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Spatial control of astrogenesis progression by cortical arealization genes

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“ 'A capa è 'na sfoglia 'e cipolla ”

ABSTRACT

Sizes of neuronal and glial complements forming the neonatal cerebral cortex largely depend on (1) rates at which pallial stem cells give rise to lineage committed progenitors and (2) proliferation/differentiation ratios peculiar to such progenitors. Generation of astrocytes from stem cells via committed progenitors has been finely investigated in its *temporal* progression. Conversely, we have only partial information about *regional* articulation of this process.

In this study, by a variety of methods including in-vitro clonal assays, gene expression profiling, lentiviral somatic transgenesis and ad-hoc rescue assays, we investigated spatial articulation of murine, pallial stem cells commitment to astrogenesis, regional progression of astroglial progenitors to differentiated astrocytes, and molecular mechanisms controlling these phenomena.

We found that neural stem cells (NSCs) originating from early (E11.5) caudo-medial (CM) pallium are more astrogenesis prone than rostro-lateral (RL)-ones. We investigated if *Emx2* and *Foxg1*, two genes showing opposite graded expression along the CM-RL pallial axis, could be responsible for this regional astrogenic bias. We found that preferential CM-NSCs progression to astrogenesis is promoted by *Emx2,* mainly via *Couptf1* up-regulation, while *Foxg1* antagonizes such progression in RL-NSCs, likely via *Zbtb20* down-regulation. Next, we found that astrogenic committed progenitors are prompted to self-renew by *Foxg1* (*Emx2* has been already documented to induce their differentiation). Consistently, with expression patterns of these factors, we found that the fraction of mature astrocytes among astroglial lineage cells is higher in neonatal hippocampus respect to age-matched neocortex.

The scenario emerging from this analysis might reflect specific geometrical/developmental constraints of the cortical primordium, where

proportionally smaller and smaller peri-ventricular sectors are in charge of generating hippocampus, neocortex and paleocortex, respectively. Not least, precocious astroglial maturation occurring in neonatal hippocampus might help dealing with special metabolic needs of this structure.

The discovery of differential regional tuning of astrogenesis deepens our knowledge of fundamental control of dorsal telencephalic histogenesis. Moreover, it suggest that a regionally unbalanced, neonatal astroglial complement might worsen neurophatological presentations peculiar to *FOXG1*-syndrome patients.

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1. INTRODUCTION

The cerebral cortex is an extremely complex and fascinating structure in charge of exerting the most diverse computational functions. Its complexity is gradually achieved during development through a stereotyped sequence of neurodevelopmental steps, leading to the progressive appearance of neuronal and glial cells and their correct integration in intricate networks. Sophisticated molecular mechanisms control cortical development, ensuring the proper temporal and spatial generation of neurons and glial cells. Here a description of most critical steps of cortical histogenesis follows, with an emphasis on the molecular players involved.

1.1 Cortical arealization

- **Early steps of brain development. From regionalization to arealization**

At very early stages of cortical development, the dorsal neuroepithelium is colonized by a pool of neuroepithelial stem cells (NESCs) that undergo symmetric cell divisions to expand the surface area of the developing telencephalon (Florio and Huttner, 2014). Initially, it is not possible to appreciate any particular morphological feature along the neuroepithelial sheet. They will arise as development proceeds and will determine the identification of specific telencephalic regions characterized by peculiar cytoarchitectonic and functional properties.

The term **regionalization** refers to early prenatal phases of the process when it is impossible to distinguish among primitive areas on the basis of morphology. The term **arealization,** by contrast, is attributed to later stages when apparent discontinuities appear between different areas

(Pallas 2001). Two main models have been proposed to describe the molecular and cellular mechanisms leading to cortical arealization. According to the "**protomap**" model, the expression of patterned genes gives regional identity to the NESCs of the early cortical proliferative sheet. Based on morphogens gradients, and thanks to the activation of area-specific transcription factors, NESCs located in different regions of the developing pallium will undergo specific proliferation, apoptosis and differentiation programs. As result, structures with different size and thickness will arise according to the intrinsic molecular identity of the respective region of the cortical primordium (Rakic 1988). According to the "**tabula rasa**" model, by contrast, the cortical primordium is considered as a "neutral reservoir" of NESCs displaying no initial positional bias. The specification of different areas would occur thanks to external afferents, mainly thalamocortical projections, that would carry all the information for region-specific differentiation program (O'Leary, 1989; Van der Loos and Woolsey, 1973). Different experimental results support both models. In particular, the "protomap" model is strengthened by the evidence that explant of early embryonic cortical tissue (E10-12 in mouse), before the wiring of thalamo-cortical projection, can retain their regional identity activating the appropriate areal markers in vitro (Arimatsu et al., 1992; Ferri and Levitt, 1993; Gitton et al., 1999; Tole and Grove, 2001; Tole et al., 1997; Vyas et al., 2003). The "tabula rasa" model, on the other side, is supported by the evidence coming from transplantation studies. After transplantation in exogenous regions, pieces of cortex can acquire new identity. For example, transplanted pieces of the embryonic occipital cortex into the parietal region acquire architectural features peculiar to the new environment (Schlaggar and O'Leary, 1991).

Despite the evident discrepancy between the two arealization models, they are both accepted by the scientific community since they highlight different, but essential aspects of the arealization program. The presently

accepted view, in fact, consider the “protomap" model the most suitable to describe the very early steps of arealization; the “tabula rasa model”, on the other side, likely reflects the refinement of cortical arealization mediated by thalamo-cortical projections taking place in mouse from E13.5 onward (Sur and Rubenstein, 2005).

- **Molecular players acting in cortical arealization**

Two classes of molecule are involved in first steps of cortical regionalization : secreted ligands (SLs) and transcription factors (TFs).

At the border of cortical field it is possible to identify three different sources of SLs represented by the cortical hem in the caudomedial region, the commissural plate, rostromedially and the cortical antihem, rostrolaterally (**Fig. 1.1a**).

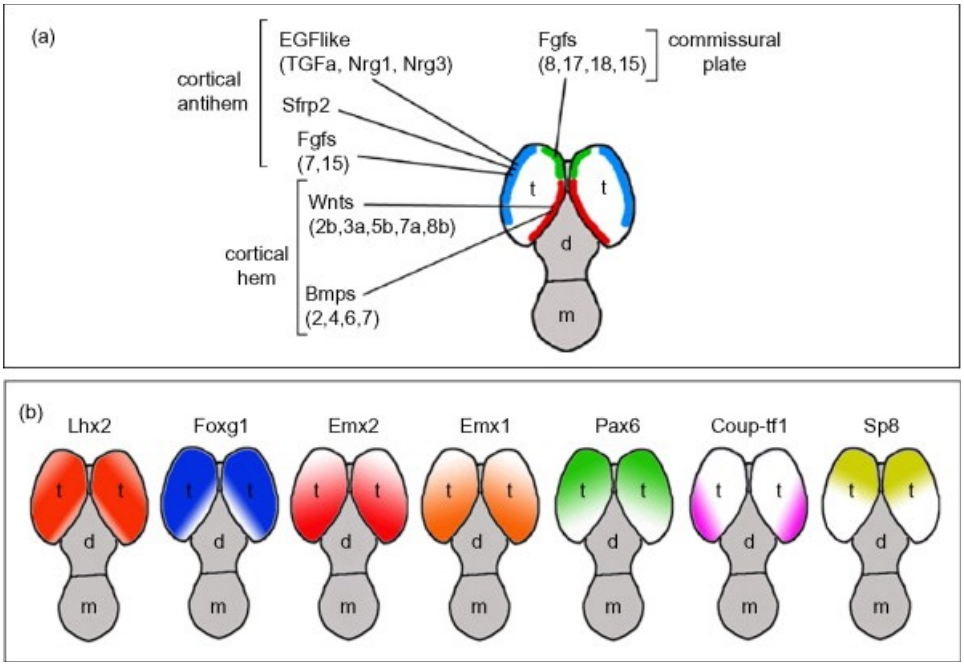

Fig. 1.1 Molecular players acting in cortical arealization. (a) main source of production, (b) gradient of expression. (Adapted from Mallamaci, 2011)

The cortical hem is a source of Wnts (combination “wingless” and “int-1” homologous proteins) and Bmps (Bone morphogenetic protein) (Grove et al., 1998)(Furuta et al., 1997), the commissural plate is the source of Fgfs

(8, 15, 17, 18) (Fibroblast growth factor)(Borello et al., 2008; Cholfin and Rubenstein, 2008) and the antihem is the source of the Wnt-chelating protein Secreted frizzled-related protein 2 (Sfrp2), Fgfs (7, 15) and of Egf-like molecules (TGFa, Nrg1, Nrg3) (Assimacopoulos et al., 2003; Borello et al., 2008) (**Fig. 1.1a**). From each source of production, these SLs diffuse along the developing telencephalon, generating variously oriented concentration gradients which result in the activation of specific molecular machinery according to the region in order. In particular :

Fgfs act at very high hierarchical level to promote rostral identity, they bind with different affinities the three membrane receptors FgfR1,2, and 3, expressed in different isoforms by telencephalic precursors thus activating an intracellular transduction machinery involving MAPK and PI3K-Akt (Ford-Perriss and Murphy, 2001, Borello et al., 2008).

Wnt signalling promotes caudomedial fate. Wnts bind to Fzd/Lrp receptors and inhibit the GSK3b-dependent phosphorylation of beta-catenin which, in turn, enters the nucleus, forms a complex including Lef/Tcf cofactors and activates its target genes (Muzio et al., 2005, Ciali and Salinas 2005).

Bmp signaling is involved in the promotion of dorsal identity in the rhombospinal domain and probably in similar aspects of rostral structure (Mallamaci 2011). The pathway is activated upon binding of the ligands to heterodimer membrane receptors with ser/thr kinase activity. The intracellular transducer is represented by cytoplasmic Smad1,5,8 proteins that, upon phosphorylation, translocate to the nucleus and activate specific genes.

Sfrp2 and **Egf-like** molecules have not been completely characterized in their contribution to cortical arealization. The first might be involved in the control of Wnt gradient, thus indirectly contributing to medio-lateral

specification (Mallamaci 2011). The latter has been reported to activate paleo-cortical markers (Levitt et al., 1997).

NESCs localized in different regions of the developing cortex and exposed to different concentration of each SL, will activate, following a “dose-dependent” behavior the expression of specific TFs. This gradient expression of TFs sets the stage for differential activation of cortical arealization programs.

Among key TFs involved in this context there are: **Lhx2, Foxg1, Emx1, Emx2, Pax6, Couptf1, Sp-8.** Emx1, Emx2 and Lhx2 show a decreasing caudomedial to rostrolateral gradient of expression while Pax6 and Foxg1 show an opposite rostrolateral to caudomedial decreasing gradient. Couptf1 expression is very high in the caudolateral region decreasing along the rostromedial axis while Sp8 show a decreasing rostromedial to caudolateral gradient (**Fig. 1.1b**) (Briata et al., 1996; Bulchand et al., 2001; Dou et al., 1999; Englund et al., 2005; Gulisano et al., 1996; Mallamaci et al., 1998; Liu et al., 2000; Monuki et al., 2001; Muzio et al., 2002; Sahara et al., 2007; Stoykova et al., 1997).

Experimental results based on the use of gain-of-funcition (GOF) and loss-of-function (LOF) mutants, revealed the contribution of each TF to specific moment of the arealization program. A brief summary of role of Lhx2, Pax6, Coup-tf1, Sp-8 is here provided and more detailed description of **Emx2** and **Foxg1** functions, throughout development, is provided in the following paragraphs.

Lhx2 acts as “pan-cortical specifier” prior to E10.5, indeed its absence leads to the loss of pallial identity and to the mis-specification of dorsal telencephalic structures to cortical hem and ventral fates (Bulchand et al., 2001; Monuki et al., 2001).

Pax6 generally promotes paleocortical fate against neo- and archicortical ones, however its specific contribution to arealization program is still not very clear. Both GOF and LOF approach, in fact, revealed that there is no

linear relationship between Pax6 levels and rostrocaudal positional values (Pinon et al. 2008; Mallamaci, 2011).

Coup-tf1 is involved in both dorsoventral and rostrocaudal patterning of the cortical field, furthermore it directly antagonizes the markers of archicortical field. Conditional *couptf1*-KO are known to have reduced caudal cortical areas and a concomitant expansion of rostral areas (Armentano et al. 2007). Coup-tf1 regulates cortical patterning by repressing Mapk/Erk signaling, which is likely downstream of Fgf signaling (Faedo et al. 2008). For this reasons it counteracts the expression of Sp8 which is typically induced by FgFs. **Sp8**, in fact, is expressed in a complementary pattern to Coup-tf1 and is required for the specification of rostral identity (Sahara et al. 2007).

- **Role of Emx2 in cortical arealization**

Emx2 is a homeobox regulatory gene encoding for an evolutionary ancient transcription factor (restricted to the homeobox, its homology with the Drosophila melanogaster gene empty spiracle (*ems*) is about 82%) (Dalton et al., 1989; Cohen and Jürgens, 1990). In mouse, *Emx2* is localized on chromosome 19 and includes three exons. *Emx2*$^{-/-}$ mutant mice die soon after birth because of the absence of urogenital system. Furthermore they show an enlargement of rostral neo- and paleo-cortex and a strong reduction of visual cortex and hippocampus, including dentate gyrus agenesy (Pellegrini et al., 1996; Yoshida et al., 1997, Bishop et al 2002). Emx2 expression starts at E8.5 in the anterior dorsal neuroectoderm, it represents, in fact, one of the earliest dorsal markers for the developing cerebral cortex, subsequently, around E10, Emx2 is also detectable in cortical neuroepithelium and in the olfactory placodes (Gulisano et al.,1996). Around E12.5, a typical rostro-lateral ^{low} - caudo medial ^{high} gradient of expression of Emx2 starts to be detectable for both

mRNA and protein and it becomes more pronounced from E14.5 onwards (Simeone et al., 1992; Gulisano et al., 1996). The graded expression is a feature shared with other transcription factors involved in early steps of cortical arealization and confirms the contribution of *Emx2* to cell identity and patterning of cerebral cortex. More in detail, Emx2 is involved in the patterning along the the rostro-caudal and the dorso-ventral axis together with *Otx2* and *Pax6*. *Emx2*$^{-/-}$*;Otx2*$^{-/+}$ mutants, show a shrinkage of the anterior prosencephalon and an enlargement of the tectum and of the rombencephalon (Kimura et al., 2005). In *Emx2*$^{-/-}$*;Pax6*$^{-/-}$ mutants, conversely, the cortex evolves to a striatum-like structure (Muzio et al., 2002). Along the antero-posterior axis, in mouse models loss of function for *Emx2*, the caudomedial areas (such as V1) are almost completely shrunken in favour of the rostrolateral ones, which are enlarged (Mallamaci et al., 2000a). Opposite distortions of the areal profile are displayed by gain-of-function mutants (Hamasaki et al., 2004).

