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THESIS TITLE

Role of the transcription factors *COUP-TFI* and *Pax6* in neural stem cell self-renewal and neurogenesis during mouse cortical development

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Ai miei genitori

ABSTRACT

The different types of neurons that populate the neocortex play different and equally important roles aimed at satisfying the physiological functions of specific projection neurons residing in distinct neocortical areas. Each area consists of a heterogeneous group of neurons distributed in specific layers of the neocortex, as a result of a correct process of cell fate commitment during corticogenesis, which defines the thickness, identity and boundaries of neocortical radial domains. Hence, the processes controlling the generation of specific classes of neurons into distinct neocortical areas, depend on the correct balance between self-renewal of neural stem cells and commitment of precursors, in time and space.

Although great progress has been made over the last decade, the mechanisms that underlie the maintenance between stem cell self-renewal and progenitor commitment during corticogenesis remains poorly understood. Here, I addressed this question directly, by investigating the potential genetic interaction between two transcriptional regulators, COUP-TFI and Pax6, in regulating the molecular network controlling progenitor commitment during neocortical area formation. Experimental evidences indicated that COUP-TFI, a caudalizing identity factor, and Pax6 responsible for rostral fate, are expressed in opposite gradients along the rostro-caudal axis of the cortex. Analysis of constitutive mutants showed that these transcription factors are responsible for controlling areal identity and neurogenesis during development.

While it is well recognized that Pax6 plays a crucial role in neural stem cell proliferation, multipotency, and neurogenesis in many regions of the central nervous system, including the cerebral cortex, little is known on the function of COUP-TFI in these processes. In the first part of my work, I aimed to evaluate the role for COUP-TFI in the maintenance of neural stem/precursor cells in neurospehere long-term expansion cultures from mouse cortical embryonic primordia. I found that in the absence of COUP-TFI function cortical cells abnormally proliferate over time, implying that COUP-TFI is

responsible for maintaining a proper progenitor pool during development.

Next, by generating double compound mutants between COUP-TFI and Pax6, I found that lowering the gene dosage of Pax6 in COUP-TFI mutants, and vice versa, of COUP-TFI in Pax6 mutants, rescued the reciprocal mutant phenotype. This indicates that the two genes modulate each other expression levels during the transition between self-renewal and neurogenic commitment. *In vivo* analysis on cortical primordia using markers of apical (self-renewal) versus basal (neurogenic) progenitors, confirmed most of the *in vitro* results, even if the *in vivo* situation resulted to be more complex. Overall, these data strongly suggest a molecular cross-regulation between COUP-TFI and Pax6 during the maintenance of a proper balance between the stem/ progenitor and committed precursor pools, most probably acting on similar target genes.

RIASSUNTO

La neocorteccia è popolata da diverse tipologie di neuroni, ognuna delle quali in grado di svolgere ruoli diversi , ma ugualmente importanti necessari per garantire una corretta attività fisiologica di specifici neuroni di proiezione che popolano la corteccia stessa.
I neuroni di proiezione risiedono in diverse aree corticali. Ogni area consiste di un gruppo eterogeneo di neuroni distribuiti in strati specifici della neocorteccia, come risultato di una corretta acquisizione d' identità cellulare durante la corticogenesi, volta a definire l'identità, lo spessore e i confini dei domini neocorticali radiali. I processi che controllano la generazione di specifiche classi di neuroni in diverse aree corticali dipendono dal corretto equilibrio tra auto-rinnovamento delle cellule staminali neurali e la specificazione dei precursori, nel tempo e nello spazio.

Nonostante le notevoli scoperte scientifiche susseguitesi nell' ultimo decennio, i meccanismi alla base dell'equilibrio tra cellule staminali auto-rinnovabili e committment dei progenitori durante la corticogenesi rimane poco conosciuta. In questo lavoro, ho affrontato la questione in modo diretto, indagando una possibile interazione genetica tra due fattori trascrizionali, COUP-TFI e Pax6. Entrambi sono noti per il loro coinvolgimento attivo nel network molecolare responsabile del destino neuronale dei precursori durante la formazione della neocorteccia. Evidenze sperimentali indicano che COUP-TFI, importante per il destino caudale in corteccia, e Pax6 responsabile invece di un destino rostrale, sono espressi in gradienti opposti lungo l'asse rostro-caudale della corteccia. L' analisi dei rispettivi mutanti costitutivi ha dimostrato che questi fattori di trascrizione sono responsabili del controllo di identità areale e neurogenesi durante lo sviluppo.

Mentre è ben noto che Pax6 gioca un ruolo cruciale nella proliferazione delle cellule staminali neurali, multipotenza e neurogenesi in molte regioni del sistema nervoso centrale, tra cui la corteccia cerebrale, poco si sa sulla funzione di COUP-TFI in questi processi. Nella prima parte del mio lavoro, ho valutato il ruolo di COUP-TFI per il mantenimento dei precursori neuronli staminali

utilizzando colture di neurosfere espanse a lungo termine derivate dal primordio corticale murino allo stadio embrionale. I miei esperimenti dimostrano che, in assenza del gene COUP-TFI le cellule proliferano in modo anomalo nel corso del tempo, il che implica che COUP-TFI è responsabile della gestione del pool di progenitori durante lo sviluppo corticale.

Di seguito, ho generato e analizzato doppi mutanti caratterizzati da differenti dosaggi genici ottenuti dall'incrocio di COUP-TFI e Pax6 tra loro. Abbassando sperimentalmente i livelli genici di Pax6 nei mutanti COUP-TFI, e viceversa di COUP-TFI nei mutanti di Pax6, ho ottenuto un recupero del fenotipo mutante in modo reciproco. I miei esperimenti suggeriscono che i due fattori di trascrizione regolano l'un l'altro i propri livelli di espressione. Analisi *In vivo* sul primordio corticale embrionale usando marcatori di progenitori apicali (self-renewal) e per progenitori basali (neurogenici) hanno confermato la maggior parte dei risultati ottenuti in vitro, anche se la situazione in vivo è risultata essere più complicata.

Complessivamente, i miei dati suggeriscono fortemente una cross-regolazione molecolare tra i due fattori di trascrizione COUP-TFI e Pax6 nel mantenere il corretto equilibrio tra precursori auto-rinnovabili e progenitori indirizzati al destino neurogenico, molto probabilmente agendo sugli stessi geni target.

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CHAPTER 1

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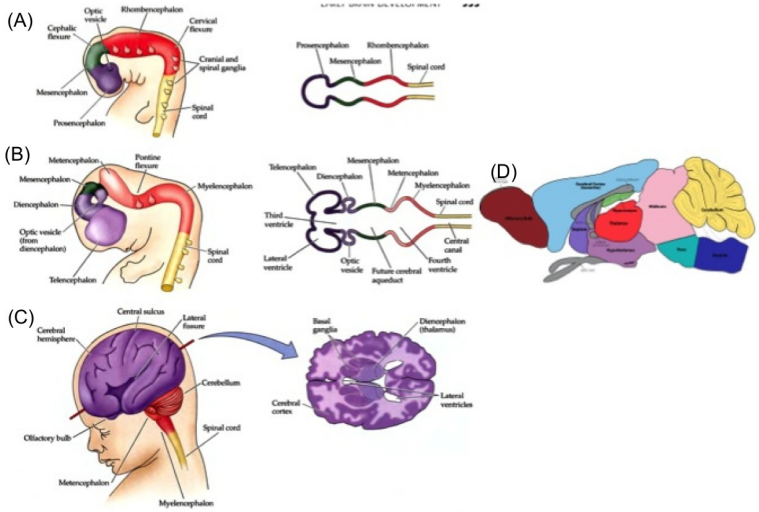
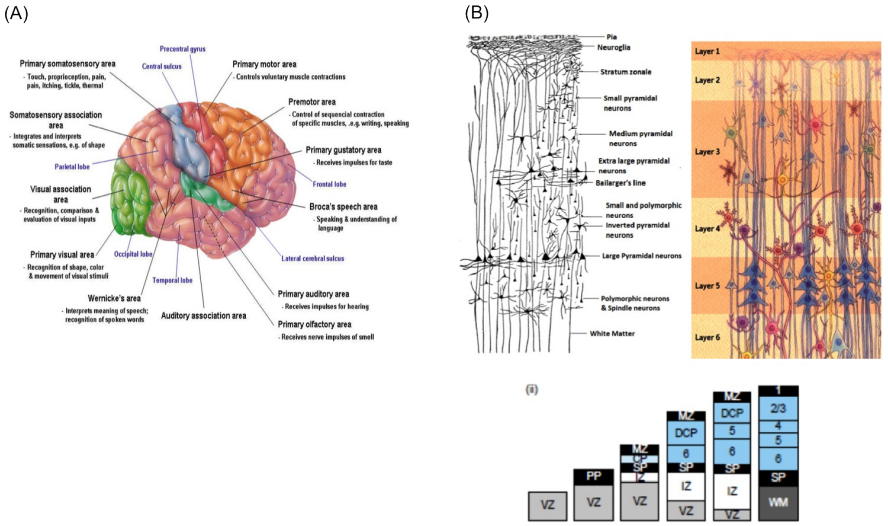
Figure 1



Regional specification of the developing brain.
(A) During early embryogenesis the neural tube becomes subdivided into the prosencephalon mesencephalon, and rhomboencephalon. To the right, a longitudinal section of the neural tube indicates the position of the major brain regions. (B) A later developmental stage, the prosencephalon gets subdivided into telencephalon and diencephalon. To the right, a longitudinal section of the neural tube indicates the position of the major brain regions shown in B. (C) The fetal human brain and spinal cord are clearly differentiated by the end of the second trimester. To the right a cross section through the forebrain at the indicated level designate the nascent sulci and gyri of the cerebral cortex, as well as the differentiation of the basal ganglia and thalamic nuclei. (D) Subdivision of the different structures in the adult mouse brain. Rodents, such as mouse and rat, do not have sulci and gyri, but the position of the different cortical and subcortical structures are maintained between human and rodent brains.

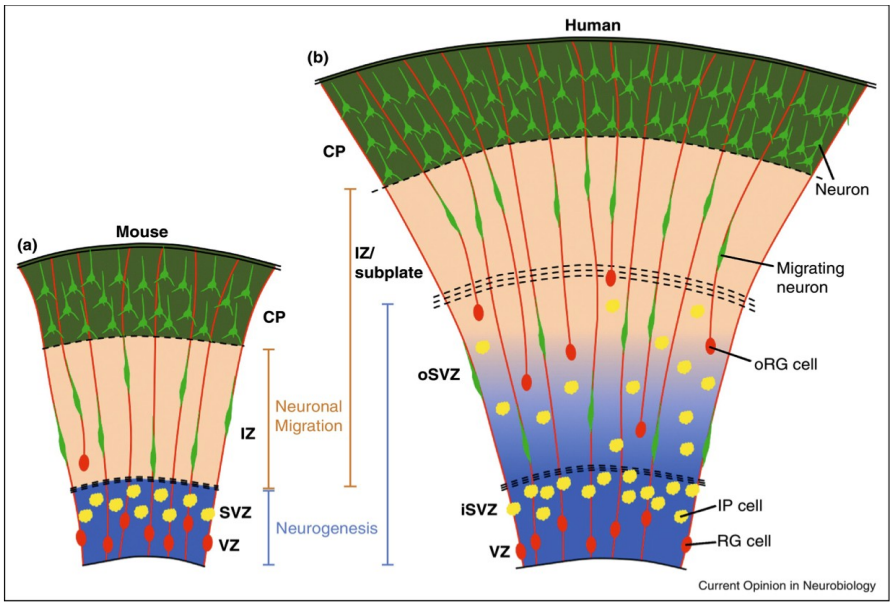
Figure 2



The six-layer structure of the cerebral cortex.

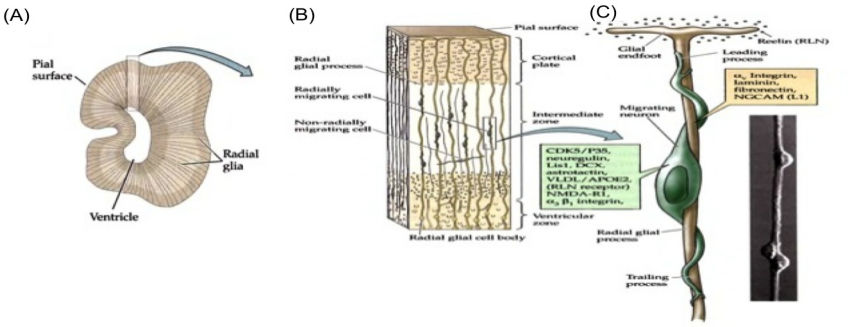
(A) Schema of a lateral view of an adult human neocortex and subdivision of the major functional areas: motor area, localized rostrally, deputed to elaborate movements on the base of sensory information, somatosensory area, deputed to collect and elaborate information on the tactile perceptions, visual areas, deputed to collect and elaborate visual information from the eyes, and auditory areas, deputed to collect and elaborate information from the ears. (B) Schema of the six layers organization of the murine neocortex, from the outermost being layer 1 to the innermost layer 6 and specific classes of migratory neuronal precursor necessary for the subsequent differentiation of distinct classes. (Bi) Schematic representation of the progressive formation of neocortical layers during development. The first structure that can properly be defined neocortex, the CP, will be divided into six layers in an inside out fashion during corticogenesis (O'Leary and Nakagawa, 2002). The first cortical projection neurons, born at ~E11.5 in mouse, migrate to settle as a layer forming the nascent cortical plate (CP), which subsequently develops into L2-L6 of the postnatal neocortex. Incoming CP neurons split the PP into the superficial marginal zone (MZ), which develops into L1 of the postnatal cortex, and the deeper subplate (SP), which is situated below L6.

Figure 3



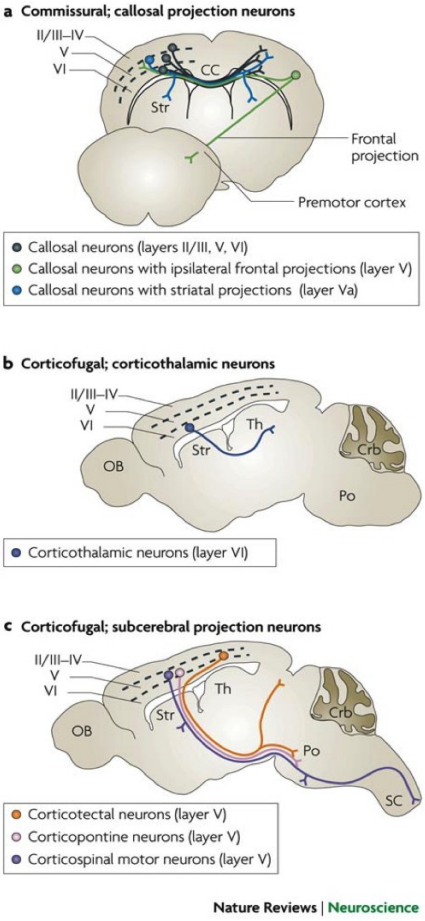
Neocortical radial organization in rodents (A) and humans (B) during development.
Rodents and humans share similar neuronal populations, although the intermediate progenitor (IP) population is strongly expanded in humans and can be subdivided into two compartments, the inner subventricular zone (ISVZ) and the outer subventricular zone (OSVZ). This is considered one the most determinant differences, which are responsible for the progressive enlargement of the total neuronal population of the human neocortex (taken from Lui et al., 2011).

Figure 4



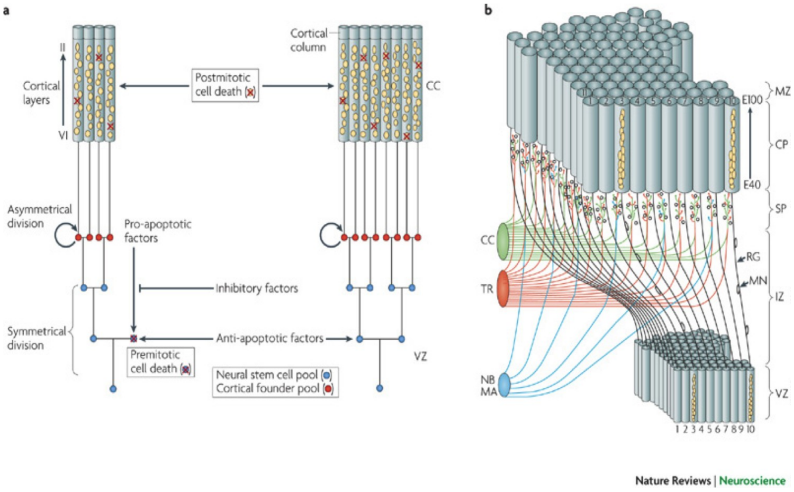
Radial migration in the developing cortex.
(A) Section through the developing forebrain showing radial glial processes from the ventricular to the pial surfaces. (B) Enlargement of boxed area in (A). Migrating neurons are intimately apposed to radial glial cells, which guide them to their final position in the cortex. Some cells take a non-radial migratory route, which can lead to wide dispersion of neurons derived from the same precursor. (C) A single neuroblast migrates upon a radial glial process (schema and electron microscope detail). Cell adhesion and other signaling molecules or receptors found on the surface of either the neuron (green) or the radial glial processes are indicated on the respective boxes (from Rakic, 1974; micrographs courtesy of E. S. Anton and P.Rakic).

Figure 5



Major cortical projection neurons and relative targets.
(A) Commissural projection neurons include contralaterally-projecting callosal neurons (layer II/III,V,VI), callosal neurons with ipsilateral and frontal projections (layerV) and with striatal projections (layer Va). (B) Corticothalamic neurons (layer VI) that target different nuclei of the thalamus. (D) Corticofugal projection neurons are constituted by all those neurons that project to targets outside the cortex: corticotectal neurons project to the tectum, corticopontine to the pons and corticospinal motor neurons to the spinal cord (all in layer V) (taken from Molyneaux et al., 2007b).

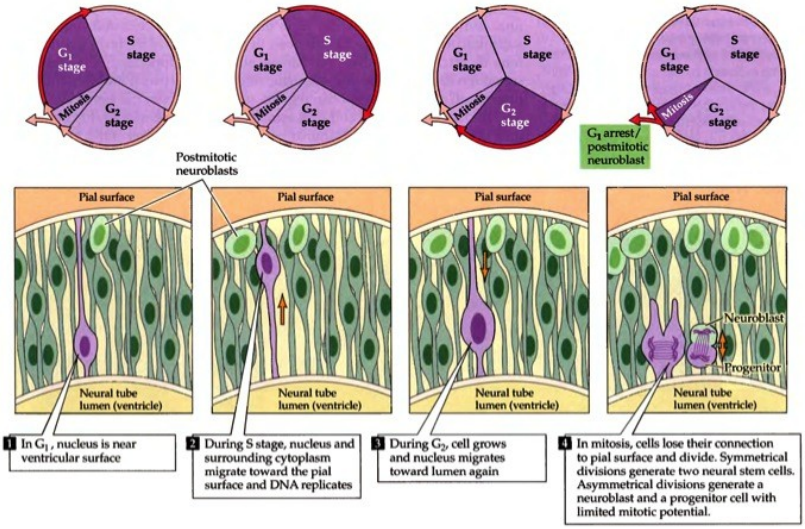
Figure 6



Radial Unit Hypothesis.

(A) The model illustrates how changes in the mode and rates of cell proliferation within the neural stem cell pool (blue circles) in the ventricular zone that divide symmetrically causes an exponential increase in the number of radial columns, which result in surface expansion of the cerebral cortex. By contrast, similar changes in proliferation kinetics occurring in the founder cells (red circles), which divide asymmetrically, cause a linear increase in the number of neurons within radial columns without a change in the cortical surface area (taken from Macmillan, 2005). (B) Model of radial neuronal migration that underlies columnar organization (taken from Elsevier, 2007).

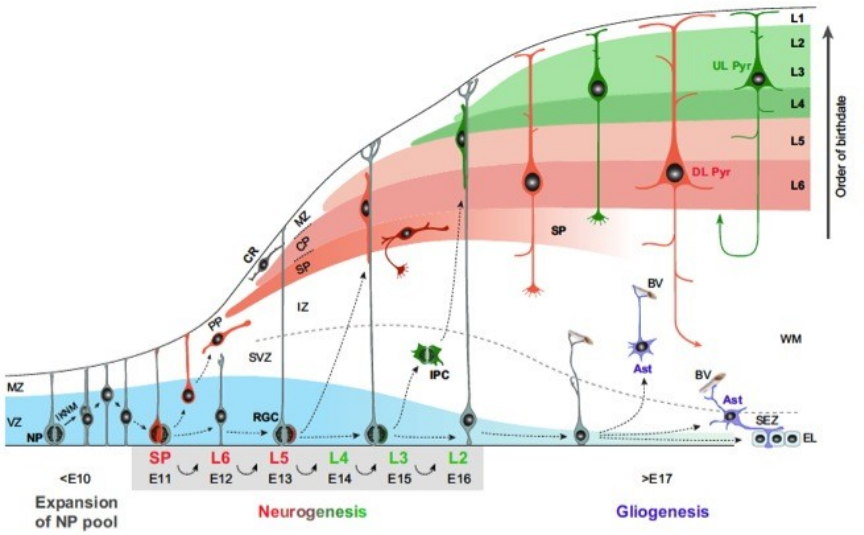
Figure 7



Interkinetic nuclear movement (IKNM).

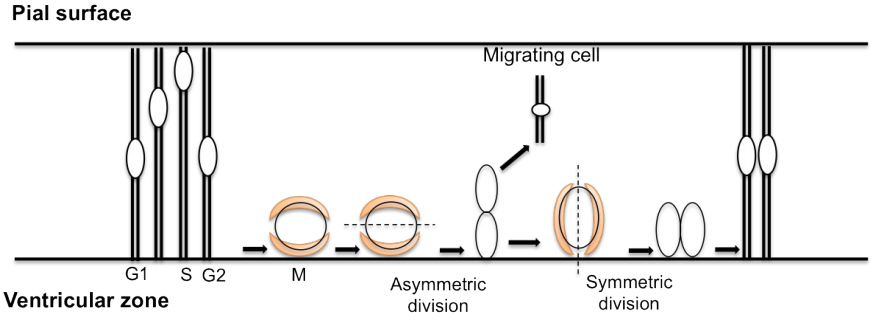
Dividing precursor cells in the vertebrate neuroepithelium are attached both to the pial surface and to its ventricular surface. The nucleus of the cell translocates between these two limits within a narrow cylinder of cytoplasm. When cells are closest to the pial surface of the neural tube, they enter a phase of DNA synthesis (S phase); after the nucleus moves back to the ventricular zone (G2 phase), the precursor cells lose their connection to the outer surface and enter mitosis (M phase). When mitosis is complete, the two daughter cells extend processes back to the outer surface of the neural tube, and the new precursor cells enter a resting (G1) phase of the cell cycle. At some point a precursor cell generates either another progenitor cell that will go on dividing and a daughter cell, a neuroblast, that will not divide further, or two post-mitotic daughter cells.

Figure 8



Schema of corticogenesis in the mouse during development.
Before neurogenesis start neural progenitors (NPs) in the ventricular zone (VZ; blue) of the developing neocortex divide symmetrically to expand the progenitor pool, undergoing interkinetic nuclear migration (IKNM-see also Figures 7, 9). At the onset of neurogenesis (at around E11), NP start to acquire molecular and morphological characteristics of glial cells and are called Radial Glial Cells (RGCC). RGCC self-renew and generate directly first preplate (PP) neurons, which split into subplate (SP) and marginal zone (MZ) neurons, and later deep layer neurons (red). RGCC generate also indirectly upper neurons by producing transit-amplifying intermediate progenitor cells (IPC) (dark green), which are positioned in the Subventricular zone (SVZ). IP cells typically divide once symmetrically then differentiate into upper layer neurons (green). RGCC transition into neurogenic subependymal zone (SEZ) and astrocytes (Ast) during the gliogenic phase is shown. In addition, radial glia migrating into the subependymal zone (SEZ), can give rise to ependymal (EL) cells, oligodendrocytes (not shown) and astrocytes (Ast in violet) pre- and perinatally during the gliogenic phase (taken from Kahl, 2012).

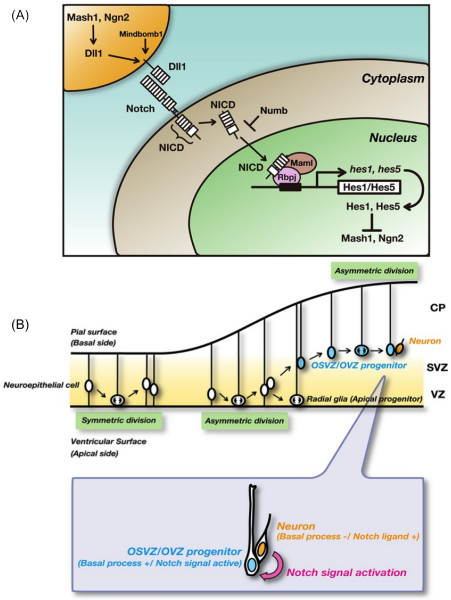
Figure 9



Nuclear translocation and symmetric versus asymmetric differentiation in cell fate acquisition.

Stem cells in the ventricular zone undergo characteristic interkinetic nuclear translocation and can divide both symmetrically and asymmetrically to generate either another stem cell and a differentiated daughter cell or two stem cells or two differentiated daughter cells with a consequent diminution of the proliferating cell pool. The ratio between symmetric and asymmetric divisions changes during development with a consequent reduction in the number of multipotent stem cells present in the adult.

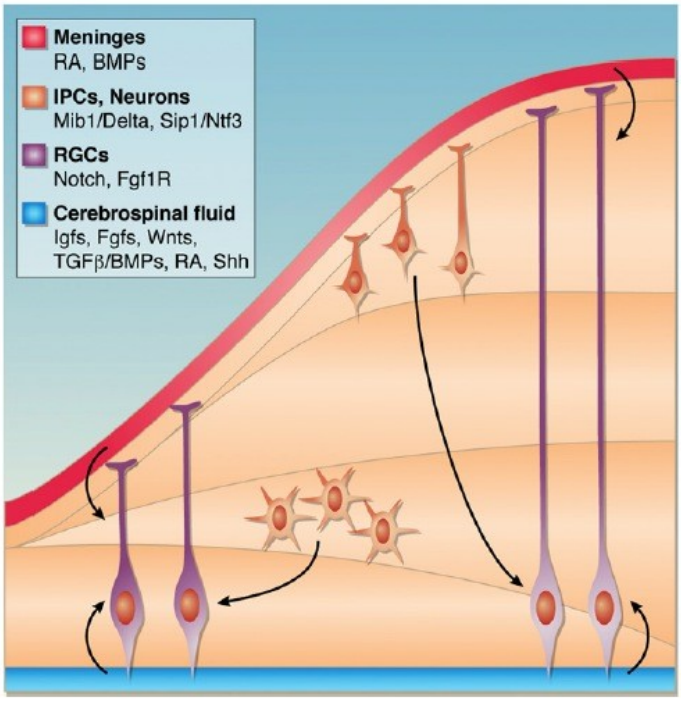
Figure 10



The core pathway of Notch signaling and his putative mechanism in cell fate acquisition.

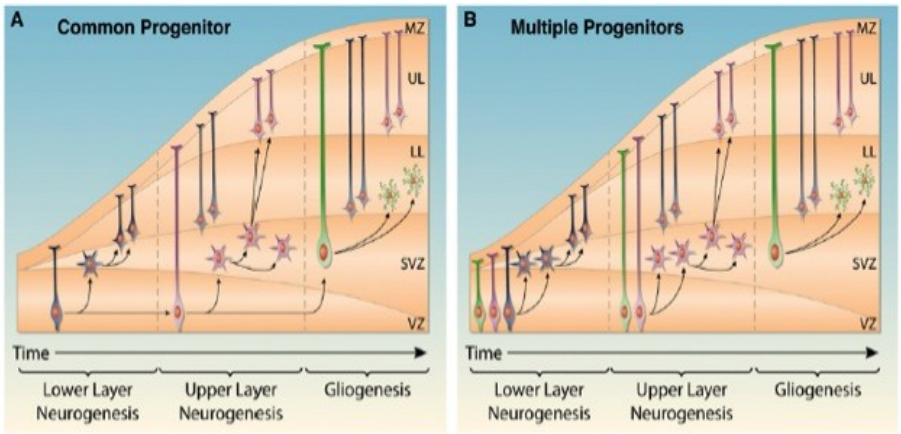
(A) Proneural genes such as *Mash1* and *Ngn2* promote neuronal differentiation and induce the expression of Dll1, which in turn activates Notch in neighboring cells. In the developing brain, upon activation of Notch, the Notch intracellular domain (NICD) is released from the transmembrane portion and transferred to the nucleus, where it forms a complex with the DNA-binding protein Rbpj and the transcriptional co-activator Maml. The NICD–Rbpj–Maml complex induces the expression of transcriptional repressor genes such as *Hes1* and *Hes5*. Hes1 and Hes5 then repress the expression of proneural genes and Dll1, thereby leading to the maintenance of neural stem/progenitor cells. Numb inhibits Notch signaling and induces neuronal differentiation. (B) Outer radial glial (OSVZ/OVZ/ORGC) progenitors have radial glia-like morphology and extend radial fibers to the pial surface, but lack apical processes. These cells undergo asymmetric cell division multiple times and generate a lot of neurons. These neurons seem to express Notch ligands and activate Notch signaling in their sibling OSVZ/OVZ progenitors (taken from H. Shimojo et al., 2011).

