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DEPARTAMENTO DE BIOLOGÍA MOLECULAR

**ROLE OF p73 AND p53 IN THE BIOLOGY OF
NEURAL STEM CELLS AND IN THE
ARCHITECTURE OF THE NEUROGENIC NICHE
IN MOUSE BRAIN**

Ph.D. Dissertation

Laura González Cano

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**FUNCIÓN DE P73 Y P53 EN LA BIOLOGÍA DE
LAS CÉLULAS TRONCALES NEURALES Y EN LA
ARQUITECTURA DE LOS NICHOS
NEUROGÉNICOS EN CEREBRO DE RATÓN**

Tesis Doctoral

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Las investigaciones correspondientes a esta Memoria de Tesis Doctoral han sido dirigidas por la Dra. M^a del Carmen Marín Vieira (Departamento de Biología Molecular, Universidad de León) y la Dra. Margarita Marqués Martínez (Departamento de Producción Animal, Universidad de León).

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Contents

TABLE OF CONTENTS

LIST OF FIGURES	V
LIST OF TABLES	IX
ABBREVIATIONS	XI
INTRODUCTION	1
1. NEUROGENESIS	4
1.1. Embryonic neurogenesis	4
1.2. Adult neurogenesis	9
1.2.1. Postnatal development of the neurogenic niches	11
1.2.2. The subventricular zone of the lateral ventricles (SVZ)	13
1.2.3. The subgranular zone of the dentate gyrus of the hippocampus (SGZ)	19
2. NEURAL STEM CELLS BIOLOGY	21
2.1. Characterization of neural stem cells in vitro: the neurosphere assay	21
2.2. Regulation of neural stem cell biology	23
2.2.1. Pathways regulating self-renewal and differentiation	23
2.2.2. Regulation by TRIM-32	25
3. THE P53 FAMILY AND ITS ROLE IN THE BIOLOGY OF NEURAL STEM CELLS	26
3.1. Structural organization of p53 tumor suppressor family	28
3.2. Functional interaction of p53 family members	31
3.2.1. Role of p73 in cancer	32
3.2.2. Role of p73 in differentiation and development	33
AIMS	37
MATERIALS AND METHODS	41
1. ANIMAL WORK.....	43
1.1. Mice strains and animal breeding	43
1.2. Mouse genotyping	43
1.3. Euthanasia and anesthesia	45
2. <i>IN VITRO</i> STUDIES	45
2.1. Establishment of neurospheres (NS) cultures from OB of 14.5d embryos	45
2.2. Self-renewal assays	46
2.3. Determination of NS size and growth kinetics	46
2.4. Differentiation assays	46
2.5. Obtention of cell pairs by blebbistatin treatment	47

3. <i>IN VIVO</i> STUDIES	48
3.1. BrdU incorporation and perfusion	48
3.2. Isolation of brain samples	48
3.3. Preparation of whole mounts from the lateral wall of the ventricles	48
4. CELL CULTURE	49
4.1. Cell lines and culture conditions	49
4.2. Derivation of primary cultures of mouse embryonic fibroblasts (MEFs)	49
4.3. Cell transfection with Lipofectamine®	50
5. RNA WORK	50
5.1. Isolation of RNA samples	50
5.2. cDNA synthesis	50
5.3. Quantitative Real Time-PCR (qRT-PCR)	51
6. DNA WORK	52
6.1. Plasmid preparations	52
6.2. Cloning of hTRIM32-luciferase reporter vector	53
7. PROTEIN WORK	53
7.1. Preparation of cellular extracts	53
7.2. Protein immunodetection by western blot	54
7.3. Immunocytochemistry	54
7.4. Free floating immunohistochemistry	55
7.5. Whole mount staining	56
8. GENE TRANSCRIPTIONAL ANALYSIS	57
9. FLOW CYTOMETRY	58
9.1. Propidium Iodide (PI) cell cycle analysis	58
9.2. Evaluation of apoptosis by Annexin V- 7AAD staining	58
10. STATISTICAL ANALYSIS	58
RESULTS AND DISCUSSION	59
1. ANALYSIS OF THE ROLE OF P53 FAMILY MEMBERS, P73 AND P53, IN THE BIOLOGY OF NEURAL STEM CELLS	61
1.1. Role of p73 in the regulation of neural stem cells self-renewal and multipotency ..	61
1.2. Functional interaction between p53 and p73 in the biology of neural stem cells ..	70
1.3. Effect of p73 loss in the regulation of asymmetric cell division	77
1.4. Effect of p73 loss in the proliferating populations of the neurogenic niches	86
1.4.1. Analysis of proliferating cellular populations in the lateral walls of the ventricles (SVZ)	86

1.4.2. Analysis of the proliferating cellular populations in the subgranular zone of the dentate gyrus of the hippocampus (SGZ)	89
2. CHARACTERIZATION OF THE P53 FAMILY FUNCTION, IN THE GENESIS AND ARCHITECTURE OF THE MURINE NEUROGENIC NICHE IN THE SEZ/SVZ	92
2.1. Comparative analysis of the different cellular populations in the SVZ between mice of the four genotypes studied	92
2.2. Analysis of the post-natal formation of the lateral wall of the lateral ventricles: transition from the radial glia cells in the ventricular zone (VZ) to the ependymal layer and subventricular zone (SVZ)	98
2.3. Comparative analysis of the SVZ architecture at different post-natal days.....	105
2.3.1. Characterization of the lateral wall of WT and p73KO mice	106
2.3.2. Comparative analysis of the lateral wall of P15 mice from WT, p73KO, p53KO and DKO mice.....	119
3. IDENTIFICATION AND ANALYSIS OF NOVEL P73 TRANSCRIPTIONAL TARGETS IN NEURAL STEM CELLS BIOLOGY.....	123
3.1. Analysis of TRIM32, a neuronal fate determinant, as a direct transcriptional target of p73	124
CONCLUSIONS	131
SUMMARY IN SPANISH	135
1. Índice	137
2. Resumen	141
2. Conclusiones	147
REFERENCES	151
APPENDIX	167

LIST OF FIGURES

Figure 1. Pre-implantation embryo development and implantation in mice	5
Figure 2. Post-implantation embryo development in mice	6
Figure 3. Neural tube formation	7
Figure 4. Lineage relationships between neuroepithelial cells	8
Figure 5. Neurogenesis during cortical development	9
Figure 6. Neurogenesis in the adult rodent brain	10
Figure 7. Transformation of RG cells during embryonic and postnatal development	12
Figure 8. Progenitors and lineages in the developing and adult brain dentate gyrus	13
Figure 9. Types of cells that comprised the adult SVZ	14
Figure 10. Three-dimensional depicting of the SVZ adult stem cell niche	17
Figure 11. Neurogenesis in the SVZ	19
Figure 12. Adult SGZ neurogenesis	20
Figure 13. Neurosphere assay	21
Figure 14. Cellular composition of NS	22
Figure 15. The p53 family as a network	28
Figure 16. Structural organization and homology between p53 family members	29
Figure 17. Schematic representation of p53 family genes and its protein structure	30
Figure 18. PCR examples of p53 and p73 genotyping	44
Figure 19. Isolation of olfactory bulbs from mouse embryos at E14.5 days <i>post coitum</i>	45
Figure 20. Stereomicroscopic images showing wholemount dissection of the lateral wall of the ventricles	49
Figure 21. Differentiation of NS into the three major cell lineages of the central nervous system	62
Figure 22. Expression analysis of p73 isoforms in WT NS	63
Figure 23. Comparative analysis of NS size from p73KO and WT cultures	63
Figure 24. Growth kinetics of NS cultures	64
Figure 25. Self-renewal capacity of NPCs in sequential passages	65
Figure 26. Cell cycle analysis of secondary NS	65
Figure 27. Expression analysis of cell cycle regulator genes	66
Figure 28. Cell death analysis in secondary NS by Annexin V / 7AAD	67
Figure 29. Comparative analysis of the differentiation kinetics of NS cultures	69
Figure 30. Analysis of p53 activation in p73 deficient NS	70

Figure 31. Comparative analysis of NS size from the four genotypes	71
Figure 32. Comparative analysis of the net cell growth of NS from the four genotypes ...	71
Figure 33. Cell cycle analysis of NS cultures from the four genotypes	72
Figure 34. Analysis of expression of pro-apoptotic and cell cycle regulator genes	73
Figure 35. Comparative analysis of the proliferating cells	73
Figure 36. Self-renewal assay of the four genotypes	74
Figure 37. Neuronal differentiation from NS of the four genotypes	75
Figure 38. Analysis of differentiated NS after 5DIV-DM	76
Figure 39. Astrocytic differentiation from NS of the four genotypes	77
Figure 40. Analysis of undifferentiated progenitors from proliferating NS of the four genotypes	78
Figure 41. Analysis of asymmetric cell divisions	79
Figure 42. Analysis of prematurely differentiating neurons in NS of the four genotypes ...	80
Figure 43. Analysis of asymmetric distribution of NICD in neural progenitors cell pairs ...	81
Figure 44. Analysis of the number of cell pairs with asymmetric distribution of TRIM32 .	82
Figure 45. Cellular localization of NICD and TRIM32 expression in cell pairs	83
Figure 46. Comparative analysis of proliferation and TRIM32 expression	83
Figure 47. Analysis of the expression of TRIM32 in premature differentiated neurons	84
Figure 48. Analysis of the presence of neurons and correlation with TRIM32 expression at early differentiation time points	85
Figure 49. Comparative analysis of the proliferating cells in the subventricular zone SVZ of P15 mice	87
Figure 50. Comparative analysis of the proliferating populations in the SVZ of P15 mice of the four genotypes	88
Figure 51. Comparative images of coronal sections from P15 mice of the four genotypes	88
Figure 52. Comparative analysis of the proliferating cells in the subgranular zone of P15 mice	90
Figure 53. Comparative analysis of the proliferating populations in the SGZ of WT, p73, and DKO mice	90
Figure 54. Comparative analysis of the population of neuroblasts in the four genotypes..	93
Figure 55. Comparative analysis of B cells in the SVZ	94
Figure 56. Comparative analysis of the ependymal layer in the four genotypes	95
Figure 57. Analysis of the proliferating ependymal cells	96
Figure 58. Comparative analysis of Noggin expression in ependymal layer	98
Figure 59. Electron microscopy analysis of the SVZ from WT and p73KO mice	99

Figure 60. Analysis of the expression of early glial markers at P7 and P15	101
Figure 61. Glial and ependymal markers colocalization in P7 mice brain	103
Figure 62. Surface map of the walls of the LV	106
Figure 63. Antero-posterior characterization of the lateral wall of P7 WT mice	107
Figure 64. Magnification of the “flower” pattern of WT P7 mice	108
Figure 65. Antero-posterior characterization of the lateral wall of P7 p73KO mice	109
Figure 66. Antero-posterior characterization of the lateral wall of P15 WT mice	111
Figure 67. Magnification of the “flower” pattern in the anterior region of P15 WT mice .	112
Figure 68. Magnification of the cilia in the lateral wall of P15 WT mice	112
Figure 69. Analysis of the small monociliated cells during development and along the antero-posterior axis	113
Figure 70. Analysis of the number of large multiciliated “ependymal” cells that express GFAP	113
Figure 71. Antero-posterior characterization of the lateral wall of P15 p73KO mice	115
Figure 72. Magnification of the aberrant pinwheel patterns in P15 p73KO mice	116
Figure 73. Details of the cilia in p73KO mice lateral wall at P15	117
Figure 74. Comparative analysis of the lateral walls of WT and p73KO adult mice	118
Figure 75. Comparative analysis of the lateral wall of P15 day old mice from the four genotypes	120
Figure 76. Comparative analysis of coronal sections of P15 day old mice from the four genotypes	121
Figure 77. TRIM32 expression in NS cultures	124
Figure 78. Analysis of TRIM32 mRNA levels in NS from the four genotype	124
Figure 79. Effect of p73 ectopic expression in TRIM32 mRNA expression levels	125
Figure 80. Transcriptional regulation of the human TRIM32 promoter by p53 family members	126
Figure 81. Transcriptional regulation of TRIM32 promoter by co-transfection of TAp73 and Δ Np73	127
Figure 82. Analysis of mRNA levels of TAp73 and TRIM32 in NPCs undergoing differentiation	128
Figure 83. Analysis of TRIM32 expression in brains from WT and p73KO mice	129

LIST OF TABLES

Table 1. PCR mixture conditions and primers sequences for mouse genotyping	44
Table 2. NS culture media and solutions	47
Table 3. Cell culture media	49
Table 4. Primers sequences	52
Table 5. Primary antibodies used for immunodetection	56
Table 6. Secondary antibodies used for immunodetection	57
Table 7. Microarray analysis of the transcriptome of K562 cells over-expressing Δ Np73 isoforms	123

ABBREVIATIONS

AD	Alzheimer's disease
AD	Antero-dorsal
AV	Antero-ventral
BDNF	Brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BLBP	Brain-lipid-binding protein
BMP	Bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
ChiP	Chromatin immunoprecipitation
CNS	Central nervous system
CSF	Cerebrospinal fluid
Dcx	Doublecortin
DIV	Days in vitro
DKO	Double knockout
DM	Differentiation media
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide phosphate
E (n°)d	Embryonic day n°
EGF	epidermal growth factor
EGFR	Epidermal growth factor receptor
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FGF2	Fibroblast growth factor 2
GCL	Granule cell layer
g	gravitational force
gDNA	Genomic DNA
GFAP	Glial fibrillary acidic protein
GLAST	Astrocyte-specific glutamate transporter
IB4	Isolectin B4
IGF1	Insulin growth factor 1
KO	Knock out
LV	Lateral ventricle
LW	Lateral wall
Mash1	Transcription factors Ascl1
MEF	Mouse embryonic fibroblast
NE	Neurite extensions
NDS	Normal Donkey Serum
NICD	Active Notch intracellular domain
NP	Neural progenitor

NPC	Neural progenitor cells
NS	Neuroespheres
NSC	Neural stem cells
OB	Olfactory Bulbs
o/n	Over night
P (n°)	Postnatal n° days
P1	Passage 1
P15	Postnatal 15 days
P2	Passage 2
PBS	Phosphate Buffer Saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PD	Posterior-dorsal
PEDF	Pigment epithelium-derived factor
p-His3	Phospho-Histone 3
PI	Propidium Iodide
PM	Proliferating media
PV	Posterior-ventral
qRT-PCR	Quantitative real time polymerase chain reaction
RE	Response element
RT	Room temperature
RG	Radial glia
RMS	Rostral migratory stream
ROS	Reactive oxygen species
SGZ	Subgranular zone
SR	Self-renewal
SVZ	Subventricular zone
TAP	Transit amplifying progenitors
Tuj-1	β-III-Tubulin
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone
WT	Wild type

Introduction

During the past decades, with the increase of the life span in the European population, the prevalence of neurodegenerative disorders, such as Alzheimer's disease, has grown dramatically. Thus, it has become a priority to strengthen efforts towards the early diagnosis and treatments for these disorders. In the recent years, the presence of neurogenesis in the adult brain has been described. Specific areas of the mammalian adult brain harbors neural stem cells (NSC) and progenitors that continuously proliferate and differentiate, and it is suspected that such processes might be affected in neurodegenerative diseases. Furthermore, the intrinsic properties of neural stem cells (unlimited self-renewal, as well as the ability to differentiate into different cell types), make them an excellent and appealing resource for application in regenerative medicine. Nowadays, several studies are trying to use neural stem cells as cell replacement therapy for damaged cells in neurodegenerative diseases. It has been suggested that stem cell therapy for neurodegenerative diseases might represent a potential cure, compared with small-molecule-based therapy, which can ameliorate symptoms but cannot correct the underlying genetic or structural problems. For all these reasons, increasing the knowledge of the mechanisms that regulate neurogenesis during development, and of adult NSCs and their cellular niches, is not only important to understand the physiology of neurogenesis, but also for the development of new therapeutic applications using adult NSCs.

The ability of maintaining neurogenesis throughout life is a direct result of neural stem cell capacity to self-renew and, under specific micro-environmental stimuli, to differentiate and generate different neural cell lineages. Thus, the identification of the genes implicated in the regulation of such processes appears to be critical for the development of new cellular therapies. In this context, the members of the p53 family of tumor suppressor genes has been suggested as possible players in neuronal survival and stem cell biology, by the integration of multiple processes such as cell survival, differentiation, maintenance of the genomic integrity, and maybe others not yet described.

1. Neurogenesis

The idea that the adult nervous system contained stem cells was viewed as impossible in the not-so-distant past. Classically, the adult nervous system was thought to be “something fixed, ended, and immutable”, and brain development was thought to be ended at birth, having essentially no regenerative capacity. However, we now know that neurogenesis, defined as the generation of neurons and glial cells on an ongoing basis from neural stem cells, occurs during development as well as in the adult brain. These discoveries lead to the generation of an important field of neural stem cells biology that would allow us to understand CNS development, brain functioning and, in addition, it would allow us to design novel regenerative therapies.

The CNS is a highly organized structure assembled from different cell types. In order to form functional neuronal networks cell must be generated in a precise number, spatial and temporal hierarchy, as well as be specifically positioned within the developing nervous system.

1.1. Embryonic neurogenesis

In mammals, embryonic development begins with the fertilization of the oocyte and consists of four stages: Cleavage, Patterning, Differentiation and Growth. The mammalian oocyte is released from the ovary and swept by the fimbriae into the oviduct, where fertilization occurs and zygote is formed. After fertilization and while the cilia in the oviduct is pushing the embryo towards the uterus, meiosis is completed and first cleavage begins about a day later to obtain 2-cell stage (E1.5d). Following the 8-cells stage, the embryo undergoes a Ca^{2+} -mediated compaction to form the morula (E2.5d) where all cells are still totipotent. Then, some of the cells from the morula differentiate to become the trophoectoderm (TE), an outer layer surrounding the inner cell mass (ICM). The cells from the TE secrete fluid, which will coalesce and expand to form the blastocoel cavity, and the embryo will become a blastocyst (E3.5d). The blastocoel gradually pushes the ICM cells to one pole resulting in an eccentric localization of the ICM while the opposite mural TE initiates uterine implantation (E4.5d). The cells of the ICM are pluripotent stem cells that can give rise to all cell types of the three embryonic layers (ectoderm, mesoderm and endoderm), the germ cell lineage, as well as to the non-trophoblast tissues that support the developing embryo (Krupinski et al., 2011; Li et al., 2010; Wang and Dey, 2006)(Figure 1).

Figure 1.- Pre-implantation embryo development and implantation in mice. Representation of the pre-implantation events and its location in the reproductive tract at different developmental stages (Wang and Dey, 2006).

At this stage the formation of the primitive endoderm (PE) occurs from the surface of ICM and the pluripotent epiblast (EPI). During the immediate post-implantation period (E6), the mouse embryo changes dramatically in size and shape. The embryo becomes a cup-shaped epithelial tissue made up of two cell layers, the inner epiblast and the outer visceral endoderm, called egg cylinder in which extraembryonic and embryonic regions are well delineated. Between day 6 and 6.5 the process of gastrulation takes place. The epiblast cells undergo epithelial-mesenchymal transformation to generate the primitive streak along the future posterior axis of the mouse embryo. First, the primitive streak elongates from the rim of the cup to its distal tip, and the cells emigrating through the primitive streak give rise to the definitive endoderm and mesoderm. Following elongation at the anterior tip of the streak, a specialized structure, the node, is formed. The node is a transit structure, equivalent of the organizer in amphibians, that gives rise to the trunk notochord and, together with the anterior visceral endoderm, is involved in the specification of the anterior-posterior (A-P) axis of the embryo which becomes evident as a result of the primitive streak formation (Marikawa, 2006). The node generates axial mesoderm, such as pre-chordal plate, notochord and somites, as well as the gut endoderm. During gastrulation, two convergent extension processes drive the narrowing and lengthening of the body axis that typifies embryos in the immediate pre-neurulation period. Chordamesodermal cells emerge from the node and intercalate in the midline, as the notochordal plate narrows to form the notochord (Sulik et al., 1994). The notochordal plate is an epithelial structure that is in contact laterally with the endoderm and dorsally with the midline cells of the neural plate.

The notochordal plate separates from the endoderm to form a temporary cylinder rod-like structure, denoted notochord. The notochord develops along the dorsal surface of the embryo and connects the anterior visceral endoderm and the node, playing an important inductive role in the formation of the neural plate (Figure 2).

Figure 2.- Post-implantation embryo development in mice. Three-dimensional schematic drawing of mouse development from implantation through early neurulation stages. The dotted line indicates the separation between extraembryonic tissues (upper portion) and embryonic tissues (bottom portion); dpc: days post coitum (Hogan et al., 1994).

At E7.5d the first step on central nervous system (CNS) formation occurs with the appearance of the neural plate from the neuroectoderm that lies dorsal to the notochord. In response to signals from the underlying notochord, the neuroectoderm thickens to give rise to a pseudostratified epithelium, the neural plate (Sulik et al., 1994).

Neurulation is a complex, highly dynamic morphogenetic process during which the flat neural plate bends and fuses to form the neural tube. Mouse primary neurulation is characterized by a stereotypical pattern of neural plate bending along the spinal axis. Neural closure begins cranially at E8.5 and finishes at E10.5 when closure is completed caudally. Initially, the neural plate bends solely at the median hinge point, overlying the notochord, to form the neural groove. As neurulation progresses the neural plate folds at dorsal hinge points. Continued folding results in the neural folds meeting in the dorsal midline where they fuse to form the neural tube (Frisen et al., 1998)(Figure 3).

Figure 3.- Neural tube formation. Scheme of the events that take place during neural plate folding to produce the neural tube (Smith and Schoenwolf, 1997).

Before closure, the neural crest cells delaminate from the neural folds and these cells migrate throughout the body to form a diverse array of peripheral nervous system, pigment cells and craniofacial mesenchyme (Raible, 2006). Furthermore, during neurulation the neural groove become the lumen of the neural tube in which the neuroepithelium is composed of a single cell layer, neuroepithelial cells, which are the primary neural progenitors from which all other CNS progenitors will derive (Huttner and Kosodo, 2005).

Neurons and glial cells in the CNS have classically been thought to derive from distinct precursor pools that diverge early during embryonic development. Neurons were proposed to derive from neuroblasts, while radial glia were thought to be the precursors to astroglial cells, being their separate origins widely accepted in most of the past century. However, recent studies have shown that the radial glia and a special subpopulation of astrocytes are indeed the neural stem cells that give rise to differentiated neurons and glial cells during development and in the postnatal brain (Kriegstein and Alvarez-Buylla, 2009).

Neurons and macroglia ultimately derive from the neuroepithelial cells. During early embryonic development and before neurogenesis, the neural plate and neural tube are composed of a single cell layer, the neuroepithelial cells, which form the neuroepithelium. Neuroepithelial cells (NE) extend from the apical surface to the basal lamina, show typical epithelial features and are highly polarized along their basal-apical axis. They can be considered stem cells, since they display the main features of stem cells, which are self-renewal and the capacity to differentiate into distinct cell types. In early development, they

expand their population by symmetric cell division to generate more neuroepithelial stem cells. These divisions are followed by many asymmetric, self-renewing divisions, generating a daughter stem cell plus a more differentiated basal progenitor, as intermediate in the generation of neurons, or a neuron (Gotz and Huttner, 2005)(Figure 4).

Figure 4.- Lineage relationships between neuroepithelial cells. A) Scheme of the cell types derived from the NE cells. B) Simplified view of the relationship between neuroepithelial cells (NE), radial glial cells (RG) and neurons (N) with basal progenitors (BP) as cellular intermediates in the generation of neurons (Gotz and Huttner, 2005; Huttner and Kosodo, 2005).

At approximately the time when cortical neurogenesis begins (around E9-10), the neuroepithelium transforms into a tissue with numerous cell layers, with the progenitor cell bodies remaining in the most apical layer -known as ventricular zone-, as well as in the adjacent cell layer -the subventricular zone-, while the neurons migrate to the more basal cell layers. After the onset of neurogenesis, and during this transition, neuroepithelial cells give rise to a distinct, but related cell type which exhibit residual neuroepithelial features as well as astroglial properties, the radial glial cells (RG) (Kriegstein and Alvarez-Buylla, 2009).

Radial glial cells represent more fate-restricted progenitors that extend from the apical surface of the ventricular zone through the neuronal layers of the basal lamina. In mice the transition of NE to RG cells occurs between E10, when no astroglial markers can be detected, and E12, when most of CNS is comprised by progenitors with astroglial features. These cells maintained neuroepithelial properties, such as the expression of nestin, the maintenance of an apical-basal polarity, and the basal lamina contact. During neurogenesis the tight junctions that couple NE cells convert to adherens junctions, and the cells begin to make astrocytes-like specialized contact with endothelial cells of the developing cerebral vasculature. Furthermore, they also acquire features associated to glial cells. Progressive thickening of the cortex throughout neurogenesis is accompanied by lengthening of the pial-directed radial processes of RG. These morphological changes are associated with the acquisition of microtubules and intermediate filaments within the radial fibers, as well as glycogen storage granules in the subpial end feet. The RG also begin to express astroglial

markers like the astrocytes-specific glutamate transporter (GLAST), the brain lipid-binding protein (BLBP), and the Ca^{2+} -binding protein $\text{s100}\beta$, as well as a variety of intermediate filaments proteins, including Vimentin and RC2 epitope (Gotz and Huttner, 2005; Huttner and Kosodo, 2005; Kriegstein and Alvarez-Buylla, 2009).

Summarizing, during cortical development, RG generates neurons directly through asymmetric division, or indirectly by generation of intermediate progenitors and either one round of amplification or two rounds of division and further amplification, that may be fundamental to increase cortical size (Figure 5).

Figure 5.- Neurogenesis during cortical development. Scheme of the different modes of neurogenesis during cortical development. MZ: marginal zone, NE: neuroepithelium, nIPC: neurogenic progenitor cell, oIPC: oligodendrocytic progenitor cell, CP: cortical plate, IZ: intermediate zone, SVZ: subventricular zone, VZ: ventricular zone (Kriegstein and Alvarez-Buylla, 2009).

1.2. Adult neurogenesis

Radial glial cells function as neural stem cells in the developing brain but for many years it was thought that there were no NSCs in the adult brain. However, in the mid-60s the discovery of adult neurogenesis (Altman, 1969), followed by the identification of cells that can function as NSCs generating neurons and glial cells *in vitro* (Reynolds and Weiss, 1992) and *in vivo* (Doetsch et al., 1997; Seri et al., 2004; Seri et al., 2001) demonstrated that NSCs were present in the developing brain and persisted in restricted regions of postnatal and adult brain (Figure 6).

Adult neurogenesis involves several crucial steps. Initially, asymmetric cell division of a stem cell occurs, resulting in one daughter stem cell and one with the potential to develop into a neuron (neuroblast). Next, the newly generated neuroblasts migrate to its final and appropriate destination in the brain where they mature and integrate by forming efferent and afferent connections with neighboring cells.

Figure 6.- Neurogenesis in the adult rodent brain. Sagittal and coronal views of mouse brain showing the areas implicated in neurogenesis. Red areas indicate the germinal zones in the adult mammalian brain: the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Zhao et al., 2008).

In the adult mammalian brain, NSCs which are generated from the precursors that build the nervous system during development are maintained in at least two regions, the subventricular zone (SVZ) of the lateral walls of the ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002) and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Doetsch et al., 1997). The SVZ contains a subpopulation of cells with astroglial properties (B1 cells) that function as NSCs, giving rise to intermediate progenitors (C cells), that finally generate neurons destined for the olfactory bulb (OB). In the SGZ, NSCs also correspond to astroglial cells (radial astrocytes or type I progenitors) which give rise to neurons that migrate into the granule cell layer of the dentate gyrus and become dentate granule cells.

The presence of NSCs at other regions of the postnatal and adult brain has been widely discussed. Recent studies have demonstrated that embryonic multipotent neural stem cells can be isolated from the E12.5-E14.5d olfactory bulbs (OB) (Vicario-Abejon et al., 2003). Moreover, neurogenesis in the OB is not completed at the end of the embryonic development and multipotent NSC can be obtained from the rostral migratory stream (RMS) and the OB of adult mice (Fukushima et al., 2002; Gritti et al., 2002). Despite this, adult neurogenesis *in vivo* has only been consistently found in the SVZ and SGZ, suggesting that specific factors, permissive for the differentiation and integration of new neurons, are present in the microenvironments of the neurogenic niches (Morrison and Kimble, 2006).

1.2.1. Postnatal development of the neurogenic niches

At birth, the walls of the lateral ventricles maintain many similarities to the ventricular zone of the immature neuroepithelium, and they change dramatically during postnatal development. Newborn mice periventricular regions are larger and contain more cells than in adults (Peretto et al., 1999). They comprised two distinct cellular zones, the ventricular zone (VZ) and the subventricular zone (SVZ). Shortly after birth, the embryonic niche begins a transformation into the postnatal NSC niche in the SVZ (Tramontin et al., 2003). The number of cells in these regions severely decreases between postnatal day 0 and day 7 and, by day 15 the lateral wall appears similar to the adult SVZ. At P0, the VZ is mainly comprised of radial glia, which are progenitors with cell bodies close to the ventricles and a long radial process that contacts the pial surface of the brain (Hartfuss et al., 2001; Merkle et al., 2004). At this stage some immature ependymal cells are present in the VZ. At the end of embryonic development, most RG cells begin to detach from the apical surface of the ventricle and convert into stratial astrocytes. After birth, a selected group of radial glia gets transformed into unique subpopulations of astrocytes, type B1 cells, which become the slow-cycling stem cells of the SVZ that function as primary neural progenitors (Merkle et al., 2004). Another subpopulation of radial glia will give rise to multiciliated ependymal cells that form the epithelial lining of the ventricles (Spassky et al., 2005). At P7, the proportion of RG decreases while the proportion of immature ependymal cells increases in a similar percentage. Finally, at P15 and P30 radial glia is completely absent from the VZ, which is predominantly composed of mature and immature ependymal cells (Tramontin et al., 2003)(Figure 7).

During postnatal development, the radial glia located in the lateral ventricle walls retracts their long distal process and lose expression of early astroglial markers giving rise to the type B1 astrocytes. A significant percentage of the cells contacting the ventricle are B1 cells. Although, RG and type B1 cells possess different morphology and express different markers, they share some interesting features like the fact that both cell types extend one short cilium to the ventricle lumen (Doetsch et al., 1999a; Doetsch et al., 1997), which was also present in neuroepithelial stem cells (Cohen and Meiningner, 1987).

Figure 7.- Transformation of RG cells during embryonic and postnatal development. Scheme of the relationship between the different cell types that comprised the neurogenic ventricular zone during embryonic and postnatal development. MZ: marginal zone, NE: neuroepithelium, oIPC: oligodendrocytic progenitor cell, nIPC: neurogenic progenitor cell, MA: mantle, SVZ: subventricular zone, VZ: ventricular zone (Kriegstein and Alvarez-Buylla, 2009).

Some molecular programs that regulate embryonic neuroprogenitor differentiation have also been shown to function in the postnatal SVZ niche. SVZ cellular proliferation and migration of newly generated neurons into OB is regulated by $TGF\alpha$, through activation of EGF receptor (Tropepe et al., 1997). Furthermore, Bone Morphogenetic Protein (BMP) and their antagonist Noggin, affect differentiation of SVZ progenitors differentiation during postnatal development (Lim et al., 2000). Additionally, activated Notch suppresses neuronal differentiation and prevents neuroblast migration to the OB (Chambers et al., 2001). Numb/Numbl also contribute to SVZ homeostasis by regulating ependymal integrity and survival of SVZ neuroblasts (Kuo et al., 2006).

Although there is no much information about the development of the SGZ, it is known that, in contrast to SVZ neurogenic niche with reminiscent features from embryonic SVZ, the developmental plan that generates the SGZ neurogenic niche is very different (Li and Pleasure, 2005). Around E9.5d the dentate precursor pool begins to proliferate, to expand and form the first granular cells in the primary dentate neuroepithelium, located near the ventricular zone. Then, the basis for the granular layer of the dentate gyrus (DG), that is the secondary dentate matrix, is formed. At E13.5d, cells within the secondary matrix are very proliferative and migrate to the nascent DG. By E17.5d, the tertiary matrix is formed within the future hilus and progenitors and granule cell populations begin to mix and migrate to the limbs of the DG. Finally, the granule cell layers are condensed and the neural progenitors become aligned with the SGZ. At P7, the early developmental stages are complete and the

dentate gyrus is formed and just beginning to mature into its neuronal layers (Figure 8) (Altman and Bayer, 1990; Li and Pleasure, 2007).

Figure 8.- Progenitors and lineages in the developing and adult brain dentate gyrus. Possible link between RG in the VZ contacting the lateral ventricle (dentate neuroepithelium) and the developing SGZ. RG generates astrocytes that reside in the SGZ, they presented long processes that traverses the dentate granule cell layer and branches in the deep molecular layer (Kriegstein and Alvarez-Buylla, 2009).

Although, Wnt and Shh pathways has been described to be involved in the maintenance of precursors in the postnatal and adult DG (Lai et al., 2003; Lie et al., 2005), it is not clear what factors regulate the behavior of precursors during their transit to the DG.

1.2.2. The subventricular zone of the lateral ventricles (SVZ)

The SVZ is one of the prominent neurogenic regions in the adult mammalian brain and is located along the walls of the lateral ventricles next to the ependyma, a thin cell layer that lines the ventricles. The adult SVZ is a highly organized microenvironment comprised by NSC as well as other cell types that contributes to important features of the niche, and to long-term neurogenesis.

At the beginning of the past century, Allen (1912) identified for the first time the presence of mitotic cells in the SVZ of adult rats using [^3H]thymidine to label dividing SVZ cells, demonstrating that proliferation continues throughout life (Tropepe et al., 1997). Retroviral lineages studies demonstrated that cells from the SVZ are an important source of astrocytes, oligodendrocytes and neurons in neonates (Levison and Goldman, 1993; Luskin, 1993). Furthermore, in adults, cells from the SVZ differentiate into neuronal precursors that migrate long distances to reach the olfactory bulb (Lois and Alvarez-Buylla, 1994). New

neurons are born throughout the SVZ and join a network of migrating neurons that coalesce to form the rostral migratory stream leading to the olfactory bulb, where they differentiate into granule and periglomerular neurons (Doetsch and Scharff, 2001). It has been demonstrated that NSCs from the SVZ also generate migrating young oligodendrocytes in normal and injured adult brain (Menn et al., 2006; Nait-Oumesmar et al., 1999).

Electron microscopy analysis established four main cell types in the SVZ based on their ultrastructural and immunocytochemical features (Doetsch, 2003; Doetsch et al., 1997): SVZ astrocytes (type B cells) highly proliferative transit amplifying population or intermediate progenitors (type C cells), neuroblasts (type A cells) and ependymal cells (type E cells) (Figure 9). The role of SVZ astrocytes as NSC was established from experiments in which infusion of the anti-mitotic drug cytosine-b-D- arabinofuranoside (Ara-C) resulted in elimination of all immature precursors, C and A cells, but not SVZ astrocytes and ependymal cells. After such extensive ablation, the SVZ regenerated and young migrating neurons reappeared (Doetsch et al., 1999b). Furthermore, they demonstrated that B cells were the responsible of such regeneration, thus, the NSC of the neurogenic niche. Ependymal cells were also suggested to be the adult NSCs responsible for neurogenesis in the SVZ (Johansson et al., 1999). However, posterior studies demonstrated that ependymal cells were quiescent and did not display the properties of NSCs *in vitro* (Capela and Temple, 2002; Doetsch et al., 1999a).