- **Role of Foxg1 in cortical arealization**

Forkhead box G1 (*Foxg1*), formerly known as *Brain factor*-1 (*Bf*-1) is a regulatory gene of the forkhead family encoding for a winged-helix transcription factor (TF). The name 'Forkhead' takes its origin from a study in *Drosophila* where a mutation in its ortholog, *Sloppy paired* (*Slp1*), caused the formation of an ectopic head structure that resembles a fork. In mouse *Foxg1* is localized in chromosome 12 and its composed by a single exon (Wiese et al., 1995; Bredenkamp et al., 2007). Its expression is detectable from E8.0 and is triggered by the ANR via Fgf8 secretion (Houart et al., 1998; Shimamura and Rubenstein, 1997) and by Sonic hedgehog (Shh) from the preplate, thus later resulting in a classical ventral/anterior^{high} to dorsal/posterior^{low} gradient of expression (Hatini et al., 1994).

Consistent with *Foxg1* expression profile, the subpallium of *Foxg1*-/- mice is significantly damaged and the cortical field is specified as hippocampus at the expense of neo- and pale-cortical identity (Xuan et al., 1995; Muzio et al., 2005). In particular, it has been demonstrated that *Foxg1* exerts pro-ventralizing effects through a positive feedback loop with Fgf signalling acting downstream of Shh **(Fig. 1.2)** (Rallu et al., 2002; Martynoga et al. 2005; Storm et al., 2006). The balance between Shh and Gli3 signals is crucial for the proper dorso-ventral specification of the telencephalon (Grove et al., 1998; Kuschel et al., 2003). Evidence from double mutants revealed that *Foxg1* is crucial for the sustainment of ventralizing signals, as *Foxg1*/*Gli3* co-ablation results in a complete loss of telencephalon (Hanashima et al., 2007). Furthermore, Foxg1 is also able to limit dorsal signals and counteracts *Wnt8b* expression, directly binding to its promoter (Danesin et al., 2009).

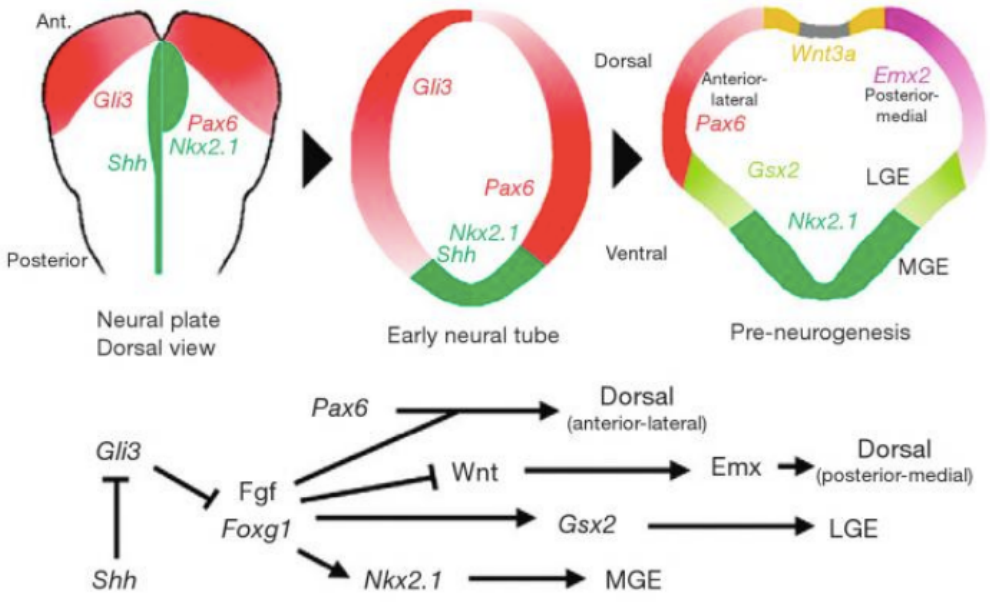

Fig. 1.2 Interaction between molecular players involved in dorso-ventral specification (Adapted from Rubenstein and Rakic, 2013).

All the players presented above follow, in general terms, the classical model of arealization according to which, building on gradients of SLs, graded expression of TFs arise, eventually leading to areal specification (Nakagawa and O'Leary, 2002). However, events leading to the proper formation of the cerebral cortex are not always unidirectional. The overall picture is complicated, in fact, by crosstalk, feedback-loop and interference between the different players. The intricate network of molecular machineries controlling development is at the bases of the complexity of the cerebral cortex.

1.2 Cortical histogenesis

Besides regional identity of cortical progenitors, another important element dictating the histogenetical program is time progression.
As mentioned above, not only - thanks to the interplay among secreted ligands and transcription factors - progenitor cells undergo different regional specifications, but within a specific region, the ability of these cells to give birth to different lineages also strongly depends on time. The *stem* potential of progenitors cells, in fact, decreases as development goes on (Luskin et al., 1994; Qian et al., 2000; McCarthy et al., 2001). In very early phases of development, NESCs, the earliest progenitors of the cerebral cortex, undergo simmetric cell division to expand their pool. With the thickening of the developing brain epithelium, at around E10 in mouse, NESCs generate more advanced neural precursor types, i.e. radial glial cells (RGCs). The transition from pure NESCs to RGCs is associated to the expression of some genes like glutamate transporter (GLAST), glial fibrillary acidic protein (GFAP) that are common glial markers (Shibata et al., 1997). These cells have a bipolar morphology with processes contacting both pial and ventricular surface and cell body

residing in the ventricular zone (VZ) (Götz and Huttner, 2005) and are now considered as *neural stem cells* (NSCs) because they are able to give rise to all the three different major cell types that colonize the mature cortex i.e neurons, astrocytes and oligodendrocytes (Kriegstein, Götz 2003; Kriegstein, Noctor, Martínez-Cerdeño 2006; Kriegstein and Alvarez-Buylla, 2009) (**Fig. 1.3**).

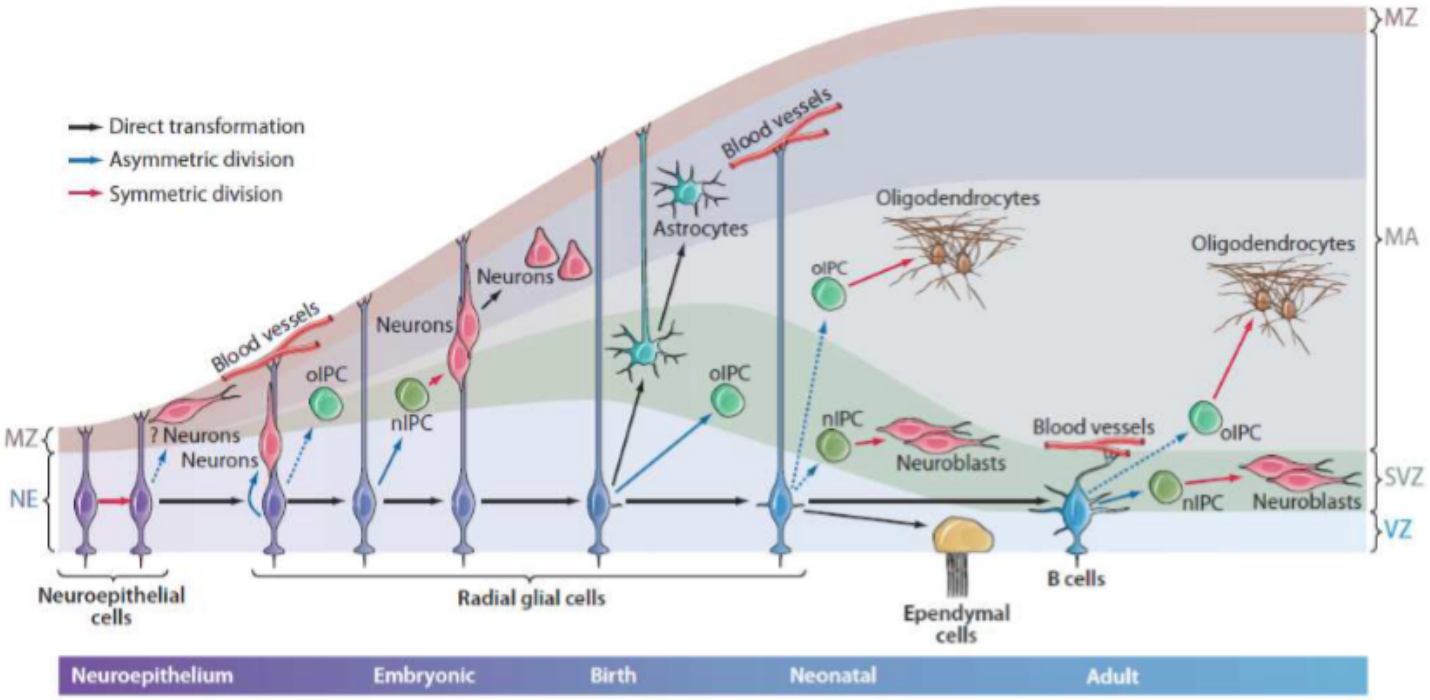

Fig. 1.3 The diversity of divisions and cells during development (Adapted from Kriegstein and Alvarez-Buylla, 2009)

- **Neuronogenesis**

During the neurogenic phase (E11-E17), RGCs undergo asymmetric cell divisions, self-renewing and generating a projection neuron (*direct neurogenesis*). As development proceeds, however, RGCs become able to asymmetrically generate intermediate progenitors (IPs), that leave the VZ, enter the sub-ventricular zone (SVZ), divide symmetrically (one or two

times) and ultimately generate two or four neurons (*indirect neurogenesis*) (Noctor et al., 2004). Early born neurons rely on somal translocation to move basally and integrate the deep layers of the cortex forming a layered structure called preplate (PP). The following phase of cortical histogenesis is characterized by an accumulation of post mitotic neurons that split this region in two layers, the more superficial marginal zone (MZ) and the deeper subplate (SP). In particular, cells that colonize the marginal zone are called *Cajal-Retzius cells*, while the deeper ones are called *subplate cells*. Between MZ and SP, the newly accumulated neurons form the cortical plate (CP) **(Fig. 1.4)**

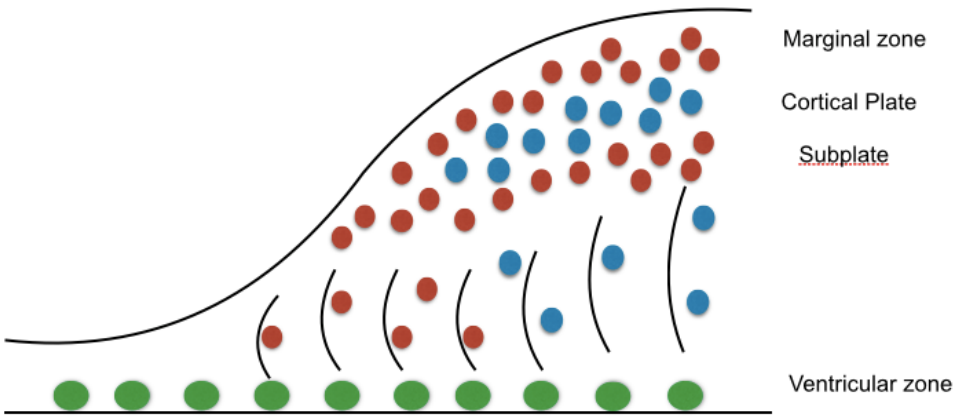

Fig. 1.4 Developing cortex. Identification of ventricual zone, subplate, cortical plate and marginal zone.

The CP, which in mammals later becomes the mature six-layered neocortex, is formed following a typical “inside-out” order (Rakic, 1974; Frantz and McConnell, 1996). As neurogenesis progress, in fact, neurons that are generated close to the ventricular zone migrate in this way, occupying more and more superficial layers, i.e. overcoming earlier born neurons. In particular, later born neurons initially transit into a multipolar morphology, next adopt a bipolar shape and finally attach the RGCs basal process to undergo locomotion towards the upper layers (Nadarajah et

al., 2001) . Since the layers of the cortex are numbered from the most superficial to the deepest one, then neurons belonging to the 6 or 5 layers are the ones that have been generated earlier, followed by neurons of layer 4, 3 and 2. **(Fig. 1.5)**.

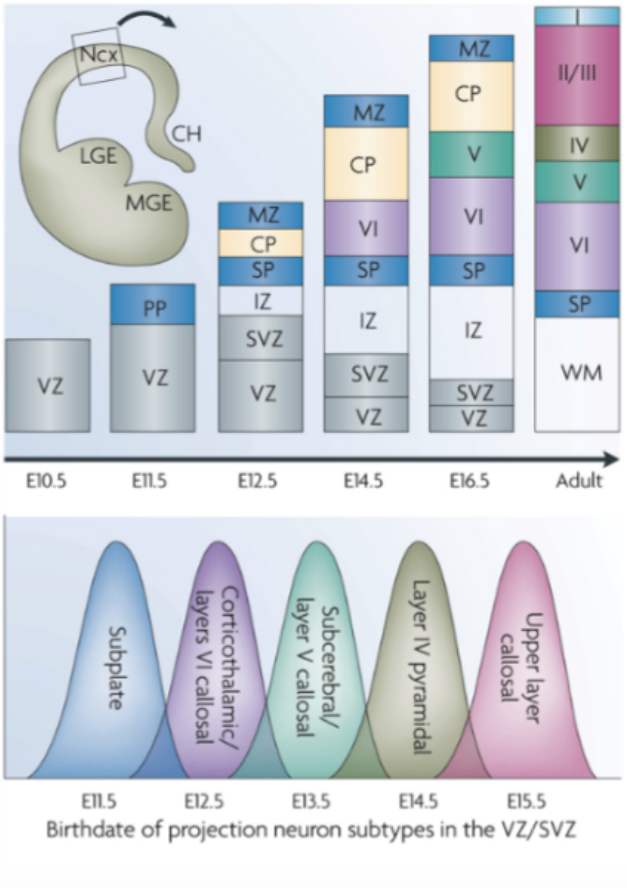

Fig. 1.5 Cerebral cortex lamination during mouse development and neuronal subtypes specification. (Adapted from Rubenstein and Rakic, 2013).

Recent data have shown that, in mouse, each RGC can give rise to 8–9 neurons stochastically distributed throughout the different layers (Gao et al, 2014). Layer-identity and cell-function are two highly interconnected aspects. Neurons in the upper layers, for example, will mainly create cortico-cortical projections required for the connection of cerebral hemispheres. Neurons in the deeper layers, on the other side, will preferentially have subcortical projections. Genetic studies have

demonstrated that the acquisition of a specific identity is dependent on the expression of some transcription factors activating dedicated programs of differentiation. Deep layer neurons are characterized by the expression of Sox5, Fezf2, and Ctip2 while Satb2 is typical of upper layer neurons. The cross-repression between the four transcription factors is sufficient to establish the different identities (Alcamo et al., 2008). A lot of research has been done in order to understand how progenitor cells activate the right program of differentiation. The first hypothesis was that the ability of RGCs to produce layer-specific subtypes of cells, meaning upper (UL) or deeper layer (DL) neurons, was mainly dependent on temporal changes in cell competence (Frantz and McConnell,1996; Desai and McConnel, 2000;). During the last years alternative views for the real mechanism orchestrating the complex cortex histogenesis came out. In particular UL-committed progenitors were found even in the earliest phases of corticogenesis (Franco et al, 2012). This may suggest that some cells have intrinsic characteristic leading to the production of one type of cell or another. Furthermore, it has been demonstrated that the entire process may rely not only on intrinsic cues, but also on important extrinsic factors. In particular the sequential acquisition of deep layer and upper layer competence seems to require the activation of a closed transcriptional cascade as well as negative feedback which is propagated from postmitotic DL neurons (Toma et al., 2014). Following the expression timing of the specific transcription factors, Tbr1 is the first to appear at the state of preplate. As the system goes on, Tbr1 expression is inhibited and cells start to express *Ctip2* and *Fezf2,* typical marker for deep layer neurons. At this point, in order to produce upper layer neurons, there must be another change in the molecular mechanism leading to the expression of *Satb2.* In this case, according to the model, a negative feedback signal coming from last produced deep layer neurons, is able to inhibit *Fezf2* resulting in the activation of *Satb2*. (Toma et al., 2014) **(Fig. 1.6)**.