Figure 11



***Signals that control radial glia cell (RGCC) Proliferative Behavior*.** A number of signaling molecules can act on RGCCs to regulate their proliferative and neurogenic behaviors. Similarly, intermediate prgenitors (IPCs) and neurons secrete signals that provide a feedback mechanism for controlling the balance between proliferation and neurogenesis in RGCCs (taken from J. Franco and U. Muller, 2012).

Figure 12



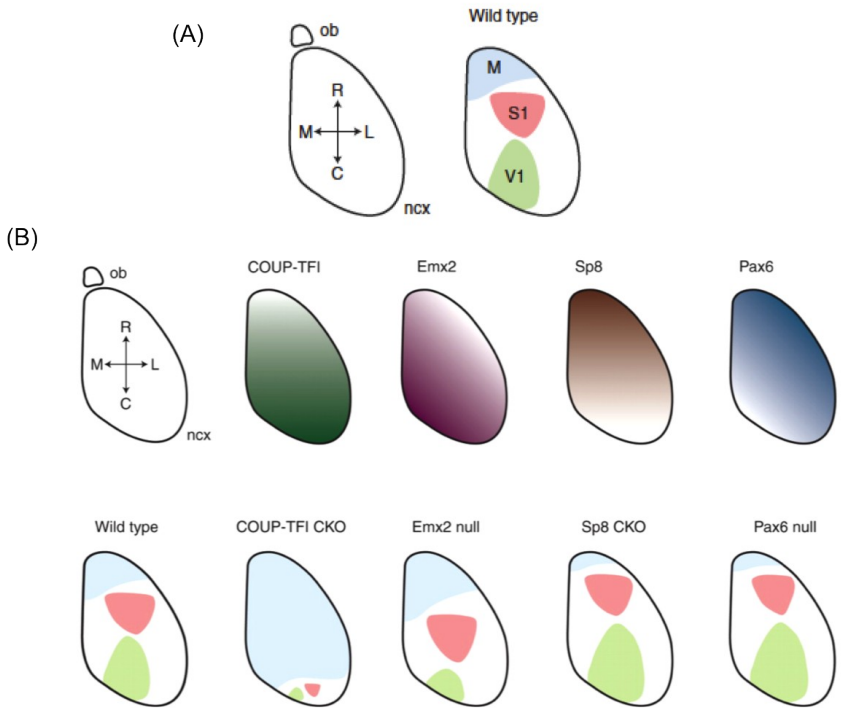
Different models of projection neuron cell-type specification in the developing neocortex.
(A) A single kind of radial glia progenitor sequentially generates all subtypes of projection neurons and macroglia. (B) Distinct subtypes of radial glia progenitors co-exist and are pre-specified to generate different subtypes of projection neurons and macroglia.

Figure 13

Table indicating cortical layer markers, their domain of expression and related phenotypes when inactivated in the mouse.

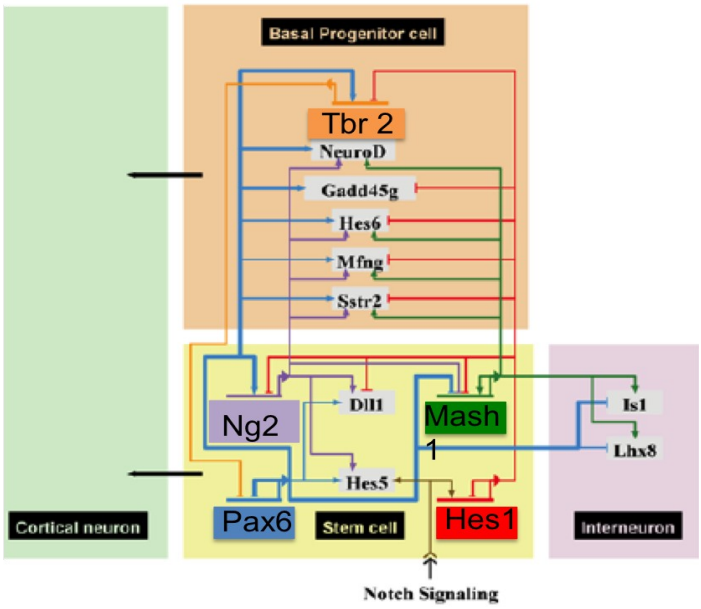
Gene	Layer of expression	Lamination phenotype	Reference
Bhhb5	Layer II/III-V	Layer IV organization CSMN formation	(Joshi et al., 2008)
CTIP2	Layer V (low in layer VI)	CSMN formation	(Arlotta et al., 2005)
Fog2	Layer VI	N/A	(Kwan et al., 2008)
Foxp2	Layer VI, V	N/A	(Ferland et al., 2003)
SATB2	Layer II/III-VI	CPN identity	(Alcamo et al., 2008; Britanova et al., 2008)
Tbr1	Layer VI	Layer VI specification	(Hevner et al., 2001)

Figure 14



The roles of FGF signaling and graded transcription factor expression in neocortical pattern formation.
(**A**) Diagram of a dorsal view of the mouse cortex (ncx, neocortex; ob, olfactory bulb; M, medial; L, lateral; R, rostral; C, caudal), with the major axes and areas labeled (M, motor; S1, somatosensory; V1, primary Visual). (**B**) The transcription factors COUP-TF1, Emx2, Sp8, and Pax6 are expressed in gradients along the rostrocaudal axis of the cortex as shown. The effects of null mutations in each transcription factor are shown (CKO, cortex-specific knockout). SP8 and Pax6 are expressed in a high rostral to low caudal gradient, Emx2 and COUP-TFI, which are expressed in opposite gradients. Pax6 and COUP-TFI, which are important regulator of neurogenesis and proliferation, in particular show specific and complementary phenotypes during areal specification.

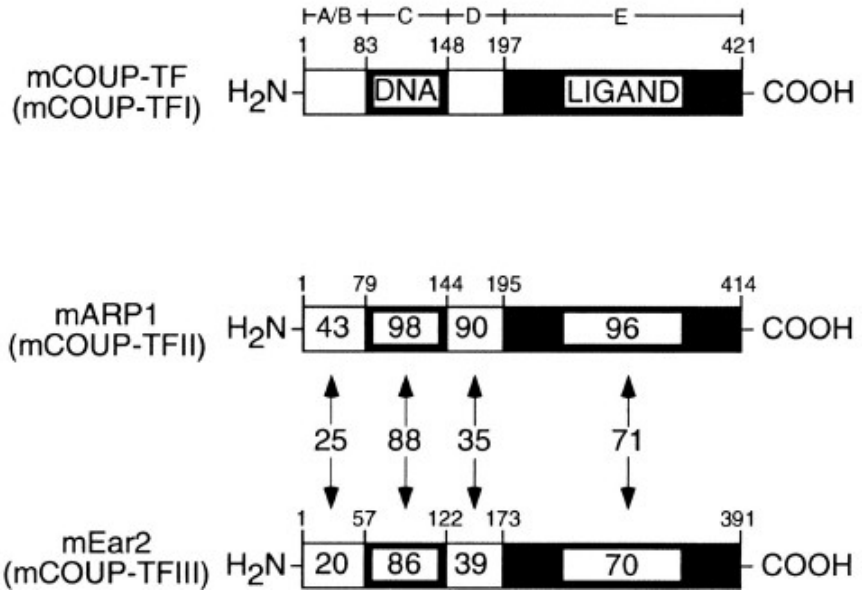
Figure 15



Neurogenesis and self-renewal regulatory molecular network operating in neocortical stem cells and controlled by the transcription factor Pax6.

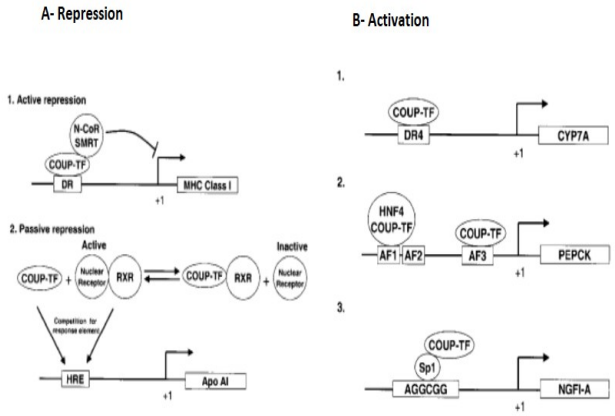
Several data on Pax6 binding and regulation properties indicate that Pax6 levels are critical in maintaining the balance between stem cell maintenance, neurogenesis and SVZ genesis, as well as enforcing cortical identity. Combining these data with published data on genes downstream of Neurog2, Mash1 and Hes1 enables construction of a basic network regulating cortical stem cell neurogenesis. Under normal conditions, Pax6 positively regulates Neurog2 and negatively regulates Mash1. Pax6 also positively regulates basal progenitor cell genes, such as Tbr2. Thick lines indicate direct regulation, thin lines indicate evidence from expression studies (Taken from Livesey et al, 2009).

Figure 16



***Relationship between the different COUP-TFs family member*.**
Mouse mCOUP-TFI is here used as a model to compare conservation of sequence with other COUP TF members. Within the DNA sequence, the regions important for binding the DNA and the ligand are also indicated. These regions are the most conserved within the members, due to their functional importance (Avram et al., 1999).

Figure 17



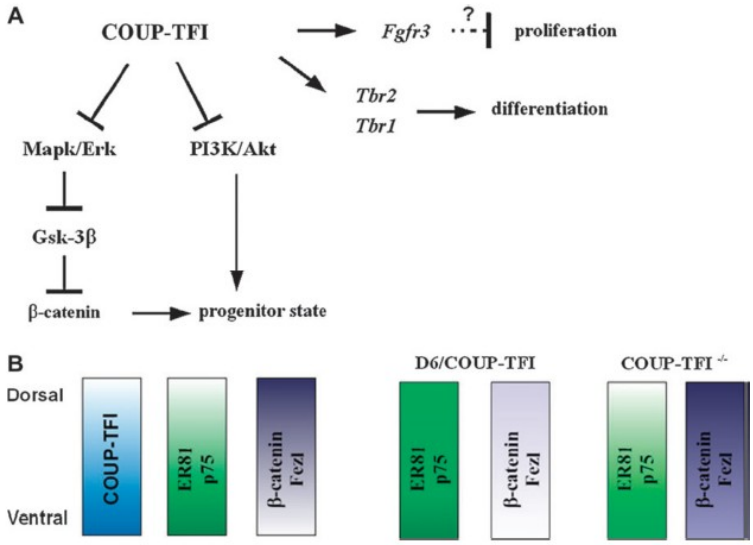
Schematic showing example of COUP-TFs studied mechanism of repression (A) and activation (B) of transcription

(LEFT – 1)Repression occurs after recruiting the co-repressors Nuclear receptor Co-repressor 1(N-CoR) and silencing mediator for retinoid and thyroid receptors (SMRT). COUP-TFs can repress expression of genes that are normally activated by RXR heterodimers that bind their related Hormone Responsive Elements (HRE).

(LEFT – 2) This repression can be achieved in a passive way, as COUP-TFs may bind RXR instead of the normal cofactor needed to activate transcription, thus competing with them.

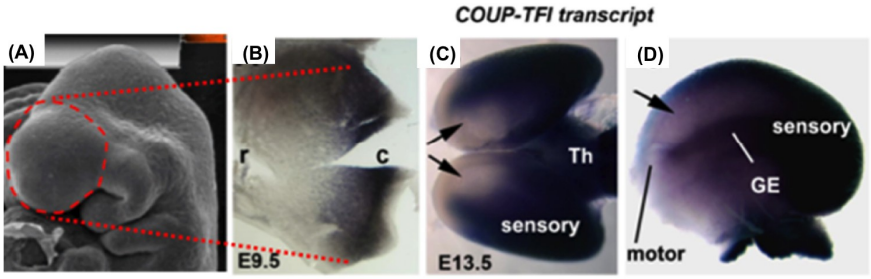
(RIGHT) The first one (1) represents a direct binding on a DR sequence followed by specific transcriptional activation, as shown for the regulation of arrestin and cholesterol 7a-hydroxylase (CYP7A). The second mechanism (2) of transcriptional activation involves a binding to the DNA and the following activation of other TF increasing their own ability to induce transcription, as demonstrated in the case of the phosphoenolpyruvate carboxykinase (PEPCK). The third (3) mechanism involves a protein- protein interaction of COUP-TFs with factors that bound to the DNA and a following transcriptional activation, as seen for the Sp1 in the Sp1 gene promoter (taken from Tsai, 2003).

Figure 18



Model for COUP-TFI function in regulating proliferation, differentiation, and patterning.
(A) COUP-TFI downregulate Mapk/Erk and PI3K/Akt activity, promoting in this way progenitor cells to leave the VZ and to differentiate either into Tbr2+ progenitors or in Tbr1+ neurons.
(B) COUP-TFI promotes ventral fate in the cortical primordium. Disruption of the COUP-TFI gradient changes dorso-ventral (D/V) molecular properties in the cortical plate, as revealed by the expression of ventral (ER81 and p75, green) and dorsal (ßcatenin and Fezl, violet) markers (taken from Faedo et al., 2008).

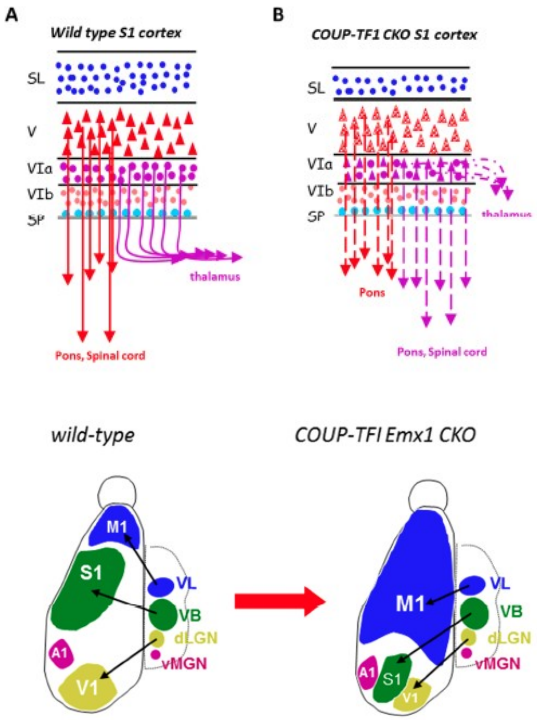
Figure 19



Whole mount in situ hybridization showing gradient expression of COUP-TFI in the developing cortex.

(A) Lateral view of a scanning electron micrograph image of a E9.5 mouse embryo in which the telencephalic vesicle is surrounded by red dashes. (B) In situ hybridization of the flattened telencephalic vesicle at early stages when COUP-TFI starts to be expressed in a high caudal-to low rostral expression domain. (C) Whole-mount in situ hybridization showing COUP-TFI expression at E13.5. Its expression gradient in the cortex is high caudally and laterally (sensory areas) and in the thalamus (Th) and low rostrally and medially (motor areas), as indicated by arrows. (D) Lateral view of the telencephalon showing the intensity of the staining that progressively reduces towards the rostro-medial domains (going from the sensory to the motor area). COUP-TFI is also expressed in a high caudal to low rostral expression domain in the ganglionic eminences (GE).

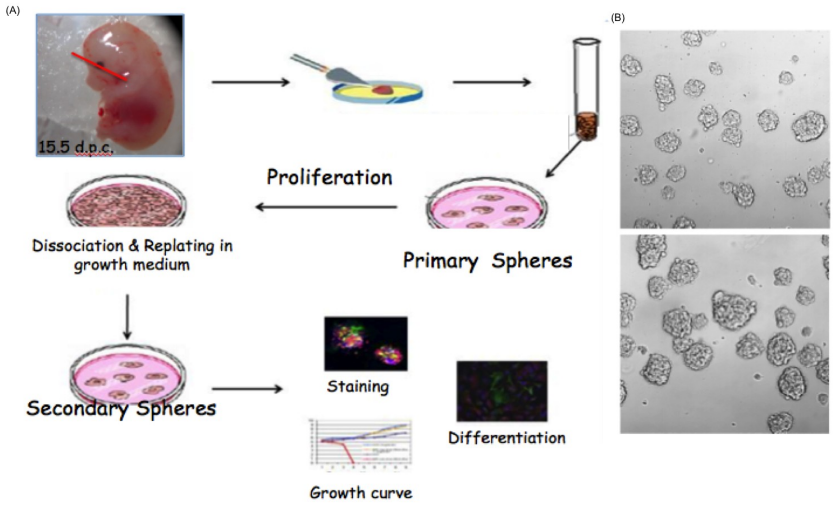
Figure 20



Outline of the main phenotype found in the COUP-TFI Emx-CKO

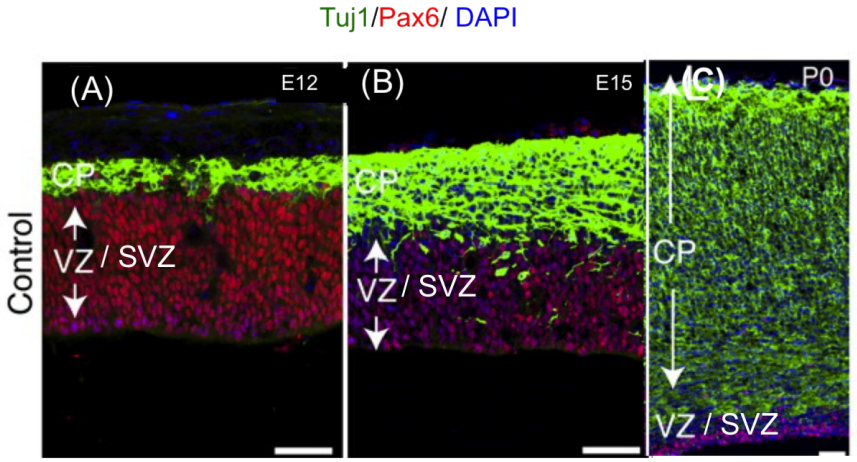
Arealization, laminar specification and connectivity are altered in the *COUP-TFI* conditional mutant (*COUP-TFI Emx-CKO*) (B) compared to control brains (A). Inactivation of COUP-TFI in the cortex shows the strongest areal phenotype studied so far, in which sensory domains are strongly reduced and shifted toward the caudal pole, while motor domains expand caudally. As a consequence also lamination is affected within the cortex, as parietal neocortex shows a reorganization of projection neurons in which a subpopulation of layer VIa project to the thalamus and ectopically to the spinal cord. Topography of thalamocortical axons is partially maintained.

Figure 21



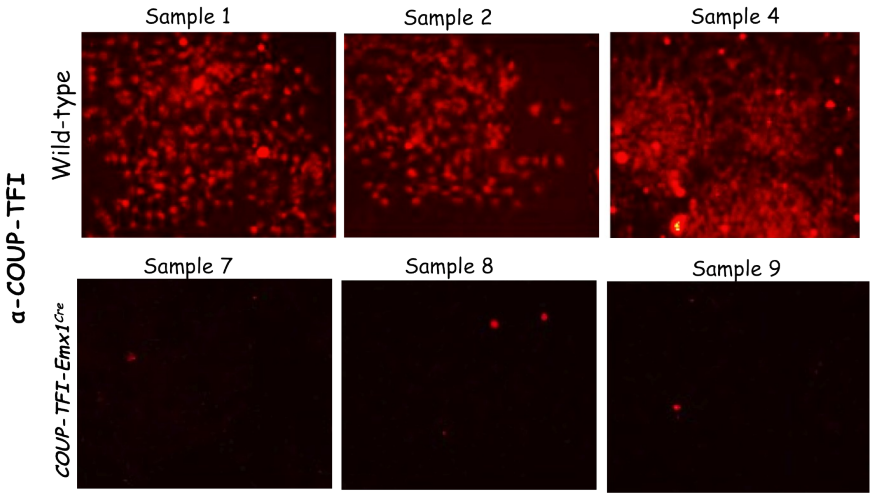
***Procedure of the neurosphere expansion assay*.**
(A) Neural/progenitor cells are isolated from the telencephalic cortex at E.15.5, excluding the ganglionic eminences (GE). After mechanical dissociation and centrifugation, single cells are cultured in appropriate conditions. Several passages are necessary to select the neural stem/progenitor population. Cells need to be harvested and subcultured in selective conditions in order to enrich the neurospheres for proliferating cells. Subsequently, it is possible to perform immunostaining on whole neurospheres, rate proliferation analysis (growth curve) and differentiation experiments on dissociated cells. (B) Example of neurosphere pictures at 20X magnification. In culture, single cells prefer to adhere to each other forming round cluster (neurospheres), which float in suspension.

Figure 22



Cortical development: proliferation versus differentiation.
Cerebral cortex at 20X magnification immunostained for Tuj1 and Pax6 to label neuronal and proliferating cells, respectively, at different embryonal stages, as indicated. Nuclei cells are stained with DAPI. The staining clearly shows how much the balance between proliferative cells in the ventricular and differentiated cells in the cortical plate is changing in thickness during development. The age of E15.5 represents a good compromise between proliferation (cells located in the VZ and SVZ) and differentiation (modified from Pereira et al., 2010).

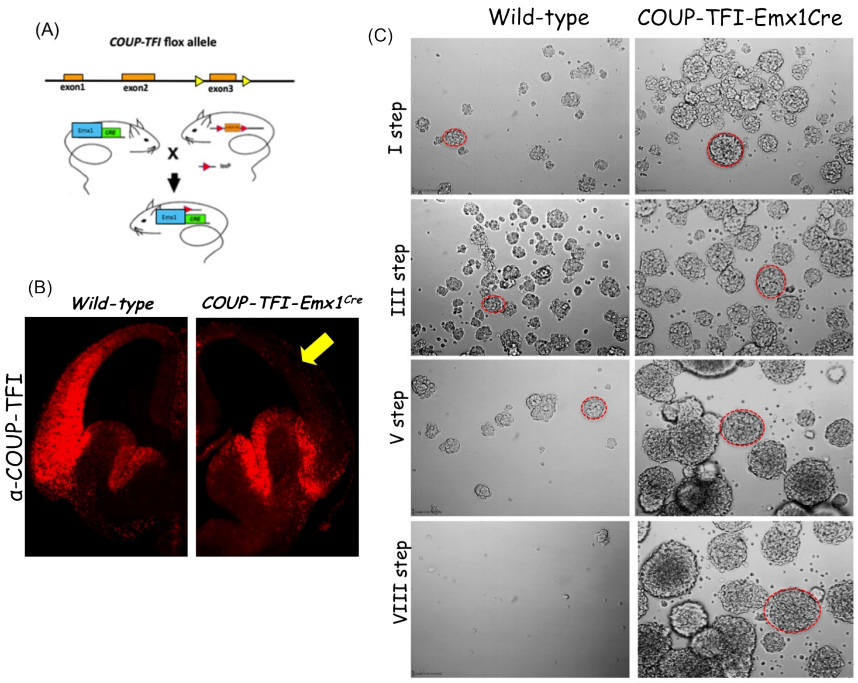
Figure 23



Absence of COUP-TFI in cortical COUP-TFI mutant neurospheres.

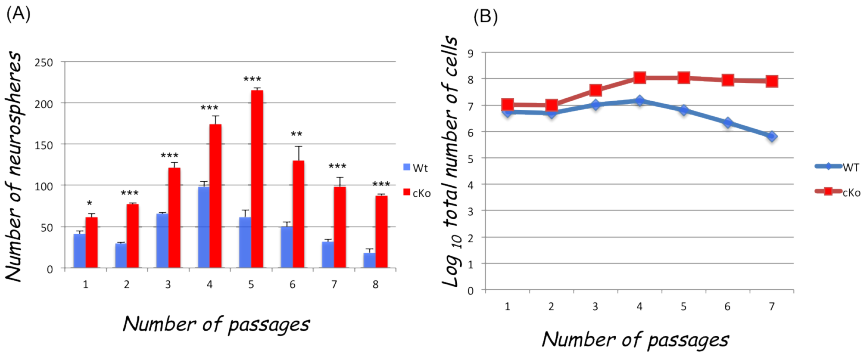
Control and COUP-TFI conditional neurospheres in culture immunostained using a COUP-TFI antibody. Soon after culturing, the neurosphere (NS) genotype is confirmed by immunofluorescence directly on the cells. After 3 days in culture, NS are seed in special dish coated with an adhesive substrate (matrigel) for few hours. Fixed for 10' with 4% PFA and stained. The first row represents wild-type samples positive for COUP-TFI, as expected. The second row confirms three mutant samples negative for COUP-TFI.

Figure 24



***COUP-TFI* controls neural stem/progenitor size and number.**
(A) Diagram showing the gene targeting strategy used to obtain the *COUP-TFI-Emx1*^{Cre} (CKO) allele after Cre recombination. The loxP sites are depicted as red triangles. Orange boxes represent exons. The mouse line positive for the *Emx-Cre* allele is mated to a *COUP-TFI floxed* mouse. The progeny will inherit both alleles. (B) Coronal sections of wild-type and homozygous embryos at E15.5 immunostained with an antibody against COUP-TFI confirm its specific deletion in the cortex. (C) Neurosphere (NS) size analysis of wild-type and CKO mutant. The neurosphere size is strongly increased in the mutant compared to the wild-type (red dotted line) already from the first passages. Wild-type NS maintain a relatively homogeneous size until the 8th step, when they go through exhaustion. Conversely mutant cells show a bigger size for the entire duration of the experimental procedure.

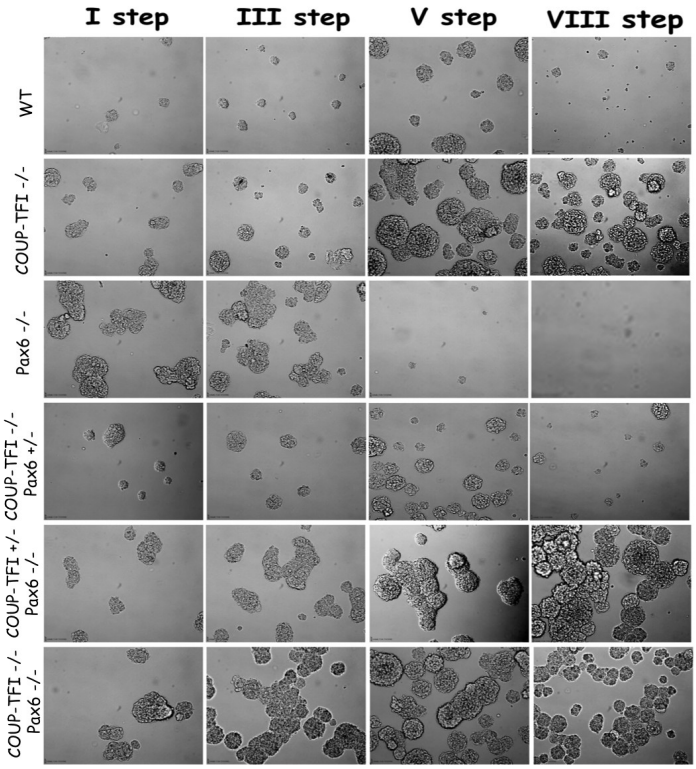
Figure 25



COUP-TFI is required in restricting the neural stem/ progenitor pool.