Figure 9.- Types of cells that comprised the adult SVZ. NSCs in the wall of the ventricles are the type B cells. B cells give rise to C cells, or intermediate progenitors, to finally produce neuroblasts (A cells) and likely oligodendrocytes (Kriegstein and Alvarez-Buylla, 2009).

During the last decade many studies have provided new insights about the anatomy and organization of NSC in the adult neurogenic sites, as well as about the nature and function of adult NSCs (Figure 10). Ependymal cells are neuroepithelial multiciliated cells that delineate the ventricles and form pinwheel-like structures around the apical processes of type B cells (Mirzadeh et al., 2008). Ependymal cells has been described to have multiple motile cilia extending from their apical surface into the ventricle (Bruni, 1998), and to display

Planar Cell Polarity (PCP), with their basal bodies underlining the directional beating of motile cilia to propel cerebrospinal fluid (CSF) flow and maintain CSF homeostasis (Mirzadeh et al., 2010; Sawamoto et al., 2006; Spassky et al., 2005). Furthermore, the motile cilia affect migration of young neurons towards OB, since they are required to create gradients of chemorepellents that guide anterior neuroblast migration along the rostral migratory stream (Nguyen-Ba-Charvet et al., 2004; Sawamoto et al., 2006). Although under normal conditions ependymal cells are quiescent and do not contribute to adult neurogenesis, it has been reported that in response to stroke they delaminate from the ependymal layer before entering cell cycle to produce neuroblasts and glial cells located in the SVZ or RMS (Carlen et al., 2009; Zhang et al., 2007). Ependymal cells are not only necessary for normal CSF flow and neuroblast migration towards the OB, but to establish and maintain the neurogenic niche microenvironment. They induce neurogenesis and suppress gliogenesis by secreting the BMP inhibitor, Noggin (Chmielnicki et al., 2004; Lim et al., 2000); they also produce Pigment Epithelium-derived Factor (PEDF) promoting *in vitro* and *in vivo* self-renewal of NSC (Andreu-Agullo et al., 2009; Ramirez-Castillejo et al., 2006).

Ependymal cells are characterized by the expression of CD24⁺, s100 β and Vimentin (Mirzadeh et al., 2008; Pastrana et al., 2009; Raponi et al., 2007). They are generated from radial glia during embryonic brain development (Spassky et al., 2005); however the mechanism by which they mature, acquiring their final characteristics, remains poorly understood. Forkhead transcription factor FoxJ1 (Jacquet et al., 2009; Yu et al., 2008) and the homeobox gene Six3 (Lavado and Oliver, 2011) have been demonstrated to be essential for ependymal cell maturation and ciliogenesis during postnatal stages of brain development. The mechanisms that guide ependymal planar cell polarity have been described as a multistep process orchestrated by the primary cilium and its basal body apparatus. Ependymal cell basal bodies display two features that correlate with the direction of fluid flow: their rotational orientation and their translational position on the apical surface. Radial glia primary cilia have an important role in the organization of the cytoskeleton before ependymal differentiation to determine the position of basal bodies (Mirzadeh et al., 2010). Additionally, RG cells display planar cell polarity, which is predictive of the polarity in their ependymal progeny. Two models have been purposed for the acquisition of planar polarity in RG cells: genetic or environmental. In the genetic model, it is hypothesized that RG lining the embryonic brain ventricles contain positional information that is projected onto the developing cortex as a spatial code through radial fiber (Rakic, 1988). Finally the planar polarity of ependymal cells may be read out of this spatial code. Alternatively, in the environmental model, the primary cilia, well known as mechanosensory

transducer (Hildebrandt et al., 2009), may be detecting signals that lead to the acquisition of planar polarity in the RG.

In ependymal cells two types of planar cell polarity have been defined: translational and rotational PCP. Translational PCP, refers to when basal bodies are present in a cluster, only partially covering the apical surface, and the position of these clusters is planar polarized. Rotational PCP can be defined as the parallel alignment of all the basal bodies within each multi-ciliated cell. While motile cilia are not required for translational PCP in ependymal cells, they are absolutely required for the rotational polarity in these cells. The translational PCP of differentiated ependymal cells instead requires the presence of non-motile primary cilia on the radial glial precursors from which these cells arise (Mirzadeh et al., 2010). Recent data suggest that radial glial cells possess a planar polarity that is then essential for the planar polarization of their ependymal cell descendants (Wallingford and Mitchell, 2011).

Several articles have linked the PCP signaling pathway to the planar polarization of ependymal cilia, showing that the core PCP proteins Vangl2, Celsr2, and Celsr3 are required for establishment of rotational and tissue-level polarity and for polarized fluid flow in the mouse brain (Tissir and Goffinet, 2012). Importantly, asymmetric Vangl2 localization reflects polarity in these cells, while Celsr2 and Celsr3 are essential for Vangl2 localization in ependymal cells (Guirao et al., 2010). A recent report has demonstrated that control of spindle orientation is essential for maintaining the population of neuroepithelial cells, but dispensable for the decision to either proliferate or differentiate. In this study, knocking out the G protein regulator LGN, randomized the orientation of normally planar neuroepithelial divisions, and the resultant loss of the apical membrane from daughter cells converted them into abnormally localized progenitors, without affecting neuronal production rate. Furthermore, overexpression of Inscuteable, a regulator of spindle orientation that induces vertical neuroepithelial divisions, shifted the fate of daughter cells (Konno et al., 2008). Thus, it has been proposed that planar mitosis ensures the self-renewal of neuroepithelial progenitors by one daughter inheriting both apical and basal compartments during neurogenesis.

Figure 10.- Three-dimensional depicting of the SVZ adult stem cell niche. Schematic depicting of the components and cell types that comprised the adult SVZ. Ependymal cells (E), C and A cells, blood vessels (Bv), microglial cells (Mg) and the extracellular matrix (Ihrie and Alvarez-Buylla, 2011).

The adult SVZ is composed by two distinct proliferative populations (Figure 10): slowly dividing progenitors B cells and focal clusters of rapidly dividing transit amplifying intermediate progenitors C cells. The B cells, the neural stem cells in this region, have been identified as a subpopulation of astrocytes derived from radial glia cells (Doetsch et al., 1999a). Based on their morphology and location in the SVZ, two types of B cells have been defined. The B1 cells frequently have apical surface in contact with the ventricle, and present a small apical process that extends towards the ventricle. In contrast, B2 cells are frequently located close to the underlying striatal parenchyma (Doetsch et al., 1997; Mirzadeh et al., 2008). The presence of B1 apical surfaces influences the geometry and organization of ependymal cells resulting in a remarkable architecture of the lateral wall. The apical surfaces of B1 cells are located in the core of E cells forming a pinwheel, a pattern that appeared to maximize the packing of E cells around the B1 apical surface. Being part of an epithelium, and having specialized apical structures are germinal niche properties retained by adult NSC, which probably must be essential for their function (Doetsch et al., 1997; Mirzadeh et al., 2008).

The astrocytic nature of adult SVZ neural stem cells has been widely demonstrated and, despite their NSC properties, they display astrocytic ultrastructural and morphology characteristics. They also express markers of astroglial cells, like the cytoskeletal proteins glial fibrillary acidic protein (GFAP), vimentin and nestin (Doetsch et al., 1999b; Hartfuss et al.,

2001; Platel et al., 2009). Although ependymal cells are also CD133 positive, the only cellular profiles in the ventricular wall that are CD133⁺/CD24⁻ are the B1 type cells (Kriegstein and Alvarez-Buylla, 2009).

NSC behavior could be modulated by multiple signals. The small apical surface is directly contacting the CSF, which contains soluble factors like Insulin-like growth factor 2 (IGF2), that regulate SVZ progenitors proliferation. BMPs, Wnts, Sonic hedgehog (SHH) and retinoic acid are also present in the CSF and may modulate B1 cells behavior (Huang et al., 2010; Lehtinen et al., 2011). Tight balance between NSC self-renewal and differentiation is essential within the neurogenic niches. The existence of a feedback mechanism between C cells and B cells to regulate the progeny produced has been suggested. Notch signaling maintains B1 cells by inhibiting the production of C cells through a possible feedback mechanism via lateral inhibition by direct cell-cell contact, maintaining the undifferentiated state (Imayoshi et al., 2010; Kopan and Ilagan, 2009). Furthermore, B1 cells received information through intercellular contacts, extracellular signals from ependymal cells, and from the extracellular matrix, as well as, from the SVZ vasculature and the surrounding neural tissue to maintain neurogenesis throughout life.

The immediate progeny of B1 astrocytes are the Type C cells, an heterogeneous population that divide actively and with a more restricted potential. They are located close to their progenitors and also in close proximity to blood vessels (Doetsch et al., 1999a; Tavazoie et al., 2008). The epidermal growth factor receptor (EGFR) and the transcription factor *Ascl1* (*Mash1*) are frequently used as Type C cells markers. Finally, after amplification of the population progenitor cells, they will give rise to neuroblasts, as well as oligodendrocyte precursors, although in lower number (Doetsch et al., 1997; Menn et al., 2006). The committed migratory neuroblasts, Type A cells, are in direct contact with B cells and express β -III-tubulin (*Tuj1*), doublecortin (*Dcx*) and polysialylated neural cell adhesion molecule (PSA-NCAM) (Memborg and Hall, 1995; Seki and Arai, 1993).

Newly formed neuroblasts migrate tangentially in chains toward the OB. The astrocytes form a glial sheath around chains of these young neurons as they migrate toward the olfactory bulb to form the RMS (Lois and Alvarez-Buylla, 1994). Once neuroblasts have reached the OB, they undergo morphological and physiological maturation into distinct subtypes of local interneurons before integrating as granule neurons in the granule cell layer (GCL), and as periglomerular neurons in the glomerular layer (GL) (Lledo et al., 2008) (Figure 11).

Figure 11.- Neurogenesis in the SVZ. Neurogenesis occurs in the SVZ and the generated neuroblasts migrate through the rostral migratory stream and incorporate into the olfactory bulb. GCL: Granular cell layer, Mi: mitral cell layer, EPL: external plexiform layer, GL: glomerular layer (Modified from Lledo, 2008 & Zhao, 2008)

Recently, it has been demonstrated that the vasculature is also an essential component of the SVZ stem cell niche, possessing unique properties that support stem cell proliferation and regeneration (Tavazoie et al., 2008). Dividing cells lie adjacent to the SVZ vascular plexus, and directly contact the vasculature at specific positions, so that signals derived from the blood can access the SVZ. Endothelial cells are a source of diffusible signals, such as vascular endothelial growth factor (VEGF), fibroblast growth factor2 (FGF2), insulin growth factor (IGF1), pigment epithelium-derived factor (PEDF) and brain-derived neurotrophic factor (BDNF). Moreover, by direct contact with endothelial and perivascular cells, the vascular lamina, as well as small molecules circulating in the blood type B and C cells received spatial cues and signals from the vasculature that regulates self-renewal and differentiation. (Tavazoie et al., 2008).

1.2.3. The subgranular zone of the dentate gyrus of the hippocampus (SGZ)

The subgranular zone of the dentate gyrus (DG) of the hippocampus is the other major neurogenic niche in the adult mammalian brain (Altman, 1965, Kaplan, 1977, Gage, 2000). Understanding the behavior and regulation of SGZ neurogenesis is of great relevance due to the fact that newly hippocampal neurons generated in the DG have been implicated in

learning and memory (Zhao et al., 2008), and aberrant neurogenesis has been linked with several neurological disorders, such as depression (Dranovsky and Hen, 2006), neuroinflammation (Monje et al., 2003) and epilepsy (Parent et al., 2006).

The SGZ is located at the interface of the granule cell layer and the hilus. The granule cell layer is composed of both mature and immature neurons, as well as astrocytes and oligodendrocytes. Hippocampal astrocytes promote neuronal differentiation of adult hippocampal progenitors and the integration of neurons derived from adult hippocampal progenitors *in vitro* (Song et al., 2002).

The SGZ contains three distinct populations. Radial astrocytes (Type I progenitors or B cells), have a radial process spanning the granule cell layer and a smaller one horizontally oriented along the SGZ (Kosaka and Hama, 1986; Seri et al., 2004). They express GLAST, GFAP, Nestin, Sox2 and BLBP (Fukuda et al., 2003; Seri et al., 2004; Steiner et al., 2006; Suh et al., 2007). Radial astrocytes give rise to type D cells, also known as Type II progenitors cells [IPC], that are non radial precursors that divide more frequently and express Sox2, PSA-NCAM and Nestin (Seri et al., 2004). Type D cells act as intermediate progenitors that progressively mature into new granule cells (type G). Newly generated neuroblasts, that express PSA-NCAM and Dcx, migrate into the adjacent granule cell layer where they mature into neurons (Figure 12).

Figure 12.- Adult SGZ neurogenesis. A) Architecture of the SGZ composed by radial astrocytes (RA) that give rise to intermediate progenitors (IPC, or type II cells) and finally differentiate into mature granule cells (GCs). B) Depicting of how neurogenesis and migration occurs within the SGZ (Fuentelba et al., 2012; Zhao et al., 2008).

2. Neural stem cells biology

Neural stem cells (NSC), as any other tissue-specific stem cell, are defined based on functional criteria by two cardinal properties: self renewal, their ability to generate a new NSC, and multipotency, the ability to differentiate giving rise to all neural lineages (Potten and Loeffler, 1990).

2.1. Characterization of neural stem cells *in vitro*: the neurosphere assay

NSCs in the adult mammalian brain were isolated for the first time by Reynolds and Weiss (Reynolds and Weiss, 1992). They dissected striatal tissue, which included the SVZ, and demonstrated that the isolated cells proliferated in the presence of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2), forming cellular aggregates of undifferentiated proliferating cells, denoted neurospheres (NS). The majority of cells within those NS expressed nestin and they could be subsequently passaged to form new NS in the presence of mitogens, demonstrating their self-renewal capacity. In each passage, these NS could also be differentiated into neurons and glial lineages, proving their multipotency (Reynolds and Weiss, 1996).

In a similar way, NSCs from olfactory bulb (OB) of E14.5d embryos cultured in the presence of EGF and FGF form neurospheres that preserved their self-renewal ability and multipotency in culture (Gritti et al., 1999; Vicario-Abejon et al., 2003).

Figure 13.- Neurosphere assay. After dissection of adult or embryonic brain tissue, cells are cultured under conditions supporting proliferation to form cellular aggregates, denoted neurospheres (NS). NSCs/NPCs within the NS display the hallmark properties of stem cells: self-renewal and multipotency (Pastrana, 2009).

The neurosphere assay allows the enrichment, characterization and expansion of stem and progenitor cells from adult and embryonic central nervous system tissues. The protocol for NS assay is detailed in the Materials and Methods, Section 2.1. Briefly, the tissue is dissected and, after enzymatic disaggregation cells are cultured in the presence of mitogens that sustained survival of NSC and neural progenitors cells. NS assay represents a selective culture system in which the more committed progenitors and differentiated mature cells are lost in each passage, whereas the undifferentiated NSC/NPC respond to mitogens and divide giving rise to neurospheres that can be dissociated and re-plated to generate subsequent NS (Galli et al., 2003).

NS have been shown to be composed of heterogenous populations of slowly dividing NSC and their progeny, a population of fast-dividing progenitor cells (Morshead et al., 2002). The capacity to generate new NS in successive passages is determined by the self-renewal capacity of the NSC in the culture. Under proliferating conditions, in the presence of EGF and bFGF, slow-dividing NSC divide symmetrically once or twice and generate fast-dividing NSC that undergo either symmetric division generating TAPs, or asymmetric division generating NSC and TAPs (Costa et al., 2011; Qian et al., 1998). Under proliferating conditions, usually the formed NS is comprised mainly by neural progenitors and few NSC. However, the maintenance of proper NS culture conditions leads to survival and active proliferation of NSC. For this, cells must be cultured at clonal densities, so that only the self-renewing cells are capable to form new NS. Besides, it is essential to culture the cells in serum-free media with EGF and/or bFGF, and in non adherent conditions to prevent differentiation (Gritti et al., 1999; Morshead et al., 2002)

Figure 14.- Cellular composition of NS. Schematic view of the cellular composition of NS. NSC within a NS will give rise to a new NS, which contains NSC and NPC upon dissociation (Modified from(Galli et al., 2003)).

The total number of NPC/NSC determines the size of a neurosphere. This way, differences in sphere size within different neurosphere populations could reflect alterations in the balance between the type of cell division (symmetric vs. asymmetric), proliferation, survival and/or differentiation status of the neural progenitors (Leone et al., 2005). Likewise, the number of newly formed neurospheres after dissociation and re-culture reflects the number of neural progenitor cells with self-renewal capacity in the culture..

In addition, withdrawal of mitogens and seeding of the NS cultures on adherent substrates leads to differentiation of the NSC/NPC giving rise to neurons, astrocytes and oligodendrocytes with a temporal sequence similar to that observed *in vivo* (Reynolds and Weiss, 1996).

The use of neurosphere assay has been controversial, since it may not reflect the *in vivo* conditions and behavior of NSC in their niches. Thus, the neurosphere assay should not be used alone to define the nature of *in vivo* stem cells. However, nowadays is accepted that, if performed carefully, it can provide a useful tool to identify populations of cells that exhibit functional properties of stem cells *in vitro* (neural stem cells and neural progenitor cells). It can also be used as a quantitative readout of the number of proliferating cells *in vivo* in a relatively simple manner (Pastrana et al., 2009). It is also a useful cellular model to study the molecular mechanisms that regulate NSC biology.

2.2.Regulation of neural stem cells biology

As we have mentioned, NSC are defined by their self-renewal and differentiation capacity; therefore, tight regulation of the balance between these processes is essential for maintaining neural stem cell biology. Self-renewal and cell fate choice of NSCs are coordinately controlled in a stage-dependent manner, but the mechanisms underlying such coordination remains poorly understood.

2.2.1. Pathways regulating self-renewal and differentiation

In the last years many genes has been described as regulators of NSC self-renewal, revealing the high complexity of this process. Some of the pathways that are necessary for self-renewal appear to regulate processes like proliferation, apoptosis, developmental potential or differentiation (Kokovay et al., 2008; Molofsky et al., 2003).

Among the genes that modulate self-renewal, there are several ones implicated in the control of cellular proliferation at cell cycle level. Progression through cell cycle is determined by the levels of cyclins and cyclin-dependent kinases (CDKs) that are negatively

regulated by cyclin-dependent kinase inhibitors (CDKIs) (Musunuru and Hinds, 1997). NSC proliferation has been demonstrated to be negatively regulated by the CDKI p27^{Kip1}, since lack of p27 selectively increased the number of the transit-amplifying progenitors, but not of the stem cells, indicating that regulation of cell-cycle is cell-type specific in neural progenitors (Doetsch et al., 2002). The CDKI p21^{Cip1}, a direct transcriptional target of p53, is another negative regulator of adult NSC proliferation and contributes to adult NSC relative quiescence, which is essential for the long maintenance of NSC self-renewal. This was concluded from experiments in which p21^{-/-} neurospheres exhibited an initial expansion of the population of neural progenitors; however, p21 loss reduced the longevity of the cultures by inducing NSC exhaustion, accompanied by a reduction in the proliferation rates (Kippin et al., 2005).

Conversely, repression of Ink4a/Arf locus by the overlapping transcriptional mechanism of the polycomb group genes Bmi1 and Hmga2, selectively promotes progenitor proliferation and stem cells self-renewal in adult stem cells, and fetal and young adult stem cells respectively. By the use of alternative reading frames, the Ink4a/Arf locus codes for p16^{Ink4a} and p19^{Arf} CDKIs (Serrano, 2003). They are important regulators of retinoblastoma and p53 pathways, respectively, and upon activation produce growth arrest, senescence or apoptosis (Lowe and Sherr, 2003; Sharpless and DePinho, 1999). Bmi1 is a potent repressor of both p16 and p19, and although p19 is the general target of Bmi1, in NSC loss of Bmi1 resulted in up-regulation of p16 which contributes to the observed phenotype (Bruggeman et al., 2005; Molofsky et al., 2003). Additionally, it has been demonstrated that Hmg2a, a member of the high-mobility group A (HMGA), negatively regulates p16 and p19 expression promoting self-renewal (Nishino et al., 2008).

The tumor suppressor p53 negatively regulates self-renewal of neural stem cells and plays an important role in cell fate determination, since p53 deficiency results in enhanced neuronal differentiation. Loss of p53 produces an increased cell proliferation in the neural stem cells *in vitro*. Additionally, it decreases the rate of apoptotic cell death, altogether resulting in an increased self-renewal. p53 regulates self-renewal by modulating survival and proliferation signals. Down-regulation of p21 is observed as the effect of p53 deficiency, giving rise to defective cell cycle arrest which leads to an increase in the proliferative capacity of neural stem cells *in vitro* (Armesilla-Díaz et al., 2009; Meletis et al., 2006). Furthermore, loss of p53 alters proliferation and cell death rates of the populations that comprised the SVZ neurogenic niche, modifying dynamics between them, and ultimately leading to neoplastic transformation (Gil-Perotin et al., 2006; Meletis et al., 2006).

Notch pathway is highly conserved and is known to play a fundamental role for the maintenance of proliferation and differentiation of stem cells in many tissues, specifically in NSC in the developing brain. Notch receptors are hetero-oligomers of large single transmembrane proteins whose extracellular domains mediate interactions with the Notch ligands (Delta, Jagged). Productive interactions with ligands within neighboring cells produce the hallmark mechanism of Notch signal transduction pathway. Upon binding, Notch signaling brings about receptor proteolysis, resulting in the release of an active Notch intracellular fragment (NICD). The NICD form a transcription complex in association with DNA binding proteins and activates downstream target genes (Kopan and Ilagan, 2009).

Multiple basic helix-loop-helix (bHLH) genes have been described to be Notch effectors and be involved in the regulation of neurogenesis (Bertrand et al., 2002; Kageyama and Nakanishi, 1997; Ross et al., 2003). *Hes1* and *Hes5* play an important role in the maintenance of neural stem cells; indeed, inactivation of these genes leads to up-regulation of pro-neural genes, acceleration of neurogenesis and premature depletion of NSC (Kageyama et al., 2008). Conversely, overexpression of *Hes* genes leads to inhibition of neurogenesis and maintenance of NSC. In contrast, pro-neural bHLH genes such as *Mash1*, *Math3*, and *Neurogenin* promote neurogenesis. At earlier stages, in the absence of these pro-neural genes, neuronal differentiation is inhibited and neural stem cells are maintained. However, at later stages, NSCs prematurely differentiate into astrocytes (Nieto et al., 2001; Tomita et al., 2000).

Furthermore, *Hes*-related bHLH genes, *Hey1* and *Hey2* have been shown to be expressed in developing brain and to share some functions in neural development with *Hes1* and *Hes5*, since their overexpression efficiently inhibits the neuronal bHLH genes *Mash1* and *Math3*, promoting neural precursor cells maintenance (Sakamoto et al., 2003).

2.2.2. Regulation of neural stem cell biology by TRIM32

TRIM32 belongs to TRIM-NHL protein family, characterized by the presence of a tripartite motif consisting of a RING finger, one or two zinc binding motifs, named B-boxes, an associated coiled-coil region, involved in protein-protein interactions, and an additional NHL C-terminal domain (Meroni and Diez-Roux, 2005; Raymond et al., 2001; Slack and Ruvkun, 1998). Although TRIM-NHL protein family members present a highly conserved structure, they are involved in a broad range of biological processes, probably due to the molecular functions of the distinct domains.

TRIM32 is a neural fate determinant strongly expressed by differentiating neurons and at lower levels, by dividing neural progenitors. Under culture conditions supporting neuronal differentiation, in which neural stem cells divide asymmetrically giving rise to differentiating neurons, TRIM32 is asymmetrically distributed and correlates with the daughter cell that undergoes neuronal differentiation. However, TRIM32 asymmetry is never observed in neural stem cells undergoing symmetric cell divisions (Schwamborn et al., 2009). TRIM32 is retained in the cytoplasm of dividing progenitors by apical membrane protein kinase C ζ (PKC ζ). During differentiation it is released and translocated to the nucleus to initiate neuronal differentiation (Hillje et al., 2011).

TRIM32 RING finger domain is strongly associated to ubiquitination, making it a novel class of E3 ubiquitin ligases involved in protein degradation. On the other hand, the NHL repeats are involved in promoting miRNA activity (Hammell et al., 2009). TRIM32 ubiquitin ligase activity targets c-myc for proteasomal degradation, while NHL domain leads to the association of TRIM32 and Ago1 to activate certain miRNAs, let-7a among them. This is known as stem cell regulator that controls proliferation and that is upregulated during neuronal differentiation (Aranha et al., 2010; Bussing et al., 2008). TRIM32 is required and sufficient for suppressing self-renewal and inducing neuronal differentiation (Schwamborn et al., 2009).

3. The p53 family and its role in the biology of neural stem cells

Some of the pathways implicated in the control of self-renewal and differentiation of stem cells appear to influence processes like proliferation, apoptosis or differentiation (Kokovay et al., 2008; Molofsky et al., 2003). In somatic cells, the members of the p53 family are deeply involved in the regulation of some of these processes. Furthermore, the discovery that the p53 family member in *Planaria*, Smed-p53, is predominantly expressed in newly made stem cell progeny supports the idea that this family plays an evolutionary conserved role in stem cell biology (Pearson and Sanchez Alvarado, 2010). Indeed, deletion of Smed-p53 leads to an initial increase of the *Planaria* stem cell population, although, the stem cell population ultimately fails to self-renew. These data demonstrate that an ancestral p53-like molecule already had functions in stem cell proliferation control and self-renewal.

The p53 family is constituted by the transcription factors p53, p63 and p73. The TP53 gene was the first member of the family identified and is the most frequently mutated gene in human tumors. Since it was cloned in 1979 (Lane and Crawford, 1979; Linzer and Levine, 1979), multiple studies have been focused in elucidating p53 function, due to its extremely

relevant role in the maintenance of the genomic integrity. The tumor suppressive function of p53 largely resides in its capacity to sense potentially oncogenic and genotoxic stress conditions, and to coordinate a complex set of molecular events leading to growth restraining responses and, ultimately, to induce senescence and/or apoptosis (Levine, 1997).

Despite its function in the maintenance of the genomic integrity, analysis of mice lacking p53 revealed that it was dispensable for embryonic development (Donehower et al., 1992), suggesting the existence of other genes besides p53 capable, at least in part, of functioning like p53. Its structural and functional homologs, p73 and p63 were not cloned until 1997 (Jost et al., 1997; Kaghad et al., 1997) and 1998 (Yang et al., 1998), respectively. They all share a similar basic structure and sequence identity. As a result, p73 and p63 can transactivate some of the p53 responsive genes, as well as other specific targets, and mediate cell cycle arrest, cellular senescence and apoptosis in response to many stimuli (De Laurenzi et al., 1998; Jost et al., 1997; Yang et al., 1998).

Thus, p53 is a central node in a molecular network controlling cell proliferation and death in response to pathological and physiological conditions in which p73 and p63, are also entangled. Upon activation, p53 family pathway induces diverse cellular outcomes, ranging from cell-cycle arrest, to senescence, to programmed cell death (apoptosis) (Figure 15). As a result, the cellular outcomes to any given conditions are influenced by the expression levels of each p53 family member, as well as by the pattern of modulators that are expressed in a given cell or tissue, and their respective expression levels. Recent studies have demonstrated the role of p53 in suppressing proliferation and promoting differentiation of embryonic stem (ES) cells, acting as important barrier to somatic reprogramming (Li et al., 2009; Marion et al., 2009). Furthermore, emerging evidence reveals the role of the p53 network in the self-renewal, proliferation and genomic integrity of adult stem cells, as reviewed in (Lin et al., 2012). The emerging picture is that of an interconnected pathway, in which p63 and p73 share many functional properties with p53, but they also claim distinct and unique biological functions in processes like neurogenesis, embryonic development and differentiation.

While many years of research have uncovered an impressive number of p53- functions, much less is known about p63 and p73 functions and mechanisms of regulation.

Figure 15.- The p53 family as a network. The p53-family pathway is activated by a wide array of signals, including potentially oncogenic stresses, as well as physiological cues. Once activated, the pathway induces diverse cellular outcomes (Collavin et al., 2010).

3.1. Structural organization of p53 tumor suppressor family

The p53 family members are modular proteins with a similar basic structure. Members share very significant homology both at the genomic and at the protein level. Each contains three functional domains (Bourdon et al., 2005) (Figure 16):

- ☞ Amino-terminal transactivation domain (TAD): responsible of transactivation of target genes.
- ☞ DNA binding domain (DBD): involved in the recognizing and binding to specific sequences of target genes.
- ☞ Oligomerization domain (OD): implicated in the formation of functional tetramers, and crucial for the biological function of p53 family members.

The TAD is the least conserved region with the lower homology among the family members. In contrast, the core DBD is the most conserved region, especially the residues that directly interact with DNA. Therefore, the three family members can bind to canonical p53 DNA-binding sites and activate transcription from p53-responsive promoters. However, growing evidence indicates that many target genes respond differently to the various family members, giving them distinct, but sometimes overlapping, functions.

Figure 16.- Structural organization and homology between p53 family members. Structural domains of p53 family members with the percentage of homology of p63 and p73 referred to p53 (Dotsch et al., 2010).

All the family members must form homo-tetramers (dimers of dimers) to be transcriptionally active. However, only p63 and p73 are capable of interacting with each other through their OD to form hetero-tetramers (Coutandin et al., 2009; Davison et al., 1999). Interestingly, although WT p53 cannot bind to p63 or p73 through the OD, p53 mutants can bind and inactivate p73 and p63 by interacting through the DBD (Marin et al., 2000).

p63 and p73 also contain a carboxy-terminal sterile alpha motif (SAM), a putative protein-protein interaction domain found in many signaling proteins and transcription factors. Additionally, they contain a transcription inhibition domain (TID) that decreases their transcriptional activity by enforcing a closed conformation through the interaction with the amino-terminal TAD (Straub et al., 2010).

All the p53 family members can give rise to several mRNA variants due to alternative splicing of C terminal end and through the use of alternative promoters within their genomic sequence. These mRNA variants will generate multiple protein isoforms some of which have completely different functions (Bourdon et al., 2005; De Laurenzi et al., 1998; De Laurenzi et al., 1999; Grob et al., 2001) (Figure 17).

The TP73 gen possesses two different promoters, P1 and P2. P1 promoter is located upstream of exon 1 and gives rise to full-length proteins that contains the N-terminal transactivation domain (TA isoforms). Utilization of the P2 promoter, located within intron 3, generates amino-terminally truncated proteins (DN isoforms) lacking the transactivation domain (Yang et al., 2000).

Figure 17.- Schematic representation of p53 family genes and protein its structure. Figure represents the structural domains of p53 family members. Location of different promoters and of alternative splicing sites are showed, including the possible isoforms obtained (Allocati, 2012).

The transcripts generated from either promoter can undergo both amino- and carboxy-terminal alternative splicing. In tumor cells, it has been described that amino-terminal alternative splicing of mRNA produced from P1 promoter gives rise to novel $\Delta Ex2$, $\Delta Ex2/3$ and $\Delta N'$ transcripts (Stiewe et al., 2002; Stiewe et al., 2004). Moreover, due to alternative splicing the p73 gene expresses at least nine C-terminal isoforms (α , β , γ , ϵ , η , η_1 and ϕ), that differ in their potential to activate target genes and induce growth suppression (De Laurenzi et al., 1998; Moll and Slade, 2004). The full-length TA-p73 proteins (TAp73 α and β) are capable of transactivating p53 targets (including p21^{Waf1/Cip1}, Bax, Mdm2 and GATA1 among others), inducing cell cycle arrest, apoptosis, senescence and differentiation (De Laurenzi et al., 2000;

Fang et al., 1999; Jost et al., 1997; Marques-Garcia et al., 2009), while the short isoforms are not capable of transactivation and they have been postulated to work as dominant negative forms in certain tumors.

The differential expression of TA/ Δ N isoforms is determined by various sets of regulatory elements within each promoter, such as E2F induction of TP73 P1 promoter (Irwin et al., 2000), while expression of carboxy-terminal splicing variants seems to be determined by tissue-specific mechanisms of alternative RNA splicing (Morgunkova, 2005).

In addition, the p53 family proteins share some common post-translational modifications, physical interactions with other proteins and reciprocal regulatory networks which may affect stability, cellular localization and their ability to transactivate specific targets thus contributing to increase their functional complexity (Figure 15) (Collavin et al., 2010).

3.2.Functional interaction of p53 family members

Multicellular organism requires a tight balance between proliferation, cell death and differentiation throughout their whole life. Proliferation is essential during development and in the adult to replace cells that are lost due to injury, while programmed cell death maintains homeostasis and is necessary to eliminate compromised cells. Finally, differentiation of stem cells is the mechanism to generate and replace specialized cells that constitute tissues and organs. The p53 family members are involved in the coordinated regulation of these crucial processes.

The intricate structural organization and regulation of p53 family members results in an enormous functional complexity. TP73 has a bimodal function derived from the existence of TA and Δ N isoforms. As we mentioned before, TAp73 full length isoforms are capable of binding to p53 response elements (p53RE), transactivating p53 target genes (Jost et al., 1997) and promoting cell cycle arrest, differentiation, senescence and apoptosis. Conversely, Δ Np73 isoforms, which lacks the TAD, can act as dominant negative over p53 and TAp73 through the formation of inactive heterotetramers, thus inhibiting their function. Δ Np73 isoforms can also compete for DNA binding by direct interaction with target promoters through p53 response element, blocking the activation of p53 target genes (Ishimoto et al., 2002; Zaika et al., 2002). However, several new lines of evidence have revealed that Δ Np73 (α and β) can be active in transactivation of specific target genes in a p53 dependent and independent manner (Kartasheva et al., 2003; Liu et al., 2004; Tanaka et al., 2004; Tanaka et al., 2006). Moreover, Δ Np73 α may either inhibit or stimulate p53 transcriptional

activity, depending on both the p53 target gene and the cellular context, suggesting that $\Delta Np73\alpha$ not only acts as an inhibitor of p53/TAp73 functions in certain tissues, but also could cooperate with p53 in playing a physiological role through the activation of specific gene targets (Goldschneider et al., 2005). Evidence from our group demonstrated that $\Delta Np73$ expression works as a positive regulator of erythroid differentiation through the stabilization of transcriptionally active, endogenous TAp73 (Marques-Garcia et al., 2009). Interestingly, both p53 and TAp73 induce $\Delta Np73$ creating an autoregulatory feedback loop (Grob et al., 2001).