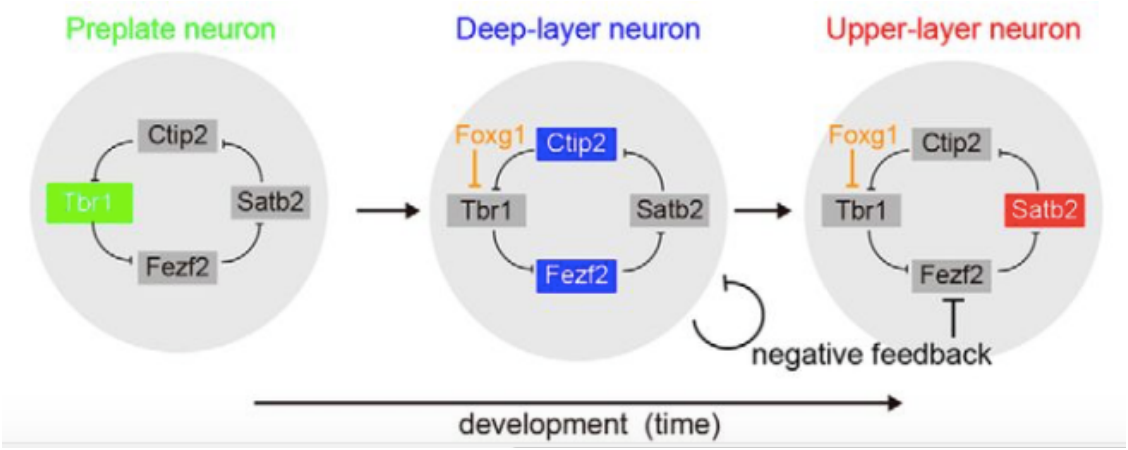

Fig. 1.6 Interaction between transcription factors in the determination of layer-specific subtypes of cells. (Adapted from Toma et al 2014).

If initially it was thought that neurons acquired the correct laminar position exclusively through vertical migration along radial glial fibers, later, it became clear that pyramidal neurons in the cortex are also able to disperse tangentially (O'Rourke et al., 1995). The migration itself it's a complex mechanism relying first of all of cell-cell interactions, but also on specific molecular changes mainly correlated to cytoskeletal modifications. During the migration, indeed, neurons transit between *bipolar* and *multipolar* morphologies (LoTurco and Bai, 2006). In the multipolar state they detach from the radial glial scaffold and initiate axonal elongation prior to entering the cortical plate (Tabata and Nakajima, 2003, Noctor et al., 2004;). Several genes play a critical role in this step of cortex development including Foxg1, UncD5 and NeuroD1 (Miyoshi and Fishell, 2012).

The final neuronal output and their correct integration in the mature cortex is strongly dependent on the ability of RGCs to properly conclude neurogenesis. Interestingly, the duration of the neurogenic phase,

presents significant variations between species (Borrell and Calegari, 2014; Sun and Hevner, 2014; Dehay et al., 2015). In the mouse IPs typically undergo up to two rounds of divisions (Noctor et al., 2004), whereas they undergo significantly more rounds in humans and other primates (Fietz et al., 2010; Betizeau et al., 2013;). Furthermore, RGCs are found in the pure subventricular zone (SBZ) in mouse while in humans they colonize an expanded outer region of the subventricular zone called outer subventricular zone (OSVZ) **(Fig. 1.7)**. This is a major difference observed between lissencephalic species such as rodents, and gyrencephalic species, such as humans and other large primates (Sun and Hevner, 2014).

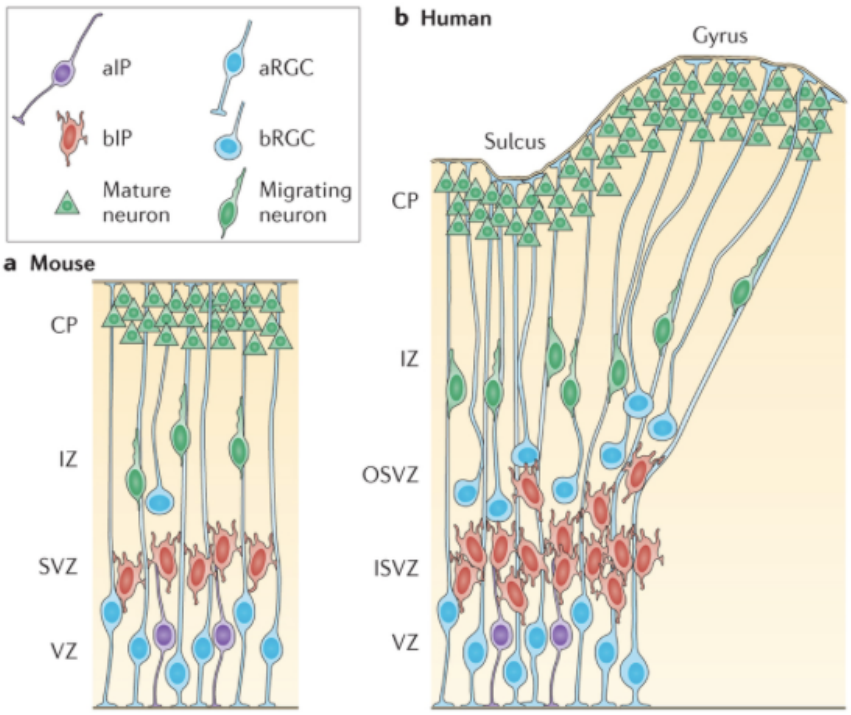

Fig. 1.7 Comparison between mouse and Human cortical neurogenesis. (Adapted from Sun and Hevner, 2014).

- **Role of Emx2 in neuronogenesis**

Several experimental evidence revealed that *Emx2* is involved in different aspect of neurogenesis. This gene, for example, can exert a bimodal impact on NSCs proliferation or differentiation (Brancaccio et al., 2010). According to its gradient of expression and to the timing of development, different important feature arise manipulating *Emx2* expression. At very early stage *Emx2* promotes cell cycle progression and inhibits premature neuronal differentiation acting through Wnt signalling. This phenomena are more pronounced in the caudomedial than in rostral pallium and, consistent with that, an impressive selective size-reduction of occipital cortex and hippocampus is detectable in $Emx2^{-/-}$ mice (Muzio and Mallamaci, 2005). In more advanced neural stem cells, on the other side, *Emx2* expression leads to the stop of proliferation and the decision to undergo neuronal differentiation (Galli et al., 2002; Gangemi et al., 2006). Furthermore, Emx2 is also involved in the lamination of the cortex. In $Emx2^{-/-}$ mutants, in fact, the generation of Cajal-Retzius cells is highly defective. Moreover, these mutants display severe alterations of the cortical plate. In absence of this gene, in fact, early cortical plate neurons do not infiltrate the preplate, which consequently is not split in marginal zone and subplate but rather forms a structure called super-plate. Furthermore, late born neurons do not follow the classical inside-out rules. The most plausible hypothesis at the basis if this impressive phenotype can be - as said above - an impaired generation or differentiation of pioneer layer I neurons which are responsible for neocortical lamination. Consistent with that, in $Emx2^{-/-}$ *Reln*-mRNA expression is strongly reduced (Mallamaci et al., 2000a,b).

- **Role of Foxg1 in neuronogenesis**

Foxg1 is involved in the control of different step of neurogenesis including the regulation of progenitors cells proliferation, migration of cortical neurons and proper lamination of the cortex. Its ability to sustain proliferation of progenitors cells is well documented. As neurogenesis goes on there is an increase in two important parameters : the length of cell cycle and the fraction of cells exiting from cell cycle. The lengthening of cell cycle is due to a prolonged G1 phase (Takahashi et al., 1995) while the increase in exiting fraction is due to a shift of progenitors from self-renewing state to a more differentiated one. It seems that lengthening of G1 can alone be sufficient to induce neuroepithelial cell differentiation and thus it can be a cause, rather than a consequence, of neurogenesis (Calegari and Huttner, 2003). Cdk-Cyclin complex inhibitors family are important regulators of this process and different experimental evidence suggest that *Foxg1* has a role in controlling this crucial aspect of neurogenesis. Foxg1 protein typically act as transcriptional repressor and it has been demonstrated that it is able to interfere with the expression of cdk-cyclin complex repressor p21Cip1. Normally, the expression of this gene is activated upon binding of FoxO-Smad complex to its regulatory region but Foxg1 is able to bind the complex and block p21Cip1 expression (Seoane et al 2004). Furthermore, Foxg1 cooperate with Polycomb factor Bmi-1, a typical repressor of the cell cycle inhibitors, to maintain the proliferative state of precursors cells. Bmi-1 overexpression, in fact, induces an increase in cells proliferation mediated by Foxg1 up-regulation-dependent p21Cip1 repression (Fasano et al., 2009). All those pieces of evidence suggest that this gene can act in a crucial point of cell cycle progression controlling the G1 to S phase transition. According to this, for example, the overexpression of *Foxg1* in neural stem compartment, is sufficient to induce a dramatic enlargement of it and

delay neurogenesis (Brancaccio et al., 2010). Consistent finding deriving from GOF experiments, in *Foxg1*$^{-/-}$ mice, the ventral telencephalic development is severely affected and progenitors cells show a reduced proliferation and increased differentiation, ultimately resulting in reduced hemispheres size (Hanashima et al., 2002; Martynoga et al., 2005; Xuan et al., 1995). As for the role of *Foxg1* in migration of pyramidal neurons, it has been demonstrated that a dynamic expression of this transcription factor is essential to have the proper integration of newborn neuron in the cortical plate. As mentioned above, pyramidal neurons undergo radial as well as tangential migration in the developing cortex (O'Rourke et al., 1992, 1995). It has been demonstrated that in order to activate the expression of Unc5D, a protein involved in the tangential migration process, it is necessary to have a temporal down-regulation of *Foxg1* levels. Subsequently, *Foxg1* is re-expressed and this is essential to induce the exit from the multipolar cell phase and to enter into the cortical plate. Thus, the dynamic expression of *Foxg1* during migration within the intermediate zone is essential for the proper assembly of the cerebral cortex. (Miyoshi and Fishell, 2012). *Foxg1* is also involved in molecular control of cortical lamination. It actively represses Tbr1, namely a step instrumental for the activation of deep layer specification program (Toma et al., 2014), and, together with *Lhx2* induced the stop of production of Cajal-Retzius cells, thus allowing the system to move to later developmental programs (Hanashima et al., 2007; Kumamoto et al., 2013).

- **Astrogenesis**

The neurogenic time window in mice extends from E11 to E17 and is followed by gliogenesis which is further subdivided in two distinct moments for the specific production of astrocytes and oligodendrocytes. The classical model of cortical histogenesis, in fact, provide three waves of generation of the different cellular subtypes showing strongly temporal separated pick but also partially overlapped time windows. **(Fig. 1.8)**

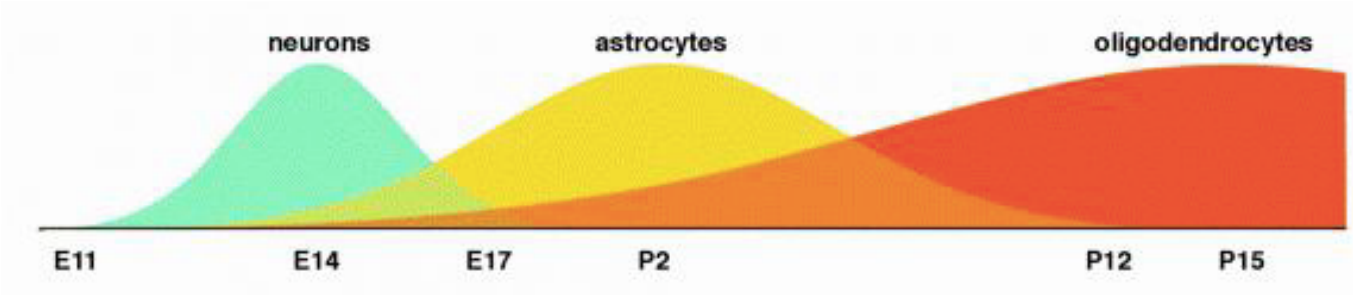

Fig. 1.8 Temporal progression of murine cortico cerebral histogenesis

Given the great morphological and functional differences between them, for long time, neurons and astrocytes were considered to take origin from different precursors. This model suggested a very early divergence of the two populations during development. However, it has been demonstrated that glial cells originate, exactly as neurons, from radial glial cells but, preferably, in a different time window. The transition from RGC to astrocyte can be accompanied with structural changes, in particular, late RGCs cells retract the pial branch and switch to unipolar morphology characterized by the appearance of radial processes (Schmechel & Rakic, 1979, Noctor *et al.*, 2004). The resulting immature astrocytes migrate to their final locations, where they undergo intensive local proliferation before terminal differentiation.

This is a very important difference respect to neurogenesis. The final abundance of neurons in the mature cortex, in fact, mainly depends on the division of RGCs in VZ or IPs in the SVZ (Borrell, Gotz, 2014; Gao et al, 2014), while the final astroglial output also rely on the local proliferation of early differentiated astrocytes (Ge et al., 2012). During the first three weeks of postnatal development, the astrocytes population expands 6–8-fold in the rodent brain (Bandeira, Lent, Herculano-Houzel, 2009). This impressive expansion can't rely only on the RGCs present in the VZ that are gradually decreasing in number after birth and thus is sustained by local astroglial proliferation (Marshall, Suzuki, Goldman, 2003; Kriegstein, Alvarez-Buylla, 2009). Important studies revealed that the switch from neurogenesis to astrogenesis is the result of at least two important factors: time and surrounding environment. It was proved that isolated E15 mouse cortico-cerebral precursors are able to produce both neurons and astrocytes, but give preferentially rise to one type of cell or another if placed on different substrate. In particular, if neural precursors were grown over E18 rat slice, they preferentially differentiated into neurons, otherwise if they were plated on P15 rat slice they gave rise only to astrocytes (Morrow et al., 2001). When the same experiment was performed using a semipermeable barrier between the tissue and precursors, the result was exactly the same, suggesting that diffusible molecules were responsible for it. This proved that cortical precursors can change their histogenetic properties over time, but this is an irreversible process. Indeed when P5 precursors were plated either over E18 or P15 slice, they eventually produced astrocytes (Morrow et al., 2001). In other words the plasticity of cortical precursors is reduced and restricted to one possible fate as development proceeds.