(A) Neurospheres (NS) counted every 3 days (3 days = 1 passage). Wild-type samples reveal an increase in the number of NS until they reach the pick at the 4th passage, after that NS start to decrease. On the contrary, conditional inactivation of COUP-TFI leads to an increased number of NS at each passage reaching a pick at the 5^{h} passage. For the following steps mutant neurospheres begin to diminish but remain always significantly higher when compared to the wt. *P<0.05, **P<0.01, ***P<0.005. (B) Semi-logarithmic curve indicating the rate of growth of dissociated NS cells reveals that in the wild-type, the total number of cells is first high but decreases after the 5th passage until full exhaustion. Differently, the number of cells is always higher in the cKO mutant than in control, and especially between the 4th and 5th passage in which mutant total cells have their maximal growth. In the following passages mutant cells they continue to growth in stable number.

Figure 26

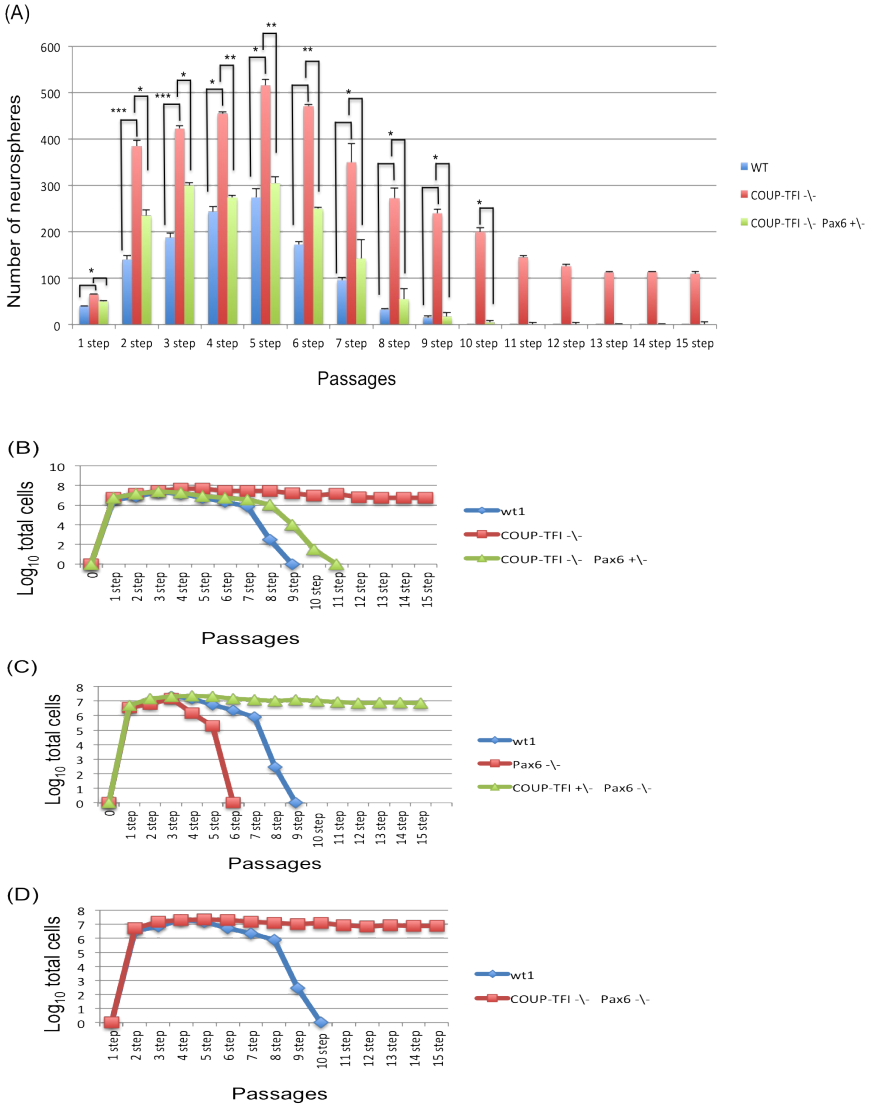


COUP-TFI versus Pax6 different dosage: Size analysis

Neurosphere size analysis for the wild-type reveals a uniformed range of size, showing a bigger size only around the 5th step, but soon after they start to decrease until they are exhausted around the 8th step. *COUP-TFI null* mutant neurospheres, similarly to *COUP-TFI* conditional mutant ones (see Figure 24), show a substantial increase in size already from the beginning reaching a pick from the 5th step onwards. Conversely, mutant Pax6 neurospheres stop to proliferate after 15 days in culture (5th passage) and cells present a characteristic chain-like structure, maybe due to adhesion defects proper of *Pax6 null* mutant described in literature (Ou, Lowes et al. 2010). Maintaining a *COUP-TFI null* background and decreasing Pax6 levels (in COUP-TFI-/-, Pax6 +/-) makes cells recovering almost to the same size of wild-type samples. Similarly, a *Pax6 null* neurosphere with decreased COUP-TFI level makes cells surviving, even if they continue to have adhesion defects. In case both transcription factors are missing, the *double null* phenotype

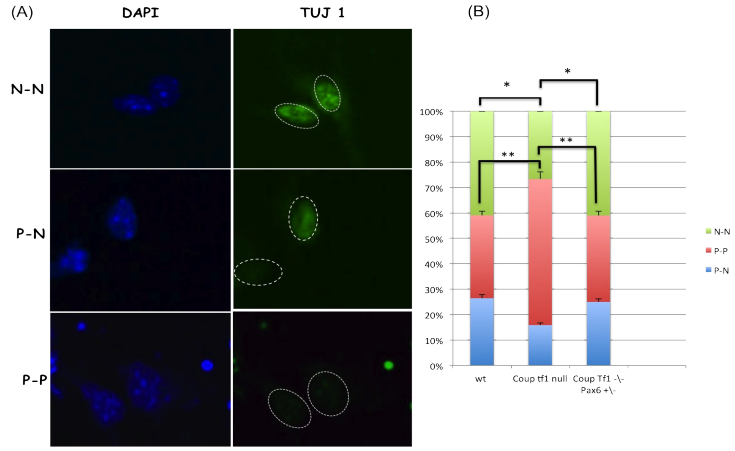
shows NS that continue to proliferate, but cells maintain a severe chain-like structure.

Figure 27


(A)
Number of neurospheres
600
500
400
300
200
100
0
1 step
2 step
3 step
4 step
5 step
6 step
7 step
8 step
9 step
10 step
11 step
12 step
13 step
14 step
15 step
Passages
WT
COUP-TFI -\-
COUP-TFI -\- Pax6 +\-
(B)
Log10 total cells
wt1
COUP-TFI -\-
COUP-TFI -\- Pax6 +\-
Passages
(C)
Log10 total cells
wt1
Pax6 -\-
COUP-TFI +\- Pax6 -\-
Passages
(D)
Log10 total cells
wt1
COUP-TFI -\- Pax6 -\-
Passages

Growth Curve analysis of the number of neurospheres and total cells.
(A) Chart indicating the total number of neurospheres (NS) counted at each passage (1 passage = 3 days in culture). The analysis shows a sharp increase of neurospheres in the complete mutant for COUP-TFI, compared to the wild-type. By decreasing Pax6 levels in a *COUP-TFI null* background, (*COUP-TFI -/- / Pax6 heterozygote*), the abnormally high number of NS is rescued almost to normal range, with the slight difference that cells stop proliferating one passage later than controls (9^{th} versus the 10^{th} step). The analysis on the total NS number has been possible only on these 3 genotypes, since in the other genotypes in which Pax6 is inactivated in both alleles, single neurospheres with their chain-like morphology were too difficult to count. Bars indicate the couple of samples considered for the statistical analysis. *P<0.05, **P<0.01, ***P<0.005. (B) Analysis of the rate of growth of the NS after dissociation in single cells for the wild-type control, *COUP-TFI null* mutants and *COUP-TFI null/Pax6 heterozygotes*. The semi-logarithmic chart reveals, a similar trend in the *COUP-TFI null* mutant than previously observed in the *COUP-TFI CKO*: the number of total cells is higher than the control and especially between the 4^{th} and the 5^{th} step in which mutant cells show their maximal peak of expansion. In this experiment, the analysis has terminated at the 15^{th} step (45 days), revealing that after reaching the peak, cells decrease a little bit, until they become stable and continue to proliferate. Similarly to what happens in the NS size analysis, lowering Pax6 levels (*COUP-TFI null/Pax6 heterozygotes*) rescued the *COUP-TFI null* phenotype to almost normal values. (C) The same analysis has been performed by comparing the *Pax6 null* phenotype and the effect in decreasing COUP-TFI levels in a *Pax6 null* background (*COUP-TFI heterozygotes/ Pax6 null*). *Pax6 null* cells, as expected, stop to proliferate after 6 passages. By decreasing COUP-TFI dosage in the *Pax6 null* background (*COUP-TFI heterozygotes/ Pax6 null*), cells survive and are pushed in an active state of proliferation. (D) Differently from what described so far, the *double null* shows a mixed phenotype: high rate of proliferation similarly to COUP-TFI constitutive mutants and a severe chain-like structure proper of the Pax6 mutant cells.

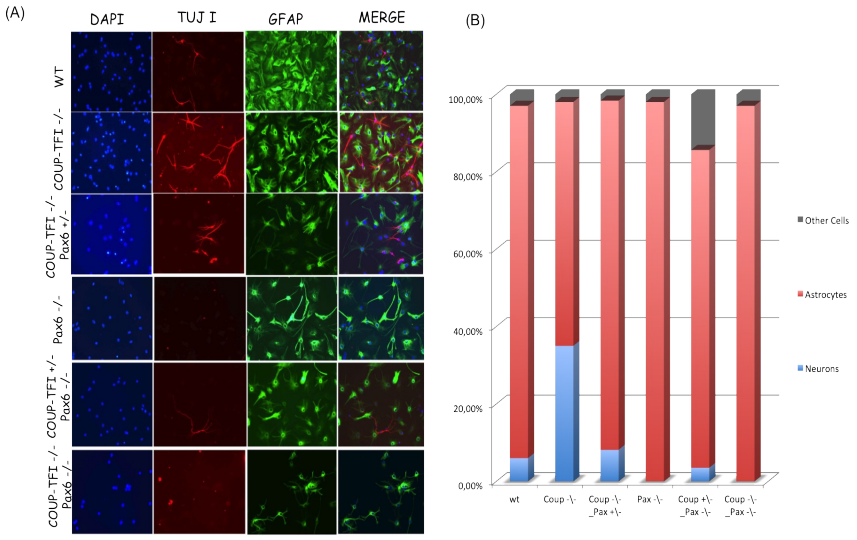
Figure 28



Pair-cell analysis: an in vitro assay to evaluate symmetric and asymmetric divisions.

(A) Stem/progenitor cells are isolated from cortices at E15.5 and plated at clonal density. After 24h in culture, cells are fixed in PFA 4% for 20' and stained for the early differentiating marker TuJ1. The technique, called "pair-cell analysis", consists in following the fate of two dividing cells by staining them with TuJ1 that selectively labels the neuronal progeny. Couple of daughter cells were analysed. When both dividing cells are negative for Tuj1, they are considered to divide in a *symmetric proliferative mode (P-P)*; when both cells are positive for Tuj1, they are dividing in a *symmetric neurogenic mode (N-N)*, and when only one cell is positive for Tuj1, the mode of division is considered to be *asymmetric (P-N)*. (B) The chart shows a statistically significant increase of symmetric divisions in the *COUP-TFI null* cells when compared to the control: here, the majority of daughter cells (56± 2,45%) are both negative for Tuj1 marker, meaning that they are neural stem/progenitor cells. Consequently, neurogenic (both cells positive for Tuj1) and asymmetric divisions (only one of the daughter cell is labeled) result strongly diminished (respectively 28± 1,63% and 16± 2,86%). Differently, the *COUP-TFI null/Pax6 heterozygous* mutant cells show an almost normal rate of symmetric proliferative (34± 1,63%) versus symmetric neurogenic (41± ,82) and asymmetric (25± 0,91%) division, when compared to wild-type cells. Brackets indicate the samples compared for statistical analysis. *P<0.05, **P<0.01, ***P<0.005.

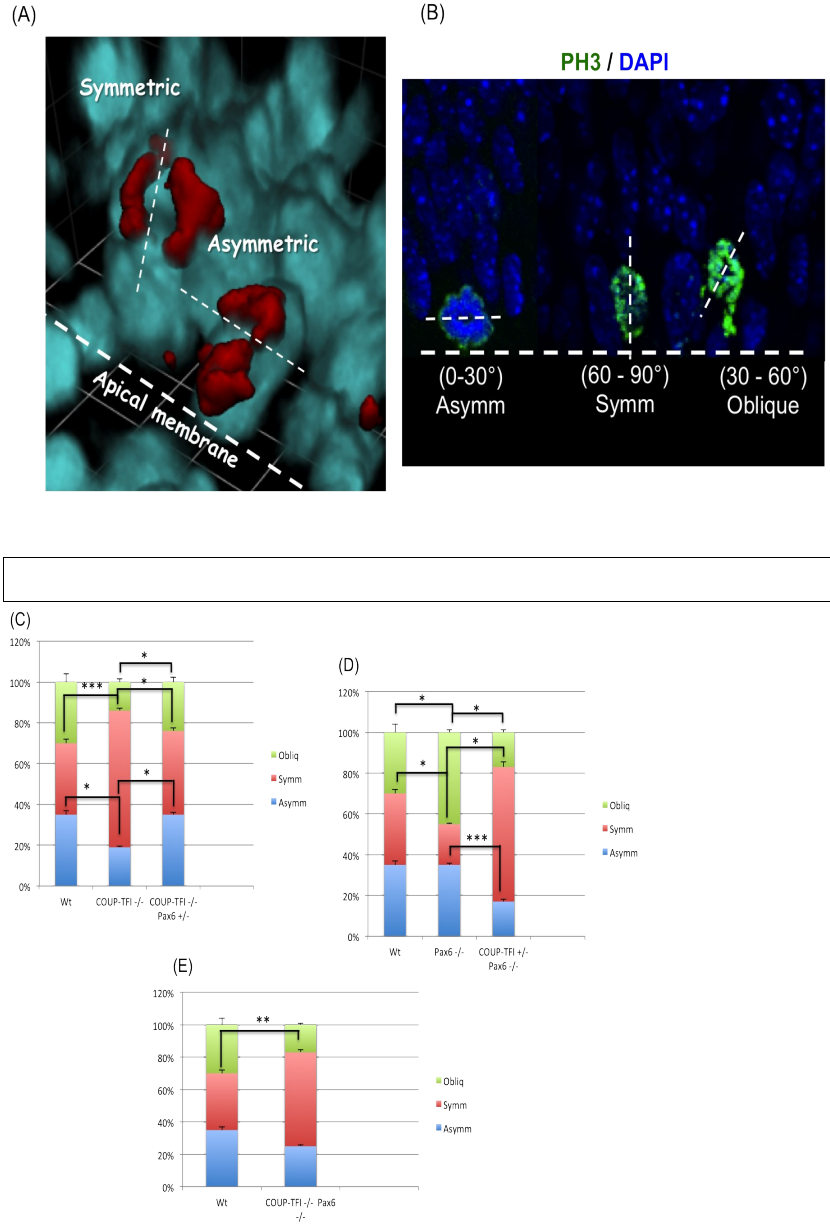
Figure 29



Neuronal differentiation.

(A) Neurospheres (NS) will differentiate to neurons, astrocytes and other types of cells (mainly oligodendrocytes) under appropriate culture conditions. After dissociation, cells are plated at clonal density, and after 2 days growth factors are removed and differentiation can be followed for 15 days. At the end of the time point, differentiated cells are processed for immunofluorescence using TuJ1, GFAP (glial fibrillary acidic protein) and O4 antibodies to identify neurons, astrocytes, and oligodendrocytes, respectively. All genotypes show a high number of astrocytes and very few neurons and oligodendrocytes , which makes it difficult to detect statistically significant differences between the three cell types. Studies indicate that in the neurosphere assay, gliogenesis becomes more prevalent than neurogenesis (Conti and Cattaneo 2010). Nevertheless, I have tried to count the three populations in the different genotypes (B). Despite the unbalance between the three populations, *COUP-TFI null* mutant cells have a number of neurons (30% more than the control) that is rescued to quite normal levels after lowering Pax6 levels (*COUP-TFI null/ Pax6 heterozygotes*): the amount of neurons is only about 2% more than the wt. No neurons could be detected in *Pax6 null* mutants, while they start to appear again after lowering COUP-TFI level (*COUP-TFI heterozygotes/ Pax6 null*): the amount of neurons is slightly increased, even if not comparable to control levels. In double null mutants, no neurons could be detected.

Figure 30

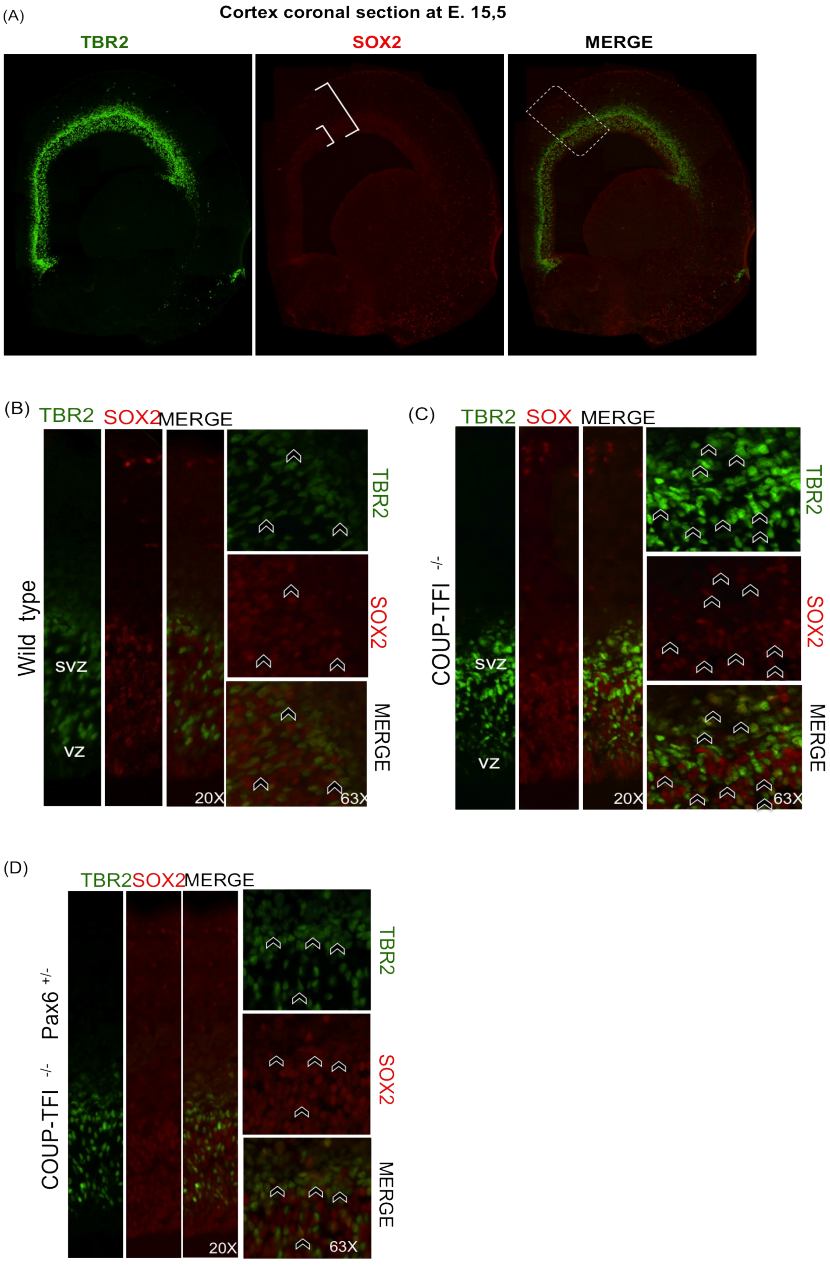

(A)
Symmetric
Asymmetric
Apical membrane
(B)
PH3 / DAPI
(0-30°)
Asymm
(60 - 90°)
Symm
(30 - 60°)
Oblique
(C)
120%
100%
80%
60%
40%
20%
0%
Wt
COUP-TFI -/-
COUP-TFI -/-
Pax6 +/-
Obliq
Symm
Asymm
(D)
Wt
Pax6 -/-
COUP-TFI +/-
Pax6 -/-
(E)
Wt
COUP-TFI -/-
Pax6
-/-

Cleavage plane analysis *in vivo*.

(A) 3-D reconstruction from a PH3 immunostaining showing the different cleavage plane orientations. Cells in symmetric divisions, show the cleavage plane (dot white line) that is perpendicular to the apical surface. Cells in asymmetric divisions present a cleavage plane parallel or oblique (not show) to the apical surface. (B) Mitotic analysis by immunostaining cortical progenitors with PH3, a marker exclusively expressed during mitosis. The progenitors taken here as an example, represent the cleavage plane angle evaluated during the analysis to discriminate between the different kinds of division and group them into three main classes: 0-30°, 60-90°, 30-60° correspond respectively to asymmetric, symmetric and oblique divisions.

(D-E-F) Statistical analysis of the cleavage planes in the different COUP-TFI and Pax6 genotypes. The analysis in wild type cortices reveals a sort of equilibrium between the three kinds of divisions with a ratio of (35± 2,1%)**:** (35± 2,1%)**: (30**± 4,1)% between vertical, horizontal and oblique divisions, respectively. (D) The majority of cells (70±1,2%) in the cerebral cortex of *COUP-TFI null* mutant cortices divide vertically, implying an increase in symmetric proliferative divisions. Oblique and horizontal divisions are detected as 10± 1,6% and 20± 0,41% of the total mitotic cells, respectively. The situation is close to normal in the mutant *COUP-TFI null/Pax6 heterozygotes*, which showed a ratio between vertical symmetric, horizontal asymmetric and oblique divisions of 41± 1,6%, 35± 1,2% and 24± 2,4%, respectively. (E) Regarding the *Pax6 null* genotype, cells have an increase of oblique divisions (45± 1, 2 at the expense of symmetric divisions (20± 0,4%), whereas the asymmetric neurogenic population does not change in the absence of Pax6 (35± 0,8%). By decreasing Pax6 expression in *COUP-TFI null* cortices (e.g. in *COUP-TFI heterozygous/Pax6 null* cortices) symmetric divisions are expanded (66± 2,4%), similarly to double *COUP-TFI/Pax6 null* cortices (58± 1,6%). Brackets indicate the samples considered for the statistical comparison. *P<0.05, **P<0.01, ***P<0.005.

Figure 31


(A)
Cortex coronal section at E. 15,5
TBR2
SOX2
MERGE
(B)
TBR2 SOX2 MERGE
Wild type
SVZ
VZ
20X
63X
TBR2
SOX2
MERGE
(C)
TBR2 SOX MERGE
COUP-TFI -/-
SVZ
VZ
20X
63X
TBR2
SOX2
MERGE
(D)
TBR2SOX2MERGE
COUP-TFI -/- Pax6 +/-
20X
63X
TBR2
SOX2
MERGE

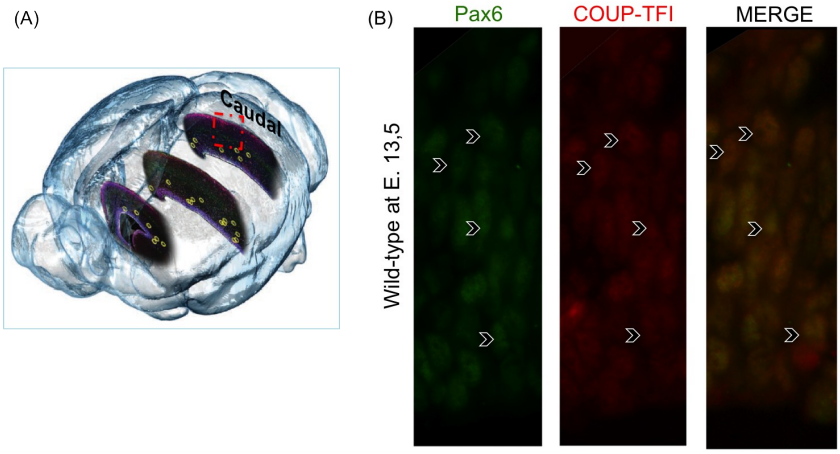
Apical and basal progenitors marker analysis.

(A) Coronal sections of E 15.5 embryos immunostained for Tbr2 and Sox2 to label the intermediate/basal and apical progenitors, respectively. The neocortical region squared in the merge represents the area considered for the analysis. The brackets in the central picture represent the measure of the radial extension of the Sox2 signal in relation to the entire extension of the cortex. The ratio between these two measurements will be considered in all genotypes. (B) Neocortical coronal sections at E15.5 are immunostained with the markers Sox2 (in red) and Tbr2 (in green) to label apical and basal/intermediate progenitor cells, respectively. Embryonic SVZ is rich in basal/intermediate progenitors. As expected, wild-type SVZ shows high expression of Tbr2 and few Tbr2+ can be seen migrating into the intermediate zone (IZ). (C) Tbr2+ cells are decreased in the SVZ of *COUP-TFI null* cortices and several Tbr2+ cells are stuck in the upper SVZ at the border with the IZ. (D) In the *COUP-TFI null/Pax6 heterozygotes*, where Pax6 expression is lowered, Tbr2+ cells stuck in the upper SVZ are diminished, but Tbr2 expression is not rescue. (B) The apical progenitor marker Sox2 is normally expressed in the VZ of the wild-type embryos with a few cells in the SVZ. (C) In mutant cortices null for COUP-TFI, apical progenitors (Sox2+ cells) are strongly increased. Sox2 areal-thickness was evaluated by considering the ratio between the Sox2+ thickness and the entire extension of the cortex from the apical to the pial surface (see the Sox2 section (A)):

$$\frac{\text{Sox2 extension}}{\text{Cortex extension}} \equiv \%\ \text{Sox2 areal thickness}$$

Area positive for Sox2 resulted increased of 6.1% in *COUP-TFI null* cortices compared to controls. Differently from Tbr2, Sox2 expression is decreased in *COUP-TFI null/Pax6 heterozygotes* although not to complete normal rates. Moreover counting the double positive cells Sox2/Tbr2, it comes out there are more cells double positive in the *COUP-TFI null* compared to the controls. Even if the number of double positive cells is lower in the *COUP-TFI null/Pax6 heterozygotes* the phenotype is once more not completely recovered.

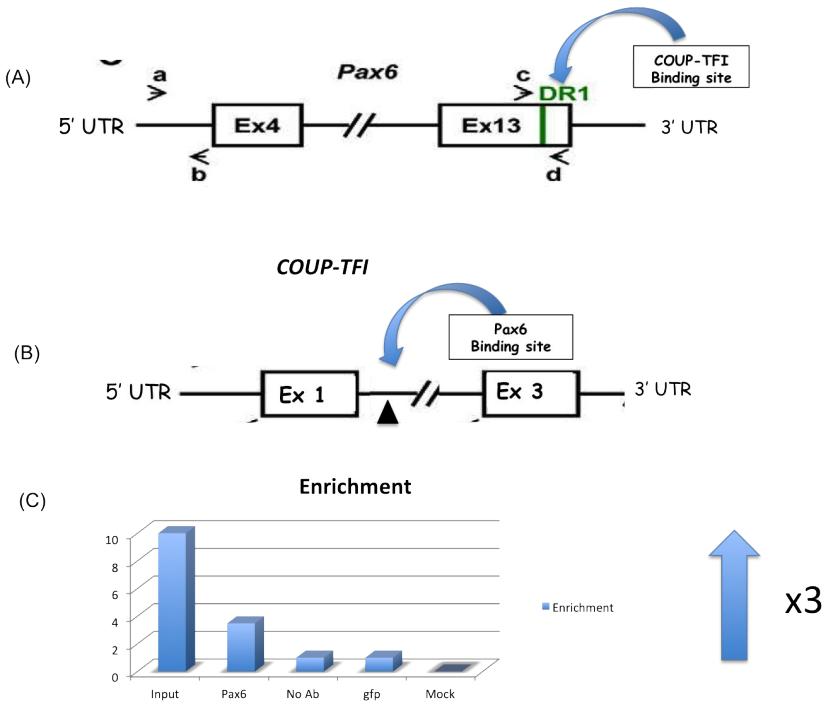
Figure 32



Expression levels of individual wild type cells immunostained for COUP-TFI and Pax6

(A) Schema of a murine brain in 3D.The neocortical anterior-posterior axis, the anterior medial and caudal part of the cortex are spatially represented. The analysis is done on the caudal part. (B) Coronal sections at 40X magnification. Wild-type tissue from embryos at E13.5 is stained with an antibody against COUP-TFI (in red) and Pax6 (in green). The arrowheads indicate the cells double positive both for COUP-TFI and Pax6. The intensity of the fluorescence within the same cells show complementary expression levels of COUP-TFI and Pax6: to a high fluorescence intensity for Pax6 corresponds a lower intensity for COUP-TFI. However, this is not observed in all cells, indicating a dynamic process occurring during corticogenesis.

