two-colored immunostaining for p73 and Reelin was carried out as described elsewhere (Abraham et al., 2004).

The following primary antibodies were used: a polyclonal antibody anti-p73α (against amino acids (aa) 427–636 of full-length p73; Kaghad et al., 1997, gift of D. Caput); a polyclonal antibody (against aa 1–15 of TAp73 (Ab5, NeoMarkers, Fremont, USA); a rabbit polyclonal anti-TAp73 antibody against aa 1–62 of human p73 (Ab14430), Abcam); a mouse monoclonal anti-p53 (CBL405, Cymbus Biotechnology); a mouse monoclonal anti-p63 (Ab 3239, Abcam); a mouse monoclonal anti-PCNA (proliferating cell nuclear antigen) (Ab-1, Clone PC10, Neomarkers); a monoclonal anti-Reelin antibody 142, raised against aa 164–405 (de Bergeyck et al., 1998) (gift of A. Goffinet); a rabbit polyclonal Tbr1 antibody (gift of R. Hevner); a rabbit polyclonal antibody against cleaved caspase-3 (Asp-175) (Cell Signalling 9661 Danvers, MA 01923); a polyclonal rabbit antibody against Glial Fibrillary Acidic Protein (anti-GFAP, Ab- 4, NeoMarkers), a rabbit antibody against aa 347–365 located at the C-terminus of human doublecortin (DCX), Sigma-Aldrich, and rabbit polyclonal antibodies against calretinin and calbindin (Swant).

Images were captured with a Zeiss Axio Imager microscope and digital camera, and photographs were prepared by using Adobe Photoshop software to adjust the levels of brightness and contrast.

4.4. *RT-PCR*

The dissected tissue from 3 subjects was quickly frozen and kept at −80 °C before total mRNA extraction. mRNA was isolated using Tripure Isolation Reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. After isolation, total RNA was treated with Amplification Grade DNAse I (Invitrogen, Carlsbad, CA) and cDNA was synthetisized with Improm-II Reverse Transcription System (Promega, Madison, WI). 5 μl of the resulting cDNA was used as a template for PCR reactions performed with Taq DNA polymerase (Roche Diagnostics, Barcelona) in 20 μl of total volume under following conditions: 94 °C for 3 min followed by 35 cycles of each 94 °C 0.5 min, 65 °C for 0.45 min, and 72 °C for 1 min. The following primers were used at 0.5 μM concentration: FW 5′-CCA TCC AGA TCA AGG TGT CC-3′ and RV 5′-GGT GAC AGG GTC ATC CAC AT-3′ (for the p73 core domain, Ensembl ID: ENST00000378295); FW 5′-ttgcccctcctagtcatttgatt-3′ and RV 5′-CCATCACCGTTCTTTG-TACTG-3′ (for the p63 core domain, Ensembl gene ID: ENSG00000073282). The product lengths are 215 base pairs for p73 and 348 base pairs for p63. GAPDH was used as control of PCR reaction (FW: 5′-ACCACAGTCCATGCCATCAC-3′ and RV: 5′-TCCACCACCCTGTTGCTGTA-3′), with a product size of 454 base pairs.

The intensity of the bands was measured by densitometry using software QuantityOne basic 4.6.3 (Bio-Rad Laboratories, Hercules, CA, USA) and normalized to GAPDH signal.

4.5. *Western blot analysis*

For total protein extraction, blocks of unfixed human temporal cortex and hippocampus from 4 subjects were homogenized with lysis buffer (62.5 mM Tris–HCl, 1% SDS, and 10% glycerol, pH 6.8). Homogenized extracts were centrifuged at 14,000 rpm during 45 min and the supernatant was collected. Protein quantification was measured in a microplate reader by using the bicinchoninic acid/cupric sulphate protocol. 5% Mercaptoethanol and 0.001% bromophenol blue were then added, and

samples were heated at 95 °C for 5 min. Equal amounts (50 μg) of each sample were electrophoresed on 10% SDS-polyacrylamide gel electrophoresis (PAGE; 10% polyacrylamide gels) and transferred to Hybond-P membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Membranes were preincubated with 5% blotting-grade blocker nonfat dry milk (Bio-Rad Laboratories, Hercules, CA) in TBS with 0.1% Tween 20 (TBS-T) at room temperature for 1 h and washed in TBS-T. Membranes were incubated with the same primary antibodies used for IHC. Mouse anti-alpha tubulin antibody (T 9026; Sigma) was used as a loading control. Membrane incubations with primary antibodies were performed overnight at 4 °C. Antibody labelling was developed by incubation with an HRP-conjugated goat anti-rabbit (1:20,000), and specific bands were visualized with the ECL plus Western Blotting Detection System kit (Amersham Biosciences). p73 (anti-TAp73 antibody against aa 1–15, Ab5, NeoMarkers, Fremont, USA) and p63 (anti-TAp63 antibody against aa 1–205, Ab 3239, Abcam) primary antibodies used for Western blot were the same described for immunohistochemistry.

The relative amounts of p73 and p63 protein expression were evaluated by densitometry, using software QuantityOne basic 4.6.3 (Bio-Rad Laboratories, Hercules, CA, USA) and normalized to the band of α-tubulin.

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