The final goal to have the proper switch from neurogenesis to astrogenesis is accomplished with the activation of specific astroglial promoters known as *Gfap* and *S100b.* This, in mouse, takes place

between E16 and E18 as result of the work of important molecular players (Deneen et al., 2006; Ge et al., 2012; Molofsky and Deneen, 2015). Following the temporal progression of development, it is possible to identify two big classes of genes involved in the regulation of astrogenesis:

1) genes controlling the accessibility of astroglial promoters.

2) genes involved in the tuning of molecular pathways impinging on astroglial gene transcription.

In the following paragraphs mechanisms controlling astroglial program activation and progression will be highlighted.

- **Molecular control of astrogenesis onset**

During neurogenesis, the chromatin of astroglial genes is in a closed configuration and both DNA methylation and histone covalent modifications seems to be involved in this process. *DNA methyltransferase* gene (Dnmt1) methylase CpG residues of astroglial genes, so as to prevent early NSCs switch from neurogenesis toward astrogenesis (Fan et al., 2005). Concomitantly, retinoic acid receptor and N-Cor repressors, work together to recruit hystone deacetylases (HDAC) to promote a repressed chromatin state **(Fig. 1.9)**.

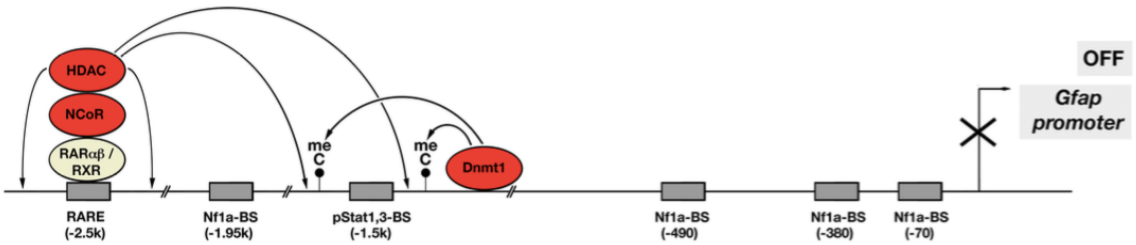

Fig. 1.9 Epigenetic regulation of the Gfap promoter. (Adapted from Mallamaci 2013).

In this respect, it has been demonstrated that the exposure of E11 cortical precursors to leukemia inhibitory factor (LIF), a well known pro-astroglial molecule, is not sufficient to induce a precocious transcriptional activation of *Gfap* promoter. In fact, LIF administration induces an increase in the level of p-Stat3, a typical activator of *Gfap* promoter, but this effector is not able to interact with its binding site. In normal conditions, in fact, such interaction starts to be allowed only from E14.5, when the site will be naturally demethylated (Takizawa *et al*., 2001).

Pro-neural genes, whose expression is very high during neurogenesis, are actively involved in the repression of astrogenesis. This negative regulation, essential to prevent a precocious onset of astrogenesis, is relieved as we approach the end of neuronogenesis. It has been demonstrated that a general open configuration of chromatin is typical of early NSCs favoring proliferation events and commitment toward neuronal lineage. On the other side, a progressive closure of chromatin push cells toward astroglial fate (Kishi *et al*., 2012). A repressive mark trimethyl-histone 3 lysine 27 (H3K27me3), in fact, becomes more and more frequent at transcription starting site (TSS) of typical pro-neural gene like Neurogenin 1 (Ngn1), moving from E11.5 to E18.5 in mouse (Hirabayashi *et al*., 2009). It has been hypothesized that the simple down-regulation of pro-neural genes expression, due to chromatin closure, could be sufficient to release the brake on astrogenesis and, as such, being instrumental for the activation of astroglial program (He *et al*., 2005).

The acquisition by the astroglial genes of the capability to get bound by their specific transactivators is a crucial point for the onset of astrogenesis (Hatada *et al*., 2008). This gradual opening of astroglial gene chormatin is a complex process which takes place while neuronogenesis is still in progress. Alrealdy at E11.5 and up to E14.5 and beyond, in fact, different transcription factors start to lead the basis for the accessibility of astroglial genes' transcription (Hatada *et al*., 2008). The most important players in

this context are : Chicken ovalbumin upstream promoter- transcription factor 1 (Couptf1), SRY (sex determining region Y)-box 9 (Sox9) , Nuclear Factor Ia (NfIa) and zinc finger and BTB domain containing 20 (Zbtb20).

Couptf1 is involved in the process through which neural precursors acquire glial competence. *Couptf1* expression levels fluctuate between E10 and E14 in mouse and it has been proposed that this could be instrumental for the sequentially regulated temporal specification of progenitors cells in mouse cortical development **(Fig. 1.10)**. In particular, *Couptf1* up-regulation around E12–14 in mouse**,** is essential for precursors cells to become able to differentiate into astrocytes in response to gliogenic cytokines in vitro (Ochiai et al., 2001). In fact, chromatin immuno-precipitation (ChIP) analysis showed that *Couptf1* dowregulation is accompanied with a greater silencing of the Stat-3 bindibg sites (Bs) on the *Gfap* promoter and with a concomitant resistance of *Couptf1* knock-down cells to pro-astroglial cytokine stimulation. *Couptf1* seems to be specifically important for the early glial competence of precursors cells, in fact, after its early transient arousal, early glial markers like glutamine synthetase (Gs) and Gfap appears in precursors cells (Naka et al., 2008).

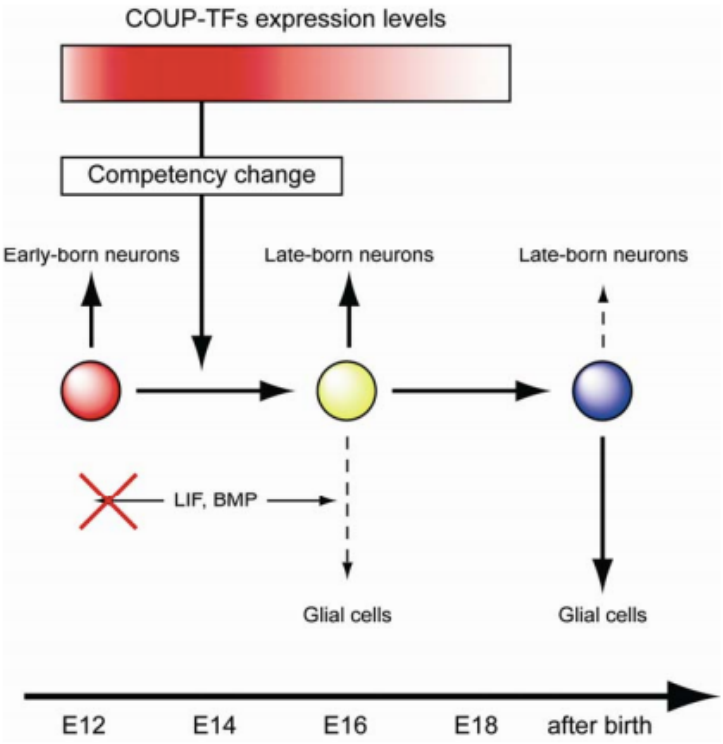

Fig. 1.10 Role of Couptf1 in the temporal specification of precursors cells in the developing cortex. (Adapted from Naka et al, 2008).

Nfia is induced in the ventricular zone (VZ) of the developing spinal cord at the onset of gliogenesis in both chick and mouse and lead to the expression of early glial marker like Glast (Deneen et al., 2006). In particular it is in involved in the regulation of Notch signaling in the binary fate decisions during neural development (Artavanis-Tsakonas et al., 1999). Notch signaling exhibit a complex context dependent role, in some cases it promotes gliogenesis, in others, it promotes neurogenesis (Udolph et al. 2001; Van De Bor and Giangrande, 2001). In absence of Nfla, Notch effectors like HES5 are unable to promote glial-fate specification. Thus, the induction of Nfla allows the switch from *pro-neuronogenic* toward *pro-astrogenic* notch signalling. In addition, at later stages, Nfia promotes migration and differentiation of astrocyte

precursors, a function that is antagonized in oligodendrocyte precursors by Olig2. (Deneen et al., 2006)**(Fig. 1.11)**.

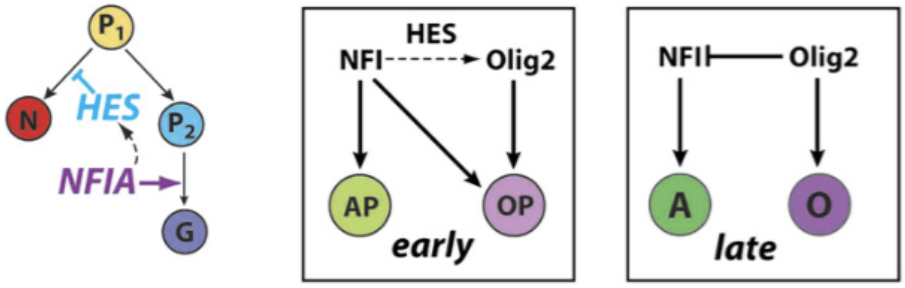

Fig 1.11 Role of Nfia and Notch signaling during development. Nfia promotes glial-fate specification via HES genes switching from progenitors with neuronogenic potential (P1) toward progenitors with gliogenic potential (P2). At early stage at early stages, Nfia promotes glial-fate specification in both the oligodendrocyte and astrocyte lineages mantainig Olig2 expression. At later stages, Nfia specifically promotes terminal astrocyte differentiation, since it is inhibited by Olig2 in the oligodendrocyte lineage (Adapted from Deneen et al, 2006).

Sox9 has been implicated in initiating and maintaining neural stem cell populations in the embryonic and adult CNS and its knockout results in an extended period of neurogenesis, coupled with a delay in the onset of oligodendrogenesis (Cheng et al., 2009; Scott et al., 2010; Stolt et al., 2005). Furthermore, Sox9 is directly involved in the induction of astrogliogenesis through its interaction with Nfia.

Sox9 is induced prior to *Nfia* in the VZ of the embryonic spinal cord confirming a hierarchical relationship between the two players.

The introduction of a dominant activator form of Sox9, for example, is sufficient to induce ectopic Nfia expression in chicken and a subsequent activation of glial marker like Glast. Similarly, in mouse, a reduced levels of Nfia expression and a delayed induction of Glast are detectable in the absence of *Sox9* confirming a conserved role of this gene in gliogenesis induction. Furthermore, Sox9 and Nfia physical interact in both mouse

models and HEK293T cells and they are able to co-regulate Apcdd1 and Mmd2 involved, during later stages of astrogenesis, in migratory and metabolic roles respectively (Kang et al. 2012) **(Fig. 1.12)**.

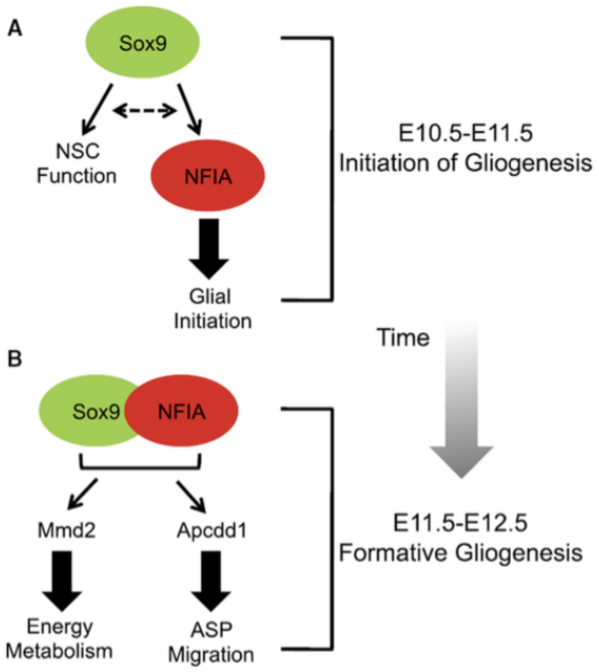

Fig. 1.12 Model of interaction of Sox9 and Nfia during gliogenesis. (A) Sox9 induction of Nfia regulates the initiation of gliogenesis. (B) The Sox9/Nfia complex controls the induction of Apcdd1 and Mmd2. (Adapted from Kang et al. 2012)

Ztbtb20 has been implicated in the control of astrogenesis onset given its interection with Nfia and Sox9. The expression level of this factor increases in progenitors cells in the VZ as development approaches the gliogenic window. Dissociated NSCs cultured in dish also conserve this Zbtb20 progressive upregulation moving from neurogenic to gliogenic window. *Zbt20* expression nicely correlates with typical astrocytic marker like Gfap, S100b and Aldh1l1 also at later stage of development and show a good degree of co-expression with *Nfia* and *Sox9*. Interestingly *Zbtb20* expression is not detectable in the the oligodendrocyte lineage,

suggesting that this factor in specifically important for astrocytes rather then for glial cells in general. The overexpression of *Zbt20* in NSCs is sufficient to induce a pronounced astroglial output without affecting proliferation or survival of precursors cells suggesting that this factors is important to determine specifically the fate commitment of NSCs. Its interaction with Nfia, in fact, represses Brn2 expression, necessary for upper-layer neuron specification, thus limiting neurogenesis in favour of astrogenesis **(Fig. 1.13)**. The promotion of astrocytes differentiation by Zbtb20 is strongly attenuated if *Nfia* and *Sox9* are concomitantly knocked-down revealing their requirement in this process. Interestingly, the overexpression of *Sox9* or *Nfia* does not increase the abundance of *Zbtb20* mRNA but, rather, it increase the percentage of Zbtb20-expressing cells suggesting that this two factors may render NPCs permissive to the induction of Zbtb20 expression in response to other cues. (Nagao et al 2016).

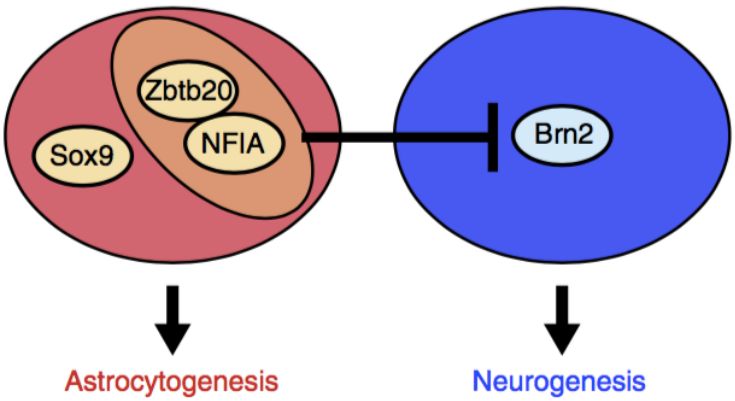

Fig. 1.13 Model for Zbtb20-dependent promotion of astrogenesis. (Adapted from Nagao et al 2016).

- **Molecular control of astrogenesis progression**

Once transcription factors acting in the early transition from neurogenesis to astrogenesis allowed the activation of astroglial program, then the final rate of production and differentiation of astroctyes depends on the balance between inhibitory and activatory players impinging on astroglial promoters. The most important molecular pathway in this context are :

- cardiotrophin-1(CT1)/Jak/Stat,
- Bmp/Smad,
- Delta/Notch,
- Neoregulin/ERBb4,
- TGFb/Smad,
- PCAP/PAC/DREAM.