Figure 33



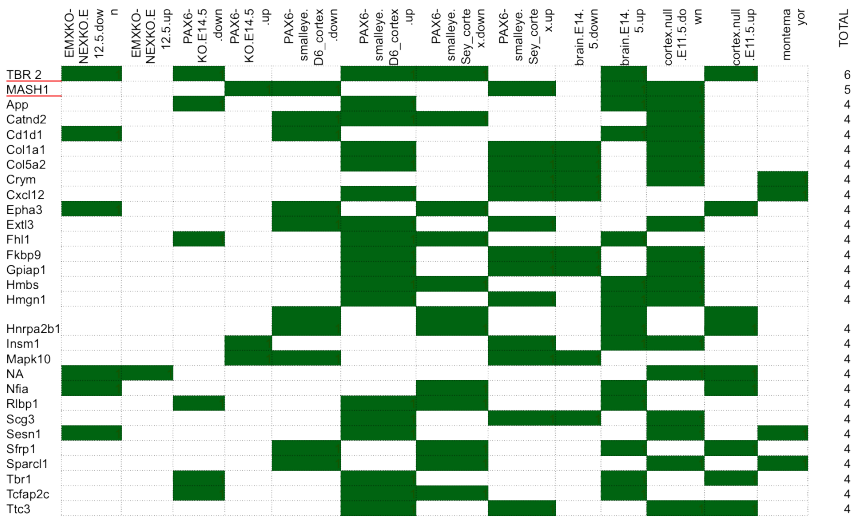
***COUP-TFI* and *Pax6* reciprocally bind to each other.**
(A) Direct binding of *COUP-TFI* on the *Pax6* locus. Highly evolutionarily conserved DR1 binding site (TGTTCACAGTCCA) located at the 3'-UTR region of the mouse Pax6 locus (taken (Tang, Xie et al. 2010)). (B) Direct binding of *Pax6* on the *COUP-TFI* locus. Evolutionary conserved *Pax6* binding site (CGTATCATTTTAT) is distant more or less 1000bp from exon one of *COUP-TFI*. (C) Real-Time PCR representation of a ChIP experiments aims to determine if *Pax6* binds to this specific *COUP-TFI* genomic region. The analysis has revealed an enrichment of the binding site about three times more than control, confirming the hypothesis that *Pax6* is recruited to the regulatory region of the *COUP-TFI* gene. The input represents the DNA that non-specifically binds the resin in the absence of antibody; no Ab is the sample without adding antibodies; gfp represents an aspecific antibody used to confirm the specificity of the result obtained; mock is a control composed by the only solution of elution, there is nothing inside (DNA and antibody); *Pax6* is the antibody of interest.

Figure 34

(A)

Downstream genes in mitotic phase Coup-TFI
Genes down-regulated in PAX6 -/- against wt
Genes up-regulated in PAX6 -/- against wt
D6-cortex down; PAX6 negative regulation
D6-cortex up; PAX6 positive regulation
Sey-PAX6 mutant cortex down; PAX6 positive regulation
Sey-PAX6 mutant cortex up; PAX6 negative regulation

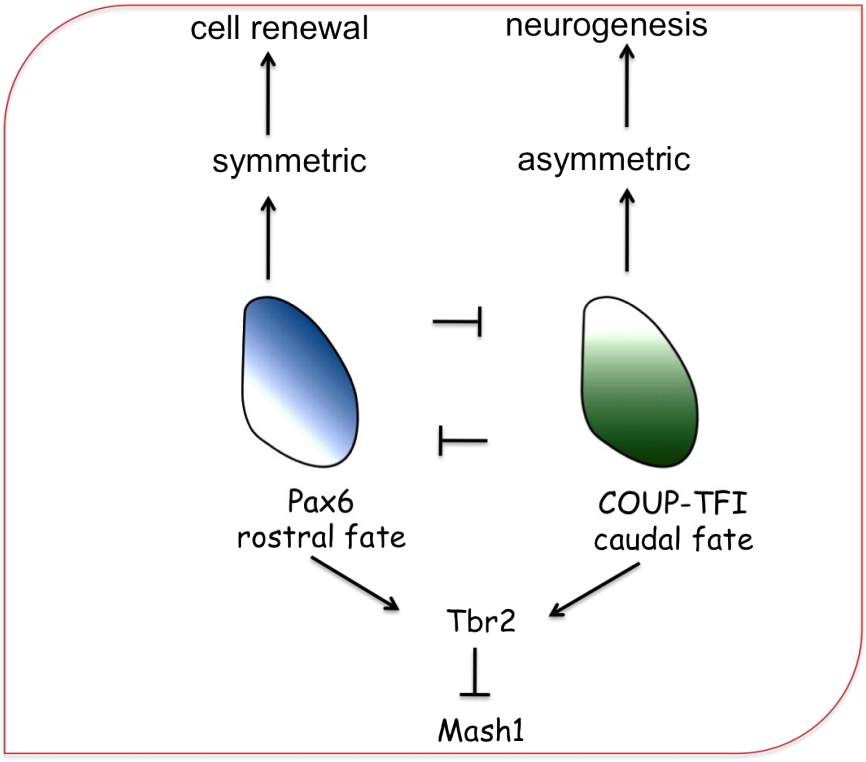
(B)



Micro array analysis shows possible common target genes of COUP-TFI and Pax6

(A) Schematic representation of a list of putative COUP-TFI downstream genes compared with other *Pax6*-specific microarray lists available in literature. The first microarray in the list was obtained with the help of the Studer lab and the functional genomic platform of the University of Nice Sophia-Antipolis (http://www.genomique.info/joomla_2.5.9/). (B) The table shows the common genes obtained by comparing microarray data from *COUP-TFI* and *Pax6* genes that have been listed according to the highest probability to be shared by both genes. This analysis was performed with the help of an external bioinformatics core.

Figure 35



Molecular model.
During areal patterning, Pax6 and COUP-TFI control their precise expression levels by co-repressing each other and influence the rate between stem cell self-renewal and progenitor commitment in an areal-dependent way. Both genes might act on similar target genes that ultimately control the balance between proliferation and differentiation.

CHAPTER 1

1. INTRODUCTION

1.1 The mammalian neocortex in the CNS

During development of the mammalian central nervous system (CNS), after the closure of the neural tube, which takes place at approximately embryonic day 7 (E7) in the mouse, a series of folding and expansions lead to the birth of the *prosencephalon*, the mesencephalon and the *rhombencephalon*, (going from rostral to caudal). Moreover, caudally to the *rhombencephalon* it is already possible to observe a structure that will become the spinal cord (SC). After these first 3 vesicles phase (E8 in the mouse), it follows a second one, characterized by a 5-vesicle structure. In this phase, the *rhombencephalon* becomes *s*ubdivided into *myelencephalon* caudally, and *mesencephalon* rostrally, while the *prosencephalon* is subdivided into *diencephalon* caudally and *telencephalon* rostrally (E8.5 in the mouse)(**Figure 1**). The *myelencephalon* will give birth to the *bulb*, the *mesencephalon* to the *pons* and *cerebellum*, and the *diencephalon* to the *thalamus*, *hypothalamus* and the neural tissues of the retina. The *telencephalon* will give rise ventrally to the *basal nuclei*, called the *basal ganglia*, rostrally to the *olfactory bulb*, and dorsally to the *archicortex, the paleocortex* and the neocortex (**Figure 1**).

At birth, the *telencephalon*, like all the neural tube, is constituted and delimited by a thin layer of pseudostratified cells called neuroepithelium (NE). At early stages the NE undergoes a significant proliferation phase, which will expand the pool of progenitors. This is also the stage in which dorsoventral patterning of the telencephalon and the first rough subdivision of the future cerebral cortex into three distinct regions (archi-, paleo- and neocortex) takes place. The ventral domain becomes the s*ubpallium* or *ganglionic emincence* (GE), which in the adult gives rise either to the *striatum*, the most medial domain of the cortex, or will become *archicortex, which* will develop to the *enthorinal* cortex, the *retrospenial* cortex, the *subiculum* and the *hippocampus*, while the dorsolateral and the most ventral regions of the cerebral cortex will give rise, respectively, to the neocortex and the *piriform* cortex (*paleocortex*) (Rubenstein and Beachy 1998; Kaas 2011).

In my project, I have been focused on a particular region (structure) of the prosencephalon, the neocortex, which is deputed to higher functions like thoughts, consciousness and voluntary movements. It is also the part of the brain that during mammalian evolution developed the largest amount of variations in size, shape and complexity. From the small mammalian ancestors, it grew in all the variety that we can see nowadays from small rodents up to primates and humans. However, despite their remarkable differences in dimensions and elaboration, mammalian neocortices share common features, functions and ontogenetic mechanisms. In all the mammals the neocortex receives the inputs coming from all the sensory systems in order to process them and endorse behavioral responses (Zhou and Walthall 1998).

A basic characteristic of the neocortex is the radial organization in a six-layer structure (**Figure 2**). Variation in thickness between different mammalian families is estimated to be up to five fold from the thinnest to the thickest, however the most striking changes among mammals can be seen in the surface area which can vary up to 2000 times from the smallest to the biggest. This amazing expansion led to the development of a gyriform cortex that limited the volume occupied by this structure initially constituted by a simple unfolded sheet. Pioneer neuro-anatomists subdivided the neocortex into different areas on the basis of their functions. This was initially studied in human patients with localized injuries in the brain. These subdivisions were maintained, although with variations in size and complexity, in all the mammals. The main areas were thus identified as: motor area, localized rostrally, deputed to elaborate movements on the base of sensory information, somatosensory area, deputed to collect and elaborate information on the tactile perceptions, visual areas, deputed to collect and elaborate visual information from the eyes, and auditory areas, deputed to collect and elaborate information from the ears. Each one of these is also subdivided in subtypes with different specific functions. Moreover, the discovery of area-specific molecular markers and the fine histological analysis showed that independently from their complexity, neocortical areas maintain their

relative positions in all mammalian species suggesting a common genetic mechanism orchestrating the formation of these areas.

1.2 Development of the layered structure of the neocortex

The initial development of the nervous system depends on an intricate interplay of inductive signals and cellular movements. In addition to the early establishment of regional identity and cellular position in the brain, substantial migration of neuronal precursors is necessary for the subsequent differentiation of distinct classes of neurons, as well as for the formation of specialized patterns of synaptic connections. The first wave of migrating neurons gives rise to the preplate (PP) a primordial layer which, later, will be split into the marginal zone (MZ) and the subplate (SP) (**Figure 3**), two important structures in neocortical development. The PP comprises also Cajal Retzius (CR) cells, the first excitatory neurons populating the cortex. They begin to invade this structure from E10.5 through tangential migration, from the cortical *hem* and pallial *septum*, two regions located at the caudo and rostro-medial pallial-subpallial boundaries (PSB), respectively, and from the *antihem* (or ventral pallium), which lies at the lateral PSB (Super and Uylings 2001). After a radial migration, guided by radial glial cells (RGCs), the CRs are found adjacent to the pia where they express *Reelin*, a gene that triggers an important pathway for neocortical lamination (Aboitiz, Morales et al. 2003).

The first cortical projection neurons, born at ~E11.5 in mouse, migrate to settle as a layer within the PP, thus forming the nascent cortical plate (CP), which subsequently develops into L2-L6 of the postnatal neocortex (Marin-Padilla 1978). Incoming CP neurons split the PP into the superficial marginal zone (MZ), which develops into L1 of the postnatal cortex, and the deeper subplate (SP), which is situated below L6 (Molliver, Kostovic et al. 1973).

The MZ, on one side, represent one of the targets of the callosal and thalamocortical axons (TCA), and harbors the apical tufts of the pyramidal neurons from all cortical layers. The SP, on the other side, is thought to give positional information to the incoming TCA. This process is crucial to direct sensory inputs, like visual or auditory stimuli, to the correct elaboration centers (areas) of the mammalian

neocortex. The neurons of the MZ and the SP, the first to achieve morphological maturity and form synapses, are thought to play crucial roles in the migration and synaptogenesis of CP neurons, as well as in the formation of proper cortical efferent and afferent projections (Molliver, Kostovic et al. 1973; Rakic 1976; Kostovic and Rakic 1980; Kostovic and Rakic 1990; Allendoerfer and Shatz 1994).

Once newborn neurons reach the pial surface, they detach from the glia and attach their leading process to the pia moving to their target position through somal translocation. Both the processes are made possible by the Reelin pathway and constitute what is called "glia-guided" migration which allows both an ordered migration of neurons to the CP and new incoming neurons, born later, to interpose between earlier settled neurons and the pia (**Figure 4**). Thanks to the "glia-guided" migration the lamination of the neocortex takes place in an inside-out fashion leading to the formation of the lower layers first and of the upper layers (the most superficial) later. These dynamic movements have been elegantly described by birth-dating experiment using tritiated Thymidine (*H3 Thymidine*) (McConnell and Kaznowski 1991). Moreover, these experiments showed that the "inside-out" formation of cortical layers is typical of mammals, whereas in the dorsal cortex of reptiles, from which the neocortex is supposed to derive, these events happen according to an opposite trend, "outside-in", so that the order of layers is inverted. This is probably due to a different responsiveness of mammalian and reptilian cells to the *Reelin* signaling (Bar, Lambert de Rouvroit et al. 2000).

1.3 The generation and classification of neocortical projection neurons

Cortical projection neurons (PN) are the major population of neurons in the neocortex as they are approximately 75% of all neocortical neurons. They are born in the dorsal germinal zone of the telencephalon, the ventricular and subventricular zones (VZ and SVZ) (Angevine and Sidman 1961) and then undergo radial migration through the intermediate zone (IZ), guided by radial glial cells (RGCs), to reach their final destination in the mantle layers (Rakic, Goldman-Rakic et al. 1988). As neurogenesis progresses, diverse

subtypes of projection neurons are generated sequentially and their migration into the mantle layers occurs in an inside-first, outside-last manner. PN are excitatory-type of neurons that express glutamate as a neurotransmitter. They have different features that allow them to classify mainly in two groups: the *commissural* and the *corticofugal* neurons. Since the molecular characterization is still not very clear, the main method used to classify them is based on the anatomical characterization of their axons and dendrites and on their targets (**Figure 5**).

Commissural neurons are so called because their projections constitute the three major cortico-cortical commissures: *anterior, posterior and callosal* (forming the Corpus Callosum, CC). The best-characterized commissural neurons are PN projecting to the *CC*. They are mainly localized in layers II/III and V and have small/medium pyramidal bodies. They can be subdivided into three subgroups depending on their axon projection patterns: single contralateral projecting neurons or *callosal projection neurons* (CPN) (II/III, V, VI layer), CPN with ipsilateral frontal projection (layer V). The last subtype is the CPN with striatal projection (layer Va) (**Figure 5**) (Molyneaux, Arlotta et al. 2007; Fame, MacDonald et al. 2011).

The second group of projection neurons, the *corticofugal* neurons, is so called because they project their axons outside the cortex, and their targets can be classified in two different subtypes. The first type are the *corticothalamic* neurons (layer VI and few in layer V), which project their axons into different nuclei of the thalamus depending on the area from which they originate (**Figure 5**) (Briggs and Usrey 2008). The second category of *corticofugal* is that of the *subcerebral* projecting neurons, which have the largest pyramidal body. They are classified depending on their targets: c*orticotectal projection neurons* (CTPN) (layer V) are mainly located in the visual area and project to the *tectum* (they also have a secondary collateral axon that targets the rostral p*ons*); the c*orticopontine* (layer V), that project principally to the *pons*; the last subtype are the corticospinal motor neurons (CSMN) (layer Vb) which project to the spinal cord (**Figure 5**). This population is mainly located in the sensorimotor area, which in the mouse is the

area located between the somatosensory and motor area M1. They project mainly to the spinal cord and are crucial for the enacting of the voluntary movements (Molyneaux, Arlotta et al. 2007; Aronoff, Matyas et al. 2010).

1.4 The radial unit hypothesis

During the sequential generation of projection neurons, the neurons that will occupy the deepest layers of the future six-layered neocortex are generated first, followed by those that will ultimately be established in more superficial layers through radial migration (Angevine and Sidman 1961; Rakic 1974; Frantz and McConnell 1996).

The radial migration of neurons into the neocortex is also ordered. Postmitotic neurons generated within the same site in the ventricular zone arrive successively at the cortical plate, where they pass by each other and become arranged vertically in the form of cell stacks, named ontogenetic or radial columns (**Figure 6**) (Rakic 1978; Rakic 1988). Thus, the radial unit consists of cells that originate from several clones that share their birthplace at the same place in the ventricular zone, migrate along a common pathway and finally settle within the same column. The radial unit hypothesis postulates that the size of the cerebral cortex depends on the number of contributing radial units, which in turn depends on the number of founder (progenitor) cells (Rakic 1988; Rakic 1995). According to this hypothesis, the number of radial columns determines the size of cortical surface, whereas the number of cells within the columns determines its thickness (**Figure 6**). Ultimately, these proliferative units form a proto-map that is subsequently refined by thalamic inputs to establish cortical areas with distinct sizes, cellular compositions and functionalities. The size of the cortex is determined in the proliferative zones before cells migrate to the cortical plate, while the total neuronal number in the cortex depends on several factors, including the number of founder cells, the time of onset of corticogenesis, the duration of the cell division cycle, the duration of the period of neurogenesis, the modes of cell division, the number of rounds of cell cycles and finally selective programmed cell death (apoptosis) (Rakic 1995).

1.5 The developing vertebrate CNS contains different types of precursor\progenitor cells

The stem\precursor cell population, which are responsible for building the brain, are located in two distinct and relatively small proliferative areas.

The first is the ventricular/germinative zone (VZ), the innermost cell layer surrounding the lumen of the neural tube, and represents a region of extraordinary mitotic activity. It has been estimated that in humans about 250 000 new neurons are generated each minute during the peak of cell proliferation during gestation. In the VZ, epithelial cells with neural stem cell (NSC) properties appear approximately at embryonic day (E) 8 and from them originate all cells of the developing and mature CNS, including adult NSCs (Alvarez-Buylla, Garcia-Verdugo et al. 2001). After a period of NSC\precursor expansion by symmetric cell division, neuroepithelial cells (NE) start to acquire radial glial features. Radial glial cells still undergo self-renewal expansion, but as neurogenesis commences, a third progenitor population starts to be generated from asymmetrically dividing RGC cells in the VZ and migrate basally. These cells, located in the SVZ, are termed intermediate progenitors or basal progenitors and divide to produce more committed cells (Reynolds and Weiss 1992; Lois and Alvarez-Buylla 1993; Doetsch and Alvarez-Buylla 1996).

1.6 Neuroepithelial cells (NE)

The neural plate is initially formed by a single layer of pseudostratified neuroepithelial cells, which undergoes symmetric self-renewal divisions to expand the pool of multipotent progenitors as well as asymmetric cell divisions to generate the earliest born neurons. NE cells have epithelial features and are highly polarized along their apico-basal axis with apical attachments to the ventricular surface and a basal fiber connecting the pial (basal) surface. Their nuclei, all along the apico-basal axis, show interkinetic nuclear movement, moving

back and forth between the apical and basal sides of the tissue during their cell cycle and undergoing mitosis at the ventricular surface (**Figure 7**). Cells that are closest to the outer surface of the neural tube enter a phase of DNA synthesis (the S phase); after the nucleus moves back to the ventricular surface (the G2 phase), the precursor cells lose their connection to the outer surface and enter in mitosis. When mitosis is complete, the two daughter cells extend processes back to the outer surface of the neural tube, and the new precursor cells enter a resting (G1) phase of the cell cycle. At some point a precursor cell generates either another progenitor cell that will go on dividing and a daughter cell – a neuroblast- that will not divide further, or two postmitotic daughter cells. Before neurogenesis starts, at E8 in the mouse, NE cells undergo a phase of massive proliferation by symmetric division (Rakic 1995). By E9, the anterior neural tube closes to form the lateral ventricles and the NE cells therefore line the ventricles as a pseudostratified neuroepithelium. At the onset of neurogenesis, they switch to an asymmetric mode of divisions allowing self-renewing and neuron generation (Gotz and Huttner 2005) (**Figure 8**).

Concomitant with the beginning of neurogenesis, between E9 and E10, NE cells in rodent and higher vertebrates begin to transform into a distinct progenitor type: radial glia cells (RGCC). During this transformation, NEs lose some of their epithelial properties in favor of certain glial characteristics, but retain contacts with the ventricular and pial surfaces that give them their radial morphology; hence the term RGCC. Among the changes characterizing the NE-to-RGCC transition are the loss of tight junctions (Aaku-Saraste, Hellwig et al. 1996), the acquisition of glycogen storage granules (Gadisseux and Evrard 1985; Rubenstein and Beachy 1998), and the expression of astroglial genes such as brain lipid-binding protein (BLBP), astrocyte-specific glutamate transporter (GLAST) and tenascin-C (Hartfuss, Galli et al. 2001; Heins, Malatesta et al. 2002; Noctor, Flint et al. 2002). RGCCs still retain many NEC characteristics, and the two cell types likely coexist for some time (Gotz and Huttner 2005). NE and RGC cells are more generally called apical progenitor cells (AP) (**Figure 8**).

1.7 Radial glial cells (RGCC)

RGC cells, one of the major class of progenitors, have long been known to have crucial roles in guiding neurons to their final locations in the cortical plate by serving as a migratory scaffolding (Rakic 2003). During the last decade, these cells have acquired a "new fame" and several experiments have demonstrated that they act as precursors of neural cells (reviewed in Malatesta and Gotz, 2012). However, unlike NEs, which typically divide symmetrically to expand the progenitor pool, RGCCs tend to divide asymmetrically to self-renew and generate a non-RGC daughter cell (Iacopetti, Michelini et al. 1999; Miyata, Kawaguchi et al. 2001; Noctor, Flint et al. 2001; Haubensak, Attardo et al. 2004; Noctor, Martinez-Cerdeno et al. 2004).

RGC cells (as well as NE) have highly polarized epithelial structures with an apico-basal polarity and extend their process from the ventricular surface to the pial surface. The nuclei of RGC cells are characterized by the periodic apico-basal translocation (interkinetic nuclear migration -IKNM) which is regulated in such a way that mitosis occurs always at the ventricular surface, while S-phase occurs at the basal most area (**Figure 8**) (Gotz and Huttner 2005; Pinto and Gotz 2007). RGCC generate pyramidal neurons either directly through mitoses at the apical surface of the VZ, or indirectly through the production of proliferating intermediate progenitors (IP) or basal progenitor cells (BP), located in the subventricular zone (SVZ).

1.8 Subventricular zone (SVZ) and basal progenitors

The SVZ, the second major germinal site, starts to form at E13.5 in the mouse and expands significantly during late corticogenesis. Cell divisions in the SVZ were thought to primarily contribute to gliogenesis, but not neurogenesis (Takahashi, Nowakowski et al. 1995). More recently, elegant studies in slice culture have shown that RGCC frequently undergo an asymmetric division to generate an intermediate or basal progenitor, which then migrates into the SVZ before pausing and undergoing a symmetric round of cells division to

produce two new neurons (Miyata, Kawaguchi et al. 2004; Noctor, Martinez-Cerdeno et al. 2004; Costa, Wen et al. 2008) (**Figure 8).** Using Tis21-green fluorescent protein (GFP) knock-in mice that express nuclear GFP in cells undergoing a neuron-producing (or neurogenic) division, progenitors dividing at the basal side of the VZ (the developing SVZ) were observed to undergo symmetric cell divisions, giving rise to two postmitotic neurons (Haubensak, Attardo et al. 2004), confirming and extending the slice culture results on a global scale.

Intermediate progenitor cells (IPCs), called also basal progenitor cells, are distinct in several important ways, perhaps the most functionally relevant difference from NE and RGC cells being that IPCs primarily undergo symmetric terminal divisions to produce pairs of neurons (Haubensak, Attardo et al. 2004; Noctor, Martinez-Cerdeno et al. 2004; Cappello, Attardo et al. 2006; Costa, Wen et al. 2008). Some IPCs can also undergo a limited (1–3) number of additional symmetric divisions by generating more IPCs before differentiating into neurons (Wu, Goebbels et al. 2005), and thus functioning as a class of neuron-restricted transient amplifying cells. Basal progenitors, located in the SVZ, are multipolar without the same epithelial structure as daughter cells of RGC cells: when produced, IP cells retract their apical attachment and basal extension, do not exhibit hallmarks of apico-basal polarity and migrate basally before they undergo mitosis. Perhaps the most functionally relevant difference from NEs and RGCCs is that IPCs primarily undergo symmetric terminal divisions to produce pairs of neurons (Miyata, Kawaguchi et al. 2001; Noctor, Martinez-Cerdeno et al. 2004; Schwamborn, Berezikov et al. 2009). Some IPCs can also undergo a limited (1–3) number of additional symmetric divisions to generate more IPCs before making neurons (Wu, Goebbels et al. 2005), thus functioning as a class of neuron-restricted transient amplifying cells. Typical IPCs in the developing neocortex express characteristic molecular markers such as Tbr2 and downregulate the RGC-specific transcription factor Pax6 (Englund, Fink et al. 2005).

Another type of neocortical progenitor has been termed short neural precursor (SNP) on the basis of its unique morphology (Gal, Morozov et al. 2006). Similar to RGCCs, SNPs divide in the VZ and have a radial morphology with an apical process in contact with the ventricular surface. Unlike RGCCs, however, the basal processes of SNPs do not reach the basal lamina. SNPs and RGCCs differ in additional ways, including their abilities to utilize the GLAST and Tαl promoters, their use of Notch downstream signaling (Mizutani, Yoon et al. 2007), their cell-cycle kinetics, and the behaviors of their immediate progeny (Stancik, Navarro-Quiroga et al. 2010). However, based on their similar neurogenic properties, it has been proposed that SNPs may represent a subset of IPCs that have not lost contact with the ventricular surface (Kowalczyk, Pontious et al. 2009). Indeed, single-cell molecular profiling of neocortical cells revealed a population of progenitors in the VZ that more closely resembled IPCs than RGCCs (Kawaguchi, Ikawa et al. 2008). Nevertheless, SNPs express Pax6 but not Tbr2 (Stancik, Navarro-Quiroga et al. 2010), indicating some molecular differences between them and IPCs.

1.9 Outer radial glial cells

It has been known that primates develop an additional germinal zone (outer subventricular zone) outside of the VZ during brain development. Recently, these progenitors were identified as a new subtype of self-renewing progenitors named outer radial glial (oRGC) cells (LaMonica, Lui et al. 2012). This new cell population is thought to arise from the division of RGC cells; they delaminate from the apical surface and translocate their nuclei in the outer portion of the SVZ where they start dividing.

ORGC cells show a distinct behaviour before mitosis, referred as mitotic somal translocation, in which the cell body moves rapidly up to the basal fiber. This type of translocation contrasts with the INM of radial glial cells, in which the nucleus moves apically and mitosis occurs at the ventricular surface, and with the mitotic behaviour of intermediate progenitor cells, which divide in place without nuclear translocation. While these cells are monopolar and do not express the

apical markers found in RGC cells, they maintain a long basal fibre connecting the basal lamina, and molecular characteristics of RGC cells such as the expression of Pax6, Nestin, GLAST, GFAP (Hansen, Lui et al. 2010). ORGC can either divide symmetrically to expand their number (Reillo, de Juan Romero et al. 2011), or asymmetrically to self-renew and produce intermediate progenitors which then contribute to the production of neurons. In this respect they are very similar to apical RGC cells. However, they appear to have a distinct behaviour and different proliferative potentialities. Just prior to division, oRGC nuclei undergo a basal translocation movement along their basal fibre. oRGC usually divide in an apico-basal fashion and the daughter cell that inherits the basal fibre remains an oRGC while the other daughter cell generally goes through a transit amplification phase. Contrary to RGC-derived IP cells, which express Tbr2 and typically divide only once to produce two neurons, oRGC-derived IP cells do not start expressing Tbr2 immediately, and live imaging shows that they go through several rounds of division before their progeny differentiates as neurons (Hansen, Lui et al. 2010).

It has been suggested that the outer subventricular zone (oSVZ) may be a primate-specific feature and a hallmark of primate corticogenesis, and that it could be an essential step in the evolution from lissencephalic to gyrencephalic brains. But recent studies have shown that oSVZ (that is, oRGC cells) also exist in non-primate species with a lissencephalic brain such as rodent and in lissencephalic primate brains (Kelava, Reillo et al. 2012).