3.2.1. Role of p73 in cancer

Initial studies reported that p73 was over-expressed in tumors such as ependymoma, neuroblastoma, breast, lung, oesophagus, stomach, colon, bladder and ovary cancers, hepatocellular carcinoma and myeloid leukaemia. However, when these studies were done, ΔN isoforms were not known and the p73 overexpression in cancer represented the overall expression of p73 mRNA. Since the discovery of ΔN variants, it became clear that TAp73 and $\Delta Np73$ were often co-expressed in tumors. Thus, it was postulated that dominant negative $\Delta Np73$ isoforms, rather than TAp73, might be physiologically relevant components of p73 over-expression in tumors (Rufini et al., 2011).

Analysis of the Trp73 mice has produced conflicting results. While inactivation of all p73 isoforms in mice does not enhance susceptibility to spontaneous tumors (Yang et al., 2000), Trp73 haploinsufficiency contributes to an increased incidence of certain spontaneous tumors, particularly when combined with Trp53 or Trp63 heterozygosity (Flores et al., 2005). Long term studies demonstrated that in mice with combinational loss of p63 and p73, one of the prevalent tumor types was myeloid leukemia (Flores et al., 2005). Furthermore, 50% of the myeloid leukemia cases in the Trp73^{+/-}/Trp63^{+/-} mice presented LOH of the TP73 remaining allele, supporting a distinct, yet undefined, role of TP73 in leukemogenesis.

The transdominant model for p73 tumor involvement, in which the dominant-negative $\Delta Np73$ isoforms, rather than TAp73, might be the physiologically relevant components of tumor-associated p73 function (Moll and Slade, 2004), has been challenged by the new mice model. In this model, 73% of the TAp73^{-/-} mice spontaneously developed malignancies, despite the lack of changes in $\Delta Np73$ in normal tissue or the tumor site. These new data suggest that the appearance of tumors was specifically due to TAp73 deletion rather than to $\Delta Np73$ deregulation (Tomasini et al., 2008). Thus, these results indicate that TAp73

isoforms mediate the tumor-suppressive function of TP73, and that loss of TAp73 alone is sufficient to promote oncogenic transformation.

Altogether, the analysis of p73 deficient mice has demonstrated that TAp73 isoforms are *bona fide* tumor suppressors, pointing to an emerging role for them in the maintenance of genomic stability that prevents both tumor formation and infertility (Tomasini et al., 2008). However, none of the published p73KO mice analysis has addressed the expression of p73 in the stem cell compartment or if TAp73 regulation of genomic stability, differentiation and apoptosis is also relevant in the context of stem cells.

Recently it has been predicted that p53 family members could act as regulators of tumor suppressor miRNAs network. They are in control of most of the known tumor suppressors miRNAs, such as let-7 and miR-34 (Boominathan, 2010). Several recent studies have implicated the miR-34 family of miRNAs in the p53 tumor suppressor network. The expression of miR-34a, miR-34b, and miR-34c is robustly induced by DNA damage and oncogenic stress in a p53-dependent manner. Thereby, miRNAs can affect tumorigenesis by working within the confines of well-known tumor suppressor pathways (He et al., 2007).

3.2.2. Role of p73 in differentiation and development

There are evidences that suggest that p73 maintains some unique functions not shared with p53, like a p73-specific role in cellular differentiation and development (De Laurenzi et al., 2000; Fernandez-Garcia et al., 2007; Stiewe, 2007). Terminal differentiation is a crucial developmental process in which cell cycle arrest is temporally and spatially coordinated with expression of specialized cellular functions and it has been described as tumor suppression mechanism (Sherr, 2004).

Many studies have indicated that TP73 plays a role in differentiation of several cell lineages such as neuronal, myogenic, myeloid or epithelial differentiation. p73 function in neural differentiation has been addressed in several studies. Overexpression of TAp73 is sufficient to induce differentiation in oligodendrocyte precursor cells (Billon et al., 2004). In contrast, overexpression of Δ Np73 inhibits differentiation in these cells and interferes with multiple developmental programs (Zhang and Chen, 2007). TP73 expression increases during retinoic acid induced differentiation of neuroblastoma cells. In addition, ectopic TAp73, but not p53, induce morphologic and biochemical markers of neuroblastoma differentiation (De Laurenzi et al., 2000).

However, it is noteworthy that most of the existent knowledge regarding the functions of p73 and p63 comes from studies in which these proteins are overexpressed, functionally

inhibited or knocked-down in transformed tumor cell lines. Thus, little is known from p73 pro-differentiation function in physiologically relevant systems like stem cells.

TP53 is involved in the differentiation of neuronal precursors (Beltrami et al., 2004), and it is active in the nucleus of neurons and primary oligodendrocytes in the hippocampus (Eizenberg et al., 1996). Furthermore, p53 act as a negative regulator of NSC proliferation, since p53KO mice show an increase of proliferation in the neurogenic niches, as well as of the self-renewal of neural stem cells (Gil-Perotin et al., 2006; Meletis et al., 2006). Interestingly, differentiation of p53 knockout-derived neurospheres was biased toward neuronal precursors, suggesting that p53 controls the proliferation and differentiation pattern of NSC (Armesilla-Diaz et al., 2009). Although p53 plays an important role in proliferation of neural stem cells, it is not essential for neuronal differentiation, based on the normal phenotype of p53KO mice (Donehower et al., 1992).

Mouse gene targeting studies have revealed that both TP63 and TP73 are required for normal embryogenesis (Mills et al., 1999; Tomasini et al., 2009; Yang et al., 2000). In the initial TP73 mice model (Trp73^{-/-}), the central DNA binding domain of p73 was deleted so all the different variants of the gene were affected. Trp73^{-/-} survive at birth, but pups have a runting phenotype and high rates of mortality with an average life span of 2-4 weeks. Most commonly, death follows massive gastrointestinal and cranial hemorrhages. Preliminary data from our group has also detected a mild anemia in the Trp73^{-/-} E14.5d embryos and young mice (P15), suggesting a defect in the erythroid system. Furthermore, the lack of p73 affects the erythroid development and other hematopoietic compartments are affected (Marques-Garcia et al., 2009).

The Trp73^{-/-} model demonstrated that p73 is critical for the development and maintenance of the nervous system (Yang et al., 2000). The hippocampus, one of the neurogenic stem cell pools, exhibit dysgenesis in these mice, with a gradual but persistent postnatal loss of neurons and greatly enlarged ventricles (hydrocephalus), together with reduced cortical tissue (Pozniak et al., 2000; Yang et al., 2000). Interestingly, most of these phenotypes can be explained by either the absence or loss of neurons; the hippocampal phenotype was associated with the absence of important developmental neurons (Cajal–Retzius cells), and the olfactory/pheromone phenotype was related with the absence of peripheral olfactory neurons. The Trp73^{-/-} animals also displayed enhanced loss of both PNS and CNS neurons postnatally. It was reported that the predominant p73-isoforms expressed in the developing brain and sympathetic ganglia were ΔNp73 variants (Pozniak et al., 2000; Yang et al., 2000). This lead to propose that ΔNp73 was an essential pro-survival protein in

developing sympathetic neurons and that lack of $\Delta Np73$ variants, but not TAp73, were the cause of the neurological defects in the $Trp73^{-/-}$ mice (Jacobs et al., 2006).

To investigate whether either of these phenotypes might be due to the loss of TAp73 isoforms, a new mice model, in which only the TA-p73 isoforms were deleted, was developed (TAp73^{-/-} mice) (Tomasini et al., 2008). In the TAp73^{-/-} mice the hippocampus dysgenesis was strikingly similar to that seen in $Trp73^{-/-}$ mice at P14. The similarity between TAp73^{-/-} and $Trp73^{-/-}$ mice on this particular phenotype suggests that it is due to TAp73 loss and not to $\Delta Np73$ loss. Thus, this data support the hypothesis in which TAp73 is essential for normal hippocampus development, while $\Delta Np73$ seems to prevent neural tissue loss due to apoptosis (Tomasini et al., 2009; Walsh et al., 2004). The requirement of TA and $\Delta Np73$ for hippocampus development and survival supports our hypothesis of a possible role of this gene in NSC biology.

Altogether, the compilation of work regarding p73 function in CNS development indicates that p73 plays a multifunctional role in this process. On one hand some of the data indicate that TAp73 is involved in neuronal and oligodendrocyte differentiation, while $\Delta Np73$ acts as a major survival factor preventing apoptosis in post mitotic neurons. On the other hand other data associate p73 to the maintenance of neurogenesis (Killick et al., 2011). Moreover, it has been demonstrated that loss of one p73 alleles, makes mice susceptible to neurodegeneration as a consequence of aging or of Alzheimer's disease (AD) (Wetzel et al., 2008). Furthermore, preliminary experiments from our laboratory indicate that there is a qualitative difference between the neurospheres obtained from the $Trp73^{-/-}$ E14.5d embryos compared with WT controls (Marta Herreros-Villanueva, PhD Dissertation Thesis). During the course of this Thesis project, several studies regarding the role of p73 in the maintenance of NSC self-renewal were published by four independent groups, including ours. These reports, which established p73 as a positive regulator of NSC/NPC self-renewal in embryonic and adult CNS neurogenesis, will be discussed as part of the Results and Discussion section of this dissertation. However, up to the beginning of this work, neither the specific p73-function in neural stem cells during development, nor the effect of p73 deficiency in the capacity of neural stem cells to differentiate and survive had been ever investigated. Thus, in this work we proposed the study of p73 function in the biology of neural stem cell and in the architecture of the neurogenic niches.

Aims

Our working hypothesis was that p53 family members, and in particular p73, play an important role in the regulation of the self-renewal and differentiation of NSC. Thus, the general goal of this Thesis was the analysis and characterization of the role of the p53 family members, p73 and p53, in the biology of neural stem cells *in vitro*, as well as *in vivo*. For this purpose, we used either primary neurospheres cultures or brain tissue from mice of the following genotypes: wild-type (WT), p73^{-/-} (p73 KO), p53^{-/-} (p53 KO), and the double knock-out mice p73^{-/-}p53^{-/-} (DKO).

The specific aims of this work were:

1. Analysis of the role of p53 family members, p73 and p53, in the biology of neural stem cells
 - 1.1. Role of p73 in the regulation of neural stem cells self-renewal and multipotency.
 - 1.2. Functional interaction between p53 and p73 in the biology of neural stem cells.
 - 1.3. Effect of p73 deficiency in the regulation of asymmetric cell division.
 - 1.4. Effect of p73 deficiency in the proliferating populations of the neurogenic niches.
2. Characterization of the p53 family functions in the genesis and architecture of the murine neurogenic niche in the subventricular zone (SVZ).
 - 2.1. Comparative analysis of the different cellular populations in the SVZ between mice of the four genotypes studied.
 - 2.2. Analysis of the post-natal formation of the lateral wall of the lateral ventricles: transition from the radial glia cells in the ventricular zone (VZ) to the ependymal layer and subventricular zone (SVZ).
 - 2.3. Comparative analysis of the SVZ architecture at different post-natal days.
3. Identification and analysis of novel p73 transcriptional targets in neural stem cells.
 - 3.1. Analysis of TRIM32, a neuronal fate determinant, as a direct transcriptional target of p73.

Materials & Methods

1. Animal work

1.1. Mice strains and animal breeding

Housing and animal experiments were conducted in agreement with European and Spanish regulations on the protection of animals used for scientific purposes (Council Directive 86/609/CEE and RD-1201/2005, respectively) with the appropriate institutional committee approval.

Mice heterozygous for Trp73 on a mixed background C57BL/6 x 129/svJae (Yang et al., 2000) were backcrossed to C57BL/6, at least five times, to enrich for C57BL/6 background. As previously published, p73 homozygous mutant mice (p73^{-/-}) usually die before reaching sexual maturity (Yang et al., 2000); thus, heterozygous animals were crossed to obtain the p73KO mice. C57BL/6 p53KO (Donehower et al., 1992) were crossed to obtain the p53KO mice. Homozygous females frequently present fertility problems; therefore we obtained p53KO by crossing p53 homozygous males with homo/heterozygous females. Finally, to generate the double Trp73; p53 knockout mice (DKO), heterozygous animals for Trp73 were initially crossed with p53KO mice obtaining the double heterozygous mice, Trp73^{+/-}; p53^{+/-}. Then double heterozygous were inter-crossed to originate the DKO animals.

1.2. Mouse genotyping

Genotyping of adult animals and embryos was performed by PCR analysis. After isolation of genomic DNA (gDNA) from tissue samples the WT and mutant alleles were amplified by PCR as previously described (Flores et al., 2005; Yang et al., 2000), followed by electrophoretic analysis of the PCR products.

- ☛ Extraction of gDNA (modified from (Laird et al., 1991)): tissue biopsies were incubated over night (o/n) at 65°C in digestion buffer (100 mM Tris-HCl pH 8.5, 200 mM NaCl, 5mM EDTA pH 8, 0.2% SDS) with 150 µg/ml proteinase K (Sigma). To remove undigested fragments, samples were centrifuged for 10 minutes at 14.000g. gDNA was precipitated from the supernatant by addition of one volume of isopropanol. Upon centrifugation for 5 minutes at 14.000g. DNA pellets were resuspended in TE (10 mM Tris-HCl, 1mM EDTA pH=8). DNA concentration was measured spectrophotometrically using *NanoDrop* (ND 1000).

- Amplification of WT and mutant alleles by PCR: 0.5 μ g of gDNA were amplified in a PCR mixture (Table1·Top panel) containing the specific primers for each reaction (Yang,2000, Flores, 2005)(Table1·Bottom panel). Amplification was performed in a Gene Amp®PCR System 2700 (APPLIED BIOSYSTEM) as follows: initial denaturing step during 5 minutes at 94°C; 30 cycles of denaturation, 1 minute at 94°C, annealing, 1,5 minutes at 60°C and elongation, 2 minutes at 72°C; with a final extension step of 5 minutes at 72°C.

Table 1.- PCR mixture conditions and primers sequence details. A) Concentrations of the different components of the PCR mixture. B) Primers sequences for p53 and p73 genotyping.

PCR mixture		
gDNA sample	0.5 μ g	
Reaction buffer with MgCl ₂	1X	
dNTPs	0.25mM	
Primer (each)	0.4mM	
UltraTools DNA Polymerase (Biotools)	1U	
Total Volume	20 μ l	

Primer	Sequence	Orientation (Location)
p73·g1 (I1)	5' GGG CCA TGC CTG TCT ACA AAG AA 3'	Sense (Exon 5)
p73·g2 (I2)	5' CCT TCT ACA CGG ATG AGG TG 3'	Antisense (Exon6)
p73·g3 (I3)	5' GAA AGC GAA GGA GCA AAG CTG 3'	Sense (pGK·Neo)
p53·g1 (p53X6.5)	5'- ACA GCG TGG TGG TAC CTT AT -3'	Sense
p53·g2 (p53X7)	5'- TAT ACT CAG AGC CGG CCT -3'	Antisense
p53·gNeo (Neo18.5)	5'- TCC TCG TGC TTT ACG GTA TC -3'	Antisense

- Electrophoretic analysis of PCR products: PCR products were loaded into a 1,8% agarose gel with ethidium bromide and separated by electrophoresis at 90V during 45 minutes. Gels were analyzed with a GelDoc imaging XR system (BIORAD). The genotype for each animal was determined by their electrophoretic mobility compared to 100 bp molecular weight marker (Figure 18).

Figure 18.- PCR examples of p53 and p73 genotyping. Characteristic PCR products from (A) WT p73 (600bp), p73 KO (375bp) and (B) WT p53 (450bp) and p53 KO (625bp).

1.3. Euthanasia and anesthesia

Animals were euthanized in agreement with European and Spanish regulations to perform *in vivo* studies and to obtain tissue samples.

Animals were fully anesthetized using an analgesic/anesthetic mixture of Medetomidine hydrochloride (Domtor, Orion Corporation), 1mg/Kg and Ketamine (Imalgene 500, Merial). Medetomidine/ Ketamine euthanasic mixture was prepared in PBS and injected intraperitoneally (75mg/Kg).

2. *In vitro* studies

2.1. Establishment of NS cultures from OB of 14.5d embryos

Pregnant females on E14.5d of gestation were euthanized. Uterus was extracted, maternal and extraembryonic tissues were removed, and embryos were dissected and transferred to 0.1M PBS (Figure 19). Then, brains were isolated from the embryos. To initiate each independent embryonic culture, the olfactory bulbs were dissected out and processed as previously described (Vicario-Abejon et al., 2003). Briefly, after incubation for 10 minutes at 37°C in washing solution (Table 2), the tissue was mechanically dissociated into single cell suspension. Cells were seeded at 200cells / μ l and incubated for four days in proliferation media at 37°C and 5% CO₂ to form primary NS (Table2).

Figure 19.- Isolation of olfactory bulbs from mouse embryos at E14.5 days post coitum. Stereomicroscopic images of E14.5d embryo and the dissected brain where the OBs are indicated by asterisks.

After 4 days-culture in proliferation media (4DIV-PM), the obtained primary NS were collected and softly centrifugated for 5 minutes at 1000rpm. Then, they were enzymatically dissociated during 10 minutes at 37°C with Accutase (Stem Cell Technologies), disaggregated and reseeded at clonal densities (20cells / μ l) to form secondary NS (P2 NS). Cellular viability was assessed by Trypan blue exclusion when counting the cells with the hemocytometer (Neubauer chamber). Enzymatic dissociation and reseeding protocol was performed every 4 days to obtain NS from successive passages.

2.2.Self-renewal assays

To determine the self-renewal capacity of NSC from E14.5d embryos, clonogenic and limiting dilution assays were performed. After 4DIV, secondary NS were enzymatically dissociated as previously mentioned. We prepared log² serial dilutions from the single cell suspension in a 10X matrix plate at densities that range from 5 cells/ μ l to 0.1 cells/ μ l. Then six replicates were seeded in flat bottom 96-well plates (p96w), with cell densities ranging from 500 to 1 cell/well. After 4DIV in proliferating media, the number of neurospheres formed in each well was quantified using phase contrast microscopy.

2.3.Determination of NS size and growth kinetics

Primary NS were seeded at low density (20 cells/ μ l) to form secondary NS and, after 4DIV, the size and growth kinetics of the NS cultures were assessed. Additionally, cells were collected to perform flow cytometry assays.

Pictures of random fields from triplicates of P2 NS cultures were acquired with NIS-Elements software in a NIKON ECLIPSE phase contrast microscope, and the diameter of the NS was measured to determine the NS size.

To determine the growth kinetics, secondary NS were enzymatically dissociated to obtain single cell suspension and the total number of viable cells was quantified in a Neubauer chamber by diluting the cell suspension with Trypan blue solution (0.4%, SIGMA).

2.4.Differentiation assays

We analyzed the differentiation capacity of whole (non dissociated) secondary NS at different time points.

Non differentiated NS were obtained by seeding individual NS onto Matrigel (BD) precoated coverslips and incubating them in proliferation media. After two hours (when NS have attached), coverslips were washed with PBS, fixed for 15 minutes with 3.7% para-formaldehyde (PFA) and stored in PBS at 4°C.

For differentiated NS, we seeded individual NS into precoated poli-L-ornithine (SIGMA) coverslips and incubated them in differentiation media (Table 2). At the indicated time points in every experiment, cells were fixed following the above described protocol .

Coverslips were precoated with Matrigel (1:60 dilution in proliferation media) for at least 2 hours at room temperature. Precoated coverslips were incubated with poli-L-ornithine for 2 hours at 37°C, and then washed with PBS just before seeding the NS.

Table 2.- NS culture media and solutions. % indicates weight/volume.

Complete Medium	Differentiation Medium	Hormone Mix (10X)	Wash Solution
Dubecco's Modified Eagle Medium (DMEM)/F12	Dubecco's Modified Eagle Medium (DMEM)/F12	Dubecco's Modified Eagle Medium	Dubecco's Modified Eagle Medium (DMEM)
Glucose 0,6%	Glucose 0,6%	Glucose 2,4%	Glucose 0.09%
Sodium Bicarbonate 0,13%	Sodium Bicarbonate 0,13%	Sodium Bicarbonate 0,11%	Sodium Bicarbonate 0.05%
HEPES 4mM	HEPES 4mM	HEPES 4mM	HEPES 0.39%
Penicillin/ Streptomycin 1%	Penicillin/ Streptomycin 1%	Sodium Selenite 0.3mM	Penicillin/ Streptomycin 1%
L-Glutamine 1%	L-Glutamine 1%	Progesterone 0.2mM	
Insuline 25µg/ml	Insuline 25µg/ml	Apo-trasferrin 1mg/ml	
rhEGF 20ng/ml	Hormone Mix 10%	Putrescine 1mg/ml	
rh-bFGF 20ng/ml	Fetal Bovine Serum (FBS) 2%		
BSA 0,4%			
Heparin 0,2%			
Hormone Mix 10%			

2.5.Obtention of cell pairs by blebbistatin treatment

For cell-pair analysis, isolated primary NS were dissociated, seeded at low density and incubated during 8 hours in proliferation media supplemented with Blebbistatin (50µM, Tocris). Then, cell-pairs were seeded on Matrigel precoated (1:100) coverslips and incubated for 10 minutes at 37°C. They were finally fixed in Cytoskeletal Buffer (137mM NaCl, 5mM KCl, 1.1 mM Na₂HPO₄, 0.4mM NaHCO₃, 0.4mM MgCl₂, 2mM EGTA, 50mM D-Glucose, 5mM PIPES pH 6.0) supplemented with 2% PFA for 10 minutes.

3. *In vivo* studies

3.1. BrdU incorporation and perfusion

The thymidine analogue BrdU (Sigma) was administered intraperitoneally (150 mg/kg) in pulse injections every 2 hours, during 8 hours. Animals were fully anesthetized with a ketamin/medetomidin mixture as previously mentioned and euthanized by transcardial perfusion. Animals were perfused with 0.1M PBS supplemented with 0.1% heparin until the draining blood became clear and then with approximately 20ml of fresh 4% PFA in 0.1 M PBS.

After perfusion, brains were dissected and post-fixed o/n in fresh 4% PFA (PBS) solution at 4°C. For cryopreservation, samples were incubated for 24h in 30% sucrose/PBS solution. Samples were then frozen by immersion in dry ice powder and stored at -80°C.

3.2. Isolation of brain samples

To obtain RNA and protein samples, postnatal mice were euthanized and brains were dissected. Brains were immediately processed following the protocols described below (Sections 5 & 7, respectively) or stored at -80°C.

3.3. Preparation of whole mounts from the lateral wall of the ventricles

Whole mounts of the lateral wall of the ventricles were dissected from postnatal mice. Animals were anesthetized, the head was cut off and brain was dissected and placed in cold 0.1M PBS. Dissection of the whole mount was made under the stereomicroscope as shown in figure 20. Briefly, the brain was divided along the interhemispheric fissure (1), then a coronally-oriented cut was made allowing the visualization of the hippocampus (3) that must be released from the overlying cortex (5) and pulled away to open the lateral wall widely. Finally the medial wall, as well as the cortex, was removed (7), so the lateral wall was completely exposed. The wholemounts were then ventricle side up transferred to a 24-well plate with 4%PFA/0.1% Triton-X100 (Tx100), fixed o/n at 4°C and stored in PBS 0.1M at 4°C.

Figure 20.- Stereomicroscopic images showing wholemounts dissection of the lateral wall of the ventricles. Arrows indicate position where the brain was cut (Mirzadeh et al., 2010).

4. Cell culture

4.1. Cell lines and culture conditions

C17.2 cells are mouse multipotent neural progenitor established from mitotic neonatal mouse cerebellum immortalized by retroviral-mediated transduction of the avian *myc* oncogene (Kitchens et al., 1994). DKO mouse embryonic fibroblasts (MEFs) were obtained following the protocol detailed below (Section 4.2.).

Cells were grown under adherent conditions at 37°C and 5% CO₂. Culture, in the culture media indicated in table 3.

Table 3.- Culture media. Composition of complete medium for C17.2 and MEFs cells.

C17.2		MEFs	
Dubecco's Modified Eagle Medium (DMEM)		Roswell Park Memorial Institute (RPMI)	
Fetal Bovine Serum (FBS)	10%	Fetal Bovine Serum (FBS)	10%
L-Glutamine	2mM	L-Gln	2mM
Penicillin/ Streptomycin	10U/ml	Penicillin/ Streptomycin	10U/ml

4.2. Derivation of primary cultures of mouse embryonic fibroblasts (MEFs)

Pregnant females were euthanized at 13.5 days post-coitum, uterus was extracted and embryos were dissected free of the maternal and extraembryonic tissues. Embryos were washed in 0.1M PBS supplemented with Penicillin/Streptomycin and Anphotericin (Sigma). We eviscerated the embryos by extracting the liver and embryos were washed before transferring them to a clean plate where we minced carefully the tissue. The tissue was then incubated in Trypsin-EDTA for 30 minutes at 37°C and 5% CO₂. Finally, the mashed tissue was mechanically dissociated into single cell suspension and cells were seeded.

4.3.Cell transfections with Lipofectamine®

The cDNAs from human pcDNA3-HA-TAp73 α , pcDNA3-HA- Δ Np73 α and pcDNA3-HA-p53 were described previously (Fernandez-Garcia et al., 2007). Cells were transfected with the indicated plasmids using Lipofectamine™ 2000(Life Technologies) following manufacturer's instructions. Briefly, the indicated amount of Lipofectamine was diluted in Opti-MEM® I Reduced Serum Medium and incubated for 5 minutes. Then, it was combined with the DNA diluted in the same volume of Opti-MEM®, mixed gently and incubated for an additional 20 minutes at room temperature to allow the DNA-Lipofectamine complexes to form. The DNA (μ g): Lipofectamine (μ l) ratio was 1:2.5. Growth medium was replaced by Opti-MEM® and, finally, the complexes were added to the cells and mixed gently by rocking the plate. Four to five hours after transfection, medium was changed and substituted for complete growth medium.

5. RNA work

5.1. Isolation of RNA samples

Total RNA was isolated from tissue samples, that were mechanically homogenized, and from C17.2 cells and P2 NS pellets. Extraction of RNA was performed using TRI®Reagent (Ambion) following the manufacturer's instructions. Briefly, samples were homogenized with 1 ml of TRI®Reagent for 5 minutes at room temperature, then 200 μ l of chloroform were added, vigorously mixed and incubated for 15 minutes at RT. Samples were centrifuged for 15 minutes at 12.000g, 4°C and the aqueous phase that contains the RNA was recovered. RNA was precipitated by addition of 1 volume of isopropanol followed by centrifugation for 15 minutes at 12.000g, 4°C. RNA pellet was washed with 75% Ethanol in H₂O/0.1% DEPC. Finally, RNA was resuspended in H₂O DEPC (RNAse free water).

RNA concentration was spectrophotometrically determined using NanoDrop (ND 1000). RNA quality was confirmed by agarose-formaldehyde gel electrophoresis.

5.2.cDNA synthesis

cDNA synthesis was performed using SuperScript™ II First-Strand Synthesis System (Invitrogen) and High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's instructions.

When using the SuperScript™ II First-Strand Synthesis System, 1µg of RNA was added to the reaction mix that contained 1X reaction buffer, 50U Superscript II enzyme, 0.5µg oligo(dT), 10mM dNTPs, 0.1M DTT, 25mM MgCl₂ and 40U of RNase out. The retrotranscription reaction was carried out at 42°C for 50 minutes. Once finished, the remaining RNA was eliminated by treatment with RNase H for 15 minutes at 37°C.

When using High Capacity RNA-to-cDNA Kit, up to 2µg of RNA was mixed with 1X retrotranscriptase buffer and 1X of the Enzyme mix that contains all the components for the retrotranscription reaction. This reaction was carried out for 60 minutes at 37°C with a final denaturation step at 95°C for 5 minutes.

In both cases, cDNA concentration was spectrophotometrically determined using NanoDrop (ND 1000).

5.3.Quantitative Real Time-PCR (qRT-PCR)

The expression levels of indicated mRNA were detected by real time quantitative RT-PCR using FastStart Universal SYBR Green Master (ROX)(Roche). A reaction mix containing 1X SYBR Green Mix and 70nM of each primer was added to 0.5µg of cDNA. Primers sequences are detailed in Table 3. Some of them were obtained from the Primer bank Database (<http://pga.mgh.harvard.edu/primerbank/index.html>) (Spandidos et al., 2008; Wang and Seed, 2003), as indicated in the table.

Amplification was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystem) as follows: 2 minutes incubation at 50°C for activation of the enzyme; an initial denaturing step, 10 minutes at 95°C; 40 cycles of denaturation for 15 seconds at 95°C, annealing for 30 seconds at 60°C, elongation for 30 seconds at 72°C; and a final extension step, 10 minutes at 72°C.

Relative gene expression was normalized to the expression of housekeeping genes using the comparative C_T method, 2^{-ΔΔC_T}. Primer efficiency was assessed by amplification of log¹⁰ serialized dilutions of control cDNA. The efficiency was determined based on the slope of the standard curve. Furthermore, quality of the PCR products was assessed by their melting curves.

Duplicates of each sample were amplified and at least three independent experiments were performed.

Table 4.- Primers sequences. In the detailed sequences the orientation of the primers are indicated as S: Sense and A: Antisense.

Gene	Sequence	Amplicon size (bp)	Reference
Bmi1	S· 5'- ATCCCCACTTAATGTGTGTCCT -3'	116	Primer Bank Database
	A· 5'-CTTGCTGGTCTCCAAGTAACG -3'		
DNp73	S· 5'- ATGCTTTACGTCCGTGACCC -3'	65	Tomasini, 2008
	A· GCACTGCTGAGCAAATTGAAC		
Hmg2a	S· 5'- AGGTGACCCCAAGAAACCAAA	108	Primer Bank Database
	A· GCAAAATTGACGGGAACCTCTG		
Noxa	S· 5'- GCAGAGCTACCACCTGAGTTC	120	Primer Bank Database
	A· CTTTTGCGACTTCCCAGGCA		
p21	S· 5'- CCTGGTGATGTCCGACCTG	103	Primer Bank Database
	A· CCATGAGCGCATCGCAATC		
TAp73	S· 5'- GCACCTACTTTGACCTCCCC	121	Tomasini, 2008
	A· GCACTGCTGAGCAAATTGAAC		
TRIM32	S· 5'- GTGGACTCGCGTCGGAGCTG	144	Kudryashova E, 2009
	A· GGTTCAGGTGAGAAGCTGCTGC		
GAPDH	S· 5'- AGGTCGGTGTGAACGGATTTG	123	Primer Bank Database
	A· TGTAGACCATGTAGTTGAGGTCA		
18s	S· 5'- AGTTCCAGCACATTTTGCGAG		Primer Bank Database
	A· TCATCCTCCGTGAGTTCTCCA		

6. DNA work

6.1. Plasmid preparation

Plasmids were obtained by standard molecular biology techniques. *E.coli* DH5 α chemically competent cells were previously prepared in accordance with Hannahan *et al.* (1989) protocol to induce competence. Bacteria were transformed with the plasmid DNA by the heat shock method. *E. coli* DH5 α were thawed on ice and incubated with 10ng of plasmid DNA for 30 minutes on ice, followed by a 1 minute heat-shock at 42°C. After a 2 minutes incubation on ice, transformed cells were grown for 1 hour in Luria Broth (LB) media without antibiotics, at 37°C with continuous shaking (220 rpm). Bacteria were plated on LB-agar plates supplemented with the appropriate antibiotic and incubated o/n at 37°C.

Resistant colonies were inoculated in LB supplemented with antibiotic and incubated o/n at 37°C with continuous shaking (220 rpm). Plasmid minipreps were prepared by the alkaline lysis method and plasmid identity was confirmed by restriction analysis. In order to

obtain large amounts of plasmid DNA, maxipreps were prepared using the QIAfilter Plasmid Maxi Kit (Qiagen) following manufacturer's instructions.

Plasmid DNA concentration was spectrophotometrically determined using NanoDrop (ND 1000).

6.2. Cloning of hTRIM32-luciferase reporter vector

The cloning of the human TRIM32 promoter was performed as part of a Master Thesis project in our group (Sandra Fuertes Álvarez, 2012). Genomic DNA was isolated from human keratinocytes and a fragment of the TRIM32 promoter was PCR-amplified. Primers were designed according to the sequence in the genome databases (ENSEMBL Gene ID: ENSG00000119401). The amplified fragment spans the region from -1351 to +270, and contains the p53 consensus binding site identified in the proximal promoter (-115 to -84), exon 1 and 218 bp from intron 1. The 1.6 kb-PCR product was cloned using the TOPO TA® Cloning kit (Invitrogen). Afterwards, it was inserted as a *HindIII-XhoI* fragment into the pGL3-Basic luciferase reporter vector. Promoter sequence identity was confirmed before and after cloning into the reporter plasmid.

7. Protein work

7.1. Preparation of cellular extracts

Either P2 NS or tissue samples were homogenized and collected by centrifugation at 7.500g for 5 minutes at 4°C. Pellets were washed with PBS, and resuspended in EBC lysis buffer (50 mM Tris pH 8, 120 mM NaCl and 0.5% NP-40) containing proteases inhibitors (Aprotinin 10 µg/ml, Leupeptin 20µg/ml, Sodium Orthovanadate 1mM and PMSF 0.1 mg/ml) and incubated in rotation for 30 minutes at 4°C. Subsequently, lysates were centrifuged at 14.000g for 12 minutes at 4°C and supernatants were transferred to fresh microfuge tube. These lysates were either loaded immediately or stored at -80°C.

Protein concentration was determined using BSA as standard protein by the colorimetric method described by Bradford (Bradford, 1976) at 595nm. Known quantities of protein samples were diluted in loading buffer (3XLB: 50 mM Tris·Cl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and incubated 3 minutes at 100°C for protein denaturation.

7.2. Protein immunodetection by western blot

Protein samples were resolved by denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE). Percentage of polyacrylamide could range from 8-12%, in this case we have used 10% polyacrylamide resolving gels. Protein samples were loaded and resolved at 70-80V in a Mini-PROTEAN III Cell (Biorad) until the dye front moved into the resolving gel. Then electrophoresis was performed at 100-120V until the front reached the edge of the gel. Proteins resolved by SDS-PAGE were transferred from the gel to nitrocellulose membranes in a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad), 80 minutes at 350mA.

Membranes were incubated 1 hour at room temperature in blocking solution (5% nonfat dried milk and 1% goat serum in TBS/0.05% Tween20 (Tw20); TBS (50mM Tris pH 7.4, 150mM Sodium Chloride pH 7.4)) to avoid non specific binding. Next, membranes were washed with TBS-0.05% Tw20 and incubated with primary antibodies diluted in 2,5% nonfat dried milk o/n at 4°C with gentle agitation on a platform shaker.

Membranes were washed and subsequently incubated with 25 ng/mL of the appropriated horseradish peroxidase-coupled secondary antibody (Pierce) in 2.5% nonfat dried milk in TBS-0.05% Tw20 for 1 hour at RT. Finally, they were washed and incubated with Super Signal West Pico Chemiluminescent Substrate (Pierce). Enhanced chemiluminiscent signal was detected by exposing the membrane to photographic films (GE) that were then developed.

Primary and secondary antibodies, as well as their dilution of use are indicated in table 5 and table 6, respectively.