A detailed description of how these pathways work and interact, activating or inhibiting astrocytes generation, follows.

The chain of molecular events known as **CTI/Jak/Stat** cascade is the principal mechanism promoting astrogenesis (Lillien et al., 1988; He *et al*., 2005). The pathway is activated by different cytokines including cardiotrophin 1 (CT1), ciliary neurotropic factor (CNTF) and LIF. These ligands are released by neurons (as well as by previously born astrocytes) and are able to bind to their specific receptors expressed by NSCs and committed progenitors, and induce their differentiation to astrocytes committed progenitors and more mature astrocytes, respectively (Bonni *et al*., 1997; Nakashima *et al*., 1999a; Ochiai *et al*., 2001; Uemura *et al*., 2002; Derouet *et al*., 2004) Upon ligand binding, gp130- and LIF-receptors undergo ethero-dimerization, followed by auto-phosporylation and activation of Jak kinase. Substrate of Jak kinase are Stat1,3. Upon phosporylation, they dimerize, enter the nucleus and transactivate both *Gfap* and *S100b* promoters (Ernst and Jenkins, 2004) **(Fig 1.14)**.

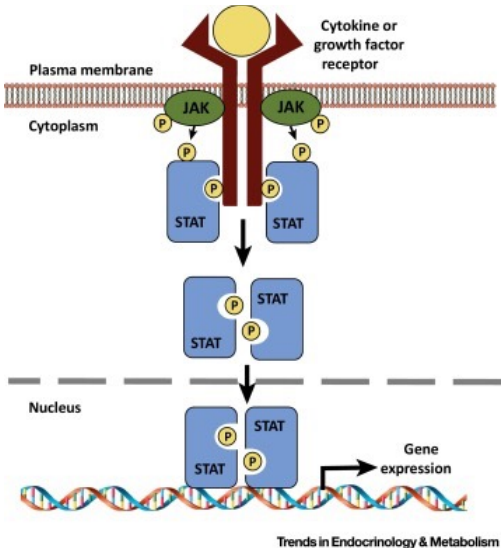

Fig 1.14 Jak-STAT pathway. (Adapted from Dodington et al 2017).

Interestingly, CNTF is able to induce the astrocytic program only in late cortical progenitors but not in early ones although its receptor and effectors are present in both types of cells (Ochiai et al., 2001). The entire mechanism, in fact, is regulated also at the epigenetic state by another effector that is the extracellular factors Fgf2 **(Fig. 1.15)**. This protein increases the methylation in H3K4 and decrease the methylation in H3K9 at the Stat-binding site of the *Gfap* promoter, thus allowing ts transactivation (Song et al., 2004). The differential responsiveness of early and late precursors to CNTF is indeed due to the epigenetic status of the *Gfap* promoter. Similar mechanisms have also been observed for the S100b promoter suggesting a common general mechanism of regulation of cell-type specific gene expression during development (Song et al., 2004).

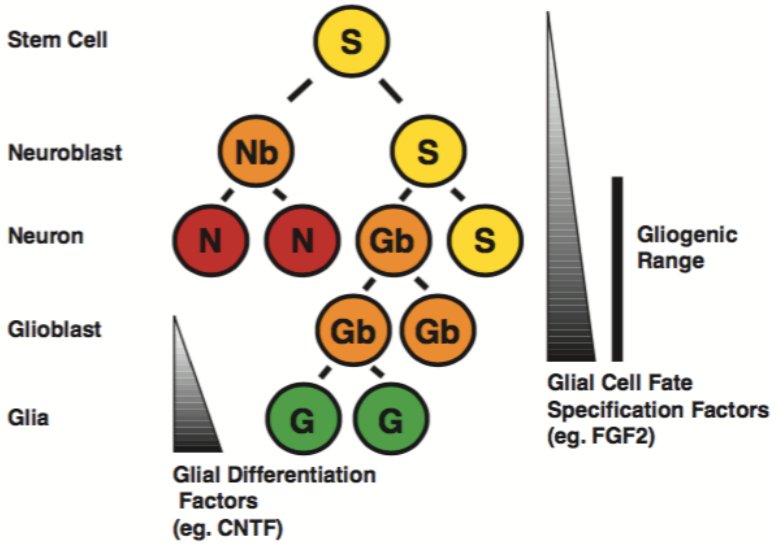

Fig. 1.15 Model of possible regulation of cell fate decisions and glial differentiation by extracellular factors in the developing cerebral cortex. (Adapted from Song et al 2004).

To properly tune its sensitivity to circulating cytokines, this pathway is provided of auxiliary circuits for positive or negative auto-regulation:

- astrogenic cytokines released by previously born astrocytes sustain the pathway, promoting further amplification of the astrogenic process.
- Phosphorylation levels of Jak2 are regulated by suppressor of cytokine signaling gene (SOCS3). This regulator binds the phosphorylated form of gp130 and LIFR and recruits a degradation complex thus eliminating the receptors already occupied by the respective ligands. SOCS3 is activated downstream the CTI/Jak/Stat pathway and as such, it represent a negative loop of control necessary to avoid a saturation of the pathway (Cao et al.,2006) **(Fig. 1.16)**.

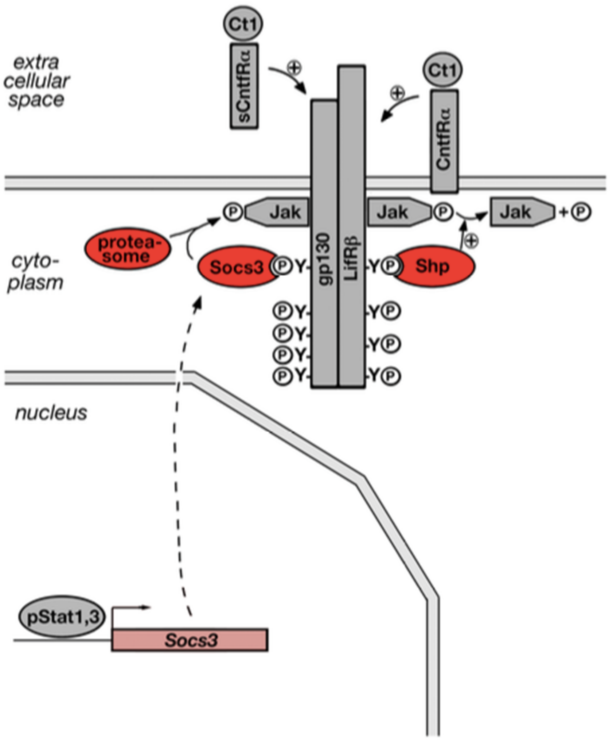

Fig. 1.16 Autologous regulatory loops modulating the astrogenic cytokine signalling axis. (Adapted from Mallamaci 2013).

- Expression levels of gp130 receptor are negatively regulated by the pro-neural machinery and positively by the Mek/Erk signaling. Neoregulin 1,2, which are strongly expressed in the neurogenic phase, repress gp130 expression confining the possibility of NSCs to robustly respond to cytokines to peri- and post-natal stages (He et al., 2005). The Mek/Erk pathway, on the other side, induces the expression of gp130 and its activation is sufficient to induce precocious astrogenesis in early- precursors (Li et al., 2012).
- Both Jak1 and Stat1,3 levels are positively activated by Egf signaling and strongly inhibited by Neurogenins (He et al., 2005). The Egf

receptor (EgfR) is a cell-surface receptor, member of the ErbB family, acting as a key regulator of astrogenesis. In fact, mice lacking EgfR show a delayed astrocyte development (Kornblum et al., 1998; Sibilia et al., 1998). A physiological increase in EgFR is detectable from E18 on in mice suggesting its pivotal involvement in the boosting of astrogenesis that takes place around birth. The expression of this receptor is a limiting step in the temporal progression of astrogenesis because it facilitates the the transmission of Ct1 signal through Jak/Stat axis, via *Stat3* upregulation (Burrows et al., 1997; Viti et al., 2003).

- Stat3 phosphorylation levels are further controlled by another pro-astrogenic pathway, namely the Delta/Notch cascade. Final effectors of this cascade, Hes1 and Hes5, facilitate phosphorylation of Stat3, acting as a bridge between it and Jak. Pro-neural genes, on the other side, inhibit Stat3 phosphorylation, balancing Hes1,5 effects (Kamakura et al., 2004).

Astroglial promoters activation is also favored by the **Bmp2/Bmp4** signaling pathway, in cross-talk with the Ct1-Jak-Stat cascade (Fukuda et al. 2007). Effectors of this pathway are Smad1,5,8 proteins, which, in their active phosphorylated form, interact with p300/CBP, creating a complex that binds the phosphorylated form of Stat3. The resulting complex binds to astroglial promoters and facilitate transcription of the corresponding genes (Adachi *et al.*, 2005). In particular, p300 acts as a bridge, interacting with with Stat3 at its amino-terminus and with Smad1 at its carboxy-terminus (Nakashima et al., 1999). It has been demonstrated that, in rat, pSmad can interact either with Stat3 or Ngn1, according to the developmental time window, thus potentially enhancing both differentiation processes. Until pro-neural factors are abundant, neuronal transcription is promoted. Approaching astrogenesis however, Ngns levels decrease and the p300/CBP/pSmad1 complex is free to

interact with Stat3, finally favoring astrocyte specification (Sun et al., 2001) **(Fig. 1.17)**.

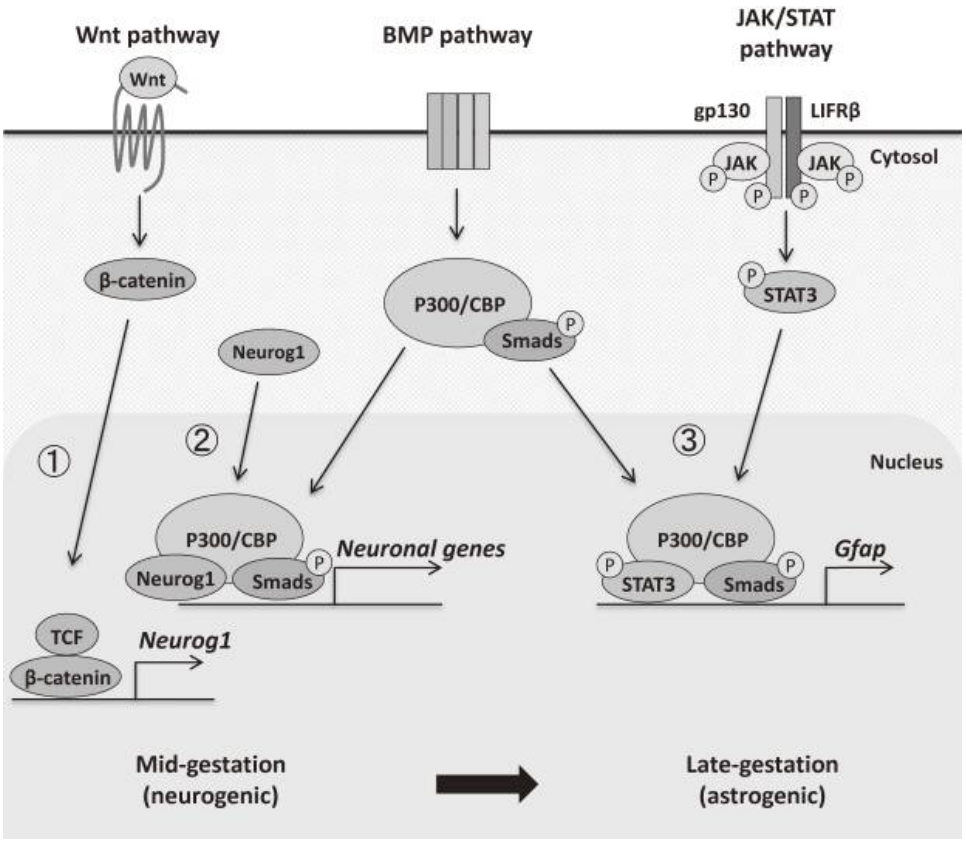

Fig. 1.17 Interaction between Jak/Stat and BMBP signaling in favor of astrogenesis. (Adapted from Takouda et al 2007).

The **DELTA/NOTCH** is another pro-astrogenic pathway. In early stages of development Notch signaling exerts essential roles in the maintenance of NSC pool cells. Later, it promotes of astrogenic differentiation at expenses of neuronal one (Louvi and Artavanis-Tsakonas, 2006)**(Fig. 1.18)**. Notch-dependent inhibition of pro-neural machinery would be, alone, instrumental for a shift toward glial fate but, apart from that, the cascade activated by the Delta ligand, is also able *per se* to favor Stat3 phosphorylation, thus enhancing pro-astrogenic pathways (Kamakura et

al., 2004). Furthermore, the central Notch signalling effector Rbpjk, directly binds to *Gfap* promoter and induces *Gfap* transcription (Ge et al., 2012)

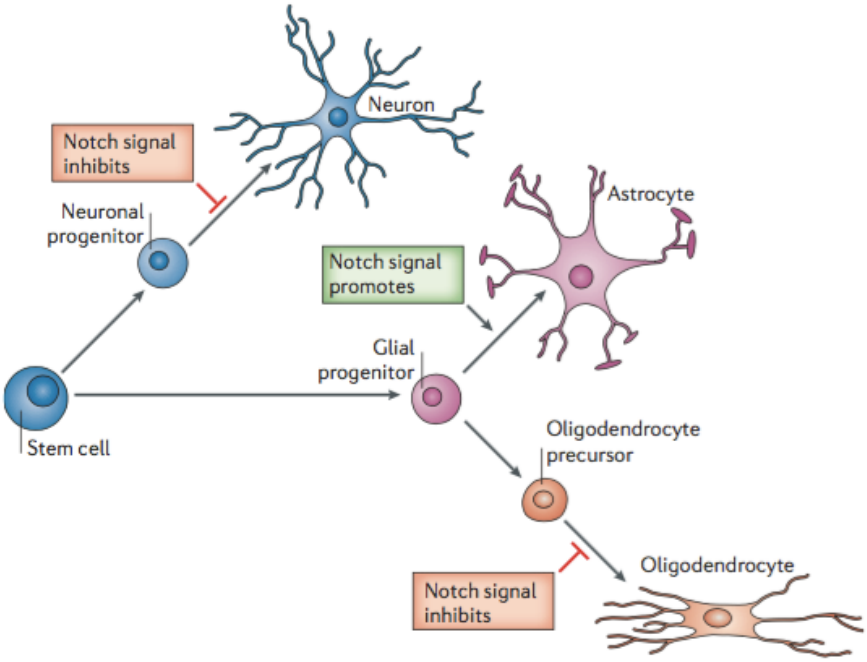

Fig. 1.18 Effect of Notch signal activation on cell fate decisions. (Adapted from Louvi and Artavanis-Tsakonas, 2006).