1.10 Signals derived from the progenitor domain: intrinsic regulation

The heterogeneity of progenitor types and their different locations in VZ and SVZ raises an important question about their molecular and cell biological differences. It is of great interest identifying the signals that establish the temporal order of neurogenesis from upper- versus lower-layer progenitors. Progenitor cells switch from symmetric to asymmetric divisions in order to produce committed neural cells. In this regard trying to understand

how asymmetric cell divisions affects progenitor self-renewal and differentiation might reveal important clues.

In the last two decades, the orientation of the axis of division in regard to the spindle orientation has been shown to correlate with the choice between symmetric and asymmetric modes of cell division in a number of systems. The use of horizontal and vertical terms in the literature on spindle orientation can be confusing, that's why is important to precise this concept (**Figure 9**). The ventricular surface is usually represented as a horizontal line so that planar divisions (divisions that occur in the plane of the endothelial surface), which give a vertical cleavage plane, are called horizontal divisions. Conversely, divisions along the apico-basal axis are vertical divisions, with a horizontal cleavage plane. Divisions with an intermediate orientation will be defined as oblique.

Early data have demonstrated that vertical spindle orientation correlates with an asymmetrical progeny, leading to models in which the unequal segregation of the apical and basal plasma membranes directs cell fate (Zhong and Chia 2008). Consistent with this mitotic spindles, vertical orientations are only found during the neurogenic phases of brain development (Haydar, Ang et al. 2003). Differently, during the early expansion phase, precise horizontal spindle orientation is crucial to maintain the neural progenitor pool. However, the frequency of vertical divisions during the neurogenic phase is too low to account for all divisions with asymmetric progeny. This could be explained by the small size of the apical membrane domain of RGCCs, such that even rare oblique mitotic spindles would give rise to cleavage planes that fail to bisect this domain resulting in its asymmetric segregation (Kosodo, Roper et al. 2004). It has been demonstrated that increasing the rate of vertical divisions can affect progenitor cell number and location (Konno, Shioi et al. 2008; Shitamukai, Konno et al. 2011).

Functional evidences demonstrate that either vertical or oblique spindle orientation is required for neurogenesis, however the mechanisms required to establish them are still unclear. The molecular

machinery for spindle orientation during neurogenesis is best understood in the fly *Drosophila melanogaster* (Siller and Doe 2009). In *Drosophila* neuroblasts orientation of the mitotic spindle along the apico-basal axis (vertical divisions) is important for asymmetric division. As Inscuteable (Insc) is a central player in *Drosophila* neuroblast apico-basal spindle orientation, the role of mammalian Insc was also explored in cortical progenitors. Postiglione et al. showed that oblique divisions are essential for generating the correct numbers of neurons in all cortical layers and that the orientation of progenitor cell divisions is important for correct lineage specification in the developing mammalian brain (Postiglione, Juschke et al. 2011).

What links asymmetric cell divisions and cell fate in *Drosophila* is the unequal segregation of polarity proteins such as Par3/Par6/aPKC apical complex (Siller and Doe 2009). Likewise, genetic studies in mice have confirmed the essential roles of many polarity proteins for proper maintenance, self-renewal and differentiation of RGC cells in vertebrate (Cappello, Attardo et al. 2006; Costa, Wen et al. 2008; Schwamborn, Berezikov et al. 2009; Kim, Lehtinen et al. 2010; Vessey, Amadei et al. 2012). In the mouse neocortex, asymmetric segregation of the apical complex protein Par3 in dividing RGCCs promotes unequal inheritance of the fate determinants Numb and Numb-like by their progeny, which in turn determines which daughter cell remains a RGC and which one becomes an IPC or neuron (Bultje, Castaneda-Castellanos et al. 2009). Numb/Numb-like guides differential daughter cell fates by inhibiting Notch signaling, possibly by controlling endocytosis and degradation of Notch (Tajbakhsh, Rocheteau et al. 2009). Notch signaling represses proneural genes and is important for maintenance and self-renewal of RGCCs during neurogenic stages of neocortical development (Gaiano, Nye et al. 2000; Nakamura, Sakakibara et al. 2000; Mizutani, Yoon et al. 2007; Imayoshi, Sakamoto et al. 2010).

In the canonical Notch signaling pathway, Notch ligands bind to the transmembrane Notch to promote release of the Notch intracellular domain, which then translocates into the nucleus to form a complex with the DNA-binding protein Rbpj/CBF1 (**Figure 10**). The NICD-

Rbpj complex subsequently activates expression of transcription factors Hes1 and Hes5, which in turn repress proneural genes. Rbpj-mediated signaling is attenuated in IPCs compared to RGCCs and knockout of Rbpj in mice abolishes Notch signaling and leads to premature depletion of RGCCs in favor of IPCs and neurons (Imayoshi, Sakamoto et al. 2010).

All these data support a model by which polarization of apical complex proteins promotes segregation of Numb/Numb-like during RGCC divisions, resulting in high Notch signaling in one daughter cell that becomes the self-renewing RGCC and low Notch signaling in the daughter that adopts an IPC or neuronal fate (Bultje, Castaneda-Castellanos et al. 2009). Since Notch signaling fails to show any relevant difference between upper- versus lower-layer progenitors at mid-neurogenesis, it will be interesting to test whether alteration in Notch signaling early in development could limit neurogenesis. Alternatively, Notch signaling in early corticogenesis could serve to enhance intrinsic differences between lower-layer RGCCs that tend to undergo neurogenic divisions and upper- layer RGCCs that preferentially undergo symmetric proliferative divisions (Franco, Gil-Sanz et al. 2012). Because the Notch ligands Delta-like1 and Delta-like3 are expressed by IPCs and neurons, but not by RGCCs (Campos, Duarte et al. 2001), one intriguing possibility is that postmitotic lower-layer neurons may signal back to symmetrically dividing upper-layer RGCCs to promote the progenitor state in both of their daughter cells (**Figure 11**).

One example of such a feed-back mechanism is given by the transcriptional repressor Sip1, which acts in newborn neurons to regulate levels of neurotrophin 3 (Ntf3) and fibroblast growth factor 9 (Fgf9), serving as signals from neurons to progenitors. Deletion of Sip1 in neurons leads to the overexpression of Ntf3 and premature production of upper-layer neurons. Although it was proposed that Sip1/ Ntf3 signaling may regulate a switch from lower- to upper-layer fate (Seuntjens, Nityanandam et al. 2009), an alternative hypothesis is that this signal may be preferentially received by upper-layer progenitors as a means to control the timing of upper-layer

neurogenesis. Such a mechanism could be integrated with a Notch pathway described above, such that Notch signaling first promotes expansion of the upper-layer progenitor pool early in development, and then once a critical number of lower-layer neurons are generated they could produce sufficiently high levels of Ntf3 to start neurogenesis from the expanded upper-layer progenitor population. In this way, an inter-lineage feedback mechanism would ensure that a correct number of projection neuron subtypes is generated at appropriate times.

1.11 Signals that come from outside the progenitor domain: diffusible signals

The microenvironment can also regulate the behaviour of neuronal progenitors through diffusible extrinsic signals and\or molecules mediating cell to cell and cell to extracellular matrix interactions. These molecules, by their nature, form gradients within the tissue and can signal in areas distant from their sources. Therefore, in each position within the developing CNS, a neural stem cell\ progenitor would be exposed to a unique combination of signals that might instruct appropriate region-specific behaviours.

The apical domains of RGCCs line the Cerebral Spinal Fluid (CSF)-filled lateral ventricles, into which RGCCs extend their primary cilia (Cohen, Binet et al. 1988; Dubreuil, Marzesco et al. 2007). Many signaling molecules are secreted into the CSF, including FGFs, insulin-like growth factors, retinoic acid (RA), sonic hedgehog (Shh), transforming growth-factor beta/bone morphogenetic proteins (TGFb/BMPs) and Wnts (Lehtinen and Walsh 2011). CSF samples from mouse or rat lateral ventricles are sufficient to stimulate progenitor proliferation and maintenance in neocortical explants and neurosphere cultures (Lehtinen, Zappaterra et al. 2011). Signaling molecules that might be a good candidate in regulate RGCCs behavior are CSF-derived Wnts.

Wnt signaling via ß-catenin is required for early neocortical specification and maintenance of progenitor cells (Machon, van den Bout et al. 2003; Junghans, Hack et al. 2005; Zhou, Borello et al.

2006). Consistent with this idea, constitutive activation of Wnt/ ß-catenin signaling forces RGCCs to undergo excessive proliferative divisions, resulting in expansion of the RGCC pool at the expense of neurogenesis (Chenn and Walsh 2002). Conversely, blocking ß-catenin-dependent transcription forces premature differentiation of RGCCs into neurons (Woodhead, Mutch et al. 2006). These data suggest that activation of ß-catenin signaling by Wnts in the CSF could be involved in controlling the decision between self-renewal and differentiation of RGCCs. The Wnt/ ß-catenin signaling pathway may be integrated with the aforementioned apico-basal polarity mechanism that determines the RGCC proliferative behavior. Since ß-catenin is a component of cadherin-based adherens junctions between RGCCs, these specialized cell contacts could play a pivotal role in progenitor behavior by maintaining contact of the apical domains of RGCCs with the CSF, regulating ß-catenin localization and function, and serving as docking sites for Par proteins (Manabe, Hirai et al. 2002). Whether Wnt/ ß-catenin signaling or cadherin-based adherens junctions are differentially regulated in upper- versus lower-layer RGCCs remains to be explored.

In conclusion, identify the transition from the proliferative to the neurogenic mode of division is critical from the perspective of vertebrate cortex evolution. Regulating the mode of division of neural progenitors appears therefore as a key process in regulating the size and surface area of the vertebrate brain (Fish, Dehay et al. 2008; Lui, Hansen et al. 2011). Furthermore, the question of the regulation of the choice between symmetric and asymmetric divisions has been the focus of intense efforts in the last two decades because defects in this control may be a causing factor for a number of human developmental diseases. In particular, it is thought that some types of microcephaly are the result of an early exhaustion of the pool of neural stem cell through a premature switch to neurogenic divisions at the expense of stem cell proliferation (Thornton and Woods 2009).

1.12 The progressive restriction model of cell-type specification

Birth-dating studies demonstrated that cortical projection neurons and astrocytes are generated in a defined temporal sequence.

Migrating neurons are born in an inside-out order: lower layer VI and V neurons are born first, followed by layer IV, III, and II neurons. Finally, toward the end of neurogenesis, a process, called gliogenesis, start in which RGCCs generate astrocytes (McConnell and Kaznowski 1991; Pinto and Gotz 2007). Two alternative models could explain the mechanism by which this temporal order is established (**Figure 12**). In one model, the fate potential of a common progenitor might change over time to generate the different subtypes of projection neurons and astrocytes in a defined temporal order (**Figure 12A**). Alternatively, multiple progenitor types may coexist, each of which is intrinsically programmed or extrinsically triggered to generate a specific subclass of neurons or astrocytes on a progenitor-specific time line (**Figure 12B**).

Several experimental strategies have been employed to distinguish between these models. Landmark heterochronic transplantation studies in ferrets provided evidence for a common progenitor whose fate potential is restricted over time, such that it sequentially generates the different types of projection neurons in order (McConnell and Kaznowski 1991; Shen, Wang et al. 2006; Pinto and Gotz 2007; Eiraku, Watanabe et al. 2008). Early progenitors, which normally produce lower-layer neurons, are capable of producing upper-layer neurons when transplanted into older host animals (Shen, Wang et al. 2006). Older progenitors, however, are restricted in their competence and can only produce upper-layer neurons, even in a younger host environment. These studies also indicated that by a time a neuron has progressed through its final mitotic division and initiates migration, it has acquired the information necessary to migrate to the layer typical of its birth date. *In vitro* studies, the cells isolated from the developing neocortex of mice lend additional support to the concept of a common progenitor that generates projection neurons in a defined temporal order (Shen, Wang et al. 2006). With the advent of stem cell technology, attempts have been made to generate neocortical neurons in cultures from human stem cells. The *in vivo* temporal order by which stem cells generate different neocortical neurons was maintained *in vitro*, with the caveat that neuronal subtypes for upper cortical layers were largely not detectable (Eiraku, Watanabe et al.

2008). These studies have provided evidence that single VZ progenitors can generate neurons destined for multiple neocortical cell layers. Clones generated from late progenitors are confined to increasingly superficial layers, indicative of progressive restriction of progenitor fate potential ((Luskin, Pearlman et al. 1988); (Price and Thurlow 1988); (Walsh and Cepko 1988); (Reid, Liang et al. 1995); (Reid, Tavazoie et al. 1997)).

However, it was shown that the majority of progenitor clones cultured *in vitro* or labeled by retroviral vectors *in vivo* produces either neurons or macroglia, but not both. The glia-specific progenitors are further restricted and typically produce either astrocytes or oligodendrocytes (McCarthy, Turnbull et al. 2001; Malatesta, Hack et al. 2003). Only 10%–20% of cortical progenitors isolated at early embryonic stages appear to be multipotent in terms of generating neurons and glia, pointing toward a model of progenitor diversity with respect to the neuronal and macroglial lineages.

This lineage restriction is in place already at early stages of neurogenesis and is maintained *in vitro* (McCarthy, Turnbull et al. 2001; Malatesta, Hack et al. 2003; Schuurmans, Armant et al. 2004), thus raising the possibility that an early specification event may initiate an intrinsic molecular program for progenitor fate restriction.

1.13 Molecular determination of cell-type specific projection neurons during lamination

The different types of neurons that populate the neocortex perform each a different and coordinated task fulfilling together the physiological functions of a certain area, depending on their particular connections and firing properties (Fame, MacDonald et al. 2011). Different groups of neurons reside usually in specific layers of the neocortex, as a result of the correct process of lamination, which defines the thickness, identity and boundaries of the neocortical radial domains.

It is important to remember that a neurogenic gradient has been identified for the birth of neurons populating neocortical layers and that a fine control of the balance between proliferation and neurogenesis is crucial for their proper development (Rakic 1978; Zhou, Borello et al. 2006). Moreover, even if each cortical progenitor follows an internal clock in the production of all the repertoire of early- and late-born neurons which will constitute respectively lower and upper layers (Shen, Wang et al. 2006), this basic program can be modified by intrinsic and extrinsic signals suggesting that during corticogenesis neuronal progenitors undergo a restriction of their neurogenic potentials (Frantz and McConnell 1996).

Until present, the efforts to better understand the processes of lamination brought to the identification of some transcription factors (TFs) expressed in early post-mitotic neurons that play important roles in the formation of specific neuronal subpopulations. One of these factors is the family zinc finger protein 2 (*Fezf2*) a TF expressed in lower layers neurons (layer VI and V).

Fezf2 was discovered as an important gene for the specification layer V subcerebral PNs, including corticospinal motor neurons (CSMN), since in *Fezf2 null* mice, layer V neurons fail to connect to their subcerebral target and acquire instead a callosal identity (Aronoff, Matyas et al. 2010; Bedogni, Hodge et al. 2010; Franco, Gil-Sanz et al. 2012). Moreover, Fezf2 is a direct downstream activator of *COUP-TFI Interacting Protein 2 (CTIP2)* (Chen, Wang et al. 2008), another TF that has an important role in layer V subcerebral PN formation since also in *CTIP2 null* brains, CSMN, which usually send their axons to the spinal cord, fail to project beyond the *pons* and are strongly disorganized, defasciculated and ectopically positioned (Arlotta, Molyneaux et al. 2005). Another example of gene whose function has been strongly correlated to a subpopulation of neurons is the *Special AT-rich sequence-binding protein 2 (SATB2)*. This gene is expressed in callosal PN of all layers in an almost complementary fashion to CTIP2 (Alcamo, Chirivella et al. 2008). Inactivation of this gene will result in the complete absence of the *corpus callosum,* and ectopic expression of CTIP2 in callosal neurons, which will acquire

the fate of subcerebral projection neurons and project caudally to the spinal cord. In the same studies it was also demonstrated that SATB2 regulates CTIP2 expression through the direct binding of a *Matrix/Scaffold Attachment Regions (MAR*) upstream of the CTIP2 locus (Britanova, Akopov et al. 2005; Alcamo, Chirivella et al. 2008).

The *basic helix-loop-helix class B-5* (*Bhlhb5*) is another TF whose expression is very specific to particular subpopulations of cortical neurons. Bhlhb5 expression is detectable from E12.5 in post mitotic neurons and is expressed in all the compartment of the neocortex, with exception of the most anterior regions (Joshi, Molyneaux et al. 2008). After P0 its domain of expression becomes more restricted it remains strongly expressed in layers V-II/III in sensory areas, particularly in the TCA recipient layer (IV layer), and in layer V CSMN of the sensorimotor area, the most caudal region of the motor area, which overlaps with the rostral S1 area (Ross, McCord et al. 2012). Very few studies have been done on this gene during cortical lamination. Nevertheless it was shown in the *Bhlhb5 null* model that, although no particular shift in sensory area domains occurred, the fine organization of layer IV neurons that receive TCA innervation was lost, indicating that Bhlhb5 has a role in the refinement of area cytoarchitecture (Joshi, Molyneaux et al. 2008).

The *T-box brain 1 (Tbr1)* is a TF expressed early in the post-mitotic compartment (E10.5), when Cajal Retzius cells (CR) migrate to the future neocortex, maintained in postnatal stages in layer VI neurons that project to the thalamus (Hevner, Shi et al. 2001). The relevance of this TF in the lamination of the neocortex was highlighted first observing that *Tbr1 null* brains have a strong impairment of TCA that fail to reach the SP, and during specification of layer VI neurons. Layer VI neurons express ectopically layer V markers in absence of Tbr1, suggesting a prominent role of this TF in layer VI specification. Mutant mice died at birth excluding thus the possibility to follow corticogenesis and arealization at post-natal stages. However, the study of statistical variations of genes associated with positional identities of the cortex, showed that Tbr1 is important for specifying rostral identity in addition to the previously observed role in

lamination (Bedogni, Hodge et al. 2010). Interestingly, Tbr1 and Bhlhb5 seemed to be expressed in a complementary pattern in the cortical plate (Joshi, Molyneaux et al. 2008; Bedogni, Hodge et al. 2010). Another remarkable aspect found in this study is the relation between Tbr1 and FGF8 signaling, suggesting that the first may regulate the pathway of the second in the postmitotic compartment. Indeed, in the absence of Tbr1 the only rostral genes that increased their expression were *Sprouty2 (Spry2),* FGF17 and *Ets variant gene 1 (Etv1)*, all of which act downstream of FGF8 (Bedogni, Hodge et al. 2010).

Although not fully characterized, other genes specific for cortical subpopulations have been recently identified. In particular*, Forkhead-box protein 2 (Foxp2)* is expressed in layer VI neurons and in a particular subpopulation of layer V neurons, especially in the M2 area (Ferland, Cherry et al. 2003; Hisaoka 2010). Finally, *Friend of Gata-2 (Fog2),* also known as *zinc finger protein multiple 2 (ZFPM2)*, is a cofactor of the regulatory transcription complex GATA-2 and is expressed specifically in layer VI neurons of the neocortex (Kwan, Lam et al. 2008).

Thus, distinct molecular pathways may control the basic differentiation programs of lower- and upper-layer excitatory neurons. Consistent with this idea, a number of genes that are restricted to either upper- or lower-layer neurons in the mature neocortex are also enriched in subsets of progenitors during development. In the early cortex, RGCCs express markers of lower-layer neurons, including Emx2, Fezf2, Otx1, and Sox2 (Frantz, Weimann et al. 1994; Leingartner, Richards et al. 2003; Chen, Schaevitz et al. 2005; Bani-Yaghoub, Tremblay et al. 2006). In contrast, a number of genes specific for upper- layer neurons, such as Cux1 and Cux2, CTIP2, Tbr1, Foxp2, Fog2, are expressed at high levels in IPCs in the SVZ during middle and late stages of neocortical development (Tarabykin, Stoykova et al. 2001; Nieto, Monuki et al. 2004; Zimmer, Tiveron et al. 2004; Britanova, Akopov et al. 2005). In addition, the SVZ is preferentially expanded specifically during the time of upper-layer neurogenesis (Takahashi, Goto et al. 1999; Lukaszewicz, Cortay et al.

2006; Martinez-Cerdeno, Noctor et al. 2006; Dehay and Kennedy 2007).

Together, these studies led to the hypothesis that IPCs in the SVZ generate upper-layer neurons, whereas lower-layer neurons are derived directly from RGCCs in the VZ (Tarabykin, Stoykova et al. 2001; Nieto, Monuki et al. 2004; Zimmer, Tiveron et al. 2004). However, IPCs are present throughout neocortical development, even during lower-layer neurogenesis (Haubensak, Attardo et al. 2004; Wu, Goebbels et al. 2005), and the majority of neurons in all layers of the neocortex derive from IPCs, with only 10% of excitatory projection neurons coming directly from RGCCs (Kowalczyk, Pontious et al. 2009). Because IPCs are derived from RGCCs (Haubensak, Attardo et al. 2004; Miyata, Kawaguchi et al. 2004; Noctor, Martinez-Cerdeno et al. 2004), these two progenitor types therefore likely represent different states of progenitor differentiation rather than separate fate-restricted lineages.

1.14 Cortical arealization

As already mentioned, radial units are considered the simplest units of neocortical functional areas, since neurons that populate them are connected mostly to other neurons of the same column. The interactions between different columns and the properties of the single ones are, most probably, what specifies the functional features of the cortical areas. Moreover, the vertical organization of radial units changes from one area to the other, since the cell populations constituting the neocortical layers are differentially represented in the radial units of different brain regions. In the mammalian (eutherian) neocortex it is possible to distinguish mainly four different functional areas. One is the motor areas (M), mainly subdivided into M1, more lateral, and M2, more medial. Their role is to elaborate voluntary movement on the base of information from the periphery. The primary somatosensory area (S1) is localized in the parietal cortex and elaborates somatosensory inputs from the whole body. S1 is surrounded by the S2, the secondary sensory areas, which further elaborate sensory information without the same topographic relationship of the S1. The visual areas (V) are localized in the caudo-

medial regions of the neocortex and processes visual inputs. Finally the auditory areas (A) are localized between the S and V domains. Both the V and S domains are subdivided into primary (V1, A1) and secondary areas (V2, A2).

The studies on the mechanisms controlling neocortical arealization have led principally to the elaboration of two hypotheses based on experimental observations. The first one, chronologically, is the "Protocortex hypothesis", claiming that the neocortical areas are specified and shaped upon arrival of thalamocortical afferences (TCA) (O'Leary 1989). Thus their features, in terms of connections and cytoarchitecture, should depend on the type of inputs they receive from TCA. This theory was suggested by a plethora of studies conducted on animals with impaired sensory pathways (ocular deprivation, genetic or surgical deafness, etc.). In these animals a given sensory pathway was ablated and the corresponding functional area was innervated by ectopic TCA and adapted to its structural organization to the sensory neocortical areas where ectopic fibers were innervated. For example, one experiment consisted in removing at birth the visual cortex in hamsters. In these animals the visual axons from the retina did not reach the LG in the thalamus and to project after in the visual area of the cortex, but they reached both the LG and the VB nucleus, that normally carries somatosensory information. The VB in turn projected to somatosensory areas (Sur, Garraghty et al. 1988). Other relevant evidences favoring the protocortex hypothesis came from experiments in which tissues from the prospective visual area were transplanted into the future somatosensory region of embryonic mouse brains. This led to the formation, in the transplanted tissues, of structures resembling the barrels of neurons in layer IV, typical of the S1 (Dehay, Horsburgh et al. 1991; Rakic, Suner et al. 1991). These experiments clearly showed the plasticity of the neocortex and proposed TCA innervation as a crucial step for areal specification (O'Leary and Nakagawa 2002; O'Leary, Chou et al. 2007). However, it is important to remark that in all mammalians studied so far the relative positions of the main functional areas are maintained nearly unaltered, suggesting the existence of a genetic program driving their specification.

Starting from these and other considerations the "Protomap hypothesis" was formulated as an alternative to the "Protocortex hypothesis" (Rakic 1988). This hypothesis states that the commitment to a given areal fate is decided at very early stages of corticogenesis (E8.5-E10.5) due to the expression of morphogens and, later, of transcription factors which confer a given commitment to undifferentiated progenitors. Thus, newborn neurons already have a genetic signature inherited by their progenitors and that will determine many features of their final differentiation. It is quite hard, indeed, to deny that neocortical areas show a first rough specification, well before the arrival of TCA. Studies on mice in which the development of the thalamus or of TCA were impaired due to the deletion of genes such as Gbx2 (Miyashita-Lin, Hevner et al. 1999) or Achaete-scute complex-like 1 (Mash1) (Tuttle, Nakagawa et al. 1999), it was found that the pattern of expression of regionalized genes such as Cadherin-6 (Cad6), Ephrin-A7 (EphA7), Inhibitor of Dna-binding-2 (Id2) and RAR orphan receptor β (RORβ) were maintained (Nakagawa, Johnson et al. 1999). This demonstrated that some of the arealization features are already specified before the arrival of TCA. This hypothesis is also in agreement with the radial unit hypothesis.

However, it is very likely that the truth lies in the middle. It is actually possible that at their cell cycle exit, migrating neurons have an initial rough genetic program that already limits the potential differentiation that will occur. The finest differentiation and specification will instead reached only after the arrival of TCA, which direct the establishment of the main circuits of the neocortex (**Figure 13**).

1.14 Transcription factor gradients in the neocortex

To further understand the molecular pathways that may control the basic differentiation programs of lower- and upper-layer excitatory neurons, It's necessary to focalize our attention on transcription factors (TF) expressed in gradients in the neocortex, which are influenced by the secreted molecules (morphogens) expressed by different organizers of the cortex. The TFs found to be involved in

neocortical development are either induced or repressed by the morphogens expressed in cortical organizers. Four TFs were found, so far, to play crucial roles in neocortical partitioning: SP8 and Pax6, expressed in a high rostral to low caudal gradient, Emx2 and COUP-TFI, which are expressed in opposite gradients (O'Leary and Nakagawa 2002) (O'Leary, Chou et al. 2007).

Pax6, in particular, is an important regulator of neurogenesis and proliferation (Gotz, Stoykova et al. 1998; Warren, Caric et al. 1999; Heins, Malatesta et al. 2002; Stoykova, Hatano et al. 2003; Haubst, Berger et al. 2004; Berger, Berger et al. 2007; Quinn, Molinek et al. 2007; Osumi, Shinohara et al. 2008). Pax6 is a member of the paired-box and homeobox-containing gene family (Pax) of transcription factors and is highly conserved among vertebrate and invertebrate species. Pax6 is essential for the development of much of the central nervous system, including the eye, spinal cord and cerebral cortex (Hill, Favor et al. 1991; Glaser, Jepeal et al. 1994; St-Onge, Sosa-Pineda et al. 1997; Simpson and Price 2002). Detailed analyses of neocortical development in mice mutant for Pax6 have identified defects in neural stem and progenitor cell proliferation, multipotency, neurogenesis, the generation of specific types of neurons, and marked changes in spatial pattern (Marquardt, Ashery-Padan et al. 2001; Estivill-Torrus, Pearson et al. 2002; Heins, Malatesta et al. 2002). In the neocortex, loss of Pax6 function results in microcephaly, abnormal development of the secondary progenitor population (also known as basal progenitor cells, BP cells) of the SVZ, and a disproportionate reduction in the production of later-born, upper layer neurons (Bishop, Goudreau et al. 2000; Estivill-Torrus, Pearson et al. 2002; Haubst, Berger et al. 2004; Quinn, Molinek et al. 2007). First evidences for a role of Pax6 in neocortical arealization came from the study of a spontaneous mutant called *Pax6*$^{sey/sey}$ (Bishop, Goudreau et al. 2000) in which *Pax6* expression is down regulated (**Figure 14**). In these mutants expression of rostral markers is decreased and caudal markers are increased and shifted rostrally. Moreover, the neocortex is reduced one third in its thickness since the mutation of Pax6 favors early cell cycle exit where newly divided progenitors do not re-enter S-phase leading to a corresponding increase of early born neurons and

depletion of the stem cell population (Quinn, Molinek et al. 2007; Sansom, Griffiths et al. 2009).