7.3. Immunocytochemistry

Cells fixed in 3.7% PFA and stored at 4°C, were immunostained with the primary antibodies indicated in table 5 using the following protocol. Cells were permeabilized 15 minutes with PBS-0.5% Tx100, then washed and incubated with blocking solution (PBS/ 10% Normal Donkey Serum (NDS)) to block the non specific binding of antibodies. After washing, cells were incubated o/n at 4°C with the primary antibody diluted in blocking solution. Next, cells were gently washed and incubated with the appropriated fluorophore-coupled secondary antibody diluted in blocking solution for 1 hour at RT.

Once immunostained, cells were gently washed to eliminate not bound molecules of secondary antibody and incubated with 1µg/ml of (DAPI) to counterstain the nucleus. Finally, cells were washed and coverslips facedown mounted in VECTASHIELD® Mounting

Medium (Vector laboratories). Confocal microscopy images were obtained with Nikon EclipseTE2000 confocal microscope.

7.4.Free floating immunohistochemistry

Coronal sections of 30 μ m of the brains previously fixed were obtained using a Microm HM 450 sliding microtome and stored in PBS supplemented with 0.05% NaN₃ (sodium azide) at 4°C.

Sections were free floating immunostained to maximize the binding of the antibodies. Coronal sections were transferred to a p24well plate with PBS that was shaking along the whole protocol.

BrdU and Ki67 staining required a previous denaturizing step to unmask the antigen, for such cases, sections were incubated in HCl 2N for 15 minutes at 37°C. Then sections were rinsed and gently washed several times.

Sections were washed with PBS, and incubated for 1 hour at room temperature in blocking solution (PBS 0.1M/ 10% NDS/ 100 μ M Glycine and 0.2% Tx100). Then sections were incubated 24 hours at room temperature with primary antibodies diluted in blocking solution (Table 5). Afterwards, sections were gently washed and incubated with the appropriated fluorophore-coupled secondary antibody (Table 6) diluted in blocking solution for 1 hour at RT. To eliminate not bound molecules of secondary antibody sections were gently washed and then incubated with 1 μ g/ml of (DAPI) to counterstain the nucleus. Sections were washed with PBS, mounted on precoated microscope slides and allow to air-dry for 5 minutes before seal with Fluoromount-G™Slide MountingMedium and covered with a coverslip.

Confocal microscopy images were obtained with Nikon Eclipse TE2000 confocal microscope and Olympus FluoView FV10i Confocal Laser Scanning Microscope.

7.5.Whole mount staining

The wholemounts stored in PBS 0.1M at 4°C were incubated o/n with PBS 0.1M/ 0.5% Tx100 before staining (Mirzadeh et al., 2010). Wholemounts were incubated for 2 hour at room temperature in blocking solution (PBS 0.1M/ 10% NDS/ 0.5% Tx100) and next incubated 48 hours at 4°C with primary antibodies diluted in blocking solution (Table 5). Wholemounts were gently washed, rinse 3 times for 10 minutes each and 3 more times for 30 minutes each, before incubation with the appropriated fluorophore-coupled secondary

antibody (Table 6) diluted in blocking solution for 24 hours at RT. Next they were washed again following the above described protocol and nucleus counterstained for 10 minutes at room temperature with DAPI 1 μ g/ml

Table 5.- Primary antibodies used for immunodetection. Besides the dilution of use is indicated the technique in which it was used.

Antibody	Source	Dilution	Application	Reference
Actina (20-33)	Rabbit	1:10.000	IB	Sigma·A5060
BrdU	Mouse	1:200	IHC	Dako·M0744
BrdU	Mouse	1:500	IHC	BD· 347583
Dcx	Goat	1:150	IHC	Santa Cruz Biotech· sc8066
Erk 2 (C-14)	Rabbit	1:10.000	IB	Santa Cruz Biotech·sc-154
GFAP	Chicken	1:500	IHC	Chemicon·Ab5541
GFAP	Mouse	1:100	IC	Millipore·MAB360
GFAP	Rabbit	1:400	IC	Neomarkers·RB-087A1
GLAST	Rabbit	1:100	IHC	Tocris·2064
γ -Tubulin	Goat	1:200	IHC	Santa Cruz Biotech· sc7396
IB4	Mouse	1:20	IHC	Sigma·L2140
Ki67	Rabbit	1:100	IHC	Abcam·ab15580
Nestin	Mouse	1:100	IC	BD·611658
NICD	Rabbit	1:100	IC	Abcam·ab8925
Noggin	Goat	1:100	IHC	Santa Cruz Biotech· sc16627
O4	Mouse	1:50	IC	Millipore·MAB345
pHis3 (Ser10)	rabbit	1:100	IC	Millipore·06-570
p-p53 (Ser15)	Rabbit	1:500	IB	Cell signaling·9284
α 100 β	Rabbit	1:100	IHC	Dako·Z0311
β -Catenin	Rabbit	1:100	IHC	Cell signaling·9587
TRIM32	Mouse	1:100	IC	AbNova·H00022954-M09
TRIM32	Rabbit	1:1.000	IB	#3149·Knoblich·
Tuj1	Rabbit	1:1.000	IC	Covance·PRB435P-01
Tuj1	Mouse	1:1.000	IC	Covance·PRB435P-01
Vimentin	Guinea Pig	1:500	IHC	Fitzgerald·20R-VP004

For high-resolution Confocal imaging, following immunostaining the wholemounts were sub-dissected to preserve only the lateral wall of the lateral ventricle as a sliver of tissue 200-300 μ m. Then the sliver was mounted in the center of a microscope slide and Fluoromount-G™ Slide Mounting Medium was applied onto the wholemount and covered with a coverslip. The slides were then stored flat for 1-2 days before imaging to allow the coverslips to settle. Confocal microscopy images were obtained in an Olympus FluoView FV10i Confocal Laser Scanning Microscope.

Table 6.- Secondary antibodies used for immunodetection. Besides the dilution of use is indicated the technique in which it was used. IHC: immunohistochemistry, IC: Immunocytochemistry, IB: immunoblot.

Antibody	Source	Dilution	Application	Reference
Alexa Fluor 488 anti-IgG	Mouse	1:500	IHC/IC	Molecular Probes
Alexa Fluor 647 anti-IgG	Rabbit	1:500	IHC/IC	Molecular Probes
Cy3 anti-IgG	Chicken	1:1500	IHC/IC	Jackson Immunoresearch
Cy3 anti-IgG	Goat	1:1500	IHC/IC	Jackson Immunoresearch
Cy3 anti-IgG	Guinea Pig	1:1500	IHC/IC	Jackson Immunoresearch
Cy3 anti-IgG	Mouse	1:1500	IHC/IC	Jackson Immunoresearch
Cy3 anti-IgG	Rabbit	1:1500	IHC/IC	Jackson Immunoresearch
Dylight 488 anti-IgG	Mouse	1:500	IHC/IC	Jackson Immunoresearch
Dylight 488 anti-IgG	Rabbit	1:500	IHC/IC	Jackson Immunoresearch
Dylight 649 anti-IgG	Chicken	1:600	IHC/IC	Jackson Immunoresearch
Dylight 649 anti-IgG	Goat	1:600	IHC/IC	Jackson Immunoresearch
FITC anti-IgG	Goat	1:100	IHC/IC	Jackson Immunoresearch
FITC anti-IgG	Mouse	1:100	IHC/IC	Jackson Immunoresearch
FITC anti-IgG	Rabbit	1:100	IHC/IC	Jackson Immunoresearch
Streptavidin Cy2 anti-IgG	Mouse	1:300	IHC/IC	Jackson Immunoresearch
TxR anti-IgG	Mouse	1:100	IHC/IC	Jackson Immunoresearch
TxR anti-IgG	Rabbit	1:100	IHC/IC	Jackson Immunoresearch
HRP anti-IgG	Mouse	1:20.000	IB	Pierce
HRP anti-IgG	Rabbit	1:20.000	IB	Jackson Immunoresearch

8. Gene transcriptional analysis

For luciferase reporter assays, cells (10^5) were transfected with $0.125\mu\text{g}$ of the reporter luciferase pGL3-hTRIM32-luc vector plus $0.0625\mu\text{g}$ pRLNull vector (Renilla luciferase) and $0,2-0,6\mu\text{g}$ of the indicated expression vectors, using Lipofectamine™ 2000 following the protocol indicated before (Section 4.3.).

Cellular extracts were prepared 24 h after transfection using the Dual Luciferase® Reporter Assay System (Promega). Briefly, cells were washed and incubated with passive lysis buffer shaking for 15 minutes at room temperature. Then cells were harvested and homogenized to completely lysate them. Extracts were centrifuge 5 minutes at $14.000g$ and supernatant was recovered. Samples were mixed with the luciferase buffer and measured to determine the luciferase activity. Finally, renilla buffer combined with its substrate was added to determine the renilla activity. Measurements were taken in a Berthold's luminometer and normalized by the values of the renilla luciferase of the same sample.

9. Flow Cytometry

9.1. Propidium Iodide (PI) cell cycle analysis

Secondary NS were enzymatically disaggregated to single cell suspension, washed with cold PBS and the pellet was resuspended in PBS. Then cells were fixed by adding ethanol drop by drop up to 70% ethanol in PBS. Cells were post-fixed at -20°C for at least 4 hours. Once fixated cells were washed twice with PBS 0.1M to eliminate ethanol and finally resuspended in Staining Solution that contains: 0.3M Sodium citrate, 0.2 mg/ml RNase A, 0.2 mg/ml PI, and incubated 15 minutes at room temperature in the dark.

Samples were analyzed by flow cytometry in a CyAn™ ADPy (Beckman Coulter), and data were analyzed using Summit™ v4.3 (Dako).

9.2. Evaluation of apoptosis by Annexin V- 7AAD staining

Annexin-V Binding Assay labeling was performed according to manufacturer's instructions (BD). Briefly, secondary NS were enzymatically disaggregated to single cell suspension and washed twice with cold PBS. Cells were resuspended in Binding Buffer (BB) 1X at a final cellular density of 1×10^6 cells/ml, 100.000 cells were double labeled with Annexin-V and 7-AAD, for 15 minutes at room temperature in the dark.

Finally, samples were diluted (1:5) in BB 1X and were analyzed by flow cytometry in a CyAn™ ADPy (Beckman Coulter), and data were analyzed using Summit™ v4.3 (Dako).

10. Statistical analysis

For quantification purposes, three mice for each group were used and serial sections corresponding to the same SVZ region for all mice (separated by 250 μ m) were chosen and examined. Student's two-tailed t-test was performed to determine statistical differences.

For the molecular experiments statistical analysis (Student's two-tailed t-test) was performed using triplicates from three independent experiments.

Results & Discussion

1. Analysis of the role of p53 family members, p73 and p53, in the biology of neural stem cells

In the adult central nervous system of mammals the ability of maintaining neurogenesis throughout life is a direct result of NSC function. NSCs are generated from the precursors that build the nervous system during development. They are able to self-renew throughout life and, in response to micro-environmental stimuli, to differentiate and generate different neural cell lineages. Thus, self-renewal and cell fate choice of NSCs are coordinately controlled in a stage-dependent manner, but the mechanisms underlying such coordination remains poorly understood.

Some of the pathways that are involved in self-renewal appear to regulate processes like proliferation, apoptosis or differentiation. In somatic cells, these processes are controlled, at least in part, by the members of the p53 family. Some of the members of this gene family have been associated to the regulation of differentiation and survival of neuronal precursors and mature neurons of the CNS. Furthermore, the profound neurological defects observed in the *Trp73^{-/-}* mice (p73KO from now on), in particular the hippocampal dysgenesis, denoted the relevance of p73 function in neural development and suggested its possible role in neurogenesis. The possibility that p73 deficiency could lead to defective neurogenesis, prompted us to hypothesize that lack of p73 would affect the neural stem cells which sustain the neurogenic process.

1.1. Role of p73 in the regulation of neural stem cells self-renewal and multipotency

To elucidate the effect of p73 deficiency in the biology of NSC, we used the neurosphere assay. Multipotent self-renewing neural stem cells and neural progenitors from both the embryonic and mature mammalian central nervous system (Reynolds and Weiss, 1992, 1996) can be propagated *in vitro* as clonal aggregates denoted neurospheres (NS). In a similar way, NSCs from embryo olfactory bulb (OB), cultured in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), form NS that preserve NSC self-renewal and multipotency. In the neurospheres assay, NSC self-renewal can be measured by the capacity of each NS to form a new one upon dissociation and culture under clonal

conditions. On the other hand, multipotency is analyzed by the ability of the NSC within each NS to differentiate in the three major cell lineages of the central nervous system.

In the presence of mitogens, primary NS cultures obtained from OBs of p73KO and WT E14.5 embryos could be propagated successively *in vitro*, forming secondary, tertiary NS and so on. These cultures expressed the neuroepithelial marker, Nestin (Figure 21). Secondary NS were plated onto pre-coated poli-L-ornithine coverslips and maintained under differentiation conditions. Cells were fixed after 8 days *in vitro* (DIV) and immunostained for the different lineages specific markers: β -III-Tubulin (Tuj1) for neurons, Glial Fibrillary Acidic Protein (GFAP) for glia, and the surface antigen O4 for oligodendrocytes (Figure 21). We found that NS from both genotypes, WT as well p73KO, were capable of giving rise to cells from the three major neural lineages within one NS. This assay was repeated with NS from different passages with the same results, demonstrating the multipotency of the NS cultures.

Figure 21.- Differentiation of NS into the three major cell lineages of the central nervous system. Confocal microscopy images (20x, left panel & 40x, right panel), after 8DIV under differentiation conditions. We used GFAP to detect astrocytes, Tuj1 to label neurons and O4 to immunostain oligodendrocytes.

We analyzed the expression levels of the p73 isoforms (TA and DN) in WT NS cultures (Figure 22) and observed that, under proliferating conditions, both TAp73 and DNp73 were expressed, with TAp73 being significantly more abundant than DNp73, supporting the hypothesis of a functional role of these p73 isoforms in NSCs.

Figure 22.- Expression analysis of p73 isoforms in WT NS. qRT-PCR analysis of TA and DNp73 expression levels in secondary WT NS.

Next, we sought to evaluate the effect of p73 loss on NSC self-renewal. To this purpose, we first quantified the number of NS and measured the size of secondary NS from both genotypes. We observed a decrease in the number and size of NS in the p73KO derived cultures in 60% of the analyzed embryos (Figure 23). The diameter of p73KO neurospheres was significantly reduced compared to that of WT cells (Figure 23). The difference in NS size was maintained in successive passages. However, FACS analysis of the NS cultures demonstrated that these differences were not due to a difference in cell size (Herrerros-Villanueva, PhD dissertation, 2007). The NS size reduction indicated that the net cell growth within the neurosphere, which reflects the sum of cell divisions from self-renewing stem cells and from their progenitors, was impaired. The fact that this impairment was maintained through-out successive passages at clonal density, suggested that lack of p73 affected the proliferation capacity and/or self-renewal of the cellular populations capable of forming new NS.

Figure 23.- Comparative analysis of NS size from p73KO and WT cultures. A) Microphotographs of secondary NS (10x) after 4DIV under proliferating conditions. B) Quantification of NS size from pictures in A, expressed in μm (* $p < 0.05$).

We further evaluated the net cell growth of these NS cultures. Primary NS (P1-NS) were dissociated and re-plated at clonal density, and total cell number from the secondary NS formed was determined after 5 DIV. Consistent with the reduction in NS size, we detected a strong reduction in the total number of cells generated after 5DIV in the cultures lacking p73 (Figure 24), confirming that p73 loss was affecting the net cell growth of the pool of NPC/NSC. NSCs are multipotent and self-renewing cells, whereas neural progenitor cells (NPC) are proliferative cells with a more restricted renewal and differentiation abilities (Seaberg and van der Kooy, 2003). In the NS assay, the mitogenic culture conditions (EGF plus FGF) allows NPC to behave like NSC giving rise to heterogeneous aggregates of NSC and NPC in which the two populations are indistinguishable. Therefore, the term NPC will be used, from now on, to include both neural stem and progenitor cells in NS cultures.

Figure 24.- Growth kinetics of NS cultures. Evaluation of the net cell growth within the NS expressed by the ratio of cell production after 5DIV related to the number of cells plated at clonal density.

The decrease in cell number production might not be directly related to a decrease in self-renewal. Self-renewal is defined as the ability of a NSC within a NS to form a new multipotent NS in sequential passages. As NS expand they become heterogeneously composed by NSC as well as NPC in different states of differentiation, as well as dead cells. However, only the cells with the stem cell capacity can form new NS in successive passages, thus the number of spheres formed after each successive passage can be taken as a reliable estimation of the self-renewal capacity. To quantify the number of NS formed in each passage, we seeded P1-NS cells at clonal density (2cells/ μ l), and counted the number of NS formed after 4DIV under proliferating conditions. We arbitrarily assigned 100% to the number of NS formed in WT cultures. We observed a highly significant decrease in the number of NS formed in p73KO cultures (Figure 25). This difference was maintained in subsequent passages, demonstrating that p73 loss impairs NS self-renewal (Figure 25) and pointing out p73 as a positive regulator of NPC self-renewal.

Figure 25.- Self-renewal capacity of NPCs in sequential passages. Analysis of the capacity of NPCs to form a new NS after dissociation. We assigned 100% to the number of NS obtained in WT cultures, and calculate the relative percentage of NS obtained in p73KO cultures.

The decrease in the number of newly formed neurospheres in the p73KO cultures suggested a successive loss of cells capable of forming neurospheres in each passage. This could be due either to the death, or growth arrest, of this cellular population, or to the shutting-off of the self-renewal program in these cells and the turning-on of the differentiation program. Thus, self-renewal can be modulated by affecting different cellular processes like proliferation, survival and/or differentiation. Therefore, we went on to comparatively analyze such processes in the WT and p73KO NS cultures and to address if the observed effect on self-renewal capacity in the p73KO cultures could be due to an alteration in one or more of these processes.

To find out whether the limited growth of p73KO NSCs was caused by a defect in the cell cycle, or a consequence of a differential apoptotic rate, we investigated these parameters. We first examined cellular proliferation analysis of neurospheres cultures stained with propidium iodide (PI) by FACS. We dissociated P2-NS grown under proliferating conditions and labeled them with PI to determine the percentage of cells in each phase of the cell cycle (Figure 26). Cell cycle profiles showed no significant differences in the percentage of cells in G1, S or G2 phase (Figure 26). Therefore, the reduction in cell yield did not seem to be the consequence of a cell cycle defect.

Figure 26.- Cell cycle analysis of secondary NS. Flow cytometry analysis of secondary NS stained with propidium iodide.

Moreover, we investigated several genes implicated in the regulation of self-renewal through the control of cellular proliferation like p21^{Cip1}, Bmi1 and Hmg2a. First, we analyzed p21^{Cip1}, a direct transcriptional target of p53/p73 that has been described as a negative regulator of NSC self-renewal through its cell cycle inhibitory function (Kippin et al., 2005). Consistent with the unmodified cell cycle progression previously observed, there were no significant differences in p21^{Cip1} expression levels between the two populations (Figure 27A). Bmi1 and Hmg2a are positive regulators of self-renewal through their repression of the Ink4a/Arf locus and, in this way, they promote cell proliferation (Bruggeman et al., 2005; Molofsky et al., 2003; Nishino et al., 2008) (Figure 27B,C). Quantitative expression analysis did not showed any significant differences in the expression levels of these two genes between WT and p73KO cultures. Thus, we can conclude that the absence of p73 is affecting the net cell growth and impairing self-renewal of the NSC cultures without altering cell cycle progression.

Figure 27.- Expression analysis of cell cycle regulator genes. mRNA expression levels of p21^{Cip1} (A), Bmi1 (B) and Hmg2a (C), measured by qRT-PCR.

Coincident with the publication of this data, several independent groups reported the role of p73 in neural stem cells (Fujitani et al., 2010; Talos et al., 2010). Although all the publications agreed that p73 was necessary to maintain neural stem cells, there were slight differences in some of the results obtained by each group.

Consistent with our findings, two of the articles described a significant reduction in the NS size. Dr Melino's group obtained NS from E14.5d and reported differences in the NS size after 4 and 7 DIV (Agostini et al., 2010). Dr Ute Moll's group analyzed NS from E14d and P0 whole brains. They found that the average size of the NS was significantly smaller by passage 6 in the embryonic cultures, while when they cultured P0 NS from whole brain (or from SVZ and SGZ), the effect was visible at passage 2 or 3 and even more dramatic. They

also demonstrated that the reduction in the NS size was not due to a smaller size of individual cells (Talós et al., 2010). The effect in NS size that we observed was more severe, since we found significant differences in NS size even in secondary NS. This difference could be due to the use of embryonic OB as source of primary NS, while Talós et al. (2010) were using whole brains at this developmental stage. Confirming our data they conclude that p73 is required for NPC proliferation.

While our data did not revealed any difference in cell cycle progression of secondary p73KO NS, these two groups postulated an impaired S-phase in NS cultures from E14 and P0 whole brains. However, Dr. Kaplan's group, working with the TAp73 specific knock-out mice, demonstrated that TAp73 was not necessary for proliferation of neural progenitors (Fujitani et al., 2010)

As mentioned before, enhanced cell death could be also affecting the pool of NSC in the p73KO NS cultures; therefore, we analyzed the apoptotic cell death within these cultures. For this purpose, we performed a double staining with Annexin V, (marker of apoptosis) and 7AAD (marker excluded from intact cells but that can penetrate dead cells or cells with damaged membranes), and quantify the number of stained cells by FACS analysis (Figure 28A). Our study revealed a two fold increase in cell death rates in p73KO cultures.

Figure 28.- Cell death analysis in secondary NS by Annexin V / 7AAD. A) FACS analysis, showing the apoptotic cells in red. B) qRT-PCR analysis of Noxa expression levels.

Accordingly, levels of the pro-apoptotic p53 target gene Noxa were significantly up-regulated in cultures lacking p73 (Figure 28B), suggesting that the observed impairment of NSC self-renewal could be due to enhanced apoptosis in these cultures. This observation would be in agreement with the previously described dominant negative effect of DNp73 over p53 function (Vilgelm et al., 2008).

The role of p73 in the developing nervous system has been defined as a rheostat for survival *versus* death where DNp73 isoforms would be the major players as potent anti-apoptotic proteins which antagonize, at least in part, the pro-apoptotic function of p53 and, potentially, TAp63 (Jacobs et al., 2006; Pozniak et al., 2000). Since the Trp73^{-/-} mice lack all p73-isoforms, it was possible that an exacerbated p53 activity, due the lack of the pro-survival DNp73 function in the Trp73KO cells, could be accounted for the enhanced apoptosis and the impaired self-renewal observed in those cultures. This hypothesis will be addressed in Section 1.2. of the Results and Discussion from this thesis.

The published data so far confirmed that p73 was necessary for the maintenance of NSC self-renewal and also for the proliferation potential of neural progenitors within the NS. Furthermore, Fujitani *et al.* (2010) demonstrated that TAp73 is required for the long-term maintenance of adult NSC. We next wanted to address whether p73 might control NSC self-renewal by regulating cellular differentiation. We had already demonstrated that the p73KO NS were multipotent, since the newly formed NS in each passage were capable of differentiating into the three major neural lineages (Figure 21). However, it was not known whether lack of p73 affected the differentiation kinetics of the NS cultures in a quantitative manner.

To address this point, we performed a time course experiment in which neurospheres of WT and p73KO were cultured in differentiation medium and, at the indicated time points, Tuj1 and GFAP positive cells were assessed (Figure 29A). At the final differentiation time point (8DIV) we observed a slight, but not significant, increase in the total number of Tuj1 positive cells with no significant differences in the number of astrocytes between the two genotypes (Figure 29B). However, when we analyzed earlier time points, we detected a significantly higher percentage of large branching neurons (Tuj1 positive cells with neurite extensions larger than four times the cell body diameter) in the p73 KO derived neurospheres than in WT cultures at every time point analyzed, suggesting an advanced stage in the differentiation process, despite the identical conditions (Figure 29C). Nevertheless, when we accounted all the Tuj-1 positive cells in the cultures, we only detected significant differences after 3DIV in differentiation conditions, finding no significant differences at day 5 or 8 (Figure 29D).

Figure 29.- Comparative analysis of the differentiation kinetics of NS cultures. A) Confocal microscopy images (20x) of NS immunostained for Tuj1 and GFAP after 8DIV under differentiation conditions. B) Quantification of the number of neurons and astrocytes after 8 DIV. C & D) Quantification at 3, 5 and 8 DIV of the number of total Tuj1 positive cells (C) or only that with long neurite extensions (D). Statistical analysis of the p73KO compared to WT NS.

These results supported the idea that lack of p73 leads to a premature onset of neuronal differentiation in NSC within the NS. Furthermore, our differentiation studies demonstrated that loss of p73 did not abolish neurospheres multipotency, but rather accelerated the appearance of Tuj1+ cells with long and branched neurite extensions, congruent with a mature neuronal phenotype. These data connote that p73 deficiency is releasing the constraints that keep the embryonic NPC undergoing symmetric, proliferative division, thus hampering their self-renewal and producing a premature differentiation.

Confirming our data Talos *et al.* (2010) also demonstrated that astrocyte differentiation was unaltered in p73KO compared to WT; moreover, they quantified the same number of total Tuj-1 positive cells obtained from passage 1 E14 NS after 9 DIV under differentiation conditions. This was in agreement with our results, since we didn't find any differences in the number of total Tuj1+ cells at the end point of differentiation. However, they showed a reduction in the number of neurons obtained at later passages, probably due to an inability

of NSCs to maintain and/or produce neuronal precursors. Furthermore, they analyzed the quality of neurons and they suggested major defects in neurite outgrowth. After 7-9 days of differentiation, they found that in WT NS most neurons showed mature morphology with long Tuj1+ processes, extensive arborization and physical connections with other neurons. On the other hand, they reported that neurons obtained from p73KO NS seemed to be underdeveloped and of poor quality and that they had multiple defects in processes formation. This data seems to be in contradiction with ours; however, it is important to point out that while they evaluated quality of neurons at end point of differentiation, we have reported premature differentiation of NS cultures at early time points, 1DIV. We have also observed that this prematurely-differentiated neurons presented aberrant ramifications, thus, it is possible that those premature differentiated neurons were indeed unable to survive.

1.2. Functional interaction between p53 and p73 in the biology of neural stem cells

As discussed before, it was possible that an exacerbated p53 activity, due the lack of the pro-survival DNp73 function in the p73KO cells, could be accounted for the enhanced apoptosis and the impaired self-renewal observed in those cultures. Indeed, analysis of phospho-p53 (Ser15) in NS cultures under proliferating conditions, revealed an activation of p53 in the cells lacking p73 (Figure 30), which could explain the enhanced apoptosis in this cultures.

Figure 30.- Analysis of p53 activation in p73 deficient NS. Western blot analysis of protein extracts from secondary NS under proliferating conditions.

Therefore, the increased apoptosis in the p73KO cells, as well as Noxa enhanced expression, could be due to compensatory p53 activation. However, the lack of increased p21^{Cip1} expression (Figure 27), a direct transcriptional target of p53, in the p73KO cells pointed against this hypothesis. To address these questions we decided to analyze the role of p53 in the context of p73 deficiency using the double knock-out mice (p53^{-/-}, Trp73^{-/-}; named DKO from now on). We established NS cultures from p73KO, p53KO, DKO or WT littermate embryos and compared the cell cycle, apoptosis and self-renewal capacities.

As shown in Figure 21A, p53KO cultures presented larger neurospheres, reflecting its enhanced self-renewal (Armesilla-Diaz et al., 2009; Gil-Perotin et al., 2006; Meletis et al., 2006). Surprisingly, while p73KO cultures presented reduced size compared to WT, the DKO cultures gave rise to bigger NS than the p73KO, but with a comparable size to WT NS, despite the absence of p53 in these cells (Figure 31B). These results support the idea that the reduced size of p73KO NS was due to a compensatory activation of p53, while at the same time indicates that p73 loss hampered the effect of p53 deficiency, implying that p73

Figure 31.- Comparative analysis of NS size from the four genotypes. A) Micrographs of secondary NS from WT, p73KO, p53KO and DKO cells (10x). B) Quantification of NS size.

To further analyze the effect of p73 loss in p53KO cultures we compared the net cell growth of NS from the four genotypes. Consistent with our previous observations, while the p53KO cultures exhibited a significant increase in their net cell growth after 5DIV (compared to WT cultures), in the DKO NS cultures, which lack p53 and p73, there were no difference in the net cell growth with respect to the WT ones, indicating that lack of p73 hampered the effect of p53 in the net cell growth of neural stem cells (Figure 32).

Figure 32.- Comparative analysis of the net cell growth of NS from the four genotypes. Percentage of cell increase related to the number of cells from secondary NS seeded, normalized by the net increase of the WT population.

It has been described that p53 regulates neural stem self-renewal through the regulation of the proliferation and apoptosis rates. As p73 seems to be necessary for the enhanced growth of p53KO cultures, we wanted to determine if the cell cycle progression and apoptosis of DKO cultures, compared with those of p53KO, were affected by the absence of p73. Therefore we analyzed the cell cycle profile and sub-G1 population of these cultures by PI-staining and flow cytometry analysis. Confirming our previous results, we detected an increase in the percentage of cells in Sub-G1 (cell death) in p73KO cultures, (Figure 33). As expected, p53KO cultures showed significant lower levels of cell death compared to WT NS. The analysis of the DKO cells revealed that the enhanced cell death elicited by the lack of p73 isoforms was abolished in the absence of p53 (Figure 33).

Figure 33.- Cell cycle analysis of NS cultures from the four genotypes. NS were dissociated after 4DIV under proliferating conditions and cells were stained with propidium iodide. We show statistical analysis of the three genotypes compared to WT, at each phase of the cell cycle.

Consistent with the decrease in the cell death rates, a strong down-regulation of the expression levels of the pro-apoptotic p53 target gene Noxa was observed in DKO cultures, compared to WT (Figure 34A). Altogether these observations confirmed our hypothesis that p53 compensatory activation was accounted for the elevated apoptosis rates in p73KO NS. However, the low levels of cell death in the DKO cultures were contradictory with the decreased net cell growth previously detected. Furthermore, DKO cultures presented a significant increase in the number of cells in S-phase, with a concomitant decrease in G1 cells, which was similar to that observed in the p53KO cultures (Figure 13). Altogether these parameters indicated that the DKO cells had enhanced cellular proliferation.

Figure 34.- Analysis of expression of pro-apoptotic and cell cycle regulator genes. qRT-PCR analysis of the mRNA levels of the pro-apoptotic p53 target gene Noxa (A) and the cell cycle regulator gene p21^{Cip1} (B).

To confirm the effect of p73 loss in the proliferation ratios of these cultures we analyzed their mitotic index by quantifying the number of phospho-His3 positive cells. Histone 3 is phosphorylated during mitosis, and it can be used to assess the mitotic index of the culture. For this purpose, secondary NS were seeded on precoated Matrigel coverslips in proliferation media, we fixed them after they attached and performed an immunocytochemistry analysis (Figure 35).

Figure 35.- Comparative analysis of the proliferating cells. A) Confocal microscopy images of secondary NS (20x). PHis3 positive cells can be observed in green, and nuclei were stained with DAPI and are shown in blue. B) Quantification of the number of PHis3 positive cells for each genotype.

Confirming our previous results, the mitotic index was higher in the p53KO cultures than in WT NS while the p73KO cultures did not showed any significant difference. Interestingly, the DKO cultures showed the highest mitotic index, confirming that although lack of p73 impairs the net cell growth in the DKO compared to p53KO, the proliferation index of these cultures was not affected, neither they were dying at higher rate. Moreover

the enhanced proliferation rate also seems to be p53 dependent through regulation of cell cycle regulator genes like p21^{Cip1}, since lack of p53 abolished p21^{Cip1} expression in DKO cultures (Figure 34B).

Therefore, lack of p53 elicits enhanced cell proliferation and abolishes cell death. However despite these parameters, absence of p73 results in a decreased net cell growth, similar to that observed in the p73KO NS cultures (Figure 32), confirming that p73 function was necessary for the enhanced growth of p53 KO cultures.

Our data so far lead us to propose that while p53 act as a negative regulator of neural stem cells self-renewal, p73 might act independently of p53 as positive regulator of this process. To demonstrate that the effect of p73 deficiency on self-renewal was independent of p53, we performed a comparative self-renewal assay with NS of the four genotypes. For this purpose, we plated serial dilutions of cells at low densities (from 500-60 cells/100 μ l) and scored the number of NS obtained after 4 DIV. Consistent with our previous results, p73KO NS showed a significantly reduced self-renewal capacity while, as previously published (Armesilla-Diaz et al., 2009; Meletis et al., 2006), p53KO cultures had a significantly enhanced self-renewal compared to WT control cultures at every cellular density investigated (Figure 36). However, when p73 was absent, despite the lack of p53 and their enhanced rates of proliferation, the DKO NS cultures did not showed differences with the WT controls at most of the cellular densities analyzed, with the exception of the higher density at which we detected a slight increase over control. Furthermore, comparison of DKO with p53KO cultures revealed a significant reduction in the number of newly formed NS.

Figure 36.- Self-renewal assay of the four genotypes. Percentage of cells obtained related to the number of cells seeded from secondary NS. The symbols at the top of the bars represent the statistical differences compared to WT, while the symbols on the lines, statistical differences of DKO compared to p53KO cultures.

These data indicated that in the absence of p73, p53 KO cells were not capable of maintaining their characteristic enhanced self-renewal. Therefore, we can conclude that p73 regulates neural stem cells self-renewal independently of p53 and also that it is necessary for the enhanced growth of p53KO NS. These results are of great relevance, since they demonstrate, for the first time, that p73 has a p53-independent role in maintaining neural progenitor cells, further emphasizing the complex relationship between p53 family members.

We have demonstrated that p73 is a positive regulator of self-renewal, and that the effect of p73 deficiency in NPC was not simply due to a decrease in the proliferation rate or enhanced apoptosis due to enhanced p53 activity. Furthermore, our data supported the idea that lack of p73 leads to a premature onset of neuronal differentiation in NSC. So, we sought to elucidate if this effect of p73 deficiency would be dependent on the presence of p53 or if, by the contrary, absence of p73 would alter the differentiation fate of p53KO cells.

We have previously shown that loss of p73KO results in a premature neuronal differentiation without altering the rates of astrocytic differentiation (Figure 29). On the other hand, it has been previously described that p53KO neural stem cells render a biased differentiation pattern, with a significantly higher number of neurons, while the number of astrocytes diminishes in the culture (Armesilla-Díaz et al., 2009). Therefore, we sought to investigate if the absence of p73 would alter the differentiation rate and the fate of p53KO cells by analyzing the differentiation potential of DKO NS at different time points during the differentiation process.

Figure 37.- Neuronal differentiation from NS of the four genotypes. A) Confocal microscopy images of differentiated secondary NS after 3DIV (40x), immunostained with TuJ1 (red). B) Quantification of the TuJ1 positive cells, with and without long neurite extensions (NE). Statistical differences are indicated compared to WT NS.