The **TGFb/Smad** pathway is involved in both precursors commitment toward astroglial fate and in the differentiation and maturation of astrocytes. The pathway is canonicallt activated upon binding of transforming factors beta (TGFβs) to their threonine kinase receptors leading to the activation of Smad2,3 transcription factors (Shi and Massagué, 2003) which, as over mentioned, cooperate with Ct1/Jak/Stat signaling in favor of astrogenesis. Alternatevely, Tgf-β may induce a non-canonical Mapk/PI3K signaling which in turn stimulates astrocytic differentiation (Stipursky et al., 2012). Interestingly, Tgf-β starts to be produced by neurons just prior to the onset of astrogenesis. As such, it is

thought to be implicated in the switch from RGCs to astrocytes-committed progenitors (Stipursky and Gomes, 2007).

The pro-astrogenic **PCAP/PAC/DREAM** pathway acts at levels of cortical precursors expressing the PAC1 receptor. Upon binding, the PACAP ligand induces an increase in cellular concentration of cAMP, by activating adenylyl cyclase. In the presence of high calcium concentrations, the DREAM protein, which is normally located close to the *Gfap* promoter, undergoes a conformational change which turns it into a transactivator, contribbuting to stimulate *Gfap* transcription **(Fig. 1.19)**.

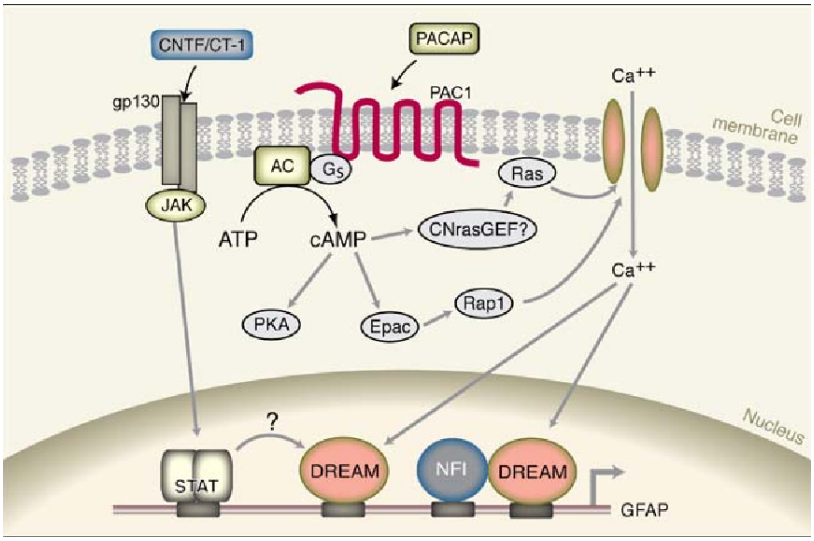

Fig 1.19 PACAP-DREAM pathway in astrogenesis. (Adapted from Vallejo 2009)

Neoregulin/ERBb4 is a typical anti-astrogenic pathway. Neuregulins (Nrg) produced by neurons bind on their receptor expressed on precursors cells and are able to activate canonical and non canonical pathways. The latter, in particular, is involved in the neuronal-dependent inhibition of astrogenesis to prevent precocious activation of astrogenesis. In this case Nrg1 binds the juxta-membrane α (jMα) isoform of the ErbB4

receptor activating the TACE protein and presenilin-dependent γ secretase. The receptor is sequentially cut and the intracellular domain (ErbB4-ICD) is released. ErbB4-ICD interacts with the NCor nuclear repressor via a Tab2 bridge, so creating a trimeric complex, which translocates to the nucleus, binds to *S100b* and *Gfap* promoters, and represses them (Sardi et al 2006) **(Fig 1.20).**

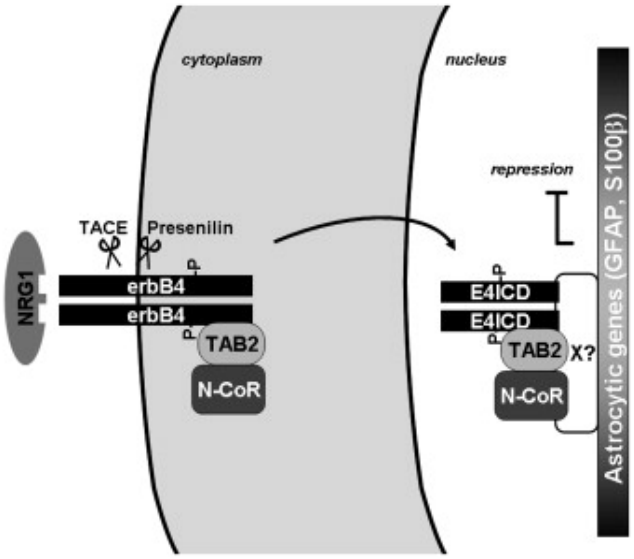

Fig. 1.20 Neoregulin-ErbB4 anti-astrogenic signaling. (Adapted from Sardi et al., 2006)

The well tuned interplay of all these different pathways is essential to finely regulate timing of astrocytes generation and ensure the right balance of neurons and astrocytes within the developing cortex.

- **Molecular control of astroblasts proliferation**

Besides the fraction of NSCs which are committed toward the astroglial lineage, another important parameter which dictates the final astroglial output is the proliferation of committed astroglial progenitors, or *astroblasts*. As aforementioned, it has been demonstrated that, much more than in case of neurons, this peculiar step of development is crucial

to the proper sizing of the astroglial pool (Ge et al., 2012). Molecular mechanisms controlling astroblasts proliferation are still not well understood but there is robust evidence that both Egf and Fgf signalling are involved.

Egf/EgfR signalling, previuosly described for its involvement in positive regulation of CTI/Jak/Stat pathway, is also promoting astroblasts proliferation. EgfR belongs to the ErbB family and, among the main pathways activated downstream of ErbB receptors, there are the Ras-Raf-Mek-Erk1/2, Stat3, and Stat5 pathways, mainly controlling proliferation and differentiation, and the PI3K-Akt-mTOR cascade, acting as a pro-survival and anti-apoptotic signalling (Yarden and Sliwkowski, 2001; Schlessinger, 2002). Those pathways are involved in the development of different tissues and organs. In case of nervous system, it has been demonstrated that EgfR knock-out mice show abnormal astrocytes development. In particular, cortical astrocytes of these mice are strongly reduced in number and activate apoptotic pathways via caspase-dependent mechanisms. As a consequence, EgfR- deficient cortical astrocytes cannot support neuronal survival in co-culture experiments. (Kornblum et al., 1998; Sibilia et al., 1998; Wagner et al, 2006).

Fgf9 is part of the big family of fibroblast growth factors (Fgfs) which are involved in many aspects of embryonic development, cell growth and morphogenesis. Regarding the nervous system, it has been shown to be to be fundamental for the expansion of the perinatal astrogenic proliferating pool and to delay the terminal differentiation of mature astrocytes (Seuntjens et al., 2009; Lum et al., 2009). It has been proposed that Fgf9-FgfR signalling might be activated by both autocrine or paracrine loops. In particular, when released by neurons, Fgfs might represent a powerful signal for induction of astrogenesis (Santos-Ocampo et al. 1996).

Consistent with the role of both Egf and Fgf signaling in promoting astroglial proliferation, strong evidence from literature show that both pathway are upregulated malignant, glial-like brain tumors, such as glioblastoma multiforme (Maxwell et al, 1991 ; Salomon et al., 1995).

- **Role of Emx2 in astrogenesis**

As aforementioned, final astrocytic outputs depend on two primary factors: progression of multipotent precursors toward the astroglial lineage and sizing of the astrogenic proliferating pool. The role of Emx2 in both steps of development has been subject of different studies but its involvement in the former is still not very clear and will be part of the work of my experimental thesis. In particular, given *Emx2* bimodal impact on NSCs proliferation/differentiation kinetics, it is very challenging to understand the biological role of this gene on delayed window of development like astrogenesis. Different evidence from literature suggest that *Emx2* overexpression in NSCs facilitate their transition from pure *neuro*- to mixed *neuro/glial*-potent progenitors suggesting a role for this gene in favor of astrogenesis (Heins et al., 2001). At the same time, however, it has also been reported that an overexpression of *Emx2*, from early developmental stage up to two weeks in culture, is accompanied with a significant decrease in astroglial output (Brancaccio et al., 2010). This apparent discrepancy in experimental results may be solved considering that *Emx2* can exert specific and different functions in NSCs rather then in astrocytes committed progenitors.

As for *Emx2* control of astroblasts kinetics, it has been extensively documented (Falcone et al. 2015). It has been demonstrated, that *Emx2* overexpression is accompanied by a strong downregulation of EgfR and Fgf9 resulting in a dampening astroblasts proliferation (Falcone et al. 2015). The molecular players orchestrating the downregulation of Egf and

Fgf signaling are Bmp and Sox9 respectively. Emx2 overexpression , in fact, promotes Bmp signaling by repressing the expression of Noggin and Fgf8 which are well know inhibitors of Bmp pathway. The resultinmg enhanced Bmp cascade, in turn, inhibits EgfR expression (Fukuchi-Shimogori and Grove, 2003; Shimogori et al., 2004; Bilican et al., 2008; Lillien and Raphael, 2000). Fgf9 expression, on the other hand, rely on Sox2 (Bani-Yaghoub et al., 2006). Upon *Emx2* overexpression, *Sox2* levels detectable at the peak of astrogenesis are strongly downregulated. This can be instrumental to the decline in *Fgf9* level oberserved upon *Emx2* overexpression. As expected, *Sox2* overexpression rescued *Fgf9* levels in an *Emx2*-GOF environment confirming its functional relevance to Emx2-dependent *Fgf9* repression. (Falcone et al. 2015) **(Fig 1.21)**.

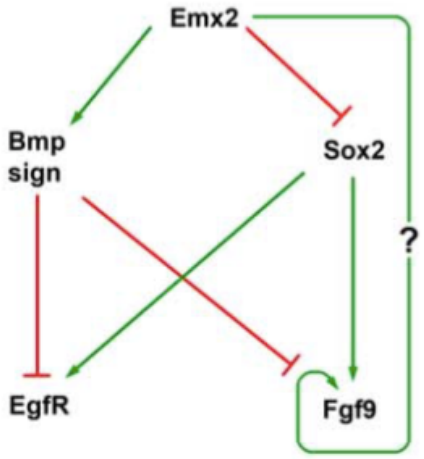

Fig. 1.21 Epistatic relationships among Emx2 and mediators of its antiastrogenic activity. (Adapted from Falcone et al 2015).

- **Role of Foxg1 in astrogenesis**

Different pieces of evidence from literature revealed that Foxg1 is not only essential for proper neurogenesis progression (Miyoshi and Fishell 2012; Toma et al., 2014; Chiola et al., 2019), but that it is also implicated in different key control points of astrogenesis. As shown in Barancaccio et al

(2010), *Foxg1* overexpression in pallial stem cells is followed by an enlargement of NSCs pool and a reduction in their astroglial output. This result is perfectly consistent with the well known role of Foxg1 in pro-proliferative mechanisms, already described in previous paragraphs (Dou et al., 1999; Seoane et al., 2004; Fasano et al 2009), and unveils Foxg1 anti-astrogenic activity, namely a very conserved feature of this gene. Indeed, Drosophila *Foxg1* orthologs, Sloppy paired-1 and -2 (*Slp1* and *Slp2*), have been shown to promote neurogenesis at the expenses of gliogenesis (Bhat et al., 2000). The reduction of astroglial output, despite the previous enlargement of NSCs pool, strongly suggest that Foxg1 anti-astrogenic activity might arise from a defective commitment of neural stem cells to glial fates. In this respect, we recently addressed this issue, investigating the impact of *Foxg1* overexpression on NSCs fate and addressing molecular mechanisms at the basis of it. (Falcone et al, 2019). In particular, we demonstrated that Foxg1 over-expression within murine neocortical stem cells antagonizes the generation of astrocytes both in-vitro and in- vivo and we identified 4 different concurrent mechanisms responsible for this effect. First, we showed that Foxg1 down-regulates the expression of key transcription factor genes like Couptf1, Sox9, Zbtb20 and Nfia which are essential to have the proper activation of astroglial program (Naka et al. 2008; Namihira et al. 2009; Kang et al. 2012; Nagao et al. 2016) and, via rescue-experiment, we proved the functional relevance of all of them to *Foxg1*-dependent anti-astrogenic read-out. Second, we showed that Foxg1, directly binds to typical astroglial promoter like Gfap, S100b and Aqp4, probabily inhibiting them. Next, we demonstrated that *Foxg1* overexpression affects the nuclear concentration of ultimate effectors of pro-astroglial and anti-astrogial pathways reducing the former and increasing the latter **(Fig. 1.22)**. Furthermore, we proved that *Foxg1* can alter the trans-activating abilities

of the pStat3–pSmad1,5,8 complex resulting in a dampening of *Gfap* promoter activation (Falcone et al, 2019).

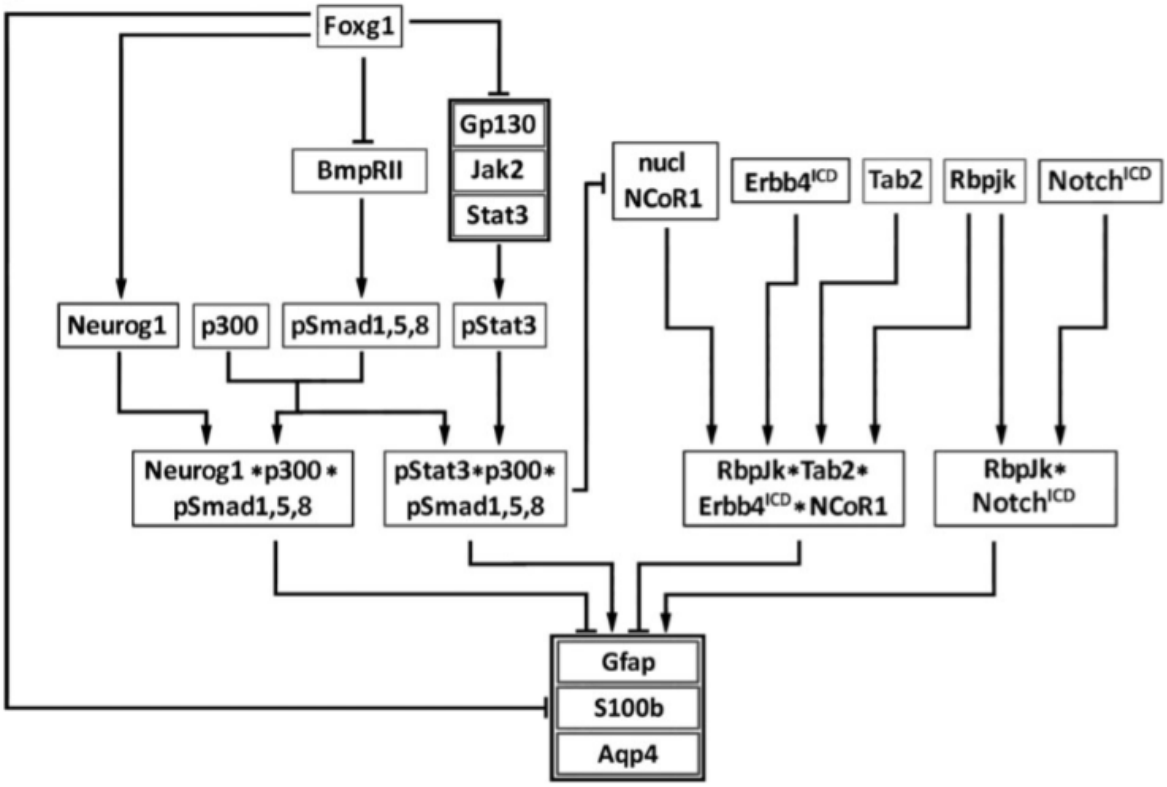

Fig. 1.22 Foxg1 modulation of genes implementing the astroglial differentiation program and their cardinal regulators (Adapted from Falcone et al 2019).