Recently, various reports show how Pax6 acts by impairing the balance between symmetric and asymmetric divisions, favoring neurogenesis and depleting the pool of progenitors, and by regulating the orientation and mode of cell division (Asami, Pilz et al. 2011). In the *Pax6*$^{sey/sey}$ neocortex, RGCCs showed a spindle orientation and a cleavage plane angle altered at mid-neurogenesis. These results, confirmed by live imaging, revealed a strong increase in non-vertical divisions amongst apical progenitors (asymmetric divisions), which lead to an increase of non-apical progenitor cells (Gotz, Stoykova et al. 1998; Asami, Pilz et al. 2011). These studies confirmed an increase of cells in the SVZ, that still retain radial glia features due to impaired interkinetic nuclear migration of RGCCs, previously demonstrated in rat *Pax6*$^{sey/sey}$ (Tamai, Shinohara et al. 2007) and then confirmed in mouse *Pax6*$^{sey/sey}$, where a subset of cells undergo cell division prior to reaching the ventricular surface (Asami, Pilz et al. 2011). However, contrasting data came out from the study of a mouse transgenic line, the *D6-Pax6* line, in which Pax6 expression levels were two fold increased in the ventricular zone and cortical plate thanks to the dorsal-specific cortical enhancer D6 (Machon, van den Bout et al. 2002; van den Bout, Machon et al. 2002; Faedo, Tomassy et al. 2008). This model failed to show a complementary phenotype to the *Pax6*$^{sey/sey}$ mutants, as expected, but rather a marked increase in the expression of Eomes/Tbr2 at E12.5, indicating an increase in the basal progenitor cell population (Sansom, Griffiths et al. 2009). The early depletion of the stem cell pool reduces the number of progenitor cells available for neurogenesis, resulting in an overall reduction in cortical size. These functions of Pax6 indicate that one of its biological roles is to promote neurogenesis by increasing the number of neocortical stem and progenitor cells, and thus neurons at their next divisions (Sansom, Griffiths et al. 2009). The combination of Pax6 binding data with the transcriptional profiling of Pax6 gain- and loss-of-function cortices enabled the delineation of Pax6 as an active controller of the cortical neurogenesis/self-renewal regulatory circuits, placing Pax6 in the context of three genes controlling cortical neurogenesis: Neurogenin2

(Neurog2), Mash1. Hes1 and Eomes/Tbr2 (**Figure 15**) (Scardigli, Baumer et al. 2003; Haubst, Berger et al. 2004; Gohlke, Armant et al. 2008).

Pax6 both positively regulates the expression of Neurog2 and also synergies with Neurog2 to promote basal progenitor cell genesis and thus production of cortical excitatory projection neurons, whereas Hes1 opposes this process by repressing many of the same genes. Ascl1 has complex functions in cortical neurogenesis: while it promotes basal progenitor cell genesis, it also drives expression of transcription factors to promote inhibitory interneuron genesis (Lhx8 and Isl1, for example). Pax6 and Hes1 both repress Ascl1, and Pax6 represses Lhx8 and Isl1, to inhibit the interneuron-producing functions of Ascl1. This network also leads to clear predictions of the normal functions of Pax6 in regulating neurogenesis and the consequences of altered Pax6 expression in the early cortex (**Figure 15**). The network indicates that Pax6 is essential for the cortical identity of the basal progenitor cells produced in the cortex, as it is an essential driver of Eomes/Tbr2 expression, a key determinant of cortical basal progenitor cell identity (Arnold, Huang et al. 2008; Sessa, Mao et al. 2008).

On the other hand, another TF required in area patterning is *Emx2*, which shows specific and complementary phenotypes during areal specification compared to Pax6 (**Figure 14).** Emx2 starts to be expressed at E8.5 and its expression is finely regulated in time and space. At E9.5 it is expressed in the rostral VZ and counteracts Wnt signaling, thus promoting cell cycle exit and differentiation (Muzio, Di Benedetto et al. 2002). Moreover it promotes IP specification contributing, then, to the increase of the progenitor pool and of the final number of neurons reaching the CP (Leingartner, Thuret et al. 2007). Its action seems to be mainly restricted to the V1. *Emx2 null* mice have reduction of the V1 area, a caudal shift of the somatosensory area and a significant enlargement of the rostral markers (**Figure 14**). Reciprocally, overexpressing Emx2 under the enhancer of *Nestin* (Nestin-Emx2), induces a strong enlargement of the V1 and a general rostral shift of sensory areas (Mallamaci, Muzio et al. 2000; Bishop, Rubenstein et al. 2002; Cecchi 2002; Hamasaki,

Leingartner et al. 2004; Sansom and Livesey 2009). From what was found until now, it is clear that during the specification of the neocortex, the morphogens create a first subdivision in rostral, ventral, caudal and dorsal domains, which in turn express gradients of TFs. These TFs seem to promote either rostral or caudal areal fates during corticogenesis and most of them carry on their role in the proliferative compartment.

1.15 COUP-TF family

1.15.1 COUP-TFs

COUP-TFI was first found as an activator of the ovalbumin gene in chicken and is a nuclear orphan receptor belonging to the family of the steroid hormone receptors. In mammals, the family of the COUP-TF genes is represented by different members, however in mice only two members, COUP-TFI and COUP-TFII have been characterized so far. Recently, it was found that these genes are evolutionary conserved and are strictly linked to the development of the nervous system from the earliest members of the holozoans (animal clade), since it has an ortholog in porifera, cnidarians and urbilaterians but not in capsaspora (Galliot and Quiquand 2011). All the genes belonging to the COUP-TF family share a common structure: at the N-terminus they show a highly variable modulatory domain, followed by the DNA-Binding-Domain (DBD), which is strictly conserved, a hinge region and a Ligand-Binding-Domain (LBD) at the C-terminus (Figure 17) (Kieback, Levi et al. 1996). The most conserved domain is the DBD, which consists of two zinc-finger domains that recognize the sequence AGGTCA.

COUP-TFs are also known to act trough homodimerization or heterodimerization with Retinoid X Receptors (RXR) (Wang, Tsai et al. 1989). Differently from the first discoveries, COUP-TFs were later found to mainly act as repressor of target genes, such as the retinoid acid receptor (RAR), the thyroid hormone receptor (TR), the vitamin D receptor (VDR) (Cooney, Tsai et al. 1992), the peroxisome proliferator-activated receptor (PPAR) and the hepatocyte nuclear factor 4 (HNF4) (Ladias, Hadzopoulou-Cladaras et al. 1992). The

action of repression of COUP-TFs can be both active and passive, the first one was described as an interaction with the DNA in the direct repeats (DR) regions containing COUP-TFs binding motive (**Figure 16**). Repression occurs after recruiting the co-repressors Nuclear receptor Co-repressor 1(N-CoR) and silencing mediator for retinoid and thyroid receptors (SMRT) (Wang, Tsai et al. 1989; Cooney, Tsai et al. 1992; Mangelsdorf and Evans 1995; Park, Tsai et al. 2003). COUP-TFs can repress expression of genes that are normally activated by RXR heterodimers that bind their related Hormone Responsive Elements (HRE) (Leng, Cooney et al. 1996). This repression can be achieved in a passive way, as COUP-TFs may bind RXR instead of the normal cofactor needed to activate transcription, thus competing with them. Three mechanisms were shown to induce COUP-TFs mediated transcriptional activation, although compared to inhibition they constitute the minority of cases (**Figure 17**). The first one is the direct binding on a DR sequence followed by specific transcriptional activation, as shown for the regulation of arrestin and cholesterol 7a-hydroxylase (CYP7A) (Lu, Salbert et al. 1994; Stroup, Crestani et al. 1997).

The second mechanism of transcriptional activation involves the binding to the DNA and the following activation of other TF increasing their own ability to induce transcription, as demonstrated in the case of the phosphoenolpyruvate carboxykinase (PEPCK) and the glucocorticoid response unit (GRU) (Hall, Sladek et al. 1995; Scott, Mitchell et al. 1996). The last known mechanism involves a protein- protein interaction of COUP-TFs with factors that bound to the DNA and a following transcriptional activation, as seen for the HNF-4 in the HNF-1a gene promoter (Ktistaki and Talianidis 1997; Park, Tsai et al. 2003).

1.15.2 COUP-TFI during neurogenesis

Insights into the role of COUP-TFI during early corticogenesis came from the discovery of its ability to control the signaling pathways of MAPK/ERK/Akt and β-catenin and to regulate corticogenesis that occurs in a ventro rostral-high to caudodorsal-low gradient (Faedo, Tomassy et al. 2008). In this study, COUP-TFI function was analyzed

in gain- and loss-of-function approaches *in vivo*. In the *D6/COUP-TFI* mouse model, in which COUP-TFI was overexpressed under the D6 gene promoter, which is normally expressed in rostral dorsomedial domains, where COUP-TFI is not or faintly expressed, neurogenesis was enhanced at the expense of the self-renewal pool of neural progenitors. This phenotype was even more evident in comparison with the *COUP-TFI null* mice, in which proliferation in the VZ increases and fewer cells exit cell cycle and begin their differentiation. This was shown by analyzing the expression of markers of different proliferative stages, like cyclin D2 (CD2) and Phospho Histone 3 (PH3), and of early differentiation markers, such as Tbr1 and Tubulin-III at E13.5 and E15.5 in both the *D6/COUP-TFI* and *COUP-TFI null* mice. Moreover, both Emx2 and Pax6, which are two genes that regulate neurogenesis, were affected in opposite ways in the two models. While in the *D6/COUP-TFI* transgenic mouse, Pax6 expression was reduced in the VZ and SVZ starting from E11.5, in the *COUP-TFI null* embryos Pax6 and Emx2 expression was increased (Faedo, Tomassy et al. 2008). Finally, in this work, it was shown that the increase in neurogenesis due to the overexpression of COUP-TFI at early stages decreases the pool of neocortical progenitors leading to a 40% increase of neurons populating lower layers and a 70% reduction of late born neurons in upper layers. This study confirms that COUP-TFI has a crucial role in the balance between neurogenesis and self-renewal. This TF induces neurogenesis in a dose dependent way and its level of expression may account for differences in radial thickness and in layer organization of the cortical areas along the rostrocaudal axis of the neocortex (**Figure 18**).

1.15.3 COUP-TFI and arealization of the neocortex

COUP-TFI is expressed early in the neocortex (E8.5 in the mouse), reaches its peak at E14.5-15.5 and is expressed in a caudo-lateral high to rostro-medial low gradient in all layers of the developing cortex, from the proliferating VZ to the post-mitotic CP (**Figure 19**).

Due to its gradient of expression in the cortex this gene was challenged for its role in neocortical arealization. COUP-TFI was first studied in constitutive *COUP-TFI null* mice (Zhou et al., 1999). Mouse null mutants die at birth due to problems of feeding since the defective development of the IX ganglion impairs suction movements of the pups. In these mutants layer IV neurons were lost in the sensory areas, a phenotype more severe than the ones observed in the mutants of other areal patterning genes. Curiously, it was not observed a significant change in proliferation or cell death in the mitotic compartment at E14.5, when most of the IV layer cells are born. Since in the *COUP-TFI null* model TCA are strongly impaired, the loss of the IV layer was proposed to be due to both, impaired specification of layer IV cells and/or absence of TCA innervation (since this process was previously shown to be important for layer IV maturation). In this initial study the shift of areal markers was not clear, while in a following study (Zhou, Tsai et al. 2001), the same group showed that regionalized markers like Id2, Rorβ and Cad8 where caudally shifted covering almost all the neocortex. It was also suggested that probably the few TCAs succeeding to reach the neocortex were caudalized due to impaired expression of signaling cues directing TCA pathfinding.

The phenotype showed in these studies was really remarkable compared to the others studied presented so far. However, it was limited by its early lethality, which did not allow the study of cortical arealization at postnatal stages, when the final specification of all cell-types and all areas occurs. Furthermore, the loss of COUP-TFI both in the cortex and in the thalamus left open the main question on the real function of this gene in the neocortical arealization process. Indeed, it might have a direct role in this process, by regulating gene expression in the neocortical territory, or an indirect one, directing thalamic nuclei maturation and axonal pathfinding.

1.15.4 The COUP-TFI$^{fl/fl}$ Emx1-Cre mouse model

An important answer to the above mentioned questions about the role of COUP-TFI arrived when Dr. Studer's lab generated and analyzed the conditional *COUP-TFI$^{fl/fl}$ Emx1-Cre* (*Emx-CKO*) mice

(Armentano, Chou et al. 2007). Differently from the previously described *COUP-TFI null* mice, this new mouse mutant took advantage of the *Cre-recombinase* activity to delete COUP-TFI specifically in the cortex. In this mouse model, indeed, Cre is expressed under the control of the Emx1 promoter, specific to the cortex, and deletes the third exon of the COUP-TFI gene, which is flanked by two flox sites. Thus COUP-TFI is ablated in the neocortex as well as in the archi- and paleocortex as early as E.10.5, but not in the thalamus (see also **Figure 24 a/b**).

It was confirmed that also in the *Emx-CKO*, similarly to the *COUP-TFI null* mouse, there is a major loss of layer IV neurons, particularly in frontal and parietal regions, but not occipital regions. This was verified at P7 both by Nissl staining and Serotonin (5-HT) immunodetection, which showed that the typical structures of the sensory areas, like the S1 barrel field (BF), are still present even though strongly caudalized and reduced. Other regionalized markers, such as Id2, Rorβ, Cad6, Cad8 and Pax6 confirmed the caudal shift of all sensory areas and the expansion of rostral/motor domains. Interestingly, although the sensory are shifted caudally, the TCA maintain their connections with the reduced sensory areas. This result was important to establish that the phenotype observed in the neocortex after ablation of COUP-TFI was not due to a defect in thalamic nuclei specification, but rather to an intrinsic neocortical defect, which affected partitioning of neocortical areas. In line with the "Protomap hypothesis" this phenotype represent strong evidence that boundaries defined genetically within the neocortex are enough to direct TCA connections topographically. This suggests that the thalamus responds to neocortical regionalization defects by rerouting their axons in function of the re-organization of cortical areas, as it probably normally happens during neocortical development.

A following study from the Studer's lab clearly showed a remarkable change in the radial organization of the neocortex associated to the massive areal reorganization observed in the *Emx-CKO* mice (Tomassy, De Leonibus et al. 2010). The typical radial laminar organization of rostral neocortical regions was now observed in

parietal regions (somatosensory area) in the *Emx-CKO* (**Figure 20**). Importantly, corticothalamic neurons of layer VIa, that normally express Tbr1, were unable to project to the thalamus, as they were projecting to subcerebral structures. Thus, it was clear that this population of neurons, due to the ectopic expression of TFs, like CTIP2, changed their fate into a different one, demonstrating the role of COUP-TFI to regulate specific neuron subset identity by regulating the timing of expression of distinct TF during corticogenesis and areal patterning. The study also showed that these mice have abnormalities in motor skilled behavior and a hyperactive repetitive behavior, probably due to the shrinkage of sensory areas and to the consequent reduction of input exchange between these areas and the motor ones. Overall, this study indicates that the laminar and the tangential organization of the neocortex are correlated and that COUP-TFI was not only involved in the areal fate commitment of the progenitors, but also in the specification of the different neuronal subpopulations. Interestingly, COUP-TFI is maintained in postmitotic cells, differently from all other genes that were studied and correlated to arealization (Pax6, Emx2, Sp8). Thus, it is not excluded that its function on neuronal specification and arealization might also depend on its expression in postmitotic neurons. Imortantly, within all the arealization genes previously discussed, COUP-TFI is the one with the strongest phenotype in terms of arealization shift (**Figure 19**).

1.16 Thesis overview: Scientific hypothesis and aim of the work

At the beginning of my PhD project, little was known about the possible involvement of the nuclear receptor COUP-TFI in neural stem/progenitor cells. Preliminary observations from the Studer and other laboratories on the constitutive null mutants indicated compromised areal identity and affected neurogenesis (Armentano, Chou et al. 2007; Faedo, Tomassy et al. 2008; Tomassy, De Leonibus et al. 2010). However the mice die at birth, which precludes any further analysis at post-natal stages where corticogenesis and arealization will be terminated.

It is believed that for obtaining correct functioning of the cerebral cortex, including proper layer and areal specification, the stem/progenitor and neural committed cell populations need to be established in proper numbers at the right time and place during cortical development. Stem progenitor cells are responsible for building the entire brain and are retained in small proliferative areas. In the neocortex, the stem cell microenvironment is constitute by the VZ, where epithelial cells with neural stem cell properties appear at Embryonic day (E) 8 and from which originate all cells of the developing and mature central nervous system. When neurogenesis start, a second progenitor population starts to be generated from asymmetrically dividing cells in the VZ and migrates basally, in the SVZ and from here to the mantle to produce more mature neurons (*Kwan et al., Development).*

The overall aim of my work was to study the molecular mechanisms underlying the biological processes leading to the correct specification of this microenvironment during corticogenesis and to follow the neural stem/precursor and neuronal committed cells deriving from the proliferating neocortical regions. For this reason I performed all my experiments at the embryonic stage (E) 15.5, since I considered it a good compromise between proliferation (occurring in the VZ and SVZ) and differentiation (occurring in the cortical plate).

My studies started by the finding that COUP-TFI was involved in the balance between proliferation and differentiation and that his

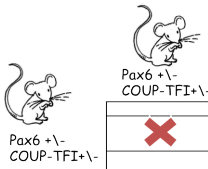
constitutive deletion caused an alteration in this balance, altered cortical lamination and areal patterning defects(Armentano, Chou et al. 2007; Faedo, Tomassy et al. 2008; Tomassy, De Leonibus et al. 2010). My project involved also another transcription factor, Pax6, known from the literature to control cortical areal identity and neurogenesis, and to directly regulated by COUP-TFI. Previous data on the *null* mutants for both transcription factors suggested an opposite phenotype between them. These evidences provided the basis for further studies to validate the hypothesis of a possible genetic interaction between the two transcription factors, an interaction that could be necessary to maintain the balance between stem cell self-renewal and progenitor commitment, known to be essential for the correct growth and shaping of the total brain structures.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Mice

COUP-TFI$^{flox/+}$ mice were generated as previously reported (Armentano, Chou et al. 2007), and propagated by backcrossing to C57BL/6 in bred mice. Homozygous COUP-TFI$^{flox/flox}$ mice were obtained by intercrossing heterozygous mice between them.
COUP-TFI$^{-/-}$ mice were generated as previously showed (Armentano, Filosa et al. 2006), and propagated by backcrossing to C57BL/6 in bred mice. Homozygous COUP-TFI$^{-/-}$ mice were obtained by intercrossing heterozygous mice between them.
Pax6$^{-/-}$ transgenic mice were a kind gift from the Max-Planck Institute, Gottinghem, Germany and they were generated as previously described (St-Onge, Sosa-Pineda et al. 1997). Homozygous Pax6$^{-/-}$ were obtained by intercrossing heterozygous mice and mated to the COUP-TFI null transgenic line to obtain transgenic mutant with a different COUP/ Pax gene dosages:



✖	Pax6 + _ Coup Tf1+	Pax6 + _ Coup Tf1-	Pax6 - _Coup Tf1 +	Pax6 - _ Coup Tf1 -
Pax6 + _ Coup Tf1+	Pax6 +\+ _ Coup Tf1+\+	Pax6+\+ _ Coup Tf1-\+	Pax6 -\+ _ Coup Tf1 +\+	Pax6 -\+ _ Coup Tf1 -\+
Pax6 + _ Coup Tf1-	Pax6 +\+ _ Coup Tf1+\-	***Pax6 +\+ _ Coup Tf1 -\-***	Pax6 -\+ _ Coup Tf1 +\-	***Pax6 -\+ _ Coup Tf1 -\-***
Pax6 - _ Coup Tf1 +	Pax6 +\- _ Coup Tf1+\+	Pax6 +\- _ Coup Tf1 -\+	***Pax6 -\- _ Coup Tf1 +\+***	***Pax6 -\- _ Coup Tf1 -\+***
Pax6 - _ Coup Tf1 -	Pax6 +\- _ Coup Tf1 +\-	Pax6 +\- _ Coup Tf1 -\-	Pax6 -\- _ Coup Tf1 +\-	***Pax6 -\- _ Coup Tf1 -\-***

We found no differences between males and females mutant mice. Genotyping was performed as follows. Midday of the day of the vaginal plug was embryonic day 0.5 (E0.5). All experiments were conducted following guidelines of the institutional Ethical Committee for animal experimentation, France.

2.2 DNA extraction from murine tissue

Mice not used for experiment were ear marked the day of the weaning and the tissue taken from the ear was used to extract the genomic DNA, while for pups and embryos the DNA was extracted from the tip of the tail cut during their sacrifice. The lysis of the sample was performed incubating the tissue overnight at +58°C, in agitation, in 500µl of lysis buffer (50mM Tris pH 7.5, 100mM EDTA pH8, 100mM NaCl, 1% SDS, 0.2µg/µl Proteinase K). The extraction of the DNA was done adding an equal volume of Isopropanol pure, the solution was mixed by inversion and then centrifuged at 20000g for 5' so that the DNA remains in the pellet, the supernatant was wasted and 1ml of ethanol 70% was added and then all was centrifuged 2' at 20000g to wash the DNA. After wasting most of the supernatant the pellet was let air drying for 10-15' and then resuspended in 70µl of H_2O milli-q for ear tissues and 500µl for tail tissues, incubating the solution for 1h at +37°C in agitation and then kept at +4°C before using or stored at -20°C.

2.3 Genotype analysis

Constitutive and conditional mutants for COUP-TFI and null mutant for Pax6 were genotyped by Polymerase Chain Reaction (PCR). Three different reactions were made in order to test the presence of the *COUP-TFI floxed* allele, the allele containing *Cre-recombinase* and a control reaction; while only one reaction was sufficient for Pax6 alleles. All reactions were done using the Green Taq (PROMEGA), a mix already containing the enzyme and the proper buffer in a 2x concentration. To genotype the *COUP-TFI* locus three primers were used to amplify a DNA sequence within the third exon of the COUP-TFI gene:

ARM531 (5' CTGCTGTAGGAATCCTGTCTC 3'),
ARM402 (5' AAGCAATTTGGCTTCCCCTGG 3'),
EX351 (5' AATCCTCCTCGGTGAGA 3').

The allele of the wild type COUP-TFI gives rise to a 250bp fragment, while the floxed COUP-TFI allele gives rise to a 350bp fragment, therefore it was possible to distinguish the homozygote wild type, one fragment of 250bp, the *heterozygote*, 2 fragment of 250 and 350bp,

and homozygote floxed, 1 fragment of 350bp. The PCR amplification program used to amplify the COUP-TFI allele was the following:

94°C – 7’	
94°C – 45’’	
60°C – 45’’	35x
72°C – 1’	
72°C – 7’	

In order to genotype the *Emx-Cre* allele the primers used were designed to amplify a fragment of DNA of the *Cre-recombinase* ORF, therefore the *Emx-Cre* was detectable. The primers that recognize the *Cre* fragment are the following:

CRE1 (5’ CAGGATATACGTAATCTGGC 3’),
CRE4 (5’ CACGGGCACTGTGTCCAGACCA 3’).

In presence of a *Cre* allele the PCR gave rise to a fragment of 200bp, as an aspecific control of the reaction the PCR was also run with two primers that amplify a fragment of the *β-actin*.
The primers were the following:

CCRmL (5’ CAACCGAGACCTTCCTGTTC 3’),
CCRmR (5’ ATGTGGATGGAGAGGAGTCG 3’).

These primers gave rise to a 250bp fragment.

The program used for these PCR was the following:

94°C – 7’	
94°C –1’	
60°C – 45’’	32x
72°C – 30’’	
72°C – 7’	

To genotype the *Pax6* locus three primers were used to amplify the DNA sequence:

AS2 (5’ CCAGAGAAAGACCTGAGACACTTAC3’),
LAC1 (5’CTGTTGGGAAGGGCGATCGGTG3’),
P6S (5’ GCATATGGGGGCAAGACTATGTG3’).

The allele of the wild type Pax6 gives rise to a 250bp fragment, while the Pax6 allele gives rise to a 300bp fragment, therefore it was possible to distinguish the homozygote wild type, one fragment of 250bp, the heterozygote, 2 fragment of 250 and 300bp, and homozygote mutant, 1 fragment of 3o0bp.
The PCR amplification program used to amplify the Pax6 allele was the following:

95°C – 5’	
95°C – 1’30’’	
60°C – 1’	10x
72°C – 1’	
95°C – 1’	
60°C – 1’	25x
72°C – 1’	
72°C – 8’	

All the reactions were done in a total volume of 10µl using 1µl (100ng-500ng) of the genomic DNA solution and 9µl of the following master mixes:

	COUP-TFI floxed		CRE-recombinase		CCR aspecific β-actin		Pax6
ARM 531	2µl 10µM	CRE1	1µl 10µM	CCR mL	1µl 10µM	AS2	2µl 10µM
ARM 402	1µl 10µM	CRE4	1µl 10µM	CCR mR	1µl 10µM	LAC1	1µl 10µM
EX351	1µl 10µM					P6S	1µl 10µM
H_2O milli-q	1µl	H_2O milli-q	3µl	H_2O milli-q	=		
Green Taq	5µl 2x	Green Taq	=	Green Taq	=		

2.4 Agarose Gel electrophoresis

Agarose gel were prepared dissolving agarose ultrapure (Invitrogen) in TBE buffer (Tris Base 89mM, Boric Acid 89mM, EDTA 2mM, pH 8.3 in H_2O milli-q adding Ethidium Bromide, a molecule able to intercalate DNA and RNA, after which his ability to absorb UV light and re-emit at 590nm is increased 20 fold compared to the free molecule. Therefore it is possible to detect on the gel bands of DNA with a sensibility of 5ng. The TBE buffer is also used to fill the vat that contains the gel, allowing the electricity to run from the negative electrode to the positive, driving also the DNA, with an inrsic negative charge, to the positive electrode. The percentage of agarose was either of 2%, in case the DNA or RNA of interest smaller than 1Kb, either of 1% in case the DNA or RNA of interest was bigger

than 1%. The electrophoresis was run applying an electric field of 100V for the 1% agarose gel and of 120V for the 2%agarose gel. Standard ladder of DNA 1kb and 100bp (Promega) were used to compare the length of samples.

2.5 Brain dissection from embryos

The plug found in the female mice put on mating the previous afternoon was considered as E0.5, at the established embryonic day the pregnant mice was sacrificed and dissected to collect the embryos and dissect the whole brain o these, from the cortex to the *hindbrain*, putting them using the Phosphate-buffered saline (PBS). The samples to be used in immunofluorescence were fixed for 2 hours, after that samples were washed in PBS 3 times, 10' each.

2.6 Cryosection

The brains to be used in Immunofluorescence were cryoprotected after the PFA fixation. The treatment was done by gradually equilibrating the brains in PBS 30% sucrose at +4°C in gentle rocking, with intermediate steps at 10% and 20% sucrose. Then, samples were first washed briefly in Optimal Cutting Temperature (OCT) in which they were also embedded and frozen with dry ice before storing at -80°C until cutting.
Samples were cut using the Cryostat (Leica Cryostat) in which they were first equilibrate at -21°C 1h and then cut at 16 µm for E15.5. The slices were placed on polarized slides (Thermo Scientific) and let dry over night at room temperature (RT) and then stored at -80°C before using. All the washings and the incubations of the slides were made in proper shanks.

2.7 Immunofluorescence on Cryosection

The slides were taken from -80°C and first let dry 1h at RT, after that they were immediately underwent to an Unmasking procedure. Slides were incubated in Unmasking Buffer (Sodium citrate 85mM,

pH6.0) in order to open the membranes of the samples and make the epitope more accessible to the antibodies. The shanks with the slides were also submerged in the Unmasking Buffer. The process was done boiling the box with the shanks twice, at first for 15'' and the second time, after changing the buffer, just until the boiling point.
Then the slides were cooled in ice 10' and washed in PBS 3 times 10' before incubating them 1h at RT with the blocking solution (PBS, 0.1% Triton, 10% Goat Serum) in order to block all the sites that would aspecifically bind antibodies. After that slides were incubated with the primary antibodies diluting them in 200 µl of PBS 0.1% Triton, 3% Goat Serum, antibody were either used in couple or in consecutive incubation depending on the antibody.
The primary antibodies used were the following:

Epitope	Working concentration	Incubation conditions	Host animal	Source
COUP-TFI polyclonal	1:500	O.N. +4°C	rabbit	Thermo Fischer
Pax6	1:500	O.N. +4°C	rabbit	Chemicon
Sox2	1:100	O.N. +4°C	goat	Santa Cruz
Tbr2	1:500	O.N. +4°C	rabbit	Abcam
Phospho-Vimentine	1:25	O.N. +4°C	mouse	Dakocytomation
Ph3	1:20	O.N. R.T.	mouse	Abcam
Gamma-Tubuline	1:500	O.N. +4°C	rabbit	Calbiochem

After primary antibody incubations slides were washed with PBS, 3 times, for10', then the secondary antibodies were incubated diluting them in 200 µl of PBS 0.1% Triton, 3% Goat Serum.