At early stages of differentiation (3DIV), the p73KO and p53 KO NS presented significant differences with respect to WT NS in the number of neurons with long neurite extensions, confirming their premature neuronal differentiation phenotype (Figure 37). In the DKO NS, the combined deficiency produced more neurons with mature phenotype than p53 or p73 single loss, suggesting an additive effect. It is noteworthy that DKO NS also presented higher number of total Tuj1 positive cells at this early stage of differentiation, supporting the additive effect of p53 and p73 deficiencies. At an intermediate stage of differentiation (5DIV), the number of Tuj1 positive cells generated in WT NS cultures had increased, but these Tuj1- positive cells still presented an immature phenotype, with very short and thin neurite extensions (Figure 38). The p73KO and DKO cells displayed Tuj1- positive neurons with a more mature phenotype, with multiple long and branched neurite extensions (Figure 18). These observations supported the idea that p73 deficiency resulted in enhanced rate, and faster kinetics, of the differentiation process independently of p53, since p53 KO cells still presented a more immature phenotype at this time point.

Figure 38.- Analysis of differentiated NS after 5DIV-DM. Confocal microscopy images of differentiated secondary NS after 5DIV (40x). Cells were immunostained with Tuj1 (red), and nuclei were counterstained with DAPI (blue).

As mentioned before, lack of p53 hinders astrocytic differentiation. Moreover, it has been proposed that p53 control of neurogenesis and gliogenesis is independent of its regulatory pathways of self-renewal (Nagao et al., 2008). Therefore, to investigate if there was a cross-talk in the regulation of cell fate decision between p53 and p73, we analyzed the differentiation potential of DKO cultures into astrocytes. After 8DIV under differentiation conditions we immunostained the cultures with GFAP and quantified the number of astrocytes for each genotype (Figure 39A). We observed that there were no differences in the number of astrocytes between WT, p73KO and DKO cultures, indicating that when p73 is absent, lack of p53 does not affect the differentiation fate decision (Figure 39 B). Consistently, the astrocytic differentiation in DKO was significantly higher than in the p53KO cultures also presenting a more mature phenotype (Figure 39A). Therefore, lack of p73 somehow rescues the uncoupling of neuronal/ astrocytic fate decision in p53KO cultures.

Figure 39.- Astrocytic differentiation from NS of the four genotypes. A) Confocal microscopy images of differentiated secondary NS after 5DIV (20x), immunostained with GFAP (green). B) Quantification of GFAP positive cells related to number of nuclei.

Surprisingly, in p73KO cultures the premature onset of neuronal differentiation did not result in an impairment of glial differentiation, even in the absence of p53. These results suggested that lack of p73 could uncouple the regulation of differentiation processes, downstream of p53. Therefore, p73 and p53 regulation of self-renewal and differentiation imply independent, yet interconnected pathways. The observation that the effect of p53-deficiency on cell fate determination is restored by p73 depletion in DKO cells, suggested a cross-talk between these genes in the regulation of this process.

We have demonstrated that DKO cultures presented premature neuronal differentiation as p73KO cultures. Furthermore, this effect of p73 loss was independent of p53 and in DKO cultures, self-renewal impairment due to p73 deficiency was dominant over the self-renewal enhancement resulted by p53 loss. Thus, p73 must control a mechanism that regulates NSC differentiation up-stream of p53.

1.3. Effect of p73 loss in the regulation of asymmetric cell division

During prenatal development, the self-renewal of neural stem cells and their progenitors occurs either by symmetric cell divisions, which generate two undifferentiated cells with the same fate or, when neurogenesis begins, by asymmetric divisions, giving rise to one progenitor and one cell that differentiates into a neuron. The transition from symmetric to asymmetric division must be tightly regulated, as excessive symmetric cell division can lead to tumorous overgrowth, whereas precocious asymmetric division results in premature differentiation. Symmetric and asymmetric cell divisions have been postulated to mediate the

balance between stem cell maintenance and neuron differentiation, respectively, during mammalian neurogenesis. Therefore, there is a need for a molecular machinery that orchestrates the coordinated balance between maintenance of self-renewal and induction of differentiation even though the mechanism of such regulation remains unclear. In this context, while the role of p53 in the regulation of self-renewal divisions has been widely documented (Cicalese et al., 2009), there is no data about p73 regulation of asymmetrical divisions of stem cells. Thus, we decided to analyze the effect of p73 deficiency on the regulation of asymmetric cell division of NSC.

NSC *in vitro* have been defined as fast dividing Nestin positive cells under proliferating conditions that clonally give rise to great numbers of Nestin positive neural progenitors that divide symmetrically to produce an equivalent progeny (Karpowicz et al., 2005). Therefore, we analyzed the number of undifferentiated NPCs under proliferating conditions. After 3DIV, we fixed the NS and immunostained them to determine the number of Nestin positive cells (Figure 40). We found that lack of p73 produced a significant reduction in the number of Nestin positive cells in p73KO and DKO cultures (Figure 40B), suggesting that the number of undifferentiated NPCs in these NS was lower compared with WT or p53 single knockout.

Figure 40.- Analysis of undifferentiated progenitors from proliferating NS of the four genotypes. A) Confocal microscopy images of NS after 3DIV under proliferating conditions (60x), immunostained with Nestin (red). Nuclei were counterstained with DAPI. B) Quantification of the number of nestin positive cells in each culture.

Closer observation of the p73KO and DKO cells revealed pairs of cells that resembled daughter cells with different nuclei size and asymmetric distribution of Nestin (Figure 41). The number of this asymmetric cell divisions was significantly lower in WT and p53KO. These results were consistent with the fact that under proliferating conditions WT neural

stem cells undergo symmetric cell divisions, and suggested that p73 deficiency may be leading to a bias towards asymmetric cell divisions.

Figure 41.- Analysis of asymmetric cell divisions. A) Magnification of the indicated areas in Figure 40, showing asymmetric cell divisions. B) Quantification of the number of asymmetric cell divisions.

Furthermore, the increased number of asymmetric cell divisions in p73KO and DKO cultures could explain the impaired self-renewal of these cells, since it would lead to a decrease in the pool of undifferentiated NPCs and to an increase in the number of differentiated cells.

For this reason, we decided to analyze the spontaneous differentiation under proliferating conditions and evaluate the number of Tuj1 positive cells for each genotype after 3DIV *in vitro*. Even though the extrinsic growth factors EGF plus FGF-2 (present in the culture media) should be sufficient to sustain symmetrical self-renewing divisions of neural stem cells (Reynolds and Weiss, 1996), we frequently observed numerous Tuj-1 positive cells with long and branched neurite extensions in the p73KO and DKO cultures, corroborating its premature differentiation under proliferating conditions. However, WT and p53KO NS rarely showed Tuj1 positive cells with short unbranched processes at such an early time point (Figure 42). Thus, lack of p73 could be affecting the frequency of self-renewing symmetrical divisions.

Figure 42.- Analysis of prematurely differentiating neurons in NS of the four genotypes. A) Confocal microscopy images of NS immunostained for Tuj1 after 3DIV under proliferating conditions (60X). B) Quantification of the premature differentiated neurons.

To further demonstrate that p73 deficiency indeed increases asymmetric cell division, we analyzed the asymmetric distribution of two markers of this process: Notch activation (by detection of the active Notch intracellular domain -NICD-) and TRIM32 segregation. Notch signaling pathway regulates binary cell-fate decisions in stem cells where the presence of NICD maintains the cell undifferentiated. Therefore, under mitotic culture conditions, WT dividing NSCs generate two daughter cells with symmetric distribution of NICD, which positively correlates with self-renewal potential. More infrequently, they can produce two daughter cells with asymmetric distribution of NICD, in which case the daughter cell without Notch activity is committed to differentiate, this way reducing self-renewal. NICD distribution was quantified in NS cultures maintained under mitotic culture conditions. We dissociated secondary NS and in the presence of mitogens (EGF& FGF) and treated the cells with Blebbistatin, a myosin II inhibitor which blocks cytokinesis (Kovacs, wang, 2003), maintaining together the newly formed cell pairs. This procedure allowed us to quantify the distribution of factors such as NICD and TRIM32 among daughter cells.

Figure 43.- Analysis of asymmetric distribution of NICD in neural progenitors cell pairs. A) Confocal microscopy pictures (100X) of NICD distribution in cell pairs. B) Quantification of the asymmetric cell divisions.

Under these conditions most of the cell divisions in the WT cultures were symmetric (Figure 23A) and they rarely displayed asymmetric distribution of NICD. However, in the absence of p73 the number of cell pairs that presented an asymmetric distribution of NICD was significantly increased (Figure 43B). It is noteworthy that we consistently observed two NICD sub-cellular localizations in the asymmetrically distributed p73KO cell pairs. Some cells displayed nuclear expression of NICD; however, we often observed cells with elevated cytoplasmic NICD expression within the higher NICD-expressing cell of the pair, suggesting that Notch signaling pathway could be impaired in those cells. This preliminary observation, which will require further investigation, would suggest that in the p73KO cultures not only the percentage of asymmetric cell division increases, augmenting the number of cell committed to differentiate, but also some of the daughter cells which inherited NICD would not be able to maintain the undifferentiated status due to the inactivation of the NICD signaling cascade.

To further demonstrate that p73 deficiency correlated with elevated levels of NPCs asymmetric divisions, we analyzed the expression of TRIM32 under the same conditions. TRIM32 is a neuronal fate determinant that is expressed at low levels in WT proliferating progenitors, and its asymmetric distribution is seldom observed (Qian et al., 1998; Qian et al., 2000; Schwamborn et al., 2009). However, in neural stem cells cultured under conditions supporting neural differentiation, TRIM32 is asymmetrically distributed, and its expression contributes to the decision of undergoing neuronal differentiation.

We analyzed TRIM32 in NPC cell pairs under proliferating conditions and found that, as reported in the literature, WT cultures rarely presented asymmetric distribution of TRIM32. Surprisingly, we observed a significantly higher percentage of cells undergoing TRIM32 asymmetric distribution in p73KO cultures (Figure 44).

Figure 44.- Analysis of the number of cell pairs with asymmetric distribution of TRIM32. Quantification of the asymmetric cell divisions by scoring TRIM32 asymmetric distributed cell pairs.

Moreover, it is important to point out that in p73KO cultures only few cells expressed enhanced levels of TRIM32, in most of which, TRIM32 is nuclear (Figure 46). However, we detected some cells that also showed high cytoplasmic TRIM32 levels, which coincidentally co-localized with NICD (Figure 45). This accumulation of NICD in the cytoplasm, and its co-localization with TRIM32, suggests that absence of p73 activates a yet unknown mechanism of Notch signal inhibition that could explain the spontaneous differentiation of NPC under mitotic conditions.

The alteration of the Notch pathway has been suggested before in p73KO cells by Dr. Ute Moll's group (Talos et al., 2010). They investigated the impact of p73 loss on this signaling pathway. They reported that overall expression of Notch receptors was reduced in p73KO NPC from E14d NS. Particularly, Notch2 expression (involved in SVZ stem cells maintenance) was strongly reduced. Altogether they found that Notch signaling pathway was profoundly altered at all levels of regulation and that its activity further diminished with successive p73KO-NS passages, suggesting that Notch pathway could be downstream the effectors of p73 function (Talos et al., 2010).

Figure 45.- Cellular localization of NICD and TRIM32 expression in cell pairs. Confocal microscopy images (100x) of NPCs cell pairs immunostained for NICD, TRIM32. Nucleus were counterstained with DAPI.

We next analyzed cell proliferation and TRIM32 distribution among daughter cells in order to address if in the p73KO cultures the daughter cell expressing TRIM32 would exit the cell cycle and be committed to differentiate. Secondary NS under proliferating conditions were subjected to confocal immunofluorescence analysis using phospho-His3 as a marker of cell proliferation (Figure 46). We observed that in WT cultures TRIM32 was expressed uniformly in most of the newly dividing cells. Surprisingly, although most of p73KO cells presented lower expression of TRIM32, we detected some cells with enhanced TRIM32 expression.

Figure 46.- Comparative analysis of proliferation and TRIM32 expression. Confocal microscopy pictures (60x) of secondary NS after 3DIV under proliferating conditions. TRIM32 was labeled in red and p-His3 in green.

Those TRIM32 positive cells in the p73KO cultures belonged to newly divided cell pairs in which one cell was p-His3 positive, and did not express TRIM32, while the other expressed TRIM32, but was negative for p-His3 staining and, therefore, was not proliferating. This suggests that in p73 deficient NPCs under proliferating conditions, the increased level of TRIM32 in one of the two daughter cells, contributes to the decision of this cell to undergo neuronal differentiation and cease to proliferate, predicting a correlation between TRIM32 expression and premature differentiation in these cultures.

Thus, we analyzed whether TRIM32 expression coincided with prematurely Tuj-1 positive differentiated cells in p73KO NS cultures under proliferating conditions. Consistent with our previous data, analysis of p73KO cultures after 4DIV under proliferating conditions revealed Tuj1 positive cells with long and branched neurite extensions, which presented enhanced TRIM32 expression in the nucleus (Figure 47).

Figure 47.- Analysis of the expression of TRIM32 in premature differentiated neurons. Confocal microscopy pictures (60x) of NS cultures under proliferating conditions.

To further evaluate if TRIM32 expression in p73KO NPCs could correlate with an accelerated differentiation rate in these cells, NS cultures were kept for only 24 hours under differentiation conditions (1DIV-DM) and TRIM32 expression, as well as the number of Tuj1 positive cells, were analyzed (Figure 48). At such an early time point of the differentiation process, WT cultures showed few Tuj-1 positive cells that usually had short lamellipodia and/or short unbranched processes. TRIM32 expression in these cells was mostly cytoplasmic confirming their immature phenotype (Hillje et al., 2011). In WT cultures, we exceptionally detected cells with neurite extensions longer than 4x their soma and with nuclear TRIM32 expression, indicating a mature phenotype. On the other hand, and consistent with our previous data, at identical culture conditions p73KO cultures presented significantly higher number of Tuj-1 positive cells with long and branched neurite extensions (Figure 48, B). All the Tuj-1 positive cells had enhanced TRIM32 expression; this was exclusively nuclear in 24% of the cells, while it was nuclear and cytoplasmic in 52% of the cells, altogether indicating a mature neuronal phenotype. These data strongly supports the idea that lack of p73 in NPCs leads to premature and accelerated differentiation.

Figure 48.- Analysis of the presence of neurons and correlation with TRIM32 expression at early differentiation time points. A) Confocal microscopy pictures of differentiated NS cultures after 1DIV under differentiation conditions..B) Quantification of the percentage of double positive cells for Tuj1 and TRIM32 expression.

Altogether, our data indicated that p73 deficiency results in enhanced asymmetric cell division of NSC/NPC cells under proliferating conditions, leading to premature differentiation and thus, impairing self-renewal. These data also revealed a more mature neuronal phenotype that, in agreement with the faster kinetics of neuronal differentiation observed in p73KO and DKO cultures, led us to hypothesize that p73 deficiency was exhausting the pool of undifferentiated neurosphere forming cells and, in this way, impairing NSC self-renewal.

The effect of p73 deficiency on the equilibrium between symmetric versus asymmetric cell division situates p73 in an up-stream regulatory point in the maintenance of NSC self-renewal *in vitro* up-stream of the regulation of pro-differentiation factors. This would explain that lack of p73 could impair the self-renewal of the p53KO NPC despite their lower rates of cell death and high mitotic index.

Altogether, our data had lead us to hypothesize that p73 function as (negative) regulator of the expression of factor/s that triggers the asymmetry of cell division. Therefore, in the absence of p73, NPCs would undergo asymmetric cell divisions at a higher rate, leading to their premature differentiation and loss of self-renewal. If this hypothesis is correct p73 deficiency should have profound effects in the development of the neurogenic niches and in the maintenance of these sites along the life of the adult organism.

1.4. Effect of p73 loss in the proliferating populations of the neurogenic niches

Adult brain contains NSCs that generate neurons and glial cells on an ongoing basis. These adult NSC, generated from the precursors that build the nervous system, are maintained in at least two niches, the SVZ and the SGZ (Miller and Gauthier-Fisher, 2009). Alterations in the mechanisms that promotes or inhibits the differentiation of embryonic neural progenitor cells would eventually deplete the pool of embryonic NSC rendering deficient neurogenic niches in the post-natal and adult mice. Since our results demonstrated that lack of p73 produces premature differentiation of embryonic neural progenitors, it is feasible to speculate that lack of p73 would deplete the pool of embryonic NPC during development resulting in a diminished population of proliferating, self-renewing cells in these sites. Therefore we analyzed the effect of p73 loss in the proliferating populations of the subventricular of the lateral walls of the ventricles (SVZ) and in the subgranular zone of the dentate gyrus of the hippocampus (SGZ), which are the two major neurogenic niches in the postnatal and adult mice brain.

1.4.1. Analysis of proliferating cellular populations in the lateral walls of the ventricles (SVZ)

We performed comparative analysis of the proliferating cells in the SVZ, which are neural stem cells, transit amplifying progenitors, and neuroblasts, of WT and p73KO mice. In our initial analysis we utilized BrdU, a synthetic analogue of thymidine that is incorporated into newly synthesized DNA by cells entering into S-phase of the cell cycle. We injected BrdU to postnatal 15 day-old mice (P15) during 8 hours, and scored the number of cells in the SVZ that had incorporated BrdU. We observed a significant reduction in the number of proliferating cells in this area in the absence of p73 (Figure 49A&C).

To confirm these results we also analyzed Histone-3 phosphorylation (pHis3) at Serine 10, which occurs during mitosis and is crucial for initiation of chromosome condensation and cell cycle progression in mammalian cells. Analysis of pHis3 in this area also revealed a severe depletion in the number of proliferating cells (Figure 49B&D).

Figure 49.- Comparative analysis of the proliferating cells in the subventricular zone SVZ of P15 mice. A&B) Panoramic of fluorescence images of the lateral ventricles of WT and p73KO mice and magnification of the indicated areas of the SVZ. Fluorescence images after BrdU injection in A, and immunostained for p-His3 in B. C&D) Quantification of the number of positive cells in A and B, respectively.

Furthermore, in collaboration with the laboratory of Dr. JM García-Verdugo (CIPF, Valencia), we performed a preliminary morphological analysis of the SVZ neurogenic niche at electronic microscopy level. When we scored the different proliferating cellular populations, we found that the number of C cells (transit amplifying progenitors) and A cells (neuroblasts) were significantly reduced in p73KO mice (data not shown). The analysis of B cells was inconclusive since these cellular population presented profound alterations and were difficult to identify which made the quantification extremely variable. A deeper analysis of the different cellular populations in the SVZ of the p73KO mice is presented in Section 2 of the Results & Discussion.

These results demonstrated that, indeed, lack of p73 was depleting the population of proliferating cells in the SVZ of P15 mice, suggesting the presence of deficient neurogenic niches in the adult mice and, therefore, resulting in an impaired neurogenesis.

Once we have demonstrated that SVZ of mice lacking p73 showed a severe reduction in the number of proliferating cells, we wanted to investigate the effect of p73 deficiency in the context of p53 deletion using the DKO mice. Thus, we quantified the number of cells that

had incorporated BrdU in P15 mice of the four genotypes. In agreement with previously published data (Meletis et al., 2006), our results from the p53KO mice showed a higher number of proliferating cell in the SVZ, consistent with their enhanced self-renewal and low apoptosis *in vitro*. As expected, the p73KO presented a significantly reduced number of proliferating cells compared to WT (Figure 50). Interestingly, the SVZ of the DKO, despite the high proliferation rates of its NSC *in vitro*, had less proliferating cells than p53KO, with a number very similar to that of the p73KO (Figure 50).

Figure 50.- Comparative analysis of the proliferating populations in the SVZ of P15 mice of the four genotypes. A) Confocal microscopy pictures (102x) of brain sections after BrdU injection . B) Quantification of the BrdU positive cells in the SVZ.

Furthermore, observation of coronal sections of the brain from the four genotypes revealed that, consistent with the existing data about p73KO brains, they showed severe hydrocephaly and extremely enlarged ventricles (Figure 51). This phenotype seems to be dominant in the DKO mice, since they also presented enlarged ventricles, and the brain architecture is very different from the p53KO or WT mice.

Figure 51.- Comparative images of coronal sections from P15 mice of the four genotypes. Confocal microscopy pictures (10x) of coronal sections from the four genotypes.

These data demonstrated, for the first time, that p73 function is required for the maintenance of the population of NSC/NPC *in vivo*, and that this effect is not due to enhanced p53 activity. Moreover, p73 function is necessary to sustain the increased proliferation, elicited by the lack of p53, in the NSC/NPC population of the SVZ.

The SVZ has been suggested as the site of origin of brain tumors (Smyth and Henderson, 1938; Vick et al., 1977). Specifically, studies in human tumors and transgenic mice have suggested the relevance of NPCs from the SVZ in the genesis of glioblastomas (Holland, 2000; Ignatova et al., 2002). Furthermore, p53 is expressed in SVZ cells and frequently deleted or mutated in glial tumors. Our discovery that p73 deficiency could restrain the enhanced proliferation of NSC/NPC lacking p53 could be of great significance for the understanding of the initiation and progression of these tumours. Many of the pathways that are found mutated in gliomas, are usually involved in the regulation of stem cell functions. Although loss of p53 by itself is not sufficient for tumor formation, it provides a proliferative advantage to the cells in the SVZ in the initiation of tumor formation. Moreover, combination of TP53 mutation/deletion in NSC/NPCs, together with other genetic aberrations like the ones in PTEN, has been proposed to be the origin of malignant gliomas (Zheng et al., 2008). Since our findings show that p73 is necessary for the enhanced self-renewal of p53KO-NSC/NPCs, it could be expected that inactivation of p73 in p53KO gliomas would be a possible therapeutic approach to repress tumor growth.

1.4.2. Analysis of the proliferating cellular populations in the subgranular zone of the dentate gyrus of the hippocampus (SGZ)

Next, we sought to analyze the other neurogenic niche in the mouse brain, which is the SGZ. The proliferating population in this area is comprised by radial NSC (A or type I progenitors), non-radial NSC (D or type II progenitors), and granule cells (neuroblasts).

Comparative analysis of the overall structure of WT and p73KO mice hippocampus, together with the quantification of proliferating populations in this region, revealed profound abnormalities in p73KO mice. They presented an aberrant structure of the hippocampus and a strong reduction in the number of proliferating cells, determined by quantification of p-His3 (Figure 52).

Figure 52.- Comparative analysis of the proliferating cells in the subgranular zone of P15 mice. A) Confocal microscopy pictures (10x) of the SGZ of the four genotypes. B) Quantification of the BrdU positive cells in the SGZ.

Finally, we performed a comparison of the SGZ of the p73KO and the DKO P15 mice. We observed that, absence of p73 in DKO mice resulted in defects of the overall structure of the SGZ, although the phenotype seems not to be as severe as the observed in the p73 single knock-out (Figure 53). Furthermore, we quantified the number of proliferating cells in this area by scoring Ki67 positive cells. We found that the number of proliferating cells in p73KO and also in DKO mice SGZ was reduced, demonstrating that p73 deficiency leads to impairment in the proliferating capacity of neurogenic niches, even in the absence of p53.

Figure 53.- Comparative analysis of the proliferating populations in the SGZ of WT, p73, and DKO mice. A) Composite confocal microscopy pictures (102x) of the SGZ, immunostained with Ki67 (in green) and GFAP (in red). B) Quantification of the Ki67 positive cells in SGZ from the three genotypes.

Our data suggests that the observed phenotype in NSC *in vitro* has severe implications *in vivo*, since the absence of p73, by itself or in the context of p53 deficiency, give rise to defects in the overall structure of the neurogenic niches and also hampers the enhanced cell proliferation of observed in p53KO mice.

These data demonstrated that lack of p73 results in defective neurogenic niches and predicted an impaired neurogenesis in the adult mice brain. Impairment of neurogenesis may compromise the extent of plasticity of the hippocampus and their associated neural circuits leading to enhanced neuronal vulnerability and functional detriment. Furthermore, recent evidence suggests that neurogenesis is impaired in animal models of Alzheimer's disease (AD), in both SVZ and SGZ (Lazarov and Marr, 2010). In this context, loss of p73 could become a risk factor for neurodegenerative diseases. In AD, compromised neurogenesis has been proposed to take place earlier than the onset of hallmark lesions or neuronal loss, and may play a role in the initiation and progression of this disease (Lazarov and Marr, 2010). This is consistent with recent reports indicating that p73 is essential to prevent neurodegeneration during aging, and that heterozygosity for p73 may be a susceptibility factor for AD or other neurodegenerative disorders (Wetzel et al., 2008). Thus, p73 could be an important player in the development of neurodegenerative diseases and therefore a relevant target for study.

2. Characterization of the p53 family functions in the genesis and architecture of the murine neurogenic niche in the subventricular zone (SVZ)

2.1. Comparative analysis of the different cellular populations in the SVZ between mice of the four genotypes studied.

We have already shown a decrease in the number of proliferating cells in the SVZ and SGZ of P15 mice. To complement these studies, we performed an in deep analysis of the effect of p73 deficiency in the cellular populations of the SVZ and their organization in the neurogenic niches.

In the SVZ, three populations of precursors, including adult NSCs, lie adjacent to a layer of ependymal cells which lines the lateral ventricle wall. During neurogenesis, B cells are relatively quiescent cells that give rise to C cells, a more rapidly dividing population that will generate the third population, the A cells. These neuroblasts migrate in glial tubes to the olfactory bulb and differentiate into olfactory interneurons that will integrate into the neural circuitry during adult stages. It has been described that the neurogenic site of the ventricular wall shows a remarkable “pinwheel” organization, where the apical surface of NSC in the core of the pinwheel is surrounded by several multi and biciliated ependymal cells. Finally the multiciliated ependymal cells form a continuous single-cell layer that covers the brain ventricles (Doetsch et al., 1997).

It has been proposed that neurogenesis in the SVZ of adult mice is regulated by ependymal cells which secrete Noggin, the bone morphogenetic protein (BMP) inhibitor, inducing neurogenesis and suppressing gliogenesis (Lim et al., 2000). These cells also secrete PEDF, which promotes self-renewal of adult NSC (Ramirez-Castillejo et al., 2006). Furthermore, the beating motion of ependymal cilia is crucial for maintaining the right flow of cerebrospinal fluid (CSF) that contains the necessary morphogens for neurogenesis, as well as to support neuroblast migration to the OB (Sawamoto et al., 2006).

To investigate in more detail the role of p73 in the SVZ organization, we characterized the different cellular populations that constitute this area using coronal sections from the brain of P15 mice from the four genotypes injected with BrdU. We first analyzed the population of proliferating neuroblasts. To identify neuroblasts we used doublecortin (Dcx) as marker of newborn neurons, combined with the proliferation markers Ki67 and BrdU (Figure 54). Comparative analysis revealed that p73KO mice had significantly less

proliferating neuroblasts than WT mice. This finding is consistent with the existence of a diminished number of NSC *in vitro* and with the impaired neurogenic potential previously proposed.

As published, p53KO mice had more proliferating neuroblasts than WT (Meletis et al., 2006). However, the percentage of proliferating neuroblasts in DKO mice was significantly reduced compared to WT or to p53KO, indicating that p73 leads to a reduction of neuroblasts in the SVZ, even in the absence of p53. We did not observe any significant difference in the number of proliferating neuroblasts between p73KO and DKO mice, indicating that p53 abolishment cannot restore the phenotype.

These results suggest an impairment of neurogenesis and, probably, a decrease in the number of new neurons that migrate to the OB. However, to demonstrate this point, quantification of neurons in the RMS and OB of the p73KO mice would be necessary. While there seems to be a decrease in the number of total Dcx positive cells in the p73KO mice, this difference it is not significant in the analyzed area. Thus, it will be of great interest to analyze the OBs of these animals as well as other areas of the brain.

Figure 54.- Comparative analysis of the population of neuroblasts in the four genotypes. A) Confocal microscopy pictures (102x) of the SVZ. B) Quantification of the proliferating neuroblasts.

Our results could seem contradictory with our previous findings about *in vitro* premature neuronal differentiation, or with the fact that we did not find any differences in the proliferation ratio between DKO and p53KO NS cultures. However, it is important to point out that from all the cellular populations that comprise the neurogenic niche, only NSC and NPC capable of self-renewal are maintained in culture in the NS assay. Thus, these

cells could be not receiving all the environmental and positional clues that regulate neurogenic niches *in vivo*. Therefore, what we observed is the direct consequence of p73 deficiency in NSC/NPC *in vitro* which is, at least in part, the deregulation of asymmetric vs. symmetric divisions and thus a premature differentiation. As discussed before, these prematurely differentiated neurons present an aberrant morphology and might not be viable *in vivo*. Furthermore, p73 regulation of asymmetric cell division may have different and more complex consequences in the context of the neurogenic niche environmental regulation *in vivo*.

We next analyzed the population of B cells, the primary progenitors identified as a subpopulation of astrocytes (GFAP positive), which are derived from radial glia in the SVZ (Merkle et al., 2004). To identify this population we scored proliferating (Ki67), GFAP positive cells. As previously published, p53KO mice presented more B cells in contact with the ventricle. Consistent with the fact that lack of p73 results in defective neurogenic niches, and that neurogenesis in these mice may be impaired, the number of proliferating GFAP positive cells was reduced in p73KO and DKO mice (Figure 55). However the most striking observation was the huge amount of non proliferating GFAP positive cells present in p73KO, as well as DKO mice, compared to WT and/or p53KO. Many of these cells had a periventricular cell soma in contact with the ventricle. They also presented GFAP positive filaments that are forming tangles surrounding the nucleus, as well as long processes that extended across the striatum towards the Pial surface. These cells resembled the morphology of radial glia cells but with expression markers of a more “mature” cell type (Figure 55 & 56).

Figure 55.- Comparative analysis of B cells in the four genotypes. Confocal microscopy pictures (102x) of the cells that comprised the SVZ of P15 mice labeled with Ki67 and GFAP.

It has been published that p73 is expressed in the ependymal cells (Hernandez-Acosta et al., 2011). Given the relevance of the ependymal layer in the organization and regulation of the SVZ niche environment, we analyzed this cellular population in our mice models. Different markers could be used for this purpose, among them $\text{s100}\beta$ and IB4, also expressed by mature astrocytes and endothelial cells, respectively. Coronal sections from the four genotypes were immunostained with $\text{s100}\beta$, as well as IB4 and GFAP antibodies (Figure 56).

Figure 56.- Comparative analysis of the ependymal layer in the four genotypes. Confocal microscopy pictures (102x) of the SVZ from the four genotypes labeled with anti GFAP, S100 β , and IB4.

WT and p53KO mice showed an organized and continuous mono-stratified epithelium of s100 β /IB4 positive cells. They also exhibited some s100 β /GFAP positive mature astrocytes in the striatum and few GFAP positive B cells (s100 β negative) in contact with the ventricle, with a higher number of B1 cells in p53KO than in WT mice (Figure 56). Surprisingly, in the absence of p73, s100 β -ependymal cells were not able to form an organized epithelium; rather than this, they presented what looks like a pseudo-stratified epithelium of s100 β positive cells distributed throughout the entire subependymal zone. This phenotype is observed even in DKO mice. In the absence of p73, only some of the s100 β positive cells are also positive for IB4, suggesting that they might not be mature *bona-fide* ependymal cells.

Furthermore, in p73 deficient mice we found that many cells that were touching the ventricle and presented long GFAP striatal processes, showed a double staining $\text{s100}\beta$ /GFAP. This points to an abnormal cell type in the SVZ, suggesting that either a big amount of mature astrocytes are getting somehow ependymal identity or that aberrantly differentiated glial cells are present. It has been published that in aged SVZ large number of astrocytes are incorporated within the ependymal layer, displaying characteristics and markers of ependymal cells. These astrocytes express GFAP as well as $\text{s100}\beta$ and it has been defined that the astrocytes integrate in the ependymal layer to repair it (Luo et al., 2006). These authors proposed a SVZ astrocyte-mediated regenerative repair of the ependymal lining, which occurs at a moderate level in the elderly brain, but can be also activated in the ependyma of young animals after injury. They hypothesized a model in which dividing SVZ astrocytes would incorporate into the ependyma to maintain the integrity of ependyma barrier. Then, the inserted astrocytes would establish cellular adherens with neighboring ependymal cells, taking on antigenic and morphologic characteristics of a mature ependymal cell, and resulting in cells that would express $\text{s100}\beta$, as well as proliferative markers, like BrdU. In accordance with this, in an inducible mice model of Numb/Numbl-like deletion that resulted in severe loss of the integrity of the ependyma wall, the damaged cells remaining in the SVZ got eventually remodeled by activation of SVZ astrocytes. Interestingly, they also observed triple-labeled cells ($\text{s100}\beta$ /GFAP/BrdU) in the remodeled ventricular wall in young mice after induction of damage (Kuo et al., 2006). However, in the p73KO mice most of the $\text{s100}\beta$ /GFAP positive cells that we observed were non proliferating cells (Figure 57).

Figure 57.- Analysis of the proliferating ependymal cells. Confocal microscopy pictures (102x) of the SVZ from WT and p73KO mice immunostained for $\text{s100}\beta$, GFAP and BrdU.

Another characteristic of the lateral wall of the ventricle in p73KO mice is that the “ependymal” cells fail to establish solid cell to cell adhesions to form the ependymal barrier and show gaps between the α 100 β positive cells and “bumps” in the ependymal layer. Invaginations of this barrier are also frequently observed in these mice (Figure 58, arrows and arrowheads, respectively). Altogether this suggests that p73 deletion results in loss of brain ventricular integrity. However in the p73KO mice there is an unsuccessful attempt of repair the ventricular integrity by recruiting activated astrocytes to this site, since analysis of the proliferation of these populations revealed few proliferating α 100 β /GFAP cells (Figure 57).

As mentioned before, ependymal cells also contribute to the neurogenic environment by expressing Noggin and potentially affecting the activity of BMP signaling in stem cells, therefore creating an environment that is permissive for neurogenesis. Furthermore, some data support a potential role for these proteins in anatomical and functional plasticity of the adult brain (Peretto et al., 2002), and it has also been described that type B cells themselves express Noggin, as well as BMP proteins, to maintain a neurogenic environment within the SVZ (Peretto et al., 2004).

Taken together, these results suggest that the expression levels and localization of BMP signaling within the adult SVZ are tightly regulated to allow production of a differentiated progeny while preserving the stem cell population. Furthermore, signaling via cell to cell contacts in the niche may allow different BMP/Noggin interactions between different cell types. Thus, we decided to analyze the expression of Noggin in the SVZ.

As observed in figure 58, in WT mice Noggin expression is coincident with ependymal α 100 β positive cells, however in the p73KO mice Noggin secretion is detected throughout the SVZ and does not completely match with the cells that express α 100 β within the SVZ, indicating that the BMP/Noggin pathway is deregulated in these animals and therefore, the appropriated maintenance of the neurogenic environment might be affected. Whether this is a direct effect of p73 deficiency, or a consequence of the alterations in the organization/maturation of this site, remains to be addressed.

Figure 58.- Comparative analysis of Noggin expression in endymal layer. Confocal microscopy pictures (102x) of the SVZ from WT and p73KO mice.

2.2. Analysis of the post-natal formation of the lateral wall of the lateral ventricles: transition from the radial glia cells in the ventricular zone (VZ) to the endymal layer and subventricular zone (SVZ).