We showed that Foxg1 levels within neocortical NSCs progressively decline prior to the neuronogenic-to-gliogenic transition, in vitro as well as in vivo. Altogether, these results suggest that *Foxg1* can work at very high hierarchal level acting as a brake to prevent precocius astrogenesis onset during neuronogenic phases and confine its burst around peri-natal window (Falcone et al, 2019). In this work we also provided evidence that Foxg1-antiastrogenic activity is conserved in humans. A detailed description of this aspect is provided in the “Appendix” section of this thesis.

1.3 Heterogeneity of astrocyte populations.

Astrocytes are a very heterogeneous population of cells essential for proper brain development and functioning. The list of roles attributed to these cells is continuously extending and, among others, they include : metabolic support to neurons, promotion of neuron survival, stimulation of synapse formation, recycling of neural transmitters, regulation of ion homeostasis and integrity of blood brain barrier (Mächler et al., 2016, Allen et al. 2014; Allen, Eroglu et al. 2017; Maragakis, Rothstein et al. 2004; Haydon, Nedergaard et al. 2015; Takano et al. 2006). Consistent with the variety of their functions, astrocytes resulted to be involved in different pathological contexts including, neurodevelopmental disorders, epilepsy, brain tumors and neurodegeneration (Lioy et al. 2011; Ballas et al. 2009; Carmignoto & Haydon, 2012; Itagaki et al 1989). Astrocytes acquire their diverse functional features as they move from immature to mature stage, which typically occurs during the first 3-4 post natal weeks in mouse and up to the first postnatal year in human (Bushong et al. 2004; Zhang, Sloan, et al. 2016). Immature and mature astrocytes are distinguishable at both morphological and molecular levels. The former are characterized by short processes and have a unipolar or bipolar appearance, the latter, in contrast, have lots of peripheral processes contacting both synapses and blood vessels, and typically have a bushy morphology (Bushong et al., 2002, 2004). Different experimental pieces of evidence suggest that functional and morphological features acquired during astrocytes maturation are sustained by transcriptome-wide changes in genes expression (Li et al 2019). In particular it has been demonstrated that both mouse and human astrocytes show an enrichment of cell cycle and cytoskeletal genes in their immature state, while, in later, more mature phases, their transcriptome is shifted toward channels and metabolic genes (Chaboub et al. 2016, Molofsky et al.

2013, Zhang et al. 2016)**(Fig. 1.23)**. These latter includes genes essential to exert basic astrocytic functions like :

- Excitatory amino acid transporters (EATT1 and EATT2), through which astrocytes control glutamate-glutamine homeostasis, accounting for 80% clearance of glutamate excess from the synapse (Anderson and Swanson, 2000).
- Glutamine synthase (GS), essential to convert glutamate up-taken by astrocytes into glutamine, then transported back into neurons, where it is transformed again into glutamate or GABA (Pow and Robinson., 1994).
- Inward rectifying potassium channels (Kir 4.1), through which astrocytes uptake K^+ ions from the synaptic cleft to maintain a low K^+ concentration in the extracellular space (Kofuji and Newman 2004).
- Connexins, involved in the formation of gap junctions and essential for cellular communication and control of synchronicity and firing of neural networks (Nicholson et al., 2002).
- Metabotropic glutamate receptor (mGlur3,5), G protein-coupled receptors, activating a series of intracellular cascades leading both to an increase in calcium concentration and accumulation of cAMP. This can propagate as waves within the astrocytes network and induce the further release of glutamate, thus enhancing neuronal excitability (Anderson & Nedergaard 2003).
- Adenosine kinase (ADK), enzyme responsible for the conversion of adenosine to AMP, prominently expressed in astrocytes and essential for the glial-neurons purinergic signaling (Aronica et al.,2011; Masino et al. 2012; Haas and Greene, 1984).

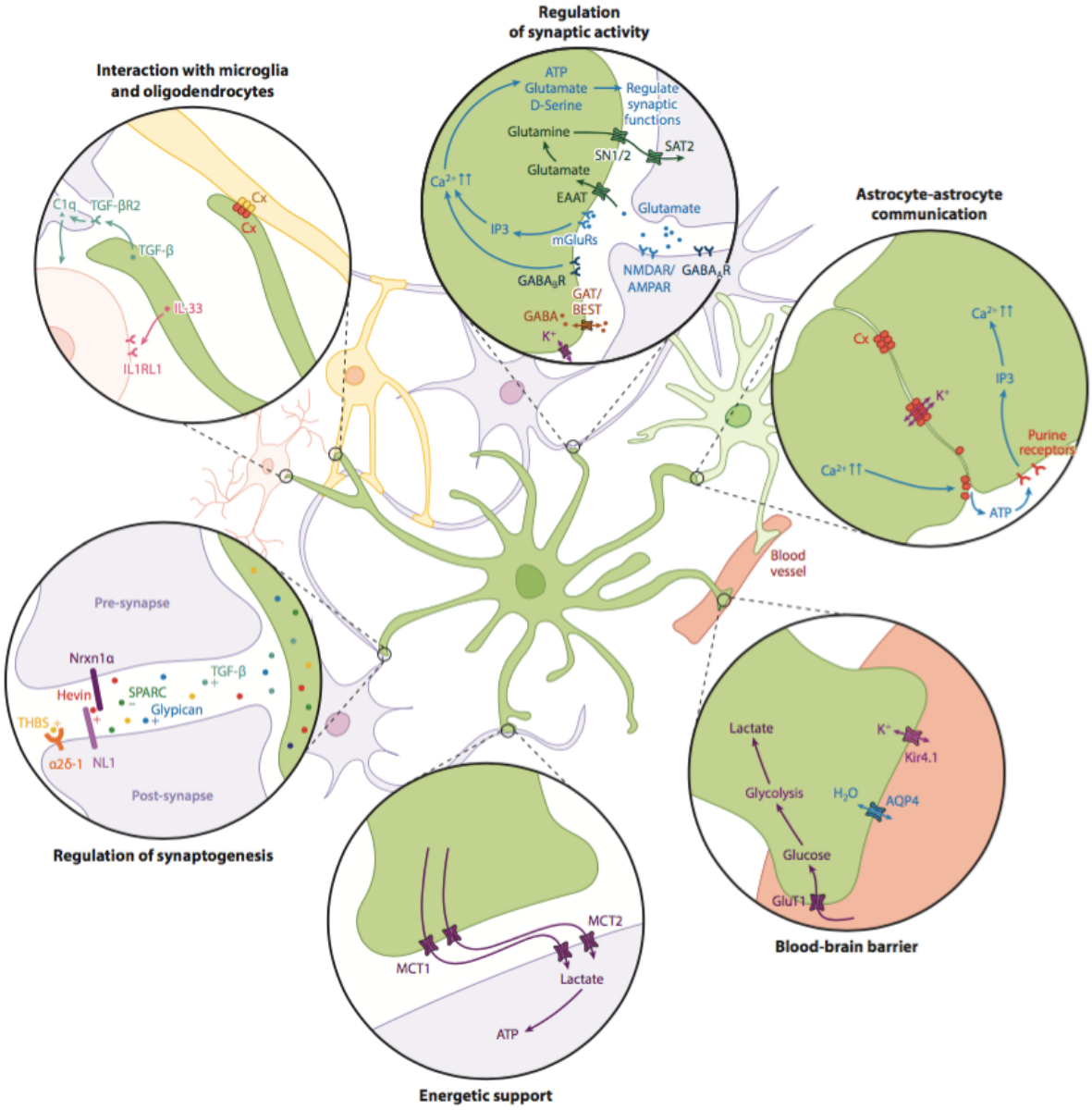

Fig 1.23 A schematic summary of astrocytes functions. (Adapted from Khakh and Deneen, 2019).

Despite some general functional features shared by different subpopulation of astrocytes, it is now very clear that these cells represent a highly heterogeneous population, showing peculiar traits according to their regional identity.

The first gross astrocytes classification takes in consideration their location, distinguishing between white- and grey-matter. Typically the

white matter astrocytes are known as “fibrous” because they have dense glial filaments. They are able to contact capillaries, extending processes known as “vascular feet” and are stained by the intermediate filament marker Gfap. The “protoplasmic” astrocytes, on the other side, typically S100β^{+} cells, are the ones found in the grey matter and have more irregular processes **(Fig. 1.24)** (Bignami et al 1972; Chaboub & Deneen 2013)

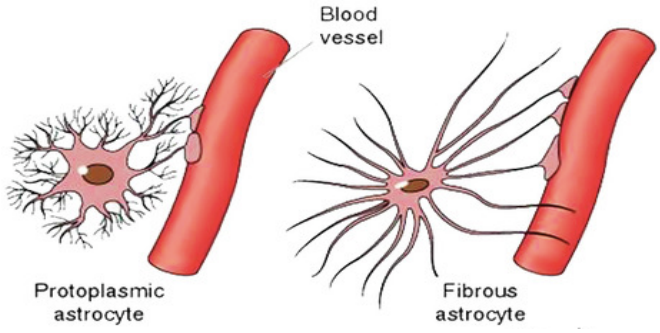

Fig 1.24 Protoplasmic and fibrous astrocytes. (Adapted from Nerve cells, Neural Circuity, and Behavior 2015).

Apart from this gross categorization, emerging evidence support the hypothesis that specific features might arise within astrocyte pools lying within different or same brain structures, leading to “regional” and “local” heterogeneity, respectively (Khakh and Deneen, 2019).

A very well known example of regional, morphological specialization is represented by astrocytes found in the cerebellum and in the retina, namely Bergman glia and Muller cells, respectively (Hoogland et al. 2010; Newman, 1996). Similarly, comparison between hippocampal and striatal astrocytes revealed significant differences in their morphology, electrophysiological properties and Ca^{2+} signaling. In particular, even if hippocampal and striatal astrocytes have equal branching complexity, the

former have much more interaction with neurons, while the latter occupy larger territories. Furthermore, spontaneous Ca^{2+} signal frequency was higher in the hippocampus than striatum confirming that different functional features might arise according to astrocytes localization (Chai et al. 2017). Differences in astrocytes proliferation dynamics have also been characterized comparing mouse cortical to sub-cortical regions. In particular, it has been shown that, during the first post-natal week, the fraction of proliferating astrocytes is much higher in the cortex compared to the hypothalamus but, hypothalamic astrocytes remains proliferative for a longer period (Shoneye et al. 2020). These results suggest that different brain regions might be characterized, at a fixed developmental point, by a specific proportion of immature and mature astrocytes according to their specific functional needs.

As for “local” diversity, very recently, distinct morphological and gene expression profiles have been found in astrocytes between different cortical layers (Lanjakornsiripan et al., 2018), furthermore, specific astrocytic regional domains were found to display graded organizational complexity in the cortex through the rostro-caudal axis (Bayraktar et al., 2020). An intersectional, FACS-based approach has also been proposed to isolate and analyze subpopulations of astrocytes sharing specific features (Lin et al. 2017). In particular, using the pan-astroglial aldehyde dehydrogenase 1 family member L1 (Aldh1l1) marker in combination with other surface antigenes, five distinct subpopulations of astrocytes can be identified. Interestingly, different brain regions are enriched for specific subclasses of astrocytes and each group maintains unique transcriptomic profiles independently of the region of origin. Furthermore, molecular profiling on post-natal cortex, revealed that each astrocytes sub-population exhibits peculiar profile of development and, consistent with that, they also differentially support synaptogenesis and exhibit different proliferative and migratory properties (Lin et al. 2017). Interestingly, these

specific subpopulations, also show signatures of enrichment for tumor- and epilepsy-associated genes, suggesting that they might have different pathological correlates (Lin et al. 2017).

Based on the emerging regional heterogeneity of astrocytes, a challenging question is to understand when and how, during development, these cells acquire their peculiar traits. Considering the little migratory ability and the substantial post-natal local expansion which characterize astrocytes (Ge et al 2012), it reasonable to suppose that their positional identity could account for the appearance of specific features. In this background, it is possible that, very early in development, patterning transcription factors instruct astrocyte-committed progenitors toward a specific molecular identity. Another, not mutually exclusive hypothesis, is that, during the peri-natal astrogenic burst, neurons surrounding astrocytes could dictate the emergence of ad-hoc functional features. Different experimental results support both options. Patterning principles have been elucidated in the generation of molecularly diverse astrocytes in the spinal cord where it has been shown that Pax6 and Nkx6.1 are selectively expressed in subsets of white matter astrocytes (Hochstim et al., 2008). Furthemore, it has been proved that both mouse and human pluripotent stem cells can be differentiated into regional specific astrocyte using diffusible morphogens in vitro (Bradley et al., 2019). As for neuronal involvement on astrocyte morphogenesis, it has been recently demonstrated that, in the cortex, it depends on the close contact between the two types of cells and rely on the interaction between astroglial-neuroligin and neuronal-neurexins (Stogsdill et al. (2017). Furthermore, it has also been demonstrated that in the cerebellum Bergmann glia require Shh from Purkinje cells to maintain their identity. In fact, in the absence of Shh, Bergmann glia acquire alternative molecular and functional features of astrocytes (Farmer et al. 2016).

Altogether these data suggest that, exactly as it happens for neurons, astrocytes generation and maturation could undergo peculiar developmental progressions within distinct brain regions, raising new interesting questions for developmental biologists. Furthermore, they confirm that the heterogeneity of astrocytes population is an important aspect to be studied in order to understand the involvement of these cells in pathological contexts, which, so far, have been investigated with a mere neurono-centric approach. In this respect, recent studies demonstrated that in mouse like in human, cortical, hippocampal and striatal astrocytes are differentially sensitive to aging-mediated transcriptional changes (Soreq et al., 2017; Clarke et al., 2018), suggesting new interesting perspective in the study of neurodegenerative disorders.

2.AIM

Main aim of my study was to investigate the *regional* articulation of mouse cortico-cerebral astrogenesis, paying special attention to :

- commitment of pallial stem cells to astrogenic fates
- subsequent progression of committed progenitors to differentiated astrocytes
- molecular mechanisms controlling such processes.

3. MATERIAL AND METHODS

- **Animal Handling and Embryo Dissection**

Animal handling and subsequent procedures were in accordance with European and Italian laws (European Parliament and Council Directive of 22 September 2010 [2010/63/EU]; Italian Government Decree of 04 March 2014, no. 26). Experimental protocols were approved by SISSA OpBA (Institutional SISSA Committee for Animal Care) and authorized by the Italian Ministery of Health (Auth. No 22DAB.N.4GU). Wild type (strain CD1, purchased from Envigo, Italy) and Aldh1l1-EGFP mice (Tg(Aldh1l1-EGFP,-DTA)D8Rth/J (jax #026033)) were maintained at the SISSA animal facility. Embryos were staged by timed breeding and vaginal plug inspection. Pregnant females were sacrificed by cervical dislocation and embryos were dissected out in ice-cold PBS (1X-phosphate buffered saline) supplemented with 0.6% glucose, under sterile conditions. Once collected, they were dissected in order to recover cortex and hippocampus from the rest of the brain.