Epitope	Working concentration	Incubation conditions	Host animal	Source
Rabbit FC 488	1:300	2h R.T.	goat	Life Technologies
Rabbit FC 594	1:300	2h R.T.	goat	Life Technologies
Goat FC 594	1:300	2h R.T.	goat	Life Technologies
Mouse FC 488	1:300	2h R.T.	goat	Life Technologies
Mouse FC 594	1:300	2h R.T.	goat	Life Technologies

After the incubation the secondary antibodies were washed in the same way of the primary antibodies, and finally the slides were mounted with the medium Vectashield with DAPI (Vector), closed with a glass coverslip using common nail polish to stick it to the slide.

2.8 DNA and RNA quantification

The instrument used to quantify either DNA or RNA was the Nanodrop spectrophotometer, able to quantify 1µl volume without the need of setting up a standard curve, but converting the absorbance at 260nm of the DNA through the Lmanbert-Beer law [$A=\varepsilon_{\lambda}lC$]. The concentration was calculated on the average of three different dilutions in the reading range of the Nanodrop.

2.9 Images acquisition

Images from immunofluorescence experiments were acquired using the LEICA DM6000 microscope.

2.10 Images analysis

E15.5 Immunofluorescence images were analyzed manually on Adobe Photoshop, both for single cells count and co-localization analysis.

2.11 Statistical analysis

All the data were statistically analyzed and graphically represented using Microsoft Office Excel software. The error bars represent s.e.m. Two-tailed Student's t-test was used for the analysis of statistical significance (*P≤0.05, **P≤0.01, ***P≤0.005). One-way analysis of variance (ANOVA) was used to compare the means among three or more groups of samples and post-hoc analysis was performed by two-tailed Student's t-test.

2.12 Neurospheres assay

2.12.1 Isolation and culture of primary spheres

Embryonic brain tissue was dissected in PBS1X and the neocortex was carefully moved in a 15ml Falcon already prepared on ice. Tissue is dissociated by triturating, until the suspension looks cloudy. Normally, 20-30 strokes are sufficient. The top cell suspensions were transferred to a clean plastic conical 15-ml tube leaving about 1ml containing the undissociated pieces. 2ml culture medium was added to the undissociated pieces, which were mechanically dissociated by trituration again until the cell suspension looked homogeneous, with no undissociated pieces left. During triturating, always avoid foaming and bubbling. All the volume was transferred to the tube containing the first round of dissociated cells. The cells were collected by centrifugation at 75g for 10 min. Supernatant was discarded and cells resuspended in 1ml culture medium. A 10ul aliquot from each sample were diluted in trypan blue, and counted in a hemocytometer. Cortex should give 2,5 $x10^6$ cells. Cells were seeded at a density of $5x10^4$ cells /cm^2 in growth medium, in 6 ml of a 25- cm^2 flask and incubated at 37°C, 5% CO2, and 95% humidity. Single cells proliferated to form spherical clusters (primary neurospheres), floating in suspension.

2.12.2Culture propagation: subculturing protocol

Flask to be passaged had been tapped on the sides to dislodge spheres. Neurospheres were transferred in 5ml fresh medium and pelleted by centrifugation at 110g for 10 min. Surnatant medium was removed and cells tritured in the left 200ul for 25-30 times. Soon after, cells were pelleted again and then 10ul were diluted in trypan blue for counting as described above. Cells were seeded at a density of $5x10^4$ cells /cm^2 in growth medium, in 6 ml of a 25- cm^2 flask and incubated at 37°C/5% CO2/95% air.

2.12.3Growth Curve

Cells were seed at clonal density for 3 days (p0 or step 0) at the described growth conditions. After 3 days of growth, spheres were analyzed by counting before to be subcultured as described above and

re-seed at the same clonal density for other 3 days. The analysis was extended for 15 passages (45 days). Then results were plotted to a semi-logarithmic chart to be analyzed.

2.12.4 Differentiation

Cells were processed as described above. After counting cells were resuspended in 10ml control medium, to remove growth factors and spinned down at 110g for 10 min. Once removed the surnatant, cells were resuspended in 0,5ml control medium, adding as growth factors only FGF-2 to avoid massive cell death. Cells were counted and resuspended in the appropriate control volume, so that $1.5x10^4$ cells/well are contained in 0,400ml 4-well chamberslide. Cells are incubated at 37°C, 5% CO2, and 95% humidity for 3 days.

2.13 Pair-Cell Analysis

Clonal culture and pair-cell analysis were done according to Qian et al. (1998). The neocortex from wild type and mutants embryo at E15.5 was dissected and dissociated in single clones as described above in the NS assay. Single cells were plated in matrigel on 8-well culture chamber-slides and incubated at 37°C, 5% CO2, and 95% humidity. Pair cells were identified after 24 hr incubation by fixing with 4% PFA for 10' and stained with TuJ1 antibody and DAPI for nuclear staining.

2.14 Chromatin Immunoprecipitation assay (ChIP)

2.14.1 Protein A preparation.

300µl of Protein A resin (SIGMA) were collect in a 15ml Falcon tube siliconized and washed in PBS 10' 3 times in slow rotator O.N. at +4°C the first day of protocol to use it the 2nd day. Before to use it, 2 washes were done in Equilibration A buffer. Then the resin was spinned down, the buffer was removed and added a volume of Equilibration A buffer equal to the volume of resin and keep at +4°C.

2.14.2 ChIP day1: Dissection, Fixation, Sonication, Antibody incubation

Tissues were dissected in HBSS and moved in a 15ml Falcon siliconized. Collected material was washed twice in 10ml of DMEM. Biological material was resuspend in a 10ml DMEM solution of PFA 1% to be fixed for 10' at R.T. in slow rotation. The fixation was stopped adding 1.1ml of Glycin 1M for 5' at R.T. in slow rotation. After cells were washed and resuspended in 6ml Lysis Buffer (to be prepared the same day: 20mM HEPES pH7.4, 1mM EDTA, 150mM NaCl, 1%SDS, 125mM Glycine, PMSF 0.2 mg/ml to be added few minutes before using). Tissues and cell membrane were tritured until the solution is clear to obtain nuclei. Soon after nuclei were spinned down and before to proceeds 10ul were diluted in trypan blue and counted in a hemocytometer chamber. Usually material from 15 embryos should be enough to obtain from 150 to 200 µl of nuclei. In the meanwhile Sonication Buffer (to be prepared the same day: 20mM HEPES pH7.4, 1mM EDTA, 150mM NaCl, 0.4% SDS, PMSF 0.2mg/ml to be added few minutes before using) was added to reach 1200µl of total volume. The solution obtained was subdivided in 3 siliconized eppendorf (300µl each). Each eppendorf was sonicated for 6 times for 5" at 10µm amplitude. Samples were spinned down at 14Kg R.T., in order to collect remaining debris. The 3 samples were respectively: the antibody of interest sample, the aspecific antibody sample, the No antibody sample. A 4^{th} eppendorf was added to the analysis with 300µl of Sonication Buffer: the Mock sample containing no DNA and no Antibody. Add to the samples the respective Antibody

(Pax6) divided it into 3 aliquots that will be used to precipitate with 2 antibody of interest (3µg of Antibody). Samples were incubated in rotation O.N. at +4°C.

2.14.3 ChIP Day2. Protein A binding, Washes, Elution, Reverse Cross-link

Protein A was added to each sample and all was incubated 3h at +4°C. Samples were then spinned down at 800g +4°c with slow accel/decel for 2' to collect the resin with the protein and DNA bund to it. The surnatant was removed and only the one from the NoAB sample was kept in order to create the INPUT stock. The resin was washed several times at R.T. with 900µl of Chilled Washing Buffer A (to be prepared the same day: 20mM HEPES pH7.4, 1mM EDTA, 500mM NaCl, 0.8% Triton X100, 0.1% SDS, PMSF 0.2mg/ml to be added few minutes before using), then 3x with chilled Washing Buffer B (to be prepared the same day: 20mM HEPES pH7.4, 1mM EDTA, 250mM LiCl, 0.5% NP40, 0.5% Deoxycholate, PMSF 0.2mg/ml to be added few minutes before using). At this point all the samples were transferred in a new eppendorf and treated with Elution Buffer (To be prepared the same day: 50mM NaHCO3, 1mM EDTA, PMSF 0.2mg/ml to be added few minutes before using) and left in rotation at R.T. for 1h in order to detach the resin from the protein and DNA fragments. At this point the resin was spinned down and the surnatant continents the eluted protein and DNA fragments was transferred in new siliconized eppendorfs. The crosslink was reversed leaving all the samples O.N. at +65°C (DO NOT put parafilm, it will melt and enter in the samples).

2.14.5 ChIP Day3. P.C.A. extraction, precipitation

DNA was extracting from the samples adding 300µl of P.C.A. (25-Phenol/24-Chloroform/1-3methylbuthanol), the upper phase was collected with the DNA in a new siliconized eppendorf and clorophorm was added before to vortex 10'' and spin. The upper phase with the DNA was collected and the samples was precipitated adding 1/10 volume of NaAc pH5.2, 1µl of glycogen and three volumes of

Ethanol ultra-pure. Precipitation was done O.N. at -20°C after mixing the solution by inversion 10 times.

2.14.6 ChIP Day4. Resuspension

Samples are spinned down at +4°C 20000g for 15'. The DNA pellet was washed adding 200µl of Ethanol 70% and spinned. After that the ethanol was removed. Samples were dried and then resuspend in 20µl of H2O milliq. Samples were stored at -20°C.

CHAPTER 3

3. RESULTS

3.1 COUP-TFI mutation impairs neural stem/progenitor maintenance

Previous data on the constitutive ("*null*") and conditional ("*CKO*") mutant mice for COUP-TFI generated in Michéle Studer laboratory (Armentano, Filosa et al. 2006; Armentano, Chou et al. 2007) indicated that COUP-TFI can repress cortical area identity associated with frontal areas, including motor areas, in progenitors of sensory cortex, allowing in this way proper specification of sensory identity in parietal and occipital cortex. Moreover, loss- and gain-of-function experiments in mice demonstrated that COUP-TFI acts by pushing proliferative cells to leave the cell cycle during cortical development (Faedo, Tomassy et al. 2008), suggesting a possible link between cell proliferation and areal formation controlled by COUP-TFI during cortical development. Therefore, since correct areal identity and neuronal formation depend on the right balance between progenitor and neuronal cells, I first evaluated whether COUP-TFI is involved in neural stem cell (NSC)/ progenitor maintenance.

To assess a putative role for COUP-TFI in NSC maintenance, I first decided to use *the neurosphere long-term expansion assay* on *COUP-TFI CKO* mutant cortical primordia in which COUP-TFI is inactivated from E10.5 exclusively in the cortical primordium (Armentano, Chou et al. 2007). The neurosphere assay evaluates the capacity of neural progenitors cells to self-renew in long-term cultures by using a defined medium. To this purpose, I have isolated cells exclusively from the neocortex of control and mutant E.15.5 embryos from the same litter and then mechanically dissociated the tissue to yield a single-cell suspension, which is then plated under quite stringent growth conditions (see also materials and methods). This procedure establishes a selective culture system in which most of the primary differentiated/differentiating central nervous system cells found in the primary tissue die out soon after plating, whereas the undifferentiated stem cells enter into a state of active proliferation. Four main conditions must absolutely be satisfied: low cell density (<5 x 104 cells/cm2), absence of serum, appropriate growth factors EGF and

FGF2). The progeny preferentially adhere to each other while dividing and form spherical clusters that float in suspension. These have been named *neurospheres*, from which comes the name of the technique (**Figure 21**).

The age of the embryos (E15.5) has been chosen because it represents a good compromise between proliferation (cells located in the VZ and SVZ) and differentiation, which occurs after cells have left the cell cycle and start to differentiate in the cortical plate (**Figure 22**). Once the cells were isolated, I confirmed that COUP-TFI protein was completely absent in the mutant cells by doing an immunofluorescence assay on wt and *COUP-TFI CKO* cells directly in the culture dish (**Figure 23**).

Large numbers of wild-type controls and COUP-TFI-deleted cortical cell populations were cultured in basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) for 1–2 passages. This step is crucial during the process of neurosphere formation, since neural stem cells will undergo symmetric cell divisions in which the two daughter cells are identical to their mother cell, but also asymmetric divisions in which one or both cell progeny will be more differentiated and thus will stop proliferating. As a consequence, each neurosphere will be a cluster of both neural stem cells and mature precursors. To increase and isolate mainly self-renewal NSC, neurospheres are thus routinely subcultured by harvesting, followed by mechanical dissociation, and by replating under the initial growth conditions. One of the most important features of this system is that, as at each subculturing step all, but the neural stem cells, will be selected away; the overall expansion of the total cell number will thus result from the division and amplification of primordially neural stem cells.

In my studies and after the subculturing procedure, I have set up a "growth curve" by plating aliquots (passage 0) of mutant and control cells in EGF and bFGF. Every three days I have dissociated and replated the cells and this for several passages in cultures. At each passage I have analysed the size of the neurospheres and successively

I have counted both the number of neurospheres and the total cells, before to seed them again at clonal density (for other three days) (see also **Figure 21** for general procedure). During the analysis, I have applied a "neurosphere size cutoff" to exclude in the counting little small spheres generated by cells that are capable of transient proliferation, such as transient amplifying cells.

My data show that the COUP-TFI-deleted cortical cultures show from the very beginning a huge increase in the size and number of neurospheres when compared to control cultures, especially around the 5th passage, which is maintained until the end of the experiment (**Figure 24**). This is not the case during the initial expansion culture, where I found little or no differences between mutant and wild-type cells; however, by step 3–4 and onwards, neurospheres and total cell number are markedly increased in mutant cultures as shown by statistical comparisons (**Figure 25**). Subsequently, around the 5th passage control cells reach a peak, but soon after they start to decrease and are completely exhausted by passages 9 or 10. This is different for COUP-TFI-mutant cells, which reach a plateau and continue to grow (proliferate) steadily in number for 14-15 passages (red in **Figure 25**). The pronounced defect in number of neural stem/progenitor cells plotted onto a semi-logarithmic graph results in a cell growth curve that clearly show a direct correlation between the trend of the curve and the proliferation/expansion of the neural stem cell pool over serial subculturing.

Overall, these data show that E15.5 cortical stem/progenitor cells have a higher rate of proliferation in the absence of COUP-TFI compared to wild-type cells. The increase in the total number of proliferating neural cells is accompanied by a strong increase in the size of neurospheres. These data demonstrate that normally COUP-TFI acts by limiting the stem/progenitor cell pool during corticogenesis.

3.2 Precise dosage of COUP-TFI and Pax6 influences stem cell survival and maintenance

Previous studies reported that the transcription factor Pax6, normally expressed in RGCCs and essential for neural stem cell proliferation, multipotency, and neurogenesis in many regions of the central nervous system, including the cerebral cortex (Gotz, Stoykova et al. 1998; Warren, Caric et al. 1999; Heins, Malatesta et al. 2002; Stoykova, Hatano et al. 2003; Haubst, Berger et al. 2004; Berger, Berger et al. 2007; Quinn, Molinek et al. 2007; Osumi, Shinohara et al. 2008), is also involved in promoting a rostral cortical identity during areal patterning (Bishop, Goudreau et al. 2000). In *Small Eye* (*Sey*) mutants in which Pax6 resulted mutated (Hill, Favor et al. 1991), neurogenesis and areal identity are compromised, resulting in a seemingly opposite phenotype to COUP-TFI mutant cortices. Given COUP-TFI role in neural stem cell maintenance, I have decided to use the same cell culture conditions as described above and assess now the function of Pax6 in neurosphere formation and maintenance. In my experiments I have used a Pax6 transgenic line in which the start codon and the entire paired domain of the Pax6 gene have been replaced by a ß-galactosidase gene leading in this way to a full protein truncation (St-Onge 1997). Even this is a different genetic model from the one used previously in the literature, homozygous Pax6-LacZ animals have an overall phenotype similar to homozygous *Small Eye* mutants (St-Onge 1997).

At first, I have evaluated the proliferative capacity of *Pax6* mutant E15.5 cortical cells in neurosphere assays in the same conditions as described above for *COUP-TFI CKO* mutant cells. Then, I have decided to compare the *Pax6 null* data with the one obtained by using a different model for COUP-TFI activation, the *COUP-TFI null* line in which COUP-TFI is inactivated constitutively similarly to *Pax6 null* mutants (Armentano, Filosa et al. 2006). This will allow me to compare two analogous genetic systems in which the two genes have been inactivated from the onset of their expression. Finally, I have mated COUP-TFI and Pax6 heterozygous mice together (homozygous mutants do not survive after birth) to obtain first double heterozygotes

and then double homozygous mutant embryos (see also crossing table in Materials and Methods).

The rationale behind all these genetic crossing is to obtain a series of embryos with different dosages of COUP-TFI and Pax6 in order to evaluate their genetic relationship during NSC maintenance in neurosphere assay (**Figure 26**). Therefore, after at least two generations of mouse crossing, I have obtained the following genotypes as assessed by PCR:

- ✓ *COUP-TFI null/Pax6 wt (COUP-TFI -/-);*
- ✓ *Pax6 null/COUP-TFI wt (Pax6 -/-);*
- ✓ *COUP-TFI null\Pax6 heterozygous (COUP-TFI-/- Pax6+/-);*
- ✓ *COUP-TFI heterozygous\Pax6 null (COUP-TFI+/- Pax6-/-);*
- ✓ *COUP-TFI null\Pax6 null (COUP-TFI-/- Pax6-/-);*

My results showed that *COUP-TFI null* cells behave like *COUP-TFI CKO* cells, as described above. The size and number of neurospheres increased dramatically and cells were able to proliferate for at least 15 passages (**Figure 27a**). On the contrary, *Pax6 null* cells start to die very quickly, during the 6th passage. Furthermore, I could notice that Pax6-deficient neurospheres tended to form chain-like structure, despite the fact that they showed a seemingly normal size (**Figure 26**). This is probably due because of adhesion defects, characteristic of *Pax6* mutant cells (Duparc, Boutemmine et al. 2006). Furthermore, my results on the *Pax6 null* mutant reproduced the one observed in *Pax6 Sey* mutants (Asami, Pilz et al. 2011).

In light of the opposite phenotypes observed in the neurosphere analysis, I hypothesized that increased expression of Pax6 in the cortical VZ, as previously described (Faedo, Tomassy et al. 2008), might interfere with the altered behaviour of *COUP-TFI null* stem/progenitor cells. To test this hypothesis, I have repeated the neurosphere assay in E15.5 *COUP-TFI null\Pax6 heterozygous* (COUP-TFI-/- Pax6+/-) cells in which approximately half of the dosage of Pax6 is decreased in a background in which COUP-TFI is completely inactivated.

Surprisingly, I observed an almost full rescue of the *COUP-TFI null* phenotype. Indeed, my analysis of the number of neurospheres and of the total number of dissociated cells showed a rate of proliferation similar to wild-type samples, with cell exhaustion at around the 11th passage (compared to wild-type cells, in which cells are exhausted at around the 9th passage). Even the size of the neurospheres is decreased, becoming comparable to the control (**Figure 26**).

Using the same logic used for the *COUP-TFI null* cells, I hypothesized that the decrease of COUP-TFI expression levels in *Pax6 null* cells might also rescue the Pax6 phenotype and extend the survival of Pax6-deficient cells in culture (in *COUP-TFI+/- Pax6-/-* cells). The neurosphere assay gave an interesting result, even if I failed to obtain a full rescue of the Pax6 null phenotype. I could observe that neurospheres continue to proliferate until the 15th passage, indicating a full rescue in terms of survival rate when compared to simple *Pax6 null* cells (**Figure 27a**). However, the neurospheres continued to show the characteristic chain-like morphology previously described in the Pax6-defiecient cells (**Figure 26**). This phenotype was even worse with time and along the different passages. This indicates that decreased dosage of COUP-TFI helps Pax6-deficient cells to continue proliferating instead of exiting the cell cycle, but that the adhesion problems observed in the absence of Pax6 cannot be compensated by decreased COUP-TFI expression. Nevertheless, I could observe that in the absence of both COUP-TFI and Pax6 (in *COUP-TFI-/- Pax6-/-* cells), the presence of huge and "sticky" neurospheres was even more exacerbated. Double mutant cells continue to proliferate until the 15th passage, maintaining the characteristic chain-like structures and forming huge and wide complexes, visible even to the unaided eye.

All the data have been plotted onto a semi-logarithmic graph and result in several cell growth curves (**Figure 27b,c,d**) that clearly show that in the absence of COUP-TFI, cells have a higher rate of proliferation, compared to the control, which is rescued to "normal" when decreasing Pax6 expression levels. Conversely, the premature

neurospheres death observed in *Pax6 null* cells, is "rescued" after decreasing COUP-TFI dosage and stem/progenitor cells enter in an active state of proliferation until the 15th passage. Taken together, these data clearly show that COUP-TFI and Pax6 synergistically interact to maintain a defined pool of stem/progenitor cell proliferation *in vitro*. They also suggest us that COUP-TFI controls the number of stem/progenitor cells by modulating Pax6 expression levels, and conversely that Pax6 controls stem-progenitor cell survival by controlling COUP-TFI expression in cortical progenitors.

3.3 Precise dosage of COUP-TFI and Pax6 influences the rate of symmetric versus asymmetric divisions in stem/progenitor cells, as evaluated by the in vitro "pair- cell analysis" assay

My previous data indicate increased proliferation of stem/progenitor cells in the absence of COUP-TFI and precocious exhaustion of the same population in the absence of Pax6. By modulating the reciprocal gene expression in the two mutant cells, I obtained a rescue in the rate of cell proliferation and in the number and size of neurospheres (**Figures 25-27**). My next question is to understand whether differences in cell proliferation observed in the various mutants might be due to changes in the mode of cell divisions, symmetric versus asymmetric, that occurs at different rates during corticogenesis. I focused my analysis on wild type, *COUP-TFI null* and *COUP-TFI null/Pax6 heterozygotes* in which I could observe a clear phenotypic rescue in the neurosphere assay. Regarding the *Pax6 null* genotypes, this analysis was not appropriate because of the high tendency of mutated cells to stick to each other.

To address this issue, I have examined the mode of cell division of cortical progenitors derived from E15.5 cortices of the different genotypes that have been plated at clonal density. The method, called "pair-cell analysis" consists in following the fate of two dividing cells by labeling them with the early neural marker TuJ1(Sahara and O'Leary 2009). After 24hr of *in vitro* culture, cells are immunostained with TuJ1 to selectively mark the neuronal progeny and distinguish from progeny that are in a proliferative state (**Figure 28a**). During the analysis of the different genotypes, each pair of daughter cells was

individually scored. I have counted on average 60 pairs of cells from each genotype and compared them with wild-type controls. When both dividing cells are negative for Tuj1, then they are considered to divide in a *symmetric proliferative mode*. When both cells are instead positive for Tuj1, then they are dividing in a *symmetric neurogenic mode*, and finally when only one cell is positive for TujI, then the mode of division is considered to be *asymmetric* (**Figure 28a**).

My data show a statistically significant increase of symmetric divisions in the *COUP-TFI null* cells when compared to control ones: here, the majority of daughter cells (56± 2,4%) are both negative for Tuj1 marker, characterizing them as neural stem/progenitor cells. Consequently neurogenic (both cells Tuj1 positive) and asymmetric division (only one of the pair of daughter cells is labeled) result strongly diminished (respectively 28± 1,6% and 16± 2,8%). Differently, the *COUP-TFI null/Pax6 heterozygous* mutant cells show an almost normal rate of symmetric proliferative (34± 1,63%)versus symmetric neurogenic (41± 0,8%) and asymmetric (25± 0,9%) division, when compared to wild-type cells (**Figure 28b**).

These data are in accordance with the previous results obtained by the neurosphere approach and confirm a crucial role for COUP-TFI in maintaining a correct balance between proliferation and differentiation during corticogenesis. In the absence of COUP-TFI, cells tend to increase their rate of self-renewal symmetric divisions at the expense of neurogenic asymmetric divisions. The ratio between symmetric and asymmetric fate is rescued by decreasing levels of Pax6 expression in a *COUP-TF1 null* background, confirming the existence of a delicate genetic dosage between COUP-TFI and Pax6 during cortical development.

3.4 Neuronal potential of differentiated neurospheres

It is well recognized that neural stem cells are multipotent (i.e., they can generate neurons, astrocytes, and oligodendrocytes), a property ought to be stably reproduced in neurosphere cultures, particularly at the clonal level when they are induced to differentiate

(Johe, Hazel et al. 1996). Differentiation from neurospheres is obtained by dissociating neurospheres and plate dissociated cells at clonal density onto a good adhesive substrate, such as matrigel. After two days, growth factors are removed and cells will naturally differentiate into the three major cell types.

After 15 days, differentiated cells were processed for immunofluorescence using TuJ1, GFAP (glial fibrillary acidic protein) and O4 antibodies to identify neurons, astrocytes, and oligodendrocytes, respectively. I have repeated this procedure in all genotypes described above (**Figure 29**). In all genotypes I have obtained a high number of astrocytes and very few oligodendrocytes and neurons. This is in accordance with other studies that indicate that in a long-term expansion culture system, such as the one of neurospheres, gliogenesis becomes more prevalent than neurogenesis. It is believed that this might be due to the switch from neurogenesis to gliogenesis that occurs during normal corticogenesis, suggesting that intrinsic cellular programs are strongly preserved *in vitro*, despite changes in environmental factors (Conti and Cattaneo 2010).

Although this approach fails to recapitulate normal corticogenesis, it nevertheless tells me that in my culture conditions neurospheres are still multipotent and able to differentiate into the three major cell types. Therefore, I decided to count the three neuronal populations (**Figure 29b).** GFAP positive cells are all comprised in a range that varies between 70% and 90% showing no particular differences. The same goes for oligodendrocytes O4 positive cells comprised in a range between 2% and 10%. On the contrary, the neuronal trend is more interesting. The analysis reveals that in the *COUP-TFI null* there is an increase number of neurons (30% more than the control) that is rescued to almost normal levels by lowering the Pax6 dosage (*COUP-TFI null/ Pax6 heterozygotes*): the amount of neurons is only about 2% higher than the wt. In the absence of Pax6 (*Pax6 null*) neurons fail to form, as previously shown (Asami, Pilz et al. 2011), however some neurons are produced by lowering COUP-TFI expression levels (*COUP-TFI heterozygotes/ Pax6 null*) even if not as many as in controls. Similarly, no neurons and very few astrocytes can

differentiate in *double null* mutant cells, indicating that severe abnormalities occur in the total absence of COUP-TFI and Pax6 functions during neurogenesis.

3.5 Precise dosage of COUP-TFI and Pax6 is required for correct cortical cell cleavage in vivo

To gain a further understanding of the characteristics of cell division modes in COUP-TFI, Pax6 and compound mutants *in vivo*, I have decided to investigate the cleavage angles of progenitor cells dividing at the apical surface of wild-type (WT) and mutant cerebral cortices at the same embryonic stage used for the neurosphere assay, i.e. E15.5, a stage corresponding to mid-neurogenesis in the mouse.

Coronal sections of E15.5 cortices of all genotypes were immunostained with the mitotic marker Phospho Histone H3 (PH3), which is specifically phosphorylated during cell divisions (**Figure 30a and 30b**). I have carefully examined the different angles of dividing cells positive for PH3 and grouped them into three main classes in function of their observed cleavage angles relative to the apical ventricular surface of the developing cortex. A vertical angle was scored when the cleavage plane of division ranged from 60° to 90°, horizontal with an angle of 0° to 30° and oblique when the angle was between 30° and 60°(**Figure 30b**).