Our data suggest that lack of p73 affects not only the number of NSCs and other cell types in the neurogenic niches, but also the structural organization of the ventricular zone. Supporting this idea, preliminary electron microscopy analysis revealed that the p73KO mice, presented important alterations in the ventricular zone. Most endymal cells display irregular shaped nuclei, with deep cytoplasmic projections of intermediate filaments into the SVZ. In contact with the ventricular lumen these cells present abundant long microvilli as

well as disorganized cilia. All these features seem to be more characteristic of P0 neonatal mice rather than P15 animals. Additionally, we observed profound abnormalities in the architecture of the neurogenic niches along the subventricular zone (Figure 59) (data obtained in collaboration with Dr. Garcia-Verdugo).

Figure 59.- Electron microscopy analysis of the SVZ from WT and p73KO mice. A & B) SVZ showing the differential morphology of type B, C, A and A/C cells. C & D) Ependymal cell (green) in the SVZ. E) Orientation of cilia in normal ependymal cells. F) Arbitrary orientation of cilia in p73^{-/-} mice P15. G) Magnification of the projections of ependymal cells showing intermediate filaments.

The organization of the germinal center of the SVZ occurs during the first postnatal days. The neonatal VZ is mainly comprised by radial glial cells (RG), with cell bodies close to the ventricles and long radial process that contact with the pial surface of the brain. During postnatal development, the RG gives rise to B1 cells (GFAP⁺/Ki67⁺) that are also touching the ventricle, to ependymal cells (s100 β ⁺), striatal astrocytes (non proliferating GFAP⁺) and to oligodendrocytes (Kriegstein and Alvarez-Buylla, 2009). Throughout this process the conversion of tight junctional complexes that couple neuroepithelial cells into adherens junctions (Aaku-Saraste et al., 1997; Stoykova et al., 1997), as well as the establishment of specialized contacts with the endothelial cells of the developing cerebral vasculature, is essential for the right organization of the SVZ.

We have observed in the P15 p73KO mice alterations in the architecture of the SVZ, as well as new a population of abnormal astrocytes-like cells that express GFAP and s100 β markers, while presenting long processes typical of radial glia cells, but they are not longer proliferating.

Therefore we decided to analyze the transition of RG cells to the different cellular types that comprise the SVZ by studying different stages of development: at 7 and 15 days after birth.

To further investigate if p73 deficiency affects the maturation of RG into ependymal cells during the formation of the lateral wall of the ventricles, we performed a comparative analysis of WT and p73KO coronal sections of mice brain using glial markers for distinct stages of maturation. GLAST is a RG marker that during later development is expressed in astrocytes and neuroblasts; however, it is absent from ependymal cells (Shibata et al., 1997). At P4, most of the cells lining the striatal side of the LV are GLAST⁺. As RG matured into ependymal cells, the number of GLAST⁺ cells lining the striatal side of the LV decreased and by P15, when the lateral wall is nearly formed, most of the cells lining the LV are GLAST negative (Lavado and Oliver, 2011). In WT mice we observed the described distribution of GLAST⁺ cells, where at P7 some cells lining the ventricle that remained GLAST⁺ (Figure 60, arrows), while at P15 all these cells have disappeared from the lateral wall of the ventricle. However, in p73KO at P7 we detected many GLAST⁺ cells in the layer that lined the ventricle (Figure 60, arrows) and many of those cells were still positive at P15 and in contact with the ventricle (Figure 60).

We also analyzed the expression of Vimentin and GFAP. Vimentin is expressed by radial fibers during embryonic development (Mirzadeh et al., 2008) and at P7 begins to be expressed by ependymal cells that would be Vimentin⁺ in the adult stages. RG cells also give rise to GFAP⁺ cells, it has been reported that between P7 and P15 the disappearance of RG markers from the SVZ correlates with the appearance of GFAP⁺ cells (Tramontin et al., 2003). As expected, WT SVZ at P7 displayed some Vimentin⁺ cells, plausibly ependymal cells, which progressively increased in number so that most of the cells lining the lateral wall were Vimentin⁺ by P15. We only detected very few cells that were GLAST⁺/Vimentin⁺ at P7, and none at P15, probably representing radial glia cells during the transition process (Figure 60). In some of the preparations, we also detected at P7 stage a third type of population Vimentin⁺/GFAP⁺. This population most likely represents an intermediate state during the transition of RG cell to ependymal cells, since it disappeared in WT P15 stage.

Figure 60.- Analysis of the expression of early glial markers at P7 and P15. Confocal microscopy pictures (102x) of the SVZ from p73KO and WT mice at 7 and 15 days after birth, stained with early glial markers. GLAST⁺, white arrows; Vimentin⁺/GLAST⁺, white arrowheads; Vimentin⁺/GFAP⁺, red arrowheads; Vimentin⁺/GFAP⁺/GLAST⁺, yellow arrow.

In the P7 p73KO mice we still observed GLAST⁺ cells touching the ventricle with radial processes towards the striatum that resemble RG cells (Figure 60, arrows). Some of those GLAST⁺ cells were also Vimentin⁺, corresponding to RG cells during the transition process (Figure 60, arrowheads). As in the WT preparation, in p73KO we also detected a Vimentin⁺/GFAP⁺ cell population, seldomly some of these cells were also GLAST⁺, underlying its immature nature. But most strikingly is the fact that this immature population, Vimentin⁺/GFAP⁺ (Figure 60, red arrowheads) described before, do not disappear in p73KO P15 mice but rather increases in number and intensity of staining. Some of these cells are also GLAST⁺ highlighting its RG origin (Figure 60, yellow arrow), suggesting that these cells could be RG cells that were not capable of an appropriated maturation into astrocytes and B cells. Surprisingly, P15 mice still present many GLAST⁺, that are GFAP⁻, and that are touching the ventricle, probably representing RG cells (Figure 60, white arrows).

Another important feature in the LW of the ventricles resulting from p73 deficiency is that, while in WT mice the Vimentin⁺ cells formed a continuous cell monolayer lining the ventricle, in p73KO mice these cells showed an aberrant organization with many disruptions (gaps) of the ependymal layer and bumps, giving the impression of depression or invaginations of the ependymal tissue, rather than a continuous mono-stratified layer (Figure 60).

Radial glia cells do not only give rise to GFAP positive cells, but they also transform into ependymal cells, passing through intermediate stages in which they express markers of RG and ependymal cells (Spassky et al., 2005).

We have shown earlier that p73 deficiency give rise to the appearance of GFAP/ α 100 β double positive cells with a disorganized distribution in the ependymal and sub-ependymal layers at 15 days after birth (Figure 57). Therefore, to further investigate the transformation of RG into ependymal cells in the absence of p73, we analyzed the expression of both ependymal markers (Vimentin/ α 100 β), as well as the astrocyte/ B cell marker GFAP, at an earlier developmental stage (7 days after birth). In P7 WT mice, most of the cells expressing Vimentin and α 100 β are forming a mono-stratified epithelium lining the ventricle. However, at this early developmental stage there are areas in which these cells still forming a pseudo-stratified layer (Figure 61, white arrows). In the P7 p73KO mice, the mono-stratified epithelium forming process appears halted. We observed chains of α 100 β ⁺/Vimentin⁺ cells that appear to progress inward rather than laterally and that result in obvious separations between the cells (Figure 61, yellow arrows and white arrowheads, respectively). Strikingly, in these cells the α 100 β expression appears as a round staining in the cell soma that extends to the radial extensions that present these cells, co-localizing with the staining of intermediate filaments, Vimentin and GFAP. Many of these triple positive cells Vimentin⁺/

GFAP⁺/s100 β ⁺ are contacting the ventricle (Figure 61, magenta arrows). These triple labeled cells most likely correspond with immature cells that are in an intermediate transitional state from RG cells to ependymal cells. Some of them might even be GLAST⁺, since in our previous experiment (Figure 60), we showed that, in the p73KO, some of the Vimentin⁺/GFAP⁺ cells that were in contact with the ventricle expressed the RG marker GLAST. Therefore, these cells express RG markers at the same time that astrocytic (GFAP) and ependymal (s100 β and Vimentin) markers, confirming their intermediate, immature phenotype.

Figure 61.- Glial and ependymal markers colocalization in P7 mice brain. Confocal microscopy pictures (102x) of the SVZ from p73KO and WT mice.

The presence of long Vimentin⁺ processes in cells lining the LV supports the idea that the p73KO mice have retained radial glia characteristics during the formation of the lateral wall. Furthermore, the presence of GLAST⁺/s100β⁺ cells showed that in the absence of p73, the cells lining the LV have abnormally mixed ependymal and radial glia characteristics. These data suggest that p73 is required for the appropriated maturation of radial glia into different cell types.

Thus we are confronted with a contradictory phenotype. We observed cells with a yet unknown identity express early glial markers as well as ependymal or mature astrocytes markers. These results had leaded us to postulate two different possibilities that could explain the phenotype. On one hand, it could be that p73 deficiency alters the correct division and transformation of radial glial cells generating intermediate state cell types, with apparently mixed identity. These cells would correspond to a more immature phenotype that can not progress into a mature ependymal cell, therefore remaining in the lateral wall of the p73KO P15 mice and halting, the maturation and organization of the SVZ of these animals. Tight regulation of the symmetric *vs.* asymmetric cell division of RG cells is necessary for the appropriated generation of the different cell types that comprised the SVZ (Kriegstein and Alvarez-Buylla, 2009). Moreover, we have previously showed that p73 is important for the regulation of symmetric cell division in NPC *in vitro*, so the observed effect could be due to the deregulation of the symmetric *vs.* asymmetric RG cells division, which would alter the cytoplasmic determinant inherited by daughter cells, and therefore affecting their differentiation fate, and, at the same time, altering the environmental and positional clues that they receive during the maturation process towards ependymal and astrocytic cells.

On the other hand, TAp73 depletion has been recently associated to accelerated aging through metabolic deregulation of mitochondrial respiration and reactive oxygen species (ROS) homeostasis, demonstrating that TAp73KO mice show more pronounced aging (Rufini et al., 2012). This relationship of p73 with accelerated aging and the presence of GFAP astrocytes integrated in the ependymal layer could suggest a premature aging phenotype in p73KO mice. However, the fact that the s100β⁺/GFAP⁺/Vimentin⁺ cells retain RG morphological characteristics as well as GLAST expression is an indication of cell immaturity rather than a sign of premature aging in which astrocytes are getting ependymal identity. Furthermore, the fact that a similar cell type existed in a transitory way, in the P7 WT mice, supports the immature and transitional nature of these cells. Nevertheless, we cannot rule out that the defects in the formation of the ependymal wall do not elicit a repair response with the concomitant recruitment of mature astrocytes (GFAP⁺/s100β⁺/GLAST⁻).

2.3. Comparative analysis of the SVZ architecture at different post-natal days.

As demonstrated, our data are consistent with an aberrant RG maturation in p73 deficient mice, confirming the idea that p73 is required for the appropriate maturation of RG into the different cell types that comprised the SVZ, but also for the correct disposition of these cells in the lateral wall of the ventricle. We have proposed that the effect of p73 in the architecture and organization of the lateral walls could be the reflection of an early developmental stage, presenting a more immature phenotype where the radial glial cells have not been capable to undergo the appropriate transformation into the ependymal or progenitors cells of the SVZ. Therefore, we sought to analyze the effect of p73 deficiency, alone or in combination with lack of p53, in the architecture of the lateral wall of the ventricles. For this purpose, we have took advantage of the whole-mount dissection technique that provide an *en face* view of the ventricular surface and facilitate the identification of ventricle-contacting cells (Mirzadeh et al., 2008). We performed a comparative analysis of whole-mounts of the lateral wall of the ventricles of mice from WT and p73KO genotypes at different developmental stages: P7, P15 and P160 (days after birth).

Recently the unique architecture of the neurogenic niche in the lateral wall of the ventricle has been described in the mouse brain (Mirzadeh et al., 2008). Three types of cells that contact the lateral ventricle have been identified: E1, multiciliated ependymal cells with large apical surface; E2, intermediate-sized biciliated ependymal cells and B1, monociliated cells with small apical surface that are identified as the NSC in the SVZ. In the neurogenic regions of mouse brain these cells are organized in special clusters, conferring a striking architecture to the ventricle wall. These clusters present a specific pattern that resembles a pinwheel, where one or more B1 cells are surrounded by E1 ependymal cells, likely to maximize the packing of E1 cells around the B1 apical surface. E1 cells shape approximated a diamond with one vertex contacting the B1 apical surface. The fact that this cellular organization is not present in the non neurogenic regions of the ventricular wall underlines the importance of these intercellular interactions and their cellular contacts with the cerebral spinal fluid (CSF) for the neurogenesis.

The B1 cells are distributed throughout the lateral wall, been the Antero-Ventral (AV) and Postero-Dorsal (PD) regions the ones with the highest number of NSC (Figure 62). In contrast, the density of E1 cells is similar among the regions, except in the PV one where cells have larger surface, resulting in lower number of cells in the region.

Figure 62.- Surface map of the walls of the LV. Confocal microscopy picture (10x) of the lateral wall of the ventricle where we the localization of B1 cells is indicated (highest to lowest number, 1>2>3>4). Based on (Mirzadeh et al., 2008).

Ependymal cells are generated during embryonic development between days E14 to E16, and its maturation occurs during the first days after birth with the formation of the cilia. Previous studies reported that ependymal cells appear in posterior and ventral regions and progressively arise in more anterior regions (Spassky et al., 2005). These authors suggested that, at birth, the ciliary budding in the developing ependymal cells has just begun, increasing the number of ependymal cells rapidly, and that they mature first in the posterior and ventral regions between day P0 and P4.

We performed the comparative analysis taking account of the differences in population distributions between the regions described before. Thus, we characterized the lateral wall following antero-ventral to posterior-dorsal direction.

2.3.1. Characterization of the lateral wall of WT and p73KO mice

P7 WT mice

In P7 WT mice we observed a gradual transformation of the lateral wall. In the more anterior region (AV), we found large quantity of cells with small monociliated apical surfaces. These correspond to radial glial cells that can be identified by the presence of a single basal body associated with a primary cilium (Figure 63). These small cells are forming organized “chains” that surround bigger cells. At this stage, in the more anterior region, we observed cells with expanded apical surface in which γ -tubulin staining marks dense punctate deposits, likely corresponding to deuterosomes (this structure is used to build multiple basal bodies) (Spassky et al., 2005) (Figure 63, left panel, yellow arrows). As previously described by Mirzadeh *et al.* (2010), these cells most likely correspond with radial glial cells undergoing ependymal transformation. There are also cells with expanded apical surface that already display multiple cilia (Figure 63, left panel, white arrows); about 50% of these cells are GFAP positive, which includes all the cells with deuterosomes and some of the multiciliated ones. However, this percentage decreases with age, since at P15 we only scored

10% GFAP-positive multiciliated cells (Figure 66). Thus, for the first time, we have described, an intermediate cellular stage of RG cells undergoing ependymal transformation. In this stage, RG cells expand the apical surface, present dense punctate tubulin positive deposits (deuterosomes) and express GFAP.

At P7, the monociliated-small apical surface radial glial cells did not display yet GFAP expression (Figure 63, white arrowhead, left panel). As we move towards more posterior regions, the multiciliated cells seem to be starting to form the pinwheel pattern, with immature large GFAP-multiciliated cells rounding groups of small monociliated cells. We will refer to these structures as “pre-pinwheels” from now on (Figure 63, middle panel, white line).

Figure 63.- Antero-posterior characterization of the lateral wall of P7 WT mice. Confocal microscopy images (102x) of WT whole mounts of the lateral wall of the ventricles.

At the posterior region the lateral wall presented higher number of pre-pinwheels, confirming a more advanced stage of maturation (Figure 63, right panel, white line). At P7 in the WT mice lateral wall most of the neighboring radial glia cells present their cilia displaced to the same side of the apical surface. This side corresponds to where their multiciliated neighbor cells concentrate their cilia (Figure 63, left panel, arrows).

It is important to highlight a “flower” pattern frequently observed in the more anterior region, and thus characteristic of immature stages. This pattern shows one deuterosome presenting GFAP positive transforming RG cell which is in the core of a group of small GFAP-negative monociliated cells (Figure 64). This “flower” pattern might be reflecting a core of radial glia cells in the process of transformation. The presence of this pattern in early developmental stages could be important in the transformation of radial glial cells in the different cell types that comprised the VZ and in the formation of the pinwheel pattern of the lateral wall of the ventricles.

Figure 64.- Magnification of the “flower” pattern of WT P7 mice. Magnification of confocal microscopy pictures (102x) of the anterior region of P7 WT mice lateral wall.

Finally, at this early post-natal stage the cells contacting the ventricle exhibited smooth cell membranes that were stained with β -catenin, most likely a reflection of the initial adherens junctions that the RG cells establish at this stage.

P7 p73KO mice

Analysis of the antero-posterior distribution of cells in the lateral wall of these mice, initially revealed a similar pattern to that of P7 WT mice, with similar numbers of small monociliated cells and GFAP-positive large surface multiciliated cells (Figure 69). However, there were significant morphological differences. While in WT mice we observed a gradual transformation through the antero-posterior axis, where the small monociliated cells got compacted in the core of pre-pinwheels, in the p73KO we seldom found pre-pinwheel patterns as we advance to the posterior region. Only occasionally we found aggregation of

cells resembling pre-pinwheels. On the contrary, the groups of large multiciliated cells form aberrant structures with many intermediate-size monociliated cells in the core, but without the organization of the pre-pinwheel structure observed in WT mice, demonstrating the lack of a specific organization (Figure 65A).

Figure 65.- Antero-posterior characterization of the lateral wall of P7 p73KO mice. A) Confocal microscopy images (102x) of p73KO whole mounts of the lateral wall of the ventricles, in the Anterior(left), middle, and posterior (right) regions. B) Magnification of the area squared in A showing small monociliated cells. C) Magnification of intermediate-size GFAP⁺ cells.

Additionally, observation of the basal bodies of the cilia in the radial glia cells revealed that often the cilia of neighboring RG cells were displaced to the opposite side of the apical

surface (Figure 65B, arrows). Furthermore, multiciliated cells revealed that their positioning was dramatically affected; some cells presented the cilia gathered in specific parts of the cell, with great variability in the orientation of basal body patches; in some cases the multiple clusters appeared in the middle of the cell (Figure 65C, arrowheads), while other cells presented the cilia distributed all over the cell (Figure 65) suggesting that lack of p73 may result in alteration in the translational Planar Cell Polarity (PCP).

P15 WT mice

We next investigated the antero-posterior region at day 15 after birth (P15). We observed a progressive modification of the organization along the antero-posterior axis. The anterior area revealed a pre-pinwheel pattern in which large multiciliated cells surrounded groups of small monociliated cells. We also observed small monociliated cells that form concatenations that contact independent pre-pinwheels. These chains of cells have a more tight appearance that the observed at P7. However, at the same stage, the posterior region displayed pinwheel patterns with few small monociliated cells in the core of large multiciliated ependymal cells. (Figure 66, white and yellow lines, respectively). This was consistent with published data describing the maturation of ependymal cells along the lateral wall, which follows an antero-posterior direction (Spassky et al., 2005). The apical surface of the monociliated cells had tightened and appeared smaller. These cells formed the core of the pinwheels and presented long GFAP positive processes, what marks them as B1 cells or NSC (Figure 66, left panel, white arrowhead). This observation is in agreement with previous reports describing that B1 cells present long GFAP fibers oriented tangentially to the epithelial surface (Mirzadeh et al., 2008).

The total number of large multiciliated cells (ependymal cells) was maintained but, at this stage, most of them ($90\% \pm 5.9\%$) were mature, ependymal GFAP negative and there were only few GFAP positive cells in the anterior area ($10\% \pm 5.9\%$). The number of small monociliated cells decreased from P7, especially in the posterior region, where now $62.9\% \pm 15.4\%$ of these cells are mature monociliated GFAP expressing B1 cells.

Figure 66. – Antero-posterior characterization of the lateral wall of P15 WT mice. Confocal microscopy images (102x) of WT whole mounts of the lateral wall of the ventricles.

At P15, the cellular clusters with “flower” pattern described at P7 were only observed in the anterior region, underlying the more immature character of this structure. At this stage, the few multiciliated cells, that still express GFAP, were surrounded by a group of very tight and small monociliated cells (Figure 67, white line).

Figure 67.- Magnification of the flower pattern in the anterior region of P15 WT mice. Magnification of confocal microscopy pictures (102x) of the anterior region of P15 WT mice lateral wall, where the “flower” pattern has almost disappeared and the cell membranes begin to present a wavy aspect.

Along the lateral wall we observed an organized distribution of the cilia. The basal bodies of the E1 cells were clustered into a well circumscribed patch and polarized to one size of the ependymal cell apical surface (Figure 68).

Figure 68.- Magnification of the cilia in the lateral wall of P15 WT mice. Magnification of confocal microscopy pictures (102x) of the anterior region of P15 WT mice lateral wall, where they presented organized distribution of the cilia.

P15 p73KO mice

The lateral wall of P15 p73KO mice presented a strikingly abnormal organization when compared to the WT mice. In general, the different cell types that contact the ventricle showed aberrant quantitative and morphological characteristics, as well as abnormal marker expression. They were in dearth of the distinctive architecture of the neurogenic site since they lacked, almost completely, the pinwheel structures and the characteristic translational planar cell polarity of the ependymal cells.

One of the most striking alterations was the immature phenotype that presents the lateral wall of the ventricle in the p73KO. While in the AV region of the WT mice the number of small monociliated cell dramatically decreased at P15, in the 73KO the number of

these cells only decreased slightly. More importantly, while in the P15 mice WT $62.9\% \pm 15.4\%$ of the small monociliated cells were GFAP-positive and corresponded to B1 cells, in the p73KO mice only $17\% \pm 3.75\%$, in the AV region, and $31.4\% \pm 6.1\%$ in the PD region express GFAP, the hallmark of B1 cells (Figure 69). These monociliated cells, which lack GFAP expression, could represent radial glial cells still undergoing ependymal maturation at this developmental stage (Figure 71, yellow arrow). Thus, most of the observed monociliated cells were not *bona-fide* B1 cells and therefore, they could not be acting as progenitor cells. This is consistent with our previous results showing that p73 leads to a reduction of neuroblasts in the SVZ and supports the hypothesis of impaired neurogenic capacity of p73KO mice brain.

Figure 69.- Analysis of the small monociliated cells during development and along the antero-posterior axis. Quantification of the small monociliated cells from confocal fields of ($124.7 \times 124.7 \mu\text{m}^2/\text{field}$), at anterior-ventral (A) region of P7 and P15 mice from WT and p73 KO mice. B) Percentage of B1 cell (small monociliated cells that expressed GFAP), from the cells quantified in A, at AV and PD regions of P15 WT and p73KO mice.

Another sign of immaturity is the presence of GFAP⁺ large multiciliated cells, presumably RG in the process of transition towards E1 cells. Rather than losing GFAP expression and becoming mature E1 cells (like it happens in the WT), most of these cells ($79.6\% \pm 5.1\%$) maintain and increase the immature GFAP expression state (Figure 70).

Figure 70.- Analysis of the number of large multiciliated “ependymal” cells that express GFAP. Percentage of the large multiciliated cells from confocal fields of ($124.7 \times 124.7 \mu\text{m}^2/\text{field}$) at anterior region of P7 and P15 mice that expressed GFAP.

This GFAP staining showed a strong round pattern in the cell soma, which was consistent with our previous observation in the coronal sections, where GFAP positive cytoplasmic processes extended towards the striatum (Figure 60). Thus, the $\text{s100}\beta^+/\text{Vimentin}^+/\text{GFAP}^+$ cells touching the ventricle identified in those sections, seems to be these large GFAP-multiciliated cells, therefore they are more likely to correspond RG in the process of transition towards E1 cells with an aberrant expression of $\text{s100}\beta$, than mature astrocytes that have been recruited to a damage area.

In the anterior and intermediate regions we could still detect cells with extended apical surfaces and dense punctate γ -tubulin positive deposits (Figure 71, white arrows). As we progressed towards the posterior regions we observed a more organized distribution, and few structures resembling pinwheels. However, the core of these pinwheels was frequently occupied by groups of GFAP-negative cells (Figure 71, white line).

Figure 71.- Antero-posterior characterization of the lateral wall of P15 p73KO mice.

Figure 71.- Antero-Posterior characterization of the lateral wall of P15 p73KO mice. Confocal microscopy pictures (102x). Multiciliated cells (white arrow); monociliated cells (yellow arrow).

Altogether these data suggest that lack of p73 results in a halt in the maturation of the lateral wall and an aberrant organization of the lateral wall of the ventricles.

In the p73KO lateral wall we frequently observed an inverted pinwheel pattern where one or two multiciliated large ependymal cells were in the core of the cluster, surrounded by a group of small GFAP negative monociliated cells, resembling the previously described “flower” pattern. We also detected aberrant pinwheel distributions in which a group of large multiciliated GFAP positive cells were surrounding a group of intermediate-sized monociliated cells, most of them being GFAP negative (Figure 72, white and yellow lines, respectively). This finding supports the idea that p73 deficiency results in a deregulation of the transition process from radial glia cells to ependymal (E1) and NSC (B1) cells, leading to the emergence of cells with abnormal identities in the ventricle. Further experiments will be required to identify the nature of these cells and the molecular mechanisms that generate them.

Figure 72.- Magnification of the aberrant pinwheel patterns in P15 p73KO mice. Magnification of confocal microscopy images (102x) of the lateral wall of P15 p73KO mice.

It is worth mentioning that the p73 deficient multiciliated cells frequently presented multiple waves and pleats in their membranes and the epithelium appeared sometimes invaginated (Figure 70). This was consistent with our previous result in coronal sections where we observed that the β 100 β ⁺/GFAP⁺ cells forming the ependymal layer did not established close cell-cell interactions but rather a loose pseudo-stratified epithelium that presented invaginations and “bumps” (Figure 60). Previous studies demonstrated the existence of a specialized intercellular junctions at the apical surfaces of B1 and E1 cells (Mirzadeh et al., 2008), describing unique junctions between B1-B1 and B1-E1 cells. The observation of a wavy membrane in our mice model suggests a deregulation of the adhesion capacity of these cells and could be one of the underlying mechanisms that alters the proper organization of the lateral wall.

The abnormal distribution of the basal bodies of the cilia is even more pronounced at P15 in the p73KO mice. Our study revealed an aberrant location, distribution and polarization of the cilia (Figure 73). While in WT mice ependymal cell basal bodies were clustered into a well circumscribed patch, p73 deficient mice presented cells either with very tight basal body patches located in one side of the apical surface (Figure 73, white arrow), or neighboring cells with the basal bodies covering all the apical surface (Figure 73, yellow arrow), or even centrally positioned basal bodies patches (Figure 73, blue arrow). The cells frequently clustered their basal bodies in groups; however, they are completely disorganized without translational planar cell polarity.

Figure 73.- Details of the cilia in p73KO mice lateral wall at P15. Magnification of confocal microscopy pictures (102x) to observe in more detail the localization of the cilia.

Altogether our data demonstrate that lack of p73 results in profound defects in the planar polarity of ependymal cells, as well as in the timing of ependymal maturation. It has been described that the position of primary cilia on the apical surface of a subpopulation of radial glia was already polarized before their transformation into ependymal cells and that the primary cilium and its basal body apparatus may orchestrate the planar polarized architecture of both radial glia and their progeny ependymal cells (Mirzadeh et al., 2010). Therefore our results suggest that p73 could play an important role on the regulation of radial glial cells biology, as well as in the primary cilia formation and regulation of its proper function in positioning of basal body patches

☞ **Adult (P160) WT and p73KO mice**

To determine whether the few p73KO mice that survive after the first couple of weeks were capable of compensating the described phenotype and completing the maturation of the ependymal layer, we characterized the lateral wall of the ventricle of adult mice and analyzed the formation of the pinwheel pattern that characterized the neurogenic niches at anterior and posterior regions. As previously published, the adult WT mice presented the pinwheel pattern in both anterior and posterior regions, with GFAP positive monociliated B1 cells and multiciliated GFAP negative E1 ependymal cells (Figure 74). In the p73KO mice most of the multiciliated cells contacting the ventricle still expressed GFAP and their membrane still presented pleats and waves as at the P15 stage.

Figure 74.- Comparative analysis of the lateral walls of WT and p73KO adult mice. Magnification of confocal microscopy pictures (102x) of the anterior and posterior region of P160 WT mice lateral wall.

The disorganization of the cilia was maintained in the ependymal cells of these animals. In the lateral wall of the p73KO mice, in which we have described before that the RG cells has an abnormal distribution of basal bodies and of its maturation, the ependymal cells never acquired the expected translational planar cell polarity, even in the adult mice. This is consistent with the idea that the translational planar polarity of differentiated ependymal cells requires the presence of non-motile primary cilia on the radial glial precursor. Therefore, if the RG cells of the p73KO mice have a defect in the regulation of the cilia distribution, it would be expected that the transition of RG cells to mature ependymal cells would be aberrant. In this context, it has been reported that defects in basal body polarity are coupled to disorganized beating of cilia and impaired directional fluid flow across the epithelium, resulting in accumulation of CSF in the brain ventricles and hydrocephaly (Ibanez-Tallon et al., 2004; Nakamura and Sato, 1993), a pathology presented by the p73KO mice (Yang et al., 2000). Furthermore, alterations in the primary cilia of RG cells, and later on in the multiciliated ependymal cells, could affect the environmental clues received by the neurogenic niche cells, affecting the maintenance of the NSC nested in them.

2.3.2. Comparative analysis of the lateral wall of P15 mice from WT, p73KO, p53KO and DKO mice

To determine whether p53 deficiency could affect the architecture of the lateral wall of the ventricle, and the possible interaction with p73 in this process, we performed a comparative study of P15 whole-mounts of the four genotypes. Our preliminary analysis of p53KO mice revealed that, at P15, lack of p53 did not affect the organization of the lateral wall, neither the planar cell polarity of the ependymal cells. The p53KO mice displayed pinwheel patterns similar to WT but, consistently with our previous data, that reported a high number of B1 cells in the absence of p53KO, we observed higher number of small GFAP positive monociliated cells (Figure 75). The large multiciliated cells in the LW of these animals were GFAP negative, as correspond with mature ependymal cells, and presented basal body patches that were polarized in one side of the apical surface (Figure 75).

Figure 75.- Comparative analysis of the lateral wall of P15 mice from the four genotypes. Confocal microscopy pictures (102x) of the anterior and posterior region of P15 mice lateral wall.

Interestingly lack of p73 in DKO mice reversed the organized structure observed in the LW of the p53KO mice. The lateral wall of DKO mice had a very similar appearance to that of the p73 single knockout. They presented high number of monociliated cells, many of them being GFAP positive, with expanded apical surface at anterior and posterior regions (Figure 75). These cells that have expanded apical surfaces and dense punctate γ -tubulin positive deposits indicative of an immature stage were RG cells that are transitioning into ependymal cells, supporting the idea that p73 is necessary for the appropriate maturation of radial glial cells. Furthermore, translational planar cell polarity is severely affected in these cells, suggesting that p73 is also necessary for the primary cilia organization in radial glial cells, as well as polarization of mature ependymal cells.

Comparative analysis of coronal sections from the four genotypes to identify the population of multiciliated cells in the lateral wall confirmed our observations. WT mice presented multiciliated cells that did not express GFAP lining the ventricles, while in p73KO mice, supporting our hypothesis, the multiciliated cells with expanded apical surface cells were expressing GFAP and presented long processes, reminiscent of their radial glia identity. Furthermore, DKO mice also showed this population of multiciliated GFAP positive cells with radial processes, and p53KO mice showed GFAP positive cells with long processes that contact the ventricle, consistent with previously published data for the B cells.

Figure 76.- Comparative analysis of coronal sections of P15 mice from the four genotypes. Confocal microscopy pictures (102x) of coronal sections immunostained with γ -tubulin to identify the population of multiciliated cells in the lateral wall.

Our findings suggest that p73 is necessary and plays multiple roles in the development and organization of the cells comprised in the neurogenic niche in the lateral wall of the ventricles and in the subependymal layer. p73 deficiency results in a temporal and spatial deregulation of the transformation process of radial glia into ependymal cells. Moreover the “ependymal” cells generated in the absence of p73 display remarkable features: they lack planar cell polarity, express GFAP at any age analyzed, have disorganized cilia and fail to form a mono-stratified epithelium. Since it has been proposed that the ependymal cells regulate neurogenesis in the SVZ of the adult mice by the secretion of morphogens and growth factors (Lim et al., 2000; Ramirez-Castillejo et al., 2006), any alteration in the appropriate function and regulation of these cells could have a profound effect in the neurogenic site and, therefore, in the neurogenic capacity. These effects of p73 appear to be not only independent of p53 function, but dominant in the absence of p53. Therefore, we have described, by the first time, a novel role of p73 in the genesis and architecture of the neurogenic site and in the regulation of planar cell polarity.

How p73 can fulfill these roles *in vivo* remains to be elucidated. However, we have previously demonstrated that one of the consequences of p73 deficiency *in vitro* is the increase in the rates of asymmetric vs. symmetric cell divisions of NPCs. Thus, it is plausible that lack of p73 *in vivo* affects the asymmetric distribution of core proteins that regulate planar cell polarity among daughter cells. It has been reported that symmetric localization of Vangl2 affects polarity of the cells (Guirao et al., 2010). That way, defects in the mechanisms regulating asymmetric cell division of radial glia cells could affect the planar cell polarity of their progeny, the ependymal cells.

Another possibility for the mechanism of p73 regulation of this process, although not mutually exclusive, is through the transcriptional regulation of the genes that encode the core proteins that regulate planar cell polarity (VANGL1, CELRS2 and CELRS3). Supporting this hypothesis a previous genome wide analysis of gene expression in cells that overexpress p73 (Table 7) had revealed these genes among others as putative p73 target genes (Marques-Garcia et al., 2009). However, future experiments will be required to address this possibility.

3. Identification and analysis of novel p73 transcriptional targets in neural stem cells

Up to this point, we have demonstrated that p73 is a positive regulator of NSC self-renewal, at least in part, by regulating the balance between symmetric and asymmetric cell divisions. We have also shown that p73 is necessary for the maintenance, differentiation and proper organization of the cells that comprise the neurogenic niche and that also plays a role in the genesis and architecture of the neurogenic site, been p73 function required for the appropriated regulation of planar cell polarity. However, the molecular mechanisms underlying p73 function remains unclear.

Based in p73 function as a transcription factor, it reasonable to consider that p73 would execute some of its multiple functions through the transcriptional regulation of cell context-specific target genes. Therefore, we attempted to identify direct transcriptional targets of p73 that could act as effectors in either the maintenance of neural stem cells, the regulation of differentiation, or the control of symmetric *versus* asymmetric cell divisions.

As part of a previous work from our laboratory, a genome-wide expression analysis through microarray hybridization was performed, using K562, a human erythroid progenitor cells, with ectopic expression of the $\Delta Np73$ isoform (Marques-Garcia et al., 2009). This analysis revealed expression differences for several genes involved in the regulation of the processes mentioned above (Table 7). Based on this information, we proceeded to investigate which of these genes were indeed direct p73 transcriptional targets. The first gene we analyzed was the neuronal fate determinant TRIM32.