My data are in agreement with the literature, which shows that apically-located cortical cells reach a sort of equilibrium between the three kinds of divisions in WT cortices with a ratio of 35:35:30 between vertical: horizontal: oblique divisions (Asami, Pilz et al. 2011) (**Figure 30**). By contrast, 70± 1,2% of cells in the cerebral cortex of *COUP-TFI null* mutant cortices opt for a vertical cleavage plane, whereas oblique and horizontal divisions are detected as 10± 1,6% and 20± 0,4% of the total mitotic cells, respectively (**Figure 30c**). Interestingly, I observed a situation close to normal in the mutant *COUP-TFI null/Pax6 heterozygotes*, which showed a ratio between vertical symmetric, horizontal asymmetric and oblique divisions of 41± 1,6%,, 35± 6,1% and 24± 2,4%, respectively. In

accordance with previous data in the literature for *Pax6 Sey* mutants (Asami, Pilz et al. 2011) *Pax6 null* cortices have an increase of oblique divisions (45± 1,2%) at the expense of symmetric divisions, which decreased to 20± 0,4%, whereas the asymmetric neurogenic population does not change in the absence of Pax6 (**Figure 30d**). This indicates that part of the symmetric divisions have been converted to oblique divisions, which are normally used by intermediate progenitors located in the basal plate (Asami, Pilz et al. 2011; Postiglione, Juschke et al. 2011). Interestingly, by decreasing Pax6 expression in *COUP-TFI null* cortices (e.g. in *COUP-TFI heterozygous/Pax6 null* cortices) symmetric divisions have massively expanded, similarly to double *COUP-TFI/Pax6 null* cortices, although in a less pronounced manner than observed in the previous genotype (**Figure 30e**). This is very intriguing and suggest that other factors involved in controlling the rate between symmetric versus asymmetric divisions have been affected when COUP-TFI and Pax6 expression levels are decreased.

Taken together, these data confirm that COUP-TFI controls the spindle orientation and cleavage plane of apical progenitors of the developing cortex by controlling Pax6 expression levels. Moreover, they also show that alterations in COUP-TFI gene dosage in the total absence of Pax6 does revert the Pax6 phenotype (reduction of symmetric divisions); however mutant cells tend to acquire an abnormally higher rate of symmetric cleavage planes than normal indicating that they must proliferate more than normally expected.

3.6 Altered distribution of apical and basal progenitors in COUP-TFI mutant cortices

To further clarify whether the alterations in the cleavage plane orientation result in alterations in the rate and/or distribution of cortical progenitor populations *in vivo*, I have immunostained E15.5 coronal sections of wild type and COUP-TFI mutant cortices with the markers Sox2 and Tbr2, which label apical and basal/intermediate progenitor cells, respectively (**Figure 31**).

In E15.5 wild-type embryos, the SVZ is rich in basal/intermediate progenitors and shows a high number of Tbr2+ cells. A few Tbr2+ cells can also be seen migrating into the intermediate zone (IZ). Differently, I observed decreased Tbr2+ cells in the lower part of the SVZ of *COUP-TFI null* cortices, and as previously described (Alfano, Viola et al. 2011) some Tbr2+ cells tend to be stuck in the upper SVZ at the border with the IZ (**Figure 31c**). This defect seems to be slightly improved by decreasing Pax6 expression levels in *COUP-TFI null* mutants (*COUP-TFI null/Pax6 heterozygotes*), particularly in the number of Tbr2+ cells stuck in the upper SVZ, although decreased expression of Tbr2 in the lower SVZ can be still observed (**Figure 31d**).

The apical progenitors stained with Sox2 are normally distributed in the VZ with a few cells in the SVZ. In mutant cortices null for COUP-TFI, Sox2 expression is increased suggesting an increased number of apical progenitors in the absence of COUP-TFI function (**Figure 31**). Because of the high cell density observed at this age, it becomes very difficult and unreliable to count single cells. Thus, I decided to evaluate the thickness of the Sox2+ domain and to calculate the ratio between the Sox2+ thickness and the entire extension of the cortex from the apical to the pial surface (see **Figure 31a**). This analysis clearly shows that the area positive for Sox2 is increased of 6.1% in *COUP-TFI null* cortices compared to controls, indicating that a higher number of apical progenitors still proliferate in the absence of COUP-TFI. Differently from Tbr2, Sox2 expression is decreased in *COUP-TFI null/Pax6 heterozygotes* although not to complete normal rates. Moreover, by counting the Sox2/Tbr2 double positive cells, I could

observe a higher rate of double positive cells in *COUP-TFI null* cortices compared to controls one. This rate of double positive cells is decreased in the *COUP-TFI null/Pax6 heterozygote* similarly to control levels.

My data above indicate an increased rate of proliferating apical progenitors and decreased mature basal progenitors in the constitutive mutant for COUP-TFI. Interestingly, a higher number of progenitors than normal, express markers of apical and basal progenitors, probably indicating a transition or hybrid identity between self-renewal and neurogenic fates of these double-positive cells. Lowering Pax6 expression in *COUP-TFI null* cortices results in a slight recovery in the number of basal progenitors and in a partial complete rescue in the accumulation of cells at the upper SVZ Importantly, the rate of cells double positive for Sox2 and Tbr2 is decreased in these mutants indicating a more normal rate between self-renewal and committed progenitors.

3.7 Do Pax6 and COUP-TFI negatively regulate each other in vivo?

The experiments described above indicate that in the absence of COUP-TFI function, the orientation of cell division results biased towards symmetrical progenitor divisions, as also indicated by the increase of the apical Sox2+ population. I also showed that this phenotype is partially rescued after decreasing Pax6 expression levels in a *COUP-TFI null* background. This suggests that the two genes could negatively regulate each other within progenitor cells and that only precise expression levels of both transcriptional regulators will commit the cell to its correct fate. To first assess whether expression levels of COUP-TFI and Pax6 are indeed complementary within single progenitor cells, I have qualitatively evaluated the expression levels of individual wild type cells immunostained for COUP-TFI and Pax6 proteins (**Figure 32**). Although some cells strongly express both TFs, I could observe that the majority has complementary expression levels of COUP-TFI and Pax6, as judged by the intensity of fluorescence within the same cells: to a high fluorescence intensity for Pax6 corresponds a lower intensity for COUP-TFI. This suggests that probably COUP-TFI and Pax6 mutually inhibit each other and that

this process is very dynamic during corticogenesis since some cells have high expression of both genes. ,Nevertheless, more experiments, particularly the ability to express at abnormally high levels one TF and assess whether the other is downregulated, will be necessary to ultimately demonstrate this point.

3.8 Identification of Pax6 binding site on COUP-TFI sequence by Chromatin Immunoprecipitation

My previous data suggest a possible reciprocal regulation between COUP-TFI and Pax6 during corticogenesis. A previous report has already demonstrated direct binding of COUP-TFI on the Pax6 locus. This consists of a highly evolutionarily conserved DR1 binding site (TGTTCACAGTCCA) located at the 3'-UTR region of the mouse Pax6 locus (Tang, Xie et al. 2010).

To evaluate whether Pax6 could also directly bind on the COUP-TFI locus, I have first searched for Pax6 binding sites on the COUP-TFI mouse locus using ECR browser, which is a browser tool designed to highlight candidate functional elements by comparing genomic sequences from several evolutionary distant organisms, such as primates and fish. This approach will likely uncover the fundamental building blocks shared by all vertebrates, while the comparative sequence analysis with closer comparisons, such as those between mice and rats, can highlight the functional structure of diverging genomic regions. Interestingly, I have been able to find one Pax6 binding site on the COUP-TFI mouse locus (CGTATCATTTTAT), which is evolutionary well conserved and which is distant more or less 1000bp from exon 1 of the gene (**Figure 33**). With the help of a post-doctoral fellow in the lab, I have carried out chromatin immunoprecipitation (ChIP) experiments that allow investigating the interactions between proteins and DNA and aims to determine if specific proteins are associated with specific genomic regions.

To test whether Pax6 could bind to the COUP-TFI locus, we have isolated the cortex from E15.5 embryos in which proteins and associated chromatin are temporarily bonded. The DNA-protein complexes (chromatin-protein) are then sheared by sonication and

DNA fragments associated with the protein of interest are selectively immunoprecipitated, using specific antibodies to the protein of interest, in my case a Pax6 polyclonal antibody. The DNA associated with the protein is identified by quantitative polymerase chain reaction (PCR). In our hands, the consecutive real time PCR analysis has revealed an enrichment of the binding site about three times higher than controls, confirming the hypothesis that Pax6 can bind to the regulatory region of the COUP-TFI gene.

3.9 Possible common target genes of COUP-TFI and Pax6

To gain further insight into the molecular mechanisms underlying the relationship between COUP-TFI and Pax6, I have tried to identify common target genes that could explain the phenotype observed *in vitro* and *in vivo*.

First, with the help of the Studer lab and the functional genomic platform of the University of Nice Sophia-Antipolis (http://www.genomique.info/joomla_2.5.9/), we have obtained a list of putative downstream genes expressed in the VZ and regulated by COUP-TFI (**Figure 34**). Then, with the help of external bioinformaticiens, we have crossed the list of putative COUP-TFI downstream genes with other Pax6-specific microarray lists available on the web and in literature **(Figure 34a)**. The common targets obtained by comparing microarray data from the two genes have been cataloged according to the highest statistical probability to be shared by both genes **(Figure 34b).** The first gene that came out from this analysis was Tbr2.

In the literature it has already been described that Pax6 can control the production of basal progenitor cells during cortical neurogenesis. In fact, loss- and gain-of-function approaches of Pax6 have revealed a molecular network controlling neural stem cell self-renewal and neurogenesis in the developing mouse cortex (Sansom and Livesey 2009). In this study, Tbr2 was found to be a direct target of Pax6 and removing Pax6 function, stem cell cortical self-renewal was reduced, resulting in an excess of early neurogenesis and a decrease of Tbr2+

cells (Quinn, Molinek et al. 2007). Regarding COUP-TFI, the results described above and a previous study (Faedo, 2008) confirm a decrease in Tbr2+ basal progenitors in *COUP-TFI null* cortices at different stages. These data seem in fact to support the hypothesis of Tbr2 as a common target of these two transcription factors, on which they could act in similar ways. If this is true, then double COUP-TFI/Pax6 cortices should completely lack Tbr2 expression, and thus production of any basal/intermediate progenitor cell.

CHAPTER 4

4. Discussion

This work provides the first description of the role for the nuclear receptor COUP-TFI in conjunction with the transcription factor Pax6 in neural stem progenitor self-renewal and cell fate determination, both fundamental processes controlling the generation of specific classes of neurons and their ultimately distribution in functional areas.

In this study, I have showed that COUP-TFI is directly involved in controlling the size and number of the stem/progenitor pool and that a precise dosage of COUP-TFI and Pax6 is necessary for correct stem/progenitor cell maintenance. These data strongly support a genetic interaction between these two transcription factors, responsible for opposite fate identity during cortical development. One of the most prominent features of the mammalian cerebral cortex is its areal identity, which relies on the proper specification and laminar distribution of several neuronal cell-types extensively interconnected into functional networks. It follows that a defect in the production of the correct number, identity and network of neurons lead to macroscopic consequences. By investigating the problem at its source, my study aims to understand whether a defect in the specification of cortical subpopulations may result from an imbalance in the ratio between stem self-renewal and progenitor committed cells.

In my thesis work I have been able to highlight the importance in maintaining a correct balance between self-renewal and committed progenitor cells in order to have a proper development of the neuronal cortical progeny. My data indicate that this balance depends on the precise genetic ratio and expression levels of two transcription factors: COUP-TFI, responsible for a caudal identity, and Pax6, responsible for a rostral identity in the cortex. The data suggest a biological model for which COUP-TFI and Pax6 may co-regulate each other in maintaining the balance between symmetric self-renewal and asymmetric neurogenic divisions, which are crucial for the correct radial and tangential expansion of the cortex during development, but also during evolution.

4.1 COUP-TFI and Pax6: a fine genic interaction to maintain the balance between self-renewal stem and progenitor cells

Although the vertebrate brain commonly derived from neuroepithelial cells, the size and complexity of the pseudostratified organization of the brain have dramatically expanded during the evolution of mammals with consequent formation of a highly folded cortex. Internal molecular controls on the balance between self-renewal stem cells and precursors are behind these events (Fish, Dehay et al. 2008). This intriguing subject has prompted me to challenge the role for COUP-TFI transcription factor in neural/stem progenitor cell commitment, since previous data showed that in its absence, areal identity, lamination and neurogenesis were impaired (Armentano, Filosa et al. 2006; Armentano, Chou et al. 2007; Faedo, Tomassy et al. 2008; Tomassy, De Leonibus et al. 2010).

My first experimental approach I have used during my studies is the *in vitro* neurospheres assay, which allowed me to identify a role for COUP-TFI in stem progenitor cell regulation. Neurospheres are a mixed and selective system. Mixed, because of concomitant inner symmetric and asymmetric divisions, resulting in the presence of committed progenitors with low proliferating profile, and neural stem cells with high proliferating capacity. What I liked most from this system is the stringent selectivity toward these two progenitor populations: the more neurospheres are subcultered by harvesting, mechanically dissociated and re-plated under the same growth conditions, the more they have a positive selection for stem self-renewal cells. In the neurosphere system most primary differentiated cells are eliminated as soon as cells are put in culture. On the contrary, undifferentiated stem cells enter into in an active proliferation state, which can be followed for days and weeks. I have showed that in the absence of COUP-TFI, cells proliferate extensively, indicating that normally COUP-TFI restricts the capacity of progenitors cells to proliferate and push these cells to take a committed fate. Further analysis showed that decreasing Pax6 level expression in a COUP-TFI null background rescued almost to the normal situation, displaying a quite right equilibrium between stem proliferating and committed cells. It's already known in literature Pax6 involvement in self-

renewal cells and neurogenesis and it's classified as a proliferating factor highly gene-dosage dependent. As expected, Pax6 null genotype leads to an extreme reduction in neural proliferating stem cells, that are instead pushed on a extreme proliferating way decreasing COUP-TFI level maintaining a Pax6 null background.
According with what is already known in literature, my data place COUP-TFI and Pax6 in a genetic interaction relationship, that took me into investigate further on their contribution about balancing neural stem/ progenitor and committed precursor cells.

4.2 COUP-TFI and Pax6 are involved in cell fate decision

Neurogenesis relies on a delicate balance between progenitor maintenance and neuronal production. This process is mainly based on the mode of cell divisions, symmetric divisions for self-renewal and expansion and asymmetric divisions for producing large numbers of diverse cortical cell types.

Given the now obvious involvement of COUP-TFI and Pax6 in regulating stem cell maintenance, I wondered whether this process was due to a differential regulation of these two transcriptional regulators in the mode of cell divisions, symmetric versus asymmetric, which are crucial for cell fate decision, and for the tangential and radial expansion of the cortex. Before the onset of neurogenesis, the neural/stem precursor cells are responsible for extensive symmetric divisions that will increase the pool of dividing cells. Subsequently, during neurogenesis symmetric divisions are coupled with increased asymmetric divisions, which will impact on cell fate acquisition by producing distinct neuronal cell types.

My data show that *COUP-TFI null* mutant have a significant increase in symmetrical self-renewal divisions at the expense of asymmetric neurogenic ones. These data were first obtained *in vitro* in the neurosphere assay and then confirmed *in vivo*: an increase of stem self-renewal cells that reflect an increase of cells that continue to proliferate, without exiting the cell cycle. Exactly like before, decreasing Pax6 levels will restore an almost normal condition leading to a balanced rate between symmetric and asymmetric divisions. Once

again, these results confirm a genetic interaction between COUP-TFI and Pax6, which mutually repress each other as a way to keep their precise expression levels during the control of symmetric proliferative, symmetric neurogenic and asymmetric neurogenic divisions.

Thus, COUP-TFI is required in maintaining a correct stem cell pool and promoting asymmetric neurogenic divisions during corticogenesis by precisely controlling Pax6 expression levels in apical progenitors.

4.3 Behaviour of neural stem and progenitor cells underlie cortical neurogenesis in COUP-TFI and Pax6 mutants background

Although the neurosphere assay suggest that COUP-TFI mutant cells continue to cycle instead of being committed to neurogenic precursors, they nevertheless retain the correct information and can start proper differentiation. By simply subtracting the growth factors, after three days of proliferative growth, cells spontaneously differentiate into the three major neuronal populations: neurons, astrocytes and oligodendrocytes. Actually, the *in vitro* differentiation of dissociated cells originating from neurospheres does not reflect the real *in vivo* ratio between these neuronal populations, but simply show the potential ability of neural stem cells to differentiate into the three neuronal populations.

In vivo, the stem/precursor cell populations are responsible for building the brain and are retained in small proliferative areas. The embryonic one is the VZ, where epithelial cells with neural stem cell properties appear at embryonic day (E) 8 and from which originate radial glial apical progenitors (RGCCs) and all cells of the developing and mature central nervous system. When neurogenesis start, a second progenitor population will be generated from asymmetrically dividing cells in the VZ, the basal or intermediate progenitors, which are more committed and migrate basally into the SVZ and from here to the cortical plate to produce mature neurons.

The ratio between apical and basal progenitors observed *in vivo* in E15.5 cortices reveals an altered relationship between the two cortical

progenitor populations in the constitutive mutant for COUP-TFI in favour of the apical (self-renewal) progenitors. In fact, basal progenitors positive for Tbr2 are decreased and tend to accumulate at the upper SVZ. Moreover, a higher number of cells expressing the apical progenitor marker Sox2 and the basal progenitor marker Tbr2 can be detected in COUP-TFI mutant cortices. This might suggest that COUP-TFI limits the production of Sox2 expression in apical progenitors, but also temporally controls the onset of Tbr2 expression and thus the appearance of basal progenitors. Alternatively, the concomitant expression of both markers might reveal the existence of a different kind of progenitor cells, a hypothesis that I plan to investigate in the future.

Nevertheless, this situation *in vivo* confirms the data obtained so far *in vitro*. The stem/progenitor cell population continues to proliferate by most probably re-entering the cell cycle resulting in more symmetric and less neurogenic divisions, and consequently, in increasing the pool of apical progenitors. *In vivo*, the phenotypic rescue after lowering Pax6 expression is not as striking as *in vitro*, although I can observe a decrease of Sox2+ apical progenitors, a recovery in the number of Tbr2+ basal progenitors and a decrease of Sox2/Tbr2 double positive progenitors.

4.4 Pax6 and COUP-TFI proteins directly regulate their expression and act on the same target genes

Neural stem cells will produce all neurons in the brain. A key feature of these cells is the ability to regulate the balance between making more neural stem cells, the process of self-renewal, and making neuronal cells, the process of neurogenesis. Increasing self-renewal will result in a brain with too few neurons and abnormal circuitry; increased neurogenesis will deplete neural stem cells too quickly, resulting in a smaller brain and neurological abnormalities. Little is currently known about how neural stem cells control this fundamental choice. I used two transcription factors, COUP-TFI and Pax6, which are both involved in neurogenesis, to dissect the molecular networks controlling neural stem cell self-renewal and neurogenesis in the developing mouse brain. The evidence that both

genes control the balance between self-renewal and neurogenesis in neural stem cells and that precise levels are required to drive the system correctly towards neurogenesis, prompted me to evaluate which kind of interaction exists between the two genes. Although my experiments on compound mutants clearly show a genetic interaction between the two genes, thanks to the chromatin immunoprecipitation technique I could confirm direct binding of Pax6 on COUP-TFI mouse sequences, and thus direct regulation of Pax6 on COUP-TFI. These data coupled with the one already reported in literature regarding a COUP-TFI binding sequence in the Pax6 locus has allowed me to corroborate direct physical interactions between these two transcription factors.

A molecular network controlling neocortical area formation defines the Pax6 proliferation factor as one of the genes responsible for rostral identity, and the COUP-TFI pro-differentiation factor responsible for a caudal fate. My data have revealed essential and non-redundant roles for COUP-TFI and Pax6 in maintaining the equilibrium between neural stem/precursor and progenitor committed cells in the embryonic cortex. More specifically, it is the precise level of expression of the two genes to be essential for controlling the balance between neural stem cell self-renewal and neurogenesis.

To further investigate their relation, I found that cortical cells tend to express complementary levels of COUP-TFI and Pax6 in progenitor cells indicating that the rostral and caudal transcription factors show a degree of mutual cross-repression in the VZ. In order to have a more complete picture, I am now trying to identify common downstream genes of the two transcription factors. By intersecting the results obtained from different microarrays analysis for COUP-TFI set up in our laboratory and those for Pax6 available in the literature, I obtained a set of genes ranked according to their probability of being downstream effectors. Among the top genes in the list appears Tbr2; a gene expressed in basal progenitor cells in the cortical SVZ, and Mash1, expressed in the ventricular zone normally in interneuron precursors. These genes are already found to be downstream targets for Pax6, positioning this gene as a major controller in a signaling

network fundamental for the neural stem/neural balance. Moreover, COUP-TFI also seems to regulate directly or indirectly (eventually via Pax6 or Sox2) Tbr2 expression. Thus, COUP-TFI and Pax6 seem to regulate similar target genes important for maintaining a proper rate between apical and basal progenitors during corticogenesis.

4.5 Perspectives

The discovery that two master genes such as COUP-TFI and Pax6 cooperate in the fine control of the balance between stem self-renewal and neuronal fate, paves the way for new exploration of the mechanisms controlling subtype-specific interactions between selected populations of projection neurons. In light of all these findings, future works are necessary to better clarify the kind of interaction between the two master genes. My experiments leads to a molecular model in which COUP-TFI and Pax6 mutually inhibit each other, and acting on similar downstream genes, which will finely regulate the balance between stem cell self-renewal and progenitor commitment (**Figure 35**).

To further clarify the molecular mechanisms controlling the balance of proliferating versus neurogenic fate progenitors, I would like to investigate the molecular interaction between COUP-TFI and Pax6, strengthen the analysis of their common target genes and clarify which could be their final function during progenitor commitment. I also plan to assess whether a recently-described population of progenitors, such as the outer radial glial cells (oRGC), thought to arise from the division of RGC cells, might be abnormally increased in the absence of COUP-TFI and Pax6: they delaminate from the apical surface and translocate their nuclei in the outer portion of the SVZ where they start dividing. Defined as the population responsible for the brain growth, they were initially thought to be specific of primate brain. But recent studies have shown that oRGC cells also exist in non-primate species with a lissencephalic brain such as rodent. After that it will be necessary to evaluate the lamination and areal fates at P0 to investigate whether changing COUP-TFI and Pax6 gene dosages can rescue or at least ameliorate the laminar and areal defects observed in the absence of one or the other gene.

CHAPTER 5

5. REFERENCES

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VERBALE DELLA RIUNIONE DEL COLLEGIO DEI DOCENTI DEL CICLO XXV DEL DOTTORATO DI RICERCA IN BIOCHIMICA E BIOLOGIA CELLULARE E MOLECOLARE TENUTASI A NAPOLI IL 22.03.2013

Il Collegio dei Docenti si è riunito in riunione telematica per discutere e deliberare sul seguente o.d.g.:
1. Esame delle relazioni dei Relatori e Controrelatori sulle tesi presentate dai dottorandi del XXV Ciclo per l'esame finale
2. Varie ed eventuali

Sono presenti: Arcari (coordinatore) Quesada (segretario) Ammendola, Aniello, Bevilacqua, Costanzo, Cubellis, Dello Russo, De Lorenzo, De Vendittis, De Simone, Esposito, Faraone-Mennella, Faraonio, Izzo, Russo.

2. Esame delle Relazioni e Controrelazioni delle tesi presentate dai dottorandi del XXV Ciclo per l'esame finale

I dottorandi del XXV Ciclo: Marcella Fiengo, Anna Lamberti, Salvatore Marco, Giorgia Montano, Rosa Pezzotti, Anna Lisa Romano, Carmen Sarcinelli, hanno presentato al Collegio, nella sua riunione del 19/02/2013, relazioni scritte e orali sull'attività svolta durante l'intero ciclo di dottorato, relazioni che in quella riunione sono state approvate dal Collegio.

Il Coordinatore propone poi che il Collegio proceda all'esame delle tesi sulla base delle relazioni che i relatori e i controrelatori hanno presentato per ciascuna tesi.

Il Collegio approva.

Il Collegio esamina quindi individualmente l'attività svolta dai dottorandi e le tesi. Dopo un'ampia discussione, il Coordinatore propone la seguente presentazione generale, da intendersi come validi per tutti i dottorandi:
"I dottorandi del XXV ciclo hanno svolto nel I e nel II anno di corso una intensa attività didattica e seminariale partecipando ai corsi di: Struttura e Fisiologia della Cellula, Struttura e Funzione delle Proteine, Genomica di Espressione, Bioinformatica degli Acidi Nucleici, Inglese in Biochimica. I risultati delle verifiche dell'attività didattica sono stati tutti positivi. I dottorandi hanno più volte esposto individualmente con seminari rivolti al Collegio e in riunioni nazionali, ed in alcuni casi internazionali, risultati sul progresso del loro lavoro sperimentale per la tesi. Il Collegio pertanto può presentare con piena soddisfazione tutti i dottorandi del XXV Ciclo.

Il Collegio approva la presentazione proposta all'unanimità.

Il Coordinatore ringrazia vivamente anche a nome del Collegio i colleghi che hanno svolto il lavoro di controrelatori.

Il Coordinatore legge le relazioni e le controrelazioni presentate per ciascuna tesi di dottorato.

Anna Lisa Romano – PhD Thesis in Biochemistry and Cellular and Molecular Biology

La dottoranda Anna Lisa Romano presenta una tesi dal titolo: *Role of the transcription factors COUP-TFI and Pax6 in neural stem cell self-renewal and neurogenesis during mouse cortical development.*

La Relatrice Dott.ssa Michèle Studer riferisce quanto segue:

Anna Lisa Romano ha iniziato a lavorare dal 1 Ottobre 2010 come studentessa Iscritta al secondo anno del corso di dottorato di ricerca in "Biochimica e Biologia Cellulare e Molecolare" dell'Università degli Studi di Napoli Federico II, presso il mio laboratorio su un progetto di ricerca di tipo cellulare e molecolare in accordo con il Dott. Elio Pizzo. Anna Lisa si è occupata di verificare sperimentalmente se il recettore nucleare COUP-TFI possa essere coinvolto nella regolazione del passaggio fra cellula corticale progenitrice a neurone corticale differenziato. A tale scopo Anna Lisa ha usato un modello genetico in cui il gene COUP-TFI è inattivato in corteccia e ha confrontato le proprietà molecolari e cellular dei progenitori corticali a un modello wild-type. Con l'aiuto di una serie di marcatori specifici per popolazioni diverse di progenitori corticali, Anna Lisa ha scoperto in vitro, tramite la tecnica delle neurosfere, ed in vivo, su sezioni adiacenti di corteccia embrionale, che COUP-TFI ha lo scopo di controllare durante le fasi di sviluppo l'espansione dei progenitori corticali multipotenti per permettere la generazione di un corretto numero di neuroni in corteccia. Infine, Anna Lisa ha potuto dimostrare geneticamente che questo controllo avviene in stretta collaborazione con il gene Pax6. Anna Lisa ha lavorato con assiduità e professionalità a questo progetto di ricerca ottenendo dei risultati inaspettati in un tempo cosi' breve (2 anni). Ritengo che la sua preparazione scientifica e la qualità dei dati ottenuti in questo periodo siano sufficienti per presentare presso la commissione dell'Università degli Studi di Napoli Federico II la sua tesi di dottorato. Inoltre, come richiesto, il suo lavoro di tesi sarà presentato in inglese.

Il controrelatore Prof. Nicola Zambrano riferisce quanto segue:

La tesi di dottorato della dott.ssa Anna Lisa Romano presenta un lavoro di ricerca originale sulle funzioni dei geni COUP-TFI e PAX6 nello sviluppo del Sistema Nervoso Centrale dei mammiferi. Il modello sperimentale utilizzato è quello murino, in particolare sono stati utilizzati ceppi mutanti per i due geni e loro incroci. Ciò ha consentito di analizzare, sia *in vitro* che *in vivo*, gli effetti del dosaggio dei due geni, ed in particolare la loro interazione genetica. Infatti, è emerso un circuito di co-regolazione dell'espressione dei due geni, che normalmente controlla il corretto equilibrio tra compartimento staminale e precursori di cellule progenitrici e di cellule indirizzate al differenziamento. Gli esperimenti effettuati sono particolarmente sofisticati, e comunque ben effettuati e controllati. I risultati ottenuti sono rilevanti per i progressi della conoscenza nel settore dello sviluppo del SNC e di sicuro impatto scientifico. La tesi è in generale ben scritta, ed espone i numerosi e convincenti dati sperimentali secondo un logico ordine sequenziale.
In definitiva, ritengo eccellenti sia il lavoro sperimentale svolto dalla dott.ssa Anna Lisa Romano, che la sua articolazione ed esposizione nella tesi di dottorato che la candidata si accinge a discutere.

Il Collegio approva all'unanimità la tesi del Dott.ssa Romano ed esprime tutta la sua soddisfazione per la qualità del lavoro svolto.

3. Varie ed eventuali
Non ve ne sono