Table 7.- Microarray analysis of the transcriptome of K562 cells over-expressing $\Delta Np73$ isoforms. Results from genes implicated in the regulation of self-renewal, differentiation, planar cell polarity as well as of asymmetric vs. symmetric cell divisions.

GENE	Fold change K562 vs. $\Delta N73$ -K562	p53/p73 RESPONSE ELEMENTS	FUNCTION
CDC42	1,69	YES	Regulation of individual cell polarity asymmetry
PAR3	1,65	NO	
PAR6A	2,44	NO	
CELSR2	1,68	NO	Planar Cell Polarity Core Proteins
CELSR3	2,05	NO	
VANG1	2,07	YES	
VANG2	3,79	YES	
TRIM32	-1,41	YES	Cell fate determinant
NUMA1	-1,43	YES	Asymmetric cell division
NUMB	-2,36	YES	
GP5M2 (LGN)	-1,75	YES	

3.1. Analysis of TRIM32, a neuronal fate determinant, as a direct transcriptional target of p73

We had previously analyzed TRIM32 expression as a marker of asymmetric cell division in p73KO NPC cultures. In those experiments, we analyzed NPC cell pairs under proliferating conditions and observed that, with the exception of the cells that were prematurely differentiating, most of p73KO cells presented lower expression levels of TRIM32 than WT cells (Figure 46). This suggested that the steady state levels of TRIM32, in the absence of p73, were lower than in WT cells. To confirm that, we analyzed TRIM32 expression from secondary neurospheres cultures after 4DIV under proliferating conditions.

Figure 77.- TRIM32 expression in NS cultures. A) Analysis by qRT-PCR of the expression levels of TRIM32 mRNA normalized by r18S expression. B) Analysis of the expression of TRIM32 by immunoblot.

We found that, although TRIM32 expression in NS cultures under mitotic conditions was variable, there was a significant reduction of TRIM32 expression in p73KO cells (Figure 77). Furthermore, TRIM32 expression analysis in NS from the four genotypes confirmed that lack of p73 resulted in reduced TRIM32 expression. We observed that lack of p53 also resulted in a significant reduction of TRIM32 levels. However, combined deletion of p53 and p73 did not appear to be additive, suggesting that they are part of the same regulatory pathway (Figure 78).

Figure 78.- Analysis of TRIM32 mRNA levels in NS from the four genotypes. qRT-PCR analysis of TRIM32 mRNA levels normalized to GAPDH gene expression.

These results, together with our microarray data, lead us to hypothesize that TRIM32 could be a transcriptional p73 target. In this context, we performed a prediction of p53-responsive elements within the human TRIM32 locus using the p53 Family-Target Genes database (Sbisa et al., 2007). Reinforcing our hypothesis, *in silico* analysis unveiled four p53-binding sites within this gene, two of them located in the 5' flanking region (at positions -1551 to-1514, and -115 to-84, relative to the transcription start site at exon 1), while the other two were within intron 1. Furthermore, a number of p53/p73-binding sites have been recently reported in mouse and human TRIM32 promoters by *in silico* analysis (Boominathan, 2010).

These data supported the idea of TRIM32 as a p73 transcriptional target. Thus, we wanted to investigate if there was a correlation and/or functional interaction between p73 and TRIM32. To address this question we first examined whether constitutive expression of TAp73 and/or Δ Np73 could regulate endogenous TRIM32. For this purpose, we transfected C17.2 immortalized mouse neural progenitor cells, with expression vectors for p73 isoforms (TA- and Δ Np73) and quantify TRIM32 expression by qRT-PCR. Ectopic expression of TAp73 resulted in a significant up-regulation of TRIM32, while Δ Np73 expression had no effect on TRIM32 basal levels (Figure 79).

Figure 79.- Effect of p73 ectopic expression in TRIM32 mRNA expression levels. qRT-PCR analysis of TRIM32 expression levels in C17.2 mouse neural progenitor cells over-expressing p73 isoforms.

Moreover, in collaboration with Dr. Jens Schwamborn's group (University of Münster, Germany) we also analyzed TRIM32 regulation by p73 isoforms in a mouse neuroblastoma cell lines, N2a (data not shown). Consistent with the results obtained in C17.2 cells, over-expression of TAp73 increased TRIM32 expression levels, while Δ Np73 slightly decreased its expression. Interestingly, when both isoforms were co-transfected at a 1:1 molar ratio, TRIM32 mRNA expression was repressed, indicating a dominant-negative effect of Δ Np73 over TAp73 induction of TRIM32. Furthermore, even in a situation where there was a ten time-molar excess of TAp73, the upregulation appeared to be impaired, suggesting an efficient Δ Np73 inhibition of TRIM32 expression.

To study whether p73 regulation of TRIM32 expression was exerted at the transcriptional level, the human TRIM32 promoter (-1351 to +270) was cloned in our laboratory. This fragment of the 5' flanking region contains the p53 consensus binding site identified in the proximal promoter (-115 to -84). Human TRIM32 promoter was amplified from human keratinocytes genomic DNA and subcloned into the pGL3-Basic plasmid, generating the hTRIM32-luc reporter vector (Materials & Methods).

Promoter reporter assays were performed in C17.2 cells with the generated construct. We co-transfected C17.2 cells with the expression vectors for p73 isoforms, the hTRIM32-luc vector, and the renilla pRL-null vector for normalization purposes. Consistent with the activation of endogenous TRIM32 promoter by ectopic expression of TAp73 in these cells, TAp73 significantly activated the hTRIM32-luc reporter, while p53 transactivated it weakly, and Δ Np73 had no effect on hTRIM32 activation (Figure 80).

Figure 80.- Transcriptional regulation of the human TRIM32 promoter by p53 family members. Luciferase assays in mouse neural progenitor cells, C17.2 (A) and DKO mouse embryonic fibroblast, DKO MEFs (B).

To address whether TAp73 could modulate TRIM32 promoter in the absence of p53, and to avoid TA *versus* Δ Np73 interactions, we used mouse embryonic fibroblasts deficient for both TP53 and TP73 genes (DKO MEFs). In these cells, both TAp73 and p53, but not Δ Np73, strongly transactivated this promoter, demonstrating that TRIM32 expression was regulated by these p53 family members (Figure 80). We also performed co-transfection analysis of Δ Np73 and TAp73 at various ratios in these cells (Figure 81). In the presence of increasing amounts of Δ Np73, we observed a significant decrease of TAp73-induced TRIM32 promoter transactivation (even at TA: Δ N molar ratios lower than 1:1), supporting the idea that Δ Np73 efficiently repress TAp73 transactivation of this promoter.

Figure 81.- Transcriptional regulation of TRIM32 promoter by co-transfection of TAp73 and ΔNp73. Luciferase assays in DKO MEFs. TAp73:ΔNp73 molar ratios varied from 1:0.6 to 1:2.

Therefore, we have shown that TRIM32 is a transcriptional target of the p53 family members, p73 and p53, and that ΔNp73 has an efficient dominant negative effect over TAp73 in DKO MEFs.

To unequivocally verify that TRIM32 was a direct transcriptional target of TA and ΔNp73, a chromatin immunoprecipitation (ChIP) assay was performed in Dr. Jens Schwamborn's laboratory. The ChIP assay in N2a transfected cells showed that both TA and ΔN p73 bound directly to the predicted p53/p73-binding site of the TRIM32 promoter, confirming that TRIM32 was a direct transcriptional target of p73 (data not shown).

Once we have demonstrated that overexpression of TA and ΔNp73 can regulate the transcriptional activity of the human TRIM32 promoter, it was important to corroborate that this regulation also occurs under more physiological conditions *in vitro* and *in vivo*. For this purpose, we sought to analyze the effect of p73 deficiency in differentiating NPC cultures and in brain tissue of WT and p73KO mice.

TRIM32 is involved in the regulation of NPC differentiation during mouse embryonic brain development and skeletal muscle stem cell differentiation (Nicklas et al., 2012). Thus, we analyzed whether p73 deficiency affected TRIM32 expression kinetics during the differentiation of embryonic NPCs. As we have shown before, both TAp73 and ΔNp73 are expressed in NPCs under proliferating conditions; however, TAp73 isoforms are significantly more abundant. During the differentiation process, TAp73 expression was up-regulated, already showing an increase 12 hours after addition of the differentiation media (DM), and augmented steadily up to 5DIV-DM (Figure 82A).

Figure 82.- Analysis of mRNA levels of TAp73 and TRIM32 in NPCs undergoing differentiation. A) qRT-PCR analysis of the expression levels of TAp73. B) Comparative analysis of TRIM32 expression during differentiation of WT and p73KO NPCs. ND: non-differentiated; d0.5-d5: days in culture with differentiation media

As expected from the behavior of a target gene, TRIM32 expression kinetics during differentiation correlated with the up-regulation of TAp73 expression (Figure 82B). Comparative analysis of TRIM32 expression during differentiation of NPCs from WT and p73KO NS cultures revealed a significant abatement of TRIM32 in the absence of p73 at every stage of differentiation, suggesting that TAp73 could be an important factor in the up-regulation of TRIM32 expression during neuronal differentiation. Nevertheless, p73KO cultures did express, and also up-regulated, TRIM32 during the differentiation process. It is noteworthy to point out that TRIM32 levels also increased during the differentiation process in the p73KO cells, therefore, other mechanisms, independent of p73, are regulating TRIM32 expression in these cultures. Furthermore, transcription independent mechanisms, like microRNAs, RNA stability, etc., might play a role regulating TRIM32 expression during differentiation.

To confirm that TRIM32 and TAp73 correlation occurs *in vivo*, we quantified TRIM32 expression in E14.5d brains from WT and p73KO mice. At this time point, the peak of mouse cortical neurogenesis is reached and TRIM32 is strongly expressed in the cortical plate and the ventricular zone, co-localizing with the expression of Tuj-1 (Schwamborn et al., 2009). qRT-PCR analysis of whole E14.5d brains demonstrated that, although p73KO E14.5d mice expressed TRIM32, there was a significant reduction in total TRIM32 expression levels in those mice when compared with control (Figure 83A). This reduction in TRIM32 expression level was also observed, albeit diminished, in the brains of P7 young mice (Figure 83B).

Figure 83.- Analysis of TRIM32 expression in brains from WT and p73KO mice. qRT-PCR analysis of TRIM32 expression levels of in E14.5d (A) and P7 mice brains(B).

Moreover, in collaboration with Dr. Jens Schwamborn's laboratory, TRIM32 expression levels in E14.5d VZ/SVZ were analyzed (data not shown). The analysis revealed that in the cortical plate, where TRIM32 levels seem to be normal, neither the gross anatomy of the cortex nor neuronal differentiation seemed to be strongly affected in the absence of p73. However TRIM32 expression levels were greatly reduced in the VZ/SVZ of p73KO mice. Furthermore, these lower levels of TRIM32 correlate with a reduction in the number of newly generated neurons in this region, suggesting that p73 deficiency results in a deregulation of TRIM32 levels that affects the initiation of neuronal differentiation *in vivo*. It is important to point out that, although at lower levels, TRIM32 is expressed in the absence of p73 *in vitro* as well as *in vivo*, indicating that there are other factors, independent of p73, that ensure TRIM32 expression.

Altogether our data establishes, for the first time, that TRIM32 expression is directly regulated by p73 at the transcriptional level. While TAp73 activates TRIM32 expression, Δ Np73 efficiently repress TAp73 transactivation of this promoter.

In differentiating neuronal progenitors, TAp73 up-regulation and the concomitant TRIM32 induced expression, correlated with *in vitro* neuronal differentiation. However, we have demonstrated (Specific aim 1) that p73 deficiency (both isoforms) resulted in enhanced asymmetric cell division of NPC/NSC under proliferating conditions, leading to premature differentiation and impaired self-renewal. Furthermore, despite the mitogenic conditions of the cultures, which should be sufficient to sustain symmetrical self-renewing divisions of NSC, TRIM32 is often asymmetrically distributed in p73 deficient cell cultures. Taken together, these apparently controversial data seems to be suggesting the existence of two levels of p73 regulation of TRIM32 activity. In multipotent, self-renewing NSC, p73 seems to be regulating the mechanism that controls the decision between symmetric *versus* asymmetric cell division, up-stream to TRIM32 up-regulation. It could be that p73 affected the mechanism underlying fate-determinant partitioning, including TRIM32, during asymmetric

cell division of NPC. In this scenario, TRIM32 induced expression would be independent of TAp73. However, in NPC that were more committed to differentiate, TAp73 would function in a cell-context dependent manner, resulting in TRIM32 transcriptional activation, up-regulation of TRIM32 expression and neuronal differentiation. This interpretation would be consistent with recent compilation of work regarding p73 function in CNS development, which indicates that p73 plays a multifunctional role in this process (Killick et al., 2011). Some of the data show that TAp73 is involved in neuronal (De Laurenzi et al., 2000; Fernandez-Garcia et al., 2007) and oligodendrocyte differentiation (Billon et al., 2004), while $\Delta Np73$ acts as a major survival factor preventing apoptosis in post mitotic neurons (Jacobs et al., 2006). Recent reports had also clearly demonstrated TAp73 requirement in the maintenance of NPC stemness by mechanisms not completely understood (Agostini et al., 2010; Fujitani et al., 2010; Gonzalez-Cano et al., 2010; Talos et al., 2010). These multiple, and apparently conflicting biological activities, may reflect the diverse transcriptional targets that this gene family can regulate depending on the cellular context in which they get activated. Our results would point to TRIM32 as an example of these TAp73 transcriptional targets.

Conclusions

AIM I: Analysis of the role of p53 family members, p73 and p53, in the biology of neural stem cells.

FIRST: p73 acts as a positive regulator of neural stem cells self-renewal. This transcription factor is necessary to maintain self-renewal and the growth rate of neural progenitor cells in the neurosphere model. This function is independent of p53, and is necessary for the enhanced growth of p53 deficient NSC/NPC *in vitro* and *in vivo*.

SECOND: p73 is necessary for the appropriate regulation of symmetric *versus* asymmetric cell division of embryonic neural progenitor cells. p73 deficiency enhances the frequency of asymmetric distribution of cell fate determinants, like NICD and TRIM32. Asymmetric distribution of TRIM32 correlates with premature differentiation of p73KO cultures; therefore, lack of p73 leads to a premature onset of neuronal differentiation *in vitro*, hampering neural stem cells self-renewal.

THIRD: p73 function is required for the maintenance of the proliferative cell populations in both neurogenic niches of mouse brain: the subventricular zone of the lateral ventricles (SVZ) and the subgranular zone of the dentate gyrus of the hippocampus (SGZ).

AIM II: Characterization of the p53 family function in the genesis and architecture of murine neurogenic niche.

FOURTH: Lack of p73 affects the maintenance of proliferating neuroblasts and neural stem cells, even in the absence of p53, in the brain of 15 days old mice.

FIFTH: p73 function is critical for the appropriate maturation of radial glial cells into the different cell types that comprise the ependymal layer and the SVZ. Therefore, it is necessary for maintaining the integrity of the lateral wall of the ventricles.

SIXTH: p73 seems to be essential for the genesis and architecture of the SVZ neurogenic niche. p73 deficiency results in profound defects in the translational planar cell polarity of radial glia and ependymal cells, as well as in the timing of the maturation of these cells.

AIM III: Identification and analysis of novel p73 transcriptional targets in neural stem cells biology.

SEVENTH: TRIM32 is a direct transcriptional target of the p53 family members, TAp73 and p53, while, Δ Np73 has an efficient dominant negative effect over TAp73 activation of TRIM32 promoter.

EIGHTH: p73 is an important factor in the regulation of TRIM32 expression during neuronal differentiation, although other mechanisms, independent of p73, must be implicated in TRIM32 expression *in vitro* and *in vivo*.

Resumen en español

ÍNDICE

ÍNDICE DE FIGURAS	IV
ÍNDICE DE TABLAS.....	V
ABREVIATURAS	VIII
INTRODUCCIÓN	1
1. NEUROGENÉISIS	4
1.1. Neurogénesis embrionaria	4
1.2. Neurogénesis Adulta	9
1.2.1. Desarrollo posnatal de los nichos neurógenicos.....	11
1.2.2. La región subventricular del ventrículo lateral (SVZ).....	13
1.2.3. La región subgranular del giro dentado del hipocampo (SGZ).....	19
2. BIOLOGÍA DE CÉLULAS TRONCALES NEURALES	21
2.1. Caracterización de células troncales neurales in vitro: ensayo de neuroesferas.....	21
2.2. Regulación de la biología de las células troncales neurales	23
2.2.1. Vías de señalización reguladoras de la auto-renovación y diferenciación celular	23
2.2.2. Regulación por TRIM-32	25
3. LA FAMILIA DE P53 Y SU FUNCIÓN EN LA BIOLOGÍA DE LAS CÉLULAS TRONCALES NEURALES	26
3.1. Organización genómica y dominios proteicos de la familia p53.....	28
3.2. Interacción funcional de los miembros de la familia p53.....	30
3.2.1. Función de p73 en cáncer	31
3.2.2. Función de p73 en la diferenciación celular y el desarrollo embrionario.....	32
OBJETIVOS	37
MATERIAL Y MÉTODOS	41
1. TRABAJO CON ANIMALES	43
1.1. Cepas de ratón y cruces	43
1.2. Genotipado de los ratones	43
1.3. Anestesia y eutanasia	45
2. ESTUDIOS <i>IN VITRO</i>	45
2.1. Establecimiento de cultivos de neuroesferas a partir de bulbo olfatorio de embriones de 14.5 días.....	45
2.2. Ensayos de auto-renovación	46
2.3. Determinación del tamaño y de la cinética de crecimiento de las neuroesferas	46

2.4. Ensayos de diferenciación	46
2.5. Obtención de parejas celulares por tratamiento con blebistatina.....	47
3. ESTUDIOS <i>IN VIVO</i>	48
3.1. Perfusión e incorporación de BrdU	48
3.2. Disección y preparación del material	48
3.3. Preparaciones completas o “ <i>whole mounts</i> ” de la pared lateral del ventrículo	48
4. CULTIVOS CELULARES	49
4.1. Líneas celulares y condiciones de cultivo	49
4.2. Derivación de cultivos primarios de fibroblastos embrionarios de ratón (MEFs) ..	49
4.3. Transfecciones celulares con Lipofectamine®	50
5. TRABAJO CON RNA	50
5.1. Aislamiento de RNA	50
5.2. Síntesis de cDNA	50
5.3. PCR Cuantitativo a Tiempo Real (qRT-PCR)	51
6. TRABAJO CON DNA.....	52
6.1. Preparación de plásmidos	52
6.2. Generación del vector indicador luciferasa dirigido por el promotor de TRIM32.	53
7. TRABAJO CON PROTEÍNAS	53
7.1. Preparación de extractos celulares	53
7.2. Inmunodetección proteica por <i>western blot</i>	54
7.3. Inmunocitoquímica	54
7.4. Inmunohistoquímica por flotación libre “Free floating”	55
7.5. Inmunohistoquímica de Preparaciones completas o “ <i>whole mounts</i> ”	56
8. ANÁLISIS TRANSCRIPCIONAL	57
9. CITOMETRÍA DE FLUJO	58
9.1. Análisis del ciclo celular por tinción con Ioduro de Propidio (PI)	58
9.2. Cuantificación de los índices apoptóticos por tinción con Annexina V/7-AAD.....	58
10. TRATAMIENTO ESTADÍSTICO DE LOS DATOS	58
RESULTADOS Y DISCUSIÓN	59
1. ANÁLISIS DE LA FUNCIÓN DE P73 Y P53, EN LA BIOLOGÍA DE LAS CÉLULAS TRONCALES NEURALES	61
1.1. Función de p73 en la regulación de la auto-renovación y multipotencia de células troncales neurales	61
1.2. Interacción funcional entre p73 y p53 en biología de células troncales neurales....	70
1.3. Efecto de la pérdida de p73 en la regulación de la división asimétrica	77

1.4. Efecto de la pérdida de p73 sobre la población de células proliferantes del nicho neurogénico.....	86
1.4.1. Análisis de la población de células proliferantes en la región subventricular de la pared lateral del ventrículo (SVZ)	86
1.4.2. Análisis de la población de células proliferantes en la región subgranular del giro dentado del hipocampo (SGZ)	89
2. CARACTERIZACIÓN DE LA FUNCIÓN DE LA FAMILIA DE P53 EN LA GÉNESIS Y ARQUITECTURA DE LOS NICHOS NEURÓGENICOS MURINOS	92
2.1. Análisis comparativo de las poblaciones celulares que integran el nicho SVZ en ratones de los cuatro genotipos	92
2.2. Análisis de la formación post-natal de la pared lateral del ventrículo: transición de la glía radial de la zona ventricular (VZ) al epéndimo y a la región subventricular (SVZ)	98
2.3. Análisis comparativo de la arquitectura del nicho SVZ en ratones de los cuatro genotipos	105
2.3.1. Caracterización de la pared lateral de ratones P7 WT y p73KO	106
2.2.2. Análisis comparativo de la pared lateral de ratones P15 WT, p73KO, p53KO y DKO	119
3. IDENTIFICACIÓN Y ANÁLISIS DE NUEVAS DIANAS TRANSCRIPCIONALES DE P73 EN CÉLULAS TRONCALES NEURALES	123
3.1. Análisis de TRIM32 como diana transcripcional de p73	124
CONCLUSIONES	131
RESUMEN EN ESPAÑOL	135
1. Índice.....	137
2. Resumen.....	141
2. Conclusiones.....	147
BIBLIOGRAFÍA	151
APÉNDICE	167

En la sociedad actual, el envejecimiento gradual de la población se ha traducido en un aumento en la incidencia de enfermedades neurodegenerativas como el Alzheimer. Esto ha llevado a incrementar los esfuerzos de la comunidad científica para mejorar los métodos de detección y terapia de estas enfermedades. En este sentido, el descubrimiento de la existencia de células troncales, con capacidad de regeneración neuronal, en el sistema nervioso central ha abierto nuevas áreas de estudio y nuevas posibilidades terapéuticas. Las células troncales neurales presentan dos características fundamentales que las hacen muy atractivas para ser empleadas en Medicina Regenerativa: la capacidad de auto-renovación ilimitada y la multipotencia. Sin embargo, para que estas terapias lleguen a ser una realidad es necesario mejorar el conocimiento tanto sobre las células troncales neurales, como de la génesis y condiciones del microambiente en el que se mantienen a lo largo de la vida del individuo, el nicho neurogénico.

La capacidad de neuro-regeneración de un individuo a lo largo de su vida depende, fundamentalmente, de la capacidad de sus células troncales neurales de mantenerse indiferenciadas, al tiempo que conservan la capacidad de diferenciarse a los distintos tipos neurales en respuesta a un estímulo sistémico. Por tanto, la identificación de los genes encargados de regular estos mecanismos es de vital relevancia en el campo de Medicina Regenerativa y de la Neurobiología en general. Entre los múltiples genes candidatos a ser reguladores de estos procesos, se encuentran los miembros de la familia génica de p53: p53, p63 y p73. Estos genes están implicados en el control de importantes procesos celulares, entre ellos el ciclo celular, la muerte celular y la diferenciación, procesos altamente regulados y de vital importancia en la biología de las células troncales.

Por ello, en esta tesis doctoral hemos estudiado el papel de los miembros de la familia de p53 (p53 y p73), en la regulación de la biología de las células troncales del sistema nervioso (NSC) y en la génesis y organización de los nichos neurogénicos.

Inicialmente, mediante estudios *in vitro* realizados con células troncales derivadas de bulbo olfatorio de embriones de 14,5 días de gestación, cultivadas como neuroesferas, se demostró que p73 actúa como regulador positivo de la auto-renovación de los progenitores neurales. De este modo, los progenitores neurales que carecen de p73

sufren una diferenciación prematura, que determina la pérdida de estas células en el cultivo, resultando en una menor capacidad de auto-renovación y menor crecimiento de las neuroesferas derivadas. Este efecto de la falta de p73 se observa en cultivos de células progenitoras procedentes de ratones que carecen, además, de p53 lo que permitió demostrar que la función de p73 en la regulación de los progenitores neurales es independiente de la función de p53. Es importante resaltar que p73 es necesario para que los cultivos de células progenitoras neurales sin p53 presenten el crecimiento descontrolado que caracteriza a las células que han perdido este gen supresor tumoral.

Demostramos, además, que la falta de p73 incrementa la frecuencia con que las células progenitoras neurales en condiciones mitogénicas segregan asimétricamente determinantes citoplasmáticos como TRIM32 y NICD, generándose de este modo un mayor número de células hijas destinadas a diferenciarse. La expresión de TRIM32, un determinante neuronal, en una de las células hijas correlacionó con la inducción del proceso de diferenciación neuronal en ésta. Estos resultados nos llevaron a proponer que p73 podría ser importante en la regulación de la expresión de factores implicados en la determinación de divisiones asimétricas. Observamos que, en ausencia de p73, los progenitores neurales *in vitro* sufren un mayor número de divisiones asimétricas, produciéndose una diferenciación prematura y, por tanto, la pérdida de la capacidad de auto-renovación.

Las células troncales del sistema nervioso adulto se localizan principalmente en dos regiones, denominadas nichos neurogénicos, localizados en la zona subventricular de los ventrículos laterales y en la zona subgranular del giro dentado del hipocampo. Las células troncales y progenitoras en estas zonas tienen capacidad de auto-renovación y también capacidad para generar precursores neurales que madurarán hasta integrarse como neuronas en el cerebro adulto. El efecto producido por la falta de p73 *in vitro* nos llevó a hipotetizar que su carencia podría afectar el mantenimiento de las células troncales neurales *in vivo*, y producir defectos en el desarrollo de los nichos neurogénicos y en el mantenimiento de la homeostasis de estos tejidos en el individuo adulto.

Un estudio inicial de las poblaciones proliferantes en los nichos neurogénicos demostró que, en ausencia de p73, la población celular con capacidad de proliferación (células troncales, progenitores neurales y neuroblastos) se veía significativamente disminuida en su conjunto. De este modo, la falta de p73 da lugar a nichos

neurogénicos “defectuosos” y hace predecir que la pérdida de estas células capaces de proliferar podría determinar graves defectos en la capacidad neurogénica.

Estos primeros análisis revelaron profundos cambios en la arquitectura de los nichos neurogénicos en los ratones carentes de p73. Por ello decidimos realizar un estudio detallado de las distintas poblaciones celulares que componen los nichos y de la organización y estructura de estos en los ratones mencionados. Llevamos a cabo un estudio comparativo de las poblaciones que componen el nicho neurogénico adulto: células troncales neurales, progenitores neurales, neuroblastos y células endoteliales que delimitan los ventrículos laterales. Nuestros resultados demostraron que la falta de p73 produce una disminución significativa de la población de células troncales neurales, así como de los neuroblastos. Además, observamos alteraciones en el número, disposición y naturaleza de las células endoteliales en ratones carentes de p73.

Durante el desarrollo embrionario, a partir de una población de células progenitoras de naturaleza glial (células de glia radial), se generarán todas las poblaciones que constituyen el nicho neurogénico adulto, incluida la capa de células endoteliales. Estas últimas son esenciales en la regulación de la homeostasis y función del nicho neurogénico. Por ello, realizamos un estudio de la transición de las células gliales a las distintas poblaciones que integran los nichos neurogénicos, analizando distintos estadios del desarrollo. Este estudio nos permitió demostrar que p73 es necesario para la correcta maduración de las poblaciones gliales, así como para la organización de las células que componen el nicho neurogénico. En ausencia de p73, las células endoteliales no son capaces de formar un epitelio mono-estratificado y presentan apariencia pseudo-estratificada y desorganizada. Además, tanto su morfología como la expresión de marcadores es aberrante, expresando tanto marcadores gliales tempranos como tardíos. Estos resultados nos llevaron a proponer que la ausencia de p73 afecta al proceso de transformación de los precursores de glia radial en las células que componen el nicho neurogénico.

Para estudiar en mayor detalle el papel que juega p73 en la organización de la pared lateral de los ventrículos laterales, así como la transformación de las poblaciones de glia radial, empleamos la técnica de “wholemout”. Esta técnica proporciona una vista frontal de las células que tapizan la pared lateral del ventrículo, así como de la organización que adoptan. Los estudios realizados anteriormente hacían suponer una maduración aberrante de las células de glia radial; por ello, decidimos realizar un

estudio comparativo de las células que tapizan el ventrículo en distintos estadios del desarrollo (concretamente analizamos ratones de 7, 15 y 160 días), de modo que pudiésemos observar la transformación del ventrículo durante su formación.

La maduración de las células endimarias que delimitan el ventrículo ocurre siguiendo un patrón posterior-anterior en el que las zonas posteriores adquieren antes un fenotipo maduro, siendo las regiones anteriores las que maduran más tardíamente. Por ello, realizamos el análisis de la pared a lo largo del eje antero-posterior, comparando los ratones normales con los ratones carentes de p73. Observamos que, en estadios tempranos, en el ratón WT las células de glia radial con una superficie apical pequeña comienzan a expandir dicha superficie, expresando transitoriamente GFAP y comenzando a multiplicar sus cilios para dar lugar, en P15, a células endimarias con gran superficie apical, múltiples cilios y sin expresión de GFAP, junto a células de superficie apical muy pequeña, un único cilio y expresión de GFAP. Estas últimas son las células B o células troncales neurales del adulto. Sin embargo, en ausencia de p73, se observa un gran número de células en estadio inmaduro, con superficie apical grande y expresión de GFAP que se mantiene, incluso en el adulto, y que presentan distintos estadios de multiplicación ciliar. Además, se detectan células monociliadas, de superficie apical intermedia y GFAP negativas, que no corresponden ni a células endimarias ni a células B, solo se detecta un pequeño número de células B monociliadas y GFAP positivas.

La arquitectura de la pared lateral del ventrículo se caracteriza por la presencia de unas estructuras denominadas molinillos. Estas estructuras son el modo de organización de las células troncales neurales adultas (células B), y parece que la correcta formación de las mismas es fundamental para el mantenimiento de la neurogenesis en el individuo adulto. En estas estructuras, un grupo de células endimarias se dispone rodeando la membrana apical de una o dos células B. En ausencia de p73, la formación de estas estructuras está profundamente afectada, observándose células endimarias que no siguen ningún patrón organizativo incluso presentaban estructuras más propias de estadios más tempranos del desarrollo.

Las células endimarias presentan múltiples cilios, implicados con su movimiento en el mantenimiento del correcto flujo del líquido ceforraquídeo. Los cuerpos basales de los cilios muestran una polarización específica imprescindible para su correcto funcionamiento. Aparecen dispuestos en una posición específica de la superficie celular,

siguiendo una direccionalidad que comparten con las células vecinas y que indica la dirección del flujo del líquido cefalorraquídeo (polaridad celular planar translacional). La polarización viene determinada desde el estadio de célula de glia radial, y los defectos en las proteínas que regulan los procesos de polarización del cilio primario en estas células conducen a una incorrecta polarización planar en las células de su progenie, las células endimarias. La observación de los cuerpos basales de los cilios en las células endimarias de los ratones carentes de p73 reveló la ausencia de una polarización correcta, dado que los cilios de células vecinas no estaban dispuestos de manera coordinada, sino de forma “aleatoria”. Se observaron células con los cilios distribuidos por toda la superficie, otras con ellos alineados en el centro de la célula, así como células con cilios agrupados en posiciones específicas de la superficie celular. En este último caso, muy frecuentemente la célula vecina presentaba los cilios con una direccionalidad opuesta, en lugar de seguir la misma dirección. Estos datos demuestran que p73 es necesario para la correcta transformación de las células de glia radial en los diferentes tipos celulares que componen los nichos, así como para la correcta regulación de la polarización de las células endimarias.

Para determinar si esta función de p73 dependía de la presencia de p53, realizamos un estudio comparativo con ratones carentes de ambos genes (DKO). Observamos que a pesar de que los ratones sin p53 (p53KO) tenían una arquitectura de la pared lateral del ventrículo semejante a la de los ratones WT, el doble mutante presentaba características similares a las observadas en ausencia únicamente de p73, demostrando que la función de p73 es independiente de p53, y que el fenotipo observado en su ausencia es dominante en ratones que carecen de p53. Esto nos llevó a concluir que p73 juega un papel fundamental en el mantenimiento y maduración de las poblaciones que conforman el nicho neurogénico, así como en la organización estructural de las mismas.

Una vez demostrado que p73 es necesario en la generación, mantenimiento, diferenciación y organización de las células troncales neurales, y dado que p73 es un factor de transcripción, tratamos de identificar nuevas dianas transcripcionales de p73 implicadas en alguno de estos procesos.

Basándonos en resultados previos de nuestro laboratorio, obtenidos durante el estudio del transcriptoma de células que sobreexpresaban isoformas de p73 (Marques-García et al., 2009), identificamos una serie de posibles dianas transcripcionales de p73, entre las que se encontraba TRIM32. TRIM32 es un determinante neural que se

distribuye asimétricamente en las células troncales neurales durante la diferenciación, de modo que la célula que expresa TRIM32 generará una neurona. Durante los estudios relacionados con la división asimétrica anteriormente descritos, observamos que, a pesar de que TRIM32 se segregaba asimétricamente de manera frecuente en células progenitoras neurales carentes de p73 comprometidas a diferenciarse, los niveles basales de los cultivos de neuroesferas p73KO presentaban niveles de TRIM32 más bajos que los cultivos WT. Todo ello nos llevó a postular que TRIM32 podría ser una diana transcripcional de p73.

Los estudios de expresión ectópica de p73 y p53, así como el análisis transcripcional del promotor humano de TRIM32, evidenciaron que p53, y más eficientemente p73 son capaces de activar la transcripción de este promotor y que además, p73 lo hace independientemente de la presencia de p53. De este modo demostramos que TRIM32 es una diana transcripcional directa de p73.

Para comprobar que estos estudios de sobre-expresión ectópica tenían relevancia fisiológica, analizamos la expresión de TRIM32 durante la diferenciación en células progenitoras neurales *in vitro*, en presencia o ausencia de p73. Observamos que la expresión de p73 aumentaba durante la diferenciación de forma paralela al aumento de la expresión de TRIM32, confirmando la relevancia de p73 en la expresión de TRIM32. Sin embargo, en ausencia de p73 los niveles de expresión de TRIM32 fueron significativamente menores. También se observó una regulación de la expresión de TRIM32 durante la diferenciación en ausencia de p73, indicando la existencia de otros factores, además de p73, capaces de regular TRIM32.

En su conjunto el presente trabajo de tesis doctoral demuestra que p73 desempeña un papel fundamental en la biología de las células troncales neurales: p73 es un regulador positivo en el mantenimiento de la capacidad de auto-renovación de las células troncales neurales, así como un regulador esencial en la transformación de las células de glía radial en los diferentes tipos celulares que componen los nichos neurogénicos y en los procesos de organización que culminan con la arquitectura típica de estos nichos.

Todo lo anterior hace de p73 un candidato a tener en cuenta en el estudio de enfermedades neurodegenerativas, así como una posible diana en el desarrollo de nuevas terapias.

OBJETIVO I: Análisis del papel de los miembros de la familia de p53, p73 y p53, en la biología de las células troncales neurales.

PRIMERA: p73 actúa como regulador positivo de la auto-renovación de las células troncales neurales. Este factor de transcripción es necesario para el mantenimiento de la capacidad de auto-renovación y el índice de crecimiento poblacional de las células progenitoras neurales en el modelo de neuroesferas. Esta función es independiente de p53, y necesaria para el crecimiento exacerbado característico de las NSC/NPC carentes de p53, tanto *in vitro* como *in vivo*.

SEGUNDA: p73 es necesario para la correcta regulación de la división simétrica versus asimétrica de las células progenitoras neurales embrionarias. La deficiencia de p73 incrementa la frecuencia de distribución asimétrica de determinantes citoplasmáticos de la identidad celular, como NICD y TRIM32. La distribución asimétrica de TRIM32 correlaciona con la diferenciación prematura de las células progenitoras neurales carentes de p73 en cultivo. De este modo, la falta de p73 conduce a una diferenciación neuronal prematura *in vitro*, restringiendo de este modo la auto-renovación de las células troncales neurales.

TERCERA: La función de p73 es imprescindible para el mantenimiento de la población proliferante en ambos nichos neurogénicos: la zona subventricular (SVZ) de los ventrículos laterales y la zona subgranular (SGZ) del giro dentado del hipocampo.

OBJETIVO II: Caracterización de la función de la familia de p53 en la génesis y arquitectura de los nichos neurogénicos murinos.

CUARTA: La falta de p73 afecta el mantenimiento de los neuroblastos proliferantes y de las células troncales neurales en cerebro de ratones de 15 días, incluso en ausencia de p53.

QUINTA: La función de p73 es crítica para la maduración de las células radiales gliales a los diferentes tipos celulares que componen la SVZ y por tanto para el mantenimiento de la integridad celular de la pared lateral de los ventrículos.

SEXTA: p73 es esencial para la génesis y arquitectura de los nichos neurogénicos en la región SVZ. Su deficiencia da lugar a profundos defectos en la polaridad celular plana

translacional de las células endoteliales, así como en la cinética de maduración de estas células. .

OBJETIVO III: Identificación y análisis de nuevas dianas transcripcionales de p73 en la biología de las células troncales neurales.

SÉPTIMA: TRIM32 es una diana transcripcional directa de TAp73 y p53, mientras que la isoforma DNp73 ejerce un efecto dominante negativo sobre la activación de TAp73 sobre el promotor de TRIM32.

OCTAVA: p73 es un factor importante en la regulación de la expresión de TRIM32 durante la diferenciación neuronal, aunque otros mecanismos, independientes de p73, deben estar implicados en la expresión de TRIM32 tanto *in vitro* como *in vivo*.

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Appendix

p73 deficiency results in impaired self renewal and premature neuronal differentiation of mouse neural progenitors independently of p53

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The question of how neural progenitor cells maintain its self-renewal throughout life is a fundamental problem in cell biology with implications in cancer, aging and neurodegenerative diseases. In this work, we have analyzed the p73 function in embryonic neural progenitor cell biology using the neurosphere (NS)-assay and showed that p73-loss has a significant role in the maintenance of neurosphere-forming cells in the embryonic brain. A comparative study of NS from Trp73^{-/-}, p53KO, p53KO;Trp73^{-/-} and their wild-type counterparts demonstrated that p73 deficiency results in two independent, but related, phenotypes: a smaller NS size (related to the proliferation and survival of the neural-progenitors) and a decreased capacity to form NS (self-renewal). The former seems to be the result of p53 compensatory activity, whereas the latter is p53 independent. We also demonstrate that p73 deficiency increases the population of neuronal progenitors ready to differentiate into neurons at the expense of depleting the pool of undifferentiated neurosphere-forming cells. Analysis of the neurogenic niches demonstrated that p73-loss depletes the number of neural-progenitor cells, rendering deficient niches in the adult mice. Altogether, our study identifies TP73 as a positive regulator of self-renewal with a role in the maintenance of the neurogenic capacity. Thus, proposing p73 as an important player in the development of neurodegenerative diseases and a potential therapeutic target.

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The mammalian neocortex develops from a pseudostratified epithelium composed by neuroepithelial cells.¹ During development these cells and their derivative progenitor will give rise to neurons, astrocytes and oligodendrocytes while maintaining their self-renewal capacity. During development of the mammalian central nervous system (mCNS), the self-renewal of neural stem cells (NSCs) and progenitors occurs either by symmetric cell divisions, which generate two undifferentiated cells with the same fate,² or by asymmetric divisions, giving rise to one progenitor and one cell that differentiates into a neuron.³ NSCs are multipotential and self-renewing, whereas neural progenitor cells (NPC) have more restricted renewal and differentiation ability.⁴ Here, the term NPC will be used to include both neural stem and progenitor cells. Self-renewal and cell fate choice of NPC are coordinately controlled in a stage-dependent manner, but the mechanisms underlying such coordination remain poorly understood. Signals that regulate stem cell maintenance and fate specification will influence the final number of neurons and glia formed during mCNS development.⁵ Such signals would be determinant for

the appropriate architecture of the neurogenic-niches in the adult brain and thus, the maintenance of the neurogenic capacity.

In adult mammalian brain NSCs are localized in two regions: the subventricular zone (SVZ) and the subgranular-cell layer zone (SGZ) of the dentate-gyrus in the hippocampus. NPC from the embryonic and mature mCNS can be propagated *in vitro* as clonal aggregates denoted neurospheres (NS).^{6,7} The NS assay represents a serum-free, selective culture in which most cells within the differentiation process rapidly die, whereas NPC respond to mitogens, divide and form NS that can be dissociated and replated to generate secondary spheres.⁸ In a similar way, NPC from olfactory bulb (OB) of E14.5 embryos have been demonstrated to be derived from the neuroepithelium, and form NS that preserve their self-renewal ability and multipotency.^{9,10}

Some of the pathways that are necessary for self-renewal appear to regulate processes like proliferation, apoptosis or differentiation.^{2,11} In somatic cells, p53-family members are deeply involved in the regulation of these processes. This

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Abbreviations: NSC, neural stem cells; NPC, neural progenitor cells; NS, neurospheres; NFC, neurospheres-forming cells; CNS, central nervous system; SVZ, subventricular zone; SGZ, subgranular-cell layer zone; OB, olfactory bulb; DIV, days *in vitro*; AD, Alzheimer's disease; BrdU, 5-bromo-2-deoxyuridine

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family is constituted by the transcription factors p53, p73 and p63. The dual nature of these genes resides in the existence of TA and DN variants. The TA-proteins are transactivation competent, inducing cell cycle arrest, apoptosis, senescence and differentiation.¹² Oppositely, the DN isoforms lack the transactivation domain and can act as dominant-negative repressors of p53 and TAp73, abrogating their ability to induce growth suppression in many cell types.^{13,14} This family has been implicated in regulation of the survival and maintenance of CNS mature neurons and neuronal precursors, with p53 functioning as a cell death protein, and DNp73 and DNp63 as major survival proteins preventing apoptosis in post-mitotic neurons and embryonic cortical precursors, respectively.^{15–17} Deficiency of p73 in the Trp73^{−/−} mice, which lacks all p73 isoforms, leads to multiple neurological abnormalities including hippocampal dysgenesis,¹⁸ denoting the relevance of p73 function in neural development and suggesting a possible role in neurogenesis. However, the role of p73 in the biology of NPC has never been addressed.

In this work, we analyzed p73 function in embryonic NPC biology using the NS assay, and demonstrate that p73 is a positive regulator of self-renewal in a p53-independent manner. This self-renewal impairment is later on reflected in a developmental retardation of the neurogenic niches and a significant depletion of the precursor pool of the SVZ and SGZ of P15-Trp73^{−/−} mice, ultimately resulting in defective neurogenic niches.

Results

Loss of p73 impairs cellular self-renewal. To address whether *TP73* ablation affected the biology of embryonic NPC, we used the NS assay.^{7,19} We utilized primary NS cultures obtained from OBs of Trp73^{−/−} and WT E14.5 embryos. NPC *in vivo* are positive for the neuroepithelial-marker Nestin.²⁰ In our *in vitro* assay, OBs cultures in the presence of mitogens form NS that express Nestin (Figure 1a). Analysis of p73-isoforms expression revealed that both, TA and DNp73, were expressed under proliferating conditions, with TAp73 being significantly more abundant than DNp73 (Figure 1b). These cell cultures were successively dissociated and reseeded at clonal density generating new NS capable of producing a differentiated progeny (Figure 1c, Supplementary Figure 1A), demonstrating their self-renewal and multipotency capacities. Moreover, TAp73 expression is upregulated during the differentiation process (Supplementary Figure 1B). The frequency of primary neurosphere-forming cells (NFC) represents the content of NPC in a given tissue.^{21–23} We observed that four times more cells were required to form primary NS from the Trp73^{−/−} neurogenic tissue, than from WT (1/125 cells in WT (0.8%) versus 1/500 cells in Trp73^{−/−} (0.2%), Supplementary Figure 1C), suggesting that lack of p73 produces a depletion of NFC in the neurogenic tissue. Interestingly, when we compared NS cultures, we consistently observed a decrease in the size and number in the Trp73^{−/−} (named p73KO from now on) NS in 60% of the analyzed embryos (Figure 1c–e). The diameter of p73KO-NS was significantly reduced compared with that of WT cells (Figures 1c and d).

To rule out the possibility that the observed phenotype was limited to a defect in the transient amplifying (TA) population, we performed long-term population analysis, for up to 10 passages, under clonal cultures conditions,⁸ and the size of the newly formed NS was evaluated at each subculturing step. The phenotype observed in the p73KO-NS was maintained, and even intensified, through the successive passages, indicating that the effect of p73 loss was not merely affecting the TA population, but rather the pool of NPC, the only capable of generating new NS (Figure 1c, NS-P8). The NS size reduction suggests that the net cell growth within the NS, which reflects the sum of cell divisions from self-renewing NSC and NPC, was impaired.

The formation of new NS after each successive passage has been widely used as a quantitative measure of the self-renewing activity of neural stem cells.^{7,24,25} We used this *in vitro* assay to examine how p73 loss affects this parameter in our cultures. To that purpose, dissociated tissue cells were seeded under serial clonal dilution and the number of NS formed in each passage was quantified. We arbitrarily assigned 100% to the NS formed in the WT cultures. We observed a highly significant decrease in the number of NS formed in p73KO cultures (Figure 1e). This difference was maintained in consecutive passages. The decrease in the number and size of newly formed NS in each passage in the p73KO cultures suggests a successive loss of NFC, and signals p73 as a positive regulator of NPC self-renewal.

To further analyze the overall growth of the p73KO cells, we determined ratios of cell production after 5 days *in vitro* (DIV) relative to number of cells plated. Growth ratios in the p73KO cultures were significantly lower than in WT (Figure 1f). Analysis of cellular proliferation and death rates demonstrated that p73 loss affected the net cell growth of the culture, without altering the cell cycle progression (Figure 1g). Consistently, there were no significant differences in the expression levels of the cell cycle inhibitor and negative regulator of self-renewal, p21^{Cip1}, neither in the expression of the positive self-renewal regulator and promoter of cell proliferation, Bmi1 (Figure 1h). We detected an increase in cell death (Figure 1i). Accordingly, levels of the proapoptotic p53 target gene *Noxa* were significantly upregulated in p73KO cultures (Figure 1j).

p73 regulates self-renewal independently of p53, but it is necessary for the enhanced self-renewal caused by p53 deficiency. The role of p73 in the developing nervous system has been defined on the basis of DNp73 function as an antiapoptotic protein that antagonizes p53 proapoptotic actions, and potentially TAp63.^{15–17} As the Trp73^{−/−} mice lack all the isoforms,¹⁸ the absence of the pro-survival DNp73 function could result in a compensatory p53 activation, which may account for the enhanced apoptosis as well as Noxa enhanced expression in the p73KO cells, and even the impaired self-renewal observed in the p73KO-NS cultures. To address this issue, we analyzed the role of p53 in NS cultures, in the context of p73 deficiency, using the double mutant: p53KO; Trp73^{−/−} mice (named DKO from now on). We observed that, although p53KO cultures presented larger NS (Figures 2a and b, $P < 0.0005$), reflecting its enhanced self-renewal,^{26,27} and the p73KO-NS were smaller ($P < 0.0005$), the DKO cells generate NS with a

Figure 1 p73 deficiency impairs cellular self-renewal and increases cell death without affecting cell cycle progression. (a–f): Successive passages of E14.5 neurospheres of the indicated genotype cultured under clonal proliferation conditions. (a) Confocal microscopy images ($10\times$) of NS of the indicated passage immunostained with anti-Nestin antibody. (b) qRT-PCR of TA- and DNp73 expression in WT-NS. (c) Bright-field photographs ($10\times$) of NS passages (P2–P8). (d) Graphical representation of NS size (P2). (e) NS self-renewal assay. Quantification of new NS was performed after 4DIV at the indicated passages. We assigned 100% to the number of WT NS. (f) NS (P2) cellular kinetics after 5DIV. (g) FACS cell cycle analysis determined by propidium iodide (PI) staining. (h) qRT-PCR analysis of p21^{Cip1} and Bmi1 expression, (i) cell death measured by the Annexin-V binding assay and (j) qRT-PCR analysis of Noxa in P2-NS cultures under proliferating conditions. Representative histograms and percentages from triplicate experiments are shown. The analyses were performed with data from two independent experiments with at least three embryos of each genotype (* $P<0.05$, ** $P<0.01$, *** $P<0.005$)

size closer to WT-NS. Up to this point, the data suggested that the reduced size in p73KO-NS could be indeed because of a p53-compensatory activation, as elimination of p53 almost rescues the size phenotype. Nevertheless, the DKO NS were considerably smaller than those formed by p53KO cells ($P<0.0005$, Figures 2a and b), indicating that p73 loss

hampered the effect of p53 deficiency, and implying that p73 function was necessary for the enhanced growth of the p53KO-NS. Cell cycle analysis of these cultures revealed that in DKO cells p53 deficiency significantly inhibited the basal apoptosis detected in the p73KO cultures, whereas enhancing cellular proliferation (significant increase in

Figure 2 p73 regulates self-renewal independently of p53, but it is necessary for the enhanced self-renewal observed in p53KO NS cultures. (a–h): NS (P2) cultures of the indicated genotypes under proliferating conditions. (a) Bright-field microphotography (10 ×) and (b) diameter quantification, (c) FACS-Pi cell cycle analysis, (d) qRT-PCR of p21^{Cip1} and Noxa expression, (e) confocal microscopy images (20 ×) of p-His3 immunostaining and (f) its quantification. (g) Cellular kinetics of NS (P2) of the indicated genotype after 4DIV. (h) Self-renewal assay: NS (P2) cells were seeded at different clonal cell densities in proliferating media from 0.6–5 cells per μl, and the formed NS were counted after 4DIV. The Trp73^{−/−}, p53^{+/−} littermates showed the same phenotype as the Trp73^{−/−} mice (data not shown). The analyses were performed with data from two independent experiments with at least three embryos of each genotype (*P < 0.05, **P < 0.01, ***P < 0.005)

S-phase and decrease in G1, compared with WT and p73KO) (Figure 2c). Consistently, p21^{Cip1} and Noxa expression were repressed in the DKO cells, demonstrating that the regulation of these genes in NPC was p53

dependent (Figure 2d). Furthermore, the mitotic index of clonally formed NS quantified by anti-phospho-Histone-3 staining supported these results, as p53KO and DKO-NS had a significantly elevated mitotic index than WT, whereas

the p73KO-NS had not (Figure 2e and f). It is important to underline that in the absence of p53, lack of DNp73 does not result in apoptosis. However, despite the enhanced proliferation rate and low apoptosis of DKO cultures, they showed an overall growth rate similar to the p73KO-NS, failing to attain p53KO growth rate (Figure 2g). This data reinforces the idea that p73KO effect in NPC was not simply due to a decrease in the proliferation rate or enhanced apoptosis.

The *in vitro* self-renewal assay revealed that p53KO cells formed significantly more NS than their WT counterparts, whereas p73KO cultures had significantly less NS, in all conditions analyzed (Figure 2h). However, p73 loss in the DKO cells abrogated the effect of p53 deficiency, as the number of NS formed in the DKO was similar to those of WT cultures, but significantly less than the observed in the p53KOs with functional p73. This was surprising, as NS obtained from the OB of p53KO mice embryos are enriched in NFC and have enhanced self-renewal.²⁶ Therefore, our data indicates that these features in p53KO cultures were dependent upon p73 functional integrity.

p73 deficiency results in premature neuronal differentiation under proliferating conditions and faster kinetics of the differentiation process. Self-renewal and differentiation are cardinal features of stem cells. A tight regulation of the facultative use of symmetric *versus* asymmetric divisions by stem cells is crucial for maintaining self-renewal capacity. It has been documented that under proliferating culture conditions, NSCs *in vitro* are fast dividing Nestin-positive cells that clonally give rise to great numbers of Nestin-positive NPC and divide symmetrically to produce equivalent progeny.²⁸ As shown in Figures 3a and b, p73 deficiency resulted in a reduction of Nestin-positive cells in NS under proliferating conditions, suggesting that these NS contained a reduced number of undifferentiated NPCs. Intriguingly, a closer observation of p73KO and DKO cells (Figure 3c) revealed pairs of cells that resembled daughter cells with different nuclei size and asymmetric distribution of Nestin (p73KO: $1.4\% \pm 0.002$ and DKO: $4.1\% \pm 0.16$, $P \leq 0.003$ and $P \leq 0.02$ compared with WT, respectively). However, WT and p53KO cultures presented few asymmetric cell pairs ($0.6\% \pm 0.009$ and $0.5\% \pm 0.1$, respectively), consistent with the notion that under proliferating culture conditions, WT-NPC divide symmetrically. Thus, impaired NS formation in the p73KO cells could reflect fewer symmetric divisions in these cells.

Increasing the number of asymmetric divisions would lead to a decrease in the pool of undifferentiated NPC and to an increase in the number of differentiated cells, with the concomitant impairment of self-renewal, whereas promoting a symmetric mode of division, like in p53KO cultures,²⁶ would result in enhanced self-renewal by keeping the NPC in an undifferentiated state. In accordance with this model, the analysis of the spontaneous neuronal differentiation in NS after 3DIV under proliferating conditions revealed that the p73KO and DKO NSs frequently contained multiple Tuj-1-positive cells (Figure 3d, Supplementary Figure 2A). These cells showed various long and branched neurite extensions, congruent with a more mature neuronal phenotype (Supplementary Figure 2B). This was surprising, as the clonally

newly-formed NS were kept under proliferating conditions and analyzed after only 3DIV, conditions at which the WT and p53KO NSs showed seldom Tuj-1-positive cells. Furthermore, few Tuj-1-positive cells detected in the latter cultures had short lamellipodia or short unbranched processes. This data indicates that p73 deficiency was prompting a premature neuronal differentiation and thus hindering the maintenance of the embryonic NPC undifferentiated population.

Our data suggest, but does not conclude, that p73 deficiency may lead to a bias towards asymmetric cell divisions, increasing the population of NPC, ready to differentiate into neurons, and thus exhausting the pool of undifferentiated NFC. If this hypothesis is correct, we would expect an enhanced rate and a faster kinetics of the differentiation process in p73KO-NS grown under conditions supporting progressive neuronal differentiation. Under these conditions,⁷ WT and p73KO cells differentiated into astrocytes and neurons after 8DIV, and oligodendrocytes were detected after 10DIV (Supplementary Figures 3A and 3B) with no significant differences in the total Tuj1⁺ cells after 5DIV or 8DIV (Figure 4b upper panel). However, despite the identical conditions, we found a significantly higher percentage of large branching neurons (Tuj1⁺ cells with long neurite extensions (Tuj1⁺ NE)) at all the time points analyzed in the p73KO-NS, suggesting an advanced stage in the differentiation process, (Figure 4a and b; lower panel and Supplementary Figure 3C). This supports the idea that p73 deficiency induces a premature onset of differentiation. When we compared the differentiation potential of p73KO, p53KO, DKO or WT-NS after 3- or 5DIV with differentiation media, we observed that either p53- or p73-deficient NS generated higher number of Tuj1⁺-NE cells compared with WT, confirming that p73 and p53 single-deficiency results in premature neuronal differentiation. Nevertheless, the combined deficiency in the DKO-NSs produced more neurons with mature phenotype than p53 or p73 single loss (Figures 4c and d), suggesting an additive effect. It is important to highlight that p53 control of neurogenesis and gliogenesis has been proposed to be independent of its regulatory pathways of self-renewal.²⁹ We analyzed if there was a cross-talk in the regulation of cell decision fate between p73 and p53. Strikingly, although lack of p53 hinders astrocytic differentiation, DKO cells showed a stronger GFAP staining and a mature astrocytic phenotype in a number similar to WT or p73KO cell, but with significantly higher number of differentiated cells than the p53KO cultures (Figure 4d), suggesting that absence of p73, rescues p53 uncoupling of neuronal fate. At a further stage of differentiation, p73KO and DKO cells displayed neurons with a clear mature phenotype with multiple branched long neurites (Figure 4e), whereas the Tuj1⁺-WT, and even the p53KO, displayed yet an immature phenotype, corroborating that p73 deficiency resulted in enhanced rate and faster kinetics of the differentiation process independently of p53.

If lack of p73 produces a premature differentiation and, in this way, depletes the pool of embryonic NPC during development, it is reasonable to expect that p73 deficiency will result in defective neurogenic-niches in the adult mice. To address this, we scored the 5-bromo-2-deoxyuridine (BrdU)-positive cells, which identify proliferating NSC and NPC in the

Figure 3 p73 deficiency results in premature neuronal differentiation under proliferating conditions. (a–e): NS (P2) cultures of the indicated genotypes under proliferating conditions (PM). (a) Confocal microscopy images ($60\times$) immunostained with the antibodies: Nestin (a–c), Tuj1-TxRed (neurons) and p-His-3-FITC (mitotic marker) (d). (b) Quantification of Nestin-positive cell in (a). (c) Magnification of boxes marked in (a) showing different nuclei size and asymmetric Nestin distribution. The p53KO pair depicted represents a symmetric distribution with similar nuclei size. (e) Quantification of Tuj1⁺ cells in (d). The analyses were performed with data from two independent experiments with at least three embryos of each genotype (** $P < 0.01$, *** $P < 0.005$)

SVZ, and found a significantly lower number of proliferating cells in Trp73^{−/−} mice (Figures 5a and c). Quantification of p-His-3-positive cells in the same region confirmed this result (Figures 5b and d). Moreover, assessment of p-His-3-positive cells in the SGZ also showed a severe depletion of the proliferating compartment in Trp73^{−/−} mice (Figures 5e and f). A morphological analysis of the neurogenic-niches at electronic microscopy level exposed profound abnormalities in the architecture along the SVZ of the Trp73^{−/−} mice.

Assessment of the different proliferating cellular subpopulations of the SVZ – astrocyte-like type-B cells (NSC), type-C cells (transit amplifying progenitors) and type-A (neuroblast)³⁰ – confirmed our BrdU data, revealing that p73 deficiency resulted in a reduction of type-C and A cells (Figures 5g and h). Furthermore, type-B cells, as well as type-A and -C cells, presented an immature morphology similar to those in newborn mice; however, the number of type-B cells was comparable with WT.

Figure 4 Lack of p73 results in enhanced rate and faster kinetics of the differentiation process. (a–e): NS (P2) cultures at the indicated DIV under differentiating conditions (DM) immunostained with the antibodies: Tuj1-TxRed (neurons) or GFAP-FITC (astrocytes). (a) Confocal microscopy images (40 ×) and (b) quantification of Tuj1⁺ cells in (a) either total Tuj1⁺ (b, upper panel) or with neurite (Tuj1⁺-NE) extensions four times bigger than the cell body diameter (b, lower panel). (c) Confocal microscopy images (40 ×, upper panel and 20 ×, lower panel) and (d) quantification of Tuj1⁺-NE and GFAP-positive cells in (c). (e) Confocal microscopy images (60 ×). The analyses were performed with data from three independent experiments with at least three embryos of each genotype and representative images are shown (**P* < 0.05, ***P* < 0.01, ****P* < 0.005)

Discussion

Learning about the mechanisms that regulate the coordinated control of proliferation and differentiation of tissue-specific stem cells is critical to understand development, cancer and neurodegeneration. In this work, we have analyzed the effect of p73 deficiency, and its functional interaction with p53, in embryonic NPC using the Ns assay. Our data reveal that p73 loss results in a depletion of the cells capable of forming NS

(NSC and NPC) contained in the neurogenic tissue of the embryos. Furthermore, this effect was maintained *in vitro* where lack of p73 resulted in impaired net cell growth, decreased capacity to form new NS under clonal conditions (self-renewal) and enhanced apoptosis without altering the cell-cycle progression.

The role of p73 in the developing nervous system has been defined as a rheostat for survival *versus* death.^{15–17} As the Trp73^{−/−} mice lack all p73 isoforms, it was possible that an

Figure 5 Lack of p73 delays the development of the SVZ and SGZ and depletes the number of proliferating cell population. (a–f): Coronal sections (10 ×) of the SVZ (a–d) or SGZ (e and f) of P15 mice brains of the indicated phenotype, immunostained with antibodies against anti-BrdU (after repetitive pulses over 8 h period) (a and c), or anti-p-Histone-3 (b, d and f) and the positive cells scored (c, d and f). Magnifications of the area included in the white squares in e are depicted in the inserts (100 ×). (g) Panel of the cellular analysis of the Trp73^{−/−} and WT mice by electron microscopy of SVZ showing the differential morphology of Type B (B), Type C (C), Type A (A) and: Ependymal cells (E). In the p73KO the cells corresponding to C and A are not marked as such as they both look alike with morphological features corresponding to more immature cells. (h) Bar graph of the average number of SVZ Type A/C cells identified per unit length in WT and Trp73^{−/−} mice (**P* < 0.05, ***P* < 0.01, ****P* < 0.005)

exacerbated p53 activity, due to the lack of the prosurvival DNp73 function, could be accounted for the enhanced apoptosis and the impaired self-renewal observed in p73KO-NS. In apparent agreement with this view, the elevated apoptotic rates of the p73KO-NS disappeared in the DKO cultures, demonstrating that, indeed, the apoptosis observed in the p73KO cells was p53 dependent, probably through the regulation of target genes like Noxa. Hence,

we confirm that p53 negatively regulates NSC self-renewal through the regulation of proliferation and survival. In this context, DNp73 is required to downregulate p53 activation of these pathways and its absence results in elevated apoptosis. Surprisingly, our data indicated that the NFC enrichment and the enhancement of the self-renewal capacity of the p53-deficient NPC, were dependent upon p73 functional integrity. Therefore, lack of p73 results in two independent,

but related, phenotypes: a smaller neurosphere size (related to the proliferation and survival of the NPC) and a decreased capacity to form new NS (self-renewal). The former seems to be the result of p53 compensatory activity, whereas the latter is p53 independent and not because of a defect in cell cycle or apoptosis regulation.

The reduction of Nestin-positive cells in p73KO and DKO-NS, and the observation that p73-deficient NS growing under proliferative conditions contained multiple Tuj1⁺ cells suggested a defective maintenance of the undifferentiated state, which is central to stem/progenitor cells. This, together with the decrease of newly formed NS in each passage in p73KO and DKO cultures, directed us to hypothesize that p73 deficiency may lead to a bias towards asymmetric cell divisions and, in this way, impair self-renewal. The role of p53 in the regulation of self-renewal divisions has been widely documented,³¹ however, there was no data on the possible function of p73 in this process. However, very recent reports have been published^{32–34} that are in-line with our results, providing an independent confirmation of p73 involvement in neuronal stemness.

Our differentiation studies demonstrated that loss of p73 did not abolish NS multipotency, but rather accelerated the appearance of Tuj1⁺ cells with long and branched neurite extensions, congruent with a mature neuronal phenotype. These data connote that p73 deficiency is releasing the constraints that keep the embryonic NPC undergoing symmetric, proliferative division, thus, hampering their self-renewal and producing a premature differentiation. Although p73 functional integrity seem to be required for the enhancement of the self-renewal capacity of p53-deficient NPCs, the effect of p53 and p73 deletion over premature differentiation seems to be additive. Therefore, p73 and p53 regulation of self-renewal and differentiation should imply independent, yet interconnected pathways. This is supported by the observation that p53 deficiency on cell fate determination is restored by p73 depletion in DKO cells, implying a cross-talk between these genes in the regulation of this process. Altogether, our work has revealed a novel p53-independent function of p73, which regulates NPC self-renewal through the regulation of cellular differentiation and proposes that this regulation is at the base of p73 function in neuronal development.

Alterations in the mechanism that promotes or inhibits the differentiation of the embryonic NPC, like p73 deficiency does, would eventually deplete the pool of embryonic NSC, rendering deficient neurogenic-niches in the adult mice. Supporting this hypothesis, the Trp73^{−/−} and TAp73^{−/−} mice models exhibited profound hippocampal dysgenesis, demonstrating that p73, and TAp73 in particular, was critical for the development and maintenance of the hippocampus, one of the neurogenic NSC pools.³⁵ Our data reveals that p73 deficiency slows down the development of both the SVZ and the SGZ niches, depletes the number of proliferating cells, and thus, renders deficient neurogenic-niches in the adult mice. Impairment in neurogenesis may compromise the extent of plasticity of the hippocampus and their associated neural circuits leading to enhanced neuronal vulnerability and functional detriment. Recent evidence suggests that neurogenesis is impaired in animal models of Alzheimer's disease (AD), in both subventricular and subgranular zones.³⁶

In AD, compromised neurogenesis has been proposed to take place earlier than the onset of hallmark lesions or neuronal loss, and may have a role in the initiation and progression of the disease. In this context, p73 loss could become a risk factor for these ailments. This is consistent with reports indicating that p73 is essential to prevent neurodegeneration during aging, and that p73 heterozygosity may be a susceptibility factor for AD.¹⁷ Thus, p73 could be an important player in the development of neurodegenerative diseases and therefore a relevant target for study.

Materials and Methods

Mice husbandry and dissection of brain tissues. Animal experiments were conducted in agreement with European and Spanish regulations for the protection of experimental animals (Council Directive 86/609/CEE and RD-1201/2005, respectively) with the appropriate institutional committee approval. Mice heterozygous for Trp73 on a mixed background C57BL/6 × 129/svJae¹⁸ were backcrossed to C57BL/6, at least five times, to enrich for C57BL/6 background. To obtain the double Trp73; p53 Knockout mice (DKO), heterozygous animals for Trp73 were crossed with p53 KO mice;³⁷ then, Trp73^{+/−}; p53^{+/−} mice were inter-crossed to obtain the DKO animals. Mice were genotyped as described before.^{18,38}

In vivo analysis. Perfusion and BrdU incorporation analysis in postnatal 15-day-old mice (P15): BrdU (Sigma-Aldrich, St. Louis, MO, USA) was administered at 150 mg/kg intraperitoneally in pulse injections every 2 h during 8 h. Animals were anesthetized with ketamin/medetomidin before transcardial perfusion. Animals were perfused with PBS, 0.1 M and subsequently with 4% paraformaldehyde (Merck, Darmstadt, Germany). After perfusion, brains were dissected, post-fixed for 24 h in 4% PFA solution at 4°C, and immersed in 30% sucrose. At this point, tissues were stored at −80°C. Coronal sections of 30 μm were obtained using a Microm HM-450 sliding microtome, and p-His-3 and BrdU immunohistochemistry (IHC) was performed as described before.²⁷

Electron microscopy analysis: P15 mice were perfused with 0.9% PBS, 1% heparin, followed by 2% paraformaldehyde/2.5% glutaraldehyde solution (PFA/GA, Electron Microscopy Sciences, Hatfield, PA, USA) and post-fixed in PFA/GA overnight. Sections of 50 μm were cut on a vibratome (Leica VT-1000, Wetzlar, Germany), embedded in araldite (Durcupan, Fluka, Hatfield, PA, USA). Semi-thin sections (1.5 μm) were cut with a diamond knife and stained lightly with 1% toluidine blue. The block with semi-thin sections was cut in ultra-thin (0.05 μm) sections with a diamond knife, stained with lead citrate and examined under a Fei Tecnai spirit electron microscopy. Pictures were taken with Soft Image System (Morada, Olympus, Tokyo, Japan) camera. For quantification purposes, three mice for each group were used and serial sections corresponding to the same SVZ region (separated by 250 μm) were chosen and examined under a FEI Tecnai Spirit electron microscopy using established protocols and criteria.³⁰

Primary culture of NPCs. To initiate each independent embryonic culture, the OBs of at least three different E14.5 embryos were dissected out, and mechanically dissociated, as previously described.¹⁰ Cells were seeded (2 × 10⁴ cells/ml) in NS complete medium (Supplementary Table 1). For each passage, spheres formed after 4 days *in vitro* (DIV) were enzymatically dissociated with Accutase (Stem Cell Technologies, Vancouver, BC, Canada) and reseeded in complete medium. Cellular viability was assessed by trypan blue exclusion. To determine the size and number of NS, cells were seeded at clonal density (2 cells per μl). For clonogenic and limiting dilution assays, NS were disaggregated and seeded in flat bottom 96-well plates at log² dilutions with a cell concentration from 500–1 cell per well. After four DIV the number of NS was determined (three independent experiments with at least three embryos of each genotype for each one). Statistical analyses were performed using the Student's two-tailed t-test.

Differentiation assays were performed seeding the NS in 15 mm coverslips covered with poli-L-ornithine (Sigma), in differentiation medium (Supplementary table 1) and incubated for 3, 5 or 8 days.

Flow cytometry assays. Cell cycle analysis was performed as described before²⁶ using single cell suspensions obtained from enzymatically disaggregated NS. Samples were analyzed by flow cytometry in a CyAn ADPc (Beckman Coulter,

Brea, CA, USA), and data were analyzed using Summit v4.3 (Dako, Glostrup, Denmark). For Annexin-V Binding Assay, NS were enzymatically disaggregated and labeled according to the manufacturer's instructions (BD, San Jose, CA, USA).

RNA isolation and real-time RT-PCR analysis. Total RNA from P2 NS was extracted with TRI reagent (Ambion, Austin, TX, USA) and cDNA was prepared using SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression of cell cycle and apoptosis markers was detected by real-time PCR in a StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) using FastStart Universal SYBR Green Master (ROX) (Roche, Basel, Switzerland). Primer sequences were obtained from Primer bank Database^{39,40} (<http://pga.mgh.harvard.edu/primerbank/index.html>) and are indicated in the Supplementary Table 2. The PCR conditions were previously described.^{39,40}

Immunocytochemistry. IHC was performed as described before²⁶ using the following antibodies: polyclonal-GFAP (1:400, Neomarkers, Labvision, Fremont, CA, USA), monoclonal-GFAP (1:100, Millipore, Billerica, MA, USA), β -Tubulin/Tuj-1 (1:1000, Covance, Princeton, NJ, USA), O4 (1:50), phospho-Histone-3 (1:100 Millipore) or Nestin (1:100, BD, Billerica, MA, USA). Images were obtained with Nikon EclipseTE2000 confocal microscope. Statistical analysis (Student's two-tailed *t*-test) was performed using triplicates from three independent experiments.

Conflict of interest

The authors declare no conflict of interest.

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