- **Lentiviral vectors packaging, titration and use.**

Third generation self-inactivating (SIN) lentiviral vectors (LVs) were generated as previously described (Follenzi and Naldini, 2002) with some adjustments. In brief, HEK293T cells were co-transfected with the transfer vector plasmid plus three auxiliary plasmids (pMD2 VSV.G; pMDLg/pRRE; pRSV-REV), in the presence of LipoD293TM (SigmaGen). The conditioned medium was collected after 24 and 48 hours, filtered and ultracentrifuged at 50000 RCF on a fixed angle rotor (JA25.50 Beckmann Coulter) for 150 min at 4°C. Lentiviral pellets were then resuspended in PBS 1X without BSA (Gibco). LVs were titrated by Real Time quantitative PCR after infection of HEK293T cells, as previously reported (Sastry et

al., 2002). One end point fluorescence titrated LV was included in each PCR titration session and PCR-titers were adjusted to fluorescence-equivalent titers throughout the study.

Where necessary, specific lentiviral plasmids were constructed with basic cloning techniques. DNA manipulations (extraction, purification and ligation), bacterial cultures and transformations were performed according to standard methods. Restriction and modification enzymes were obtained from New England Biolabs and Promega; DNA fragments were purified from agarose gel by QIAquick Gel Extraction Kit (Qiagen); plasmid preparations were done by DN PLASMID PURIFICATION KIT (Qiagen). Plasmids were grown in E. Coli, Xl1-blue or ElectroMAXTM Stbl4TM Competent Cells (Invitrogen).

LVs used for this study were referred to throughout the thesis according to the standard nomenclature: LV:pX-GOI, where pX is the promoter and GOI is the gene of interest.

They were :

- LV_BMPRE-minCMV-ZsGreen, obtained from the pLKO-H2BCFP-BRE-ZsGreen lentiviral BMP-reporter (Oshimori and Fuchs 2012)(a kind gift from Elaine Fuchs), by deleting its MscI-MscI 521bp fragment and so disrupting its pPgk1-H2B-CFP cassette:
- LV_pCMV-Coupft1 (DNasu #HsCD00877725)
- LV_pNes-rtTA-M2, aka pNes/hsp68-rtTA2S-M2 (Brancaccio et al. 2010);
- LV_pPgk1-EGFP-pri-miR.αEmx2-1.8 was generated by cloning the pre-miR.αEmx21.8 module : (5'TGCTGATTCTCCACCGGTTAATGTGGGTTTTGGCCACTGACTGA CCCACATTACGGTGGAGAAT 3') into BfuAI-cut pLVmiR.23 (Diodato et al. 2013);
- LV_pPgk1-EGFP-pri-miR.NC was obtained by transferring the SalI-XhoI cDNA fragment from ''pcDNATM-6.2-GW/EmGFP-

miR_neg_control_plasmid" (Invitrogen) into SalI-digested pCCL-SIN-18PPT.Pgk.EGFP-Wpre (Follenzi and Naldini 2002), in sense orientation.

- LV_pPgk1-EGFP-pri-miR.aFOXG1.1690 was generated by cloning the pre-miR.αFOXG1.1690 module (5' TGC TGA AAC GTT CAC TTA CAG TCT GGG TTT TGG CCA CTG ACT GAC CCA GAC TGA GTG AAC GTT TCA GG 3') into BfuAI-cut pLVmiR.23 (Diodato et al. 2013).
- LV_pPgk1-mCherry-WPRE, constructed by transferring the mCherry module form LV_pTa1-mCherry (Brancaccio et al. 2010) into LV_pPgk1-EGFP-WPRE, in place of EGFP;
- LV_pPgk1-rtTA-M2 (Spigoni et al. 2010);
- LV_pU6-antiFoxg1-shRNA (Sigma #SHCLND-NM_008241, TRCN0000081746);
- LV_pU6-ctr-shRNA, generated from LV_pU6-ctr-shRNA-pCMV-EGFP, aka pll3.7 (Addgene #11795), by removing the pCMV-Egfp containing, NotI-EcoRI fragment;
- LV_Stat3-EGFP-reporter (Addgene#110495)
- LV_TREt-EGFP-pri-miR.aFOXG1.1690 and LV_TREt-EGFPpri-miR.NC, were both built in parallel steps, as follows. The, AgeI-KpnI fragments from LV_pPgk1-EGFP-pri-miR.αFoxg1.1690. and LV_pPgk1-EGFP-pri-miR.NC were transferred into AgeI/KpnI-cut LV-TREt-IRES-EGFP-WPRE (Falcone et al. 2015), so obtaining LV_TREt-EGFP-pri-miR.αFOXG1.1690 and LV_TREt-EGFP-pri-miR.NC, respectively.
- LV_TREdarkEGFP-miR.αFOXG1.1690 and LV_TREdarkEGFP-miR.NC were obtained by replacing the AgeI-SalI EGFP fragments of LV_TREt-EGFP-pri-miR.αFOXG1.1690 and LV_TREt-EGFP-pri-miR.NC, respectively, by the AgeI-SalI darkEGFP fragment (provided by Gene Universal as a pUC57-based shuttle clone; see below), encoding for a mutant version of EGFP (including an AlaAlaVal-motif in place of the essential TreTyrGly motif), not fluorescent, however still specifically

recognized by the anti-EGFP, chicken polyclonal antibody (Abcam #137970) employed in this study (fluorescence and immunofluorescence controls not shown).

>AgeI-SalI darkEGFP fragment : [AgeI, Met, AlaAlaVal, Stop and SalI underlined]

ACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGG
GGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCAC
AAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGC
AAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGC
CCTGGCCCACCCTCGTGACCACCCTGGCCGCAGTGGTGCAGTGCTT
CAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCC
GCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGG
ACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCG
ACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGA
GGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGC
CACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGT
GAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTC
GCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGC
TGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAA
AGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTG
ACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAA
GCGGCCGCGTCGAAAAGGGAGGTAGTGAGTCGAC

- LV_TREdarkEGFP-miR.αEmx2-1.8 was obtained by transferring the BamHI-XhoI fragment from “LV_pPgk1-EGFP-pri-miR.α.Emx2-1.8” into BamHI-XhoI digested “LV_TREdarkEGFP-miR.αFoxg1.1960”.
- LV_TREt-Foxg1 (Raciti et al. 2013);

- LV_TREt-hFoxg1-wt was cloned by transferring the AgeI-SalI fragment from pUC57 “h-F1WT” plasmid provide by GeneUniversal into the AgeI-SalI digested LV_TREt-IRES2-EGFP (Falcone et al. 2015)
- LV_TREt-hFoxg1-(G224S) was cloned by transferring the AgeI-SalI fragment from pUC57 “h-F1-MUT” plasmid provide by GeneUniversal into the AgeI-SalI digested LV_TREt-IRES2-EGFP (Falcone et al. 2015)
- LV_TREt-hFoxg1-(W308X) was cloned by transferring the AgeI-(XhoI -filled) fragment from pUC57 “h-F1-W308X” plasmid provide by GeneUniversal into the AgeI-(SalI-filled) digested LV_TREt-IRES2-EGFP (Falcone et al. 2015)
- LV_TREt-Emx2 (Raciti et al. 2013);
- LV_TREt-IRES2-EGFP (Falcone et al. 2015);
- LV_TRE-NFIA (Addgene #64901);
- LV_TREt-PLAP, obtained by replacing the XhoI/SalI fragment of LV_pPgk1-EGFP by an XhoI-compatible/SalI-compatible element, including the XbaI-AgeI 0.35kb TREt fragment of P199 (Stegmeier et al. 2005) and the EcoRI/ SalI 2.2kb IRES-PLAP fragment from pCLE (Addgene #17703);

When not otherwise stated, each LV was employed each at a multiplicity of infection (moi) of 8. Murine and human neural cells were transduced at densities of 1,000 and 100 cells/µl, respectively. As previously described (Brancaccio et al. 2010), and according to our experience (not shown), these conditions are sufficient to effectively co-transduce the almost totality of neural cells (Chiola et al, 2019).

- **Cell cultures**

- Derivation of Human Neocortical Precursor Line

NPCs were derived from the cerebral cortex of a single 10.2 post conception week (PCW) human fetus, collected from routine termination of pregnancies under full ethical approval in line with Department of Health guidelines (LREC 96/085;96/085—In vitro study of postmortem human fetal neural tissue, blood and haematopoietic organs, approved by Cambridge Central Ethics Committee). Cells were grown and expanded in a chemi- cally defined, serum-free medium in the presence of Fibroblast Growth Factor 2 (Fgf2) and Epidermal Growth Factor (Egf) (10 and 20 ng/mL, respectively) and routinely assessed for multipotency, as described (Pluchino et al. 2009).

- Clonal assay

The general temporal articulation of clonal assays is the following. Pallial tissue was dissected from either E11.5 or E13.5 mouse embryos (for details see Fig. 1A,C, 3A), mechanically dissociated to single cells by gentle pipetting and kept in proliferative medium, at 600,000 cells/ml, for 4 or 2 days, respectively. At DIV4 or DIV2, cells were trypsinized, resuspended in differentiative medium and attached to poly-D-lysine-coated dishes (0,2mg/ml), at clonal density (approximately 16,800 cells/cm^2). 3 days later, LIF was administered to the culture at a concentration of 1*10^6 u/ml. 24 hours later, cells were fixed in 4%PFA for analysis.

Where needed, **(Fig 2,3,4,5, App2)** neural cells were infected with dedicated LV-sets just after the dissection (DIV 0), and TetON-controlled transgenes were kept on by 2 µg/ml doxycycline (Sigma #D9891-10G) throughout the culturing window.

- Astrocytes proliferation assay

Cortices were dissected from E12.5 mouse embryos, mechanically dissociated to single cells by gentle pipetting and kept in proliferative medium (a), at 600,000 cells/ml. Floating neurospheres were trypsinized every 3/4 days and, at DIV 10, they were ultimately dissected to single cells, resuspended in differentiative medium (b), attached to poly-L-lysine-coated dishes (0,1mg/ml), at a density of approximately 150.000 cells/cm^2 and acutely transduced with a dedicated LV mix **(Fig .6)** After one week in differentiative condition, cells were fixed in 4%PFA for analysis. TetON-controlled transgenes were kept on by 2 µg/ml doxycycline (Sigma #D9891-10G) throughout the last week in culture.

- Astrocytes maturation assay

Cortices and hippocampus were collected from P0 or P4 Aldh1l1-Egfp pups **(Fig. 6**) and dissected to single cells through mechanic dissociation followed by enzymatic digestion. Specifically, tissue was chopped to small pieces for 5 minutes, in the smallest volume of ice-cold 1X PBS - 0,6% glucose- 0,1% DNaseI solution.The minced tissue was then resuspended and digested in 0.25 mg/ml trypsin - 4 mg/ml DNAseI for 5 minutes at 37°C. Digestion was stopped by adding ≥1.5 volumes of DMEM/ F12/10%FBS. Large debris deriving from the dissociation were sedimented for 1 minute on cold ice and supernatant containing the remaining single cells was diluted with differentiative medium (c) and plated to poly-L-lysine-coated dishes (0,2 mg/ml). After two hours attached cells were gently washed with PSB to remove small debris and then fixed in in 4%PFA for analysis.

- Molecular mediator assay

Propedeutically to RNA quantification assays **(**see **Fig. 5)**, cortices were dissected from E11.5 mouse embryos, mechanically dissociated to single

cells by gentle pipetting, acutely transduced with a dedicated LV mixes and kept in proliferative medium (a), at 600,000 cells/ml. After 3 to 5 days in culture, cells were collected by centrifugation and processed for RNA extraction. TetON-controlled transgenes were kept on by 2 µg/ml doxycycline (Sigma #D9891-10G) throughout the culturing window.

- Human cortical cultures, differentiation assays

Proliferating neural precursors were expandend in culture on low-attachment plates or flasks, at 100,000 cells/ml (corresponding to 20,000-25,000 cells/cm^2), in proliferative medium (d).The resulting floating neurospheres were passaged by Accutase (Sigma #A6964-100ML) every 4-8 days, according to Manufacturer's instructions. DIV150 **(Fig. App. 1A)** and DIV120 **(Fig. App. 1C)** derivatives of post-conceptional week (PCW) 10 human pallial precursors were transduced with dedicated LV mixes, at moi=2 for each virus. Next they were acutely induced to differentiate, plating them on multiwell plates pre-coated with Matrigel (Corning), at 50,000 cells/cm^2 (100,000 cells/ml), in differentiative medium (e) which was half-changed every four days. Three days later, upon a final 24h pulse of LIF (1*10^6 u/ml), cells were blocked in 4% PFA and profiled for immunofluorescence.

- Human cortical cultures, molecular mediator assay

Propedeutically to RNA quantification assays, proliferating neural precursors were expandend in culture on low-attachment plates or flasks, at 100,000 cells/ml (corresponding to 20,000-25,000 cells/cm^2), in proliferative medium (d).The resulting floating neurospheres were passaged by Accutase (Sigma #A6964-100ML) every 4-8 days, according to Manufacturer's instructions. At the equivalent of PCW10+DIV120, dissociated neural cells were transduced with LVs for shRNA-mediated dow-regulation of *Foxg1* or control. Two days later, cells were re-

transduced with LV_Stat1,3-RE-minP-EGFP and LV_BMPRE-minCMV-ZsGreen reporters together with a LV_pPgk1-mCherry-WPRE, as a normalizer. Cells were kept under pro-proliferative condition (culturing media d) until DIV10 and finally processed for RNA extraction **(Fig. App. 1E,F)**.

- **Culturing Media**

Culturing media referred to above were as follows:

(a) mouse culture proliferative medium: 1:1 DMEM-F12, 1X N2 supplement (Invitrogen), 1 mg/ml BSA, 0.6% w/v glucose, 2 microgram/ml heparin (Stemcell technologies #7980), 20 ng/ml bFGF (Invitrogen #PHG0261), 20 ng/ml EGF (Invitrogen #PHG0311), 1X Pen/Strept (Invitrogen #15140122), 10 pg/ml fungizone (Invitrogen #15290026), as in Brancaccio et al 2010.

(b) mouse culture differentiative medium: Neurobasal A (Invitrogen), 1X B27 (Invitrogen), 5% of fetal bovine serum (FBS), 1X Glutamax (Gibco), 1X Pen/Strept (Invitrogen #15140122), 10 pg/ml fungizone (Invitrogen #15290026).

(c) acute mouse culture differentiative medium: Dulbecco's Modified Eagle Medium; Invitrogen containing 10% fetal bovine serum (FBS), and penicillin/streptomycin

(d) human proliferative medium: NeurocultTM NS-A Proliferation Kit (StemCell Technologies #05751), supplemented by 0.2% heparin (StemCell Technologies #7980), 10ng/ml human recombinant bFGF (Invitrogen #PHG0261), 20ng/ml human recombinant EGF (Invitrogen #PHG0311), 1x Pen/Strept (Invitrogen #15140122); growth factors re-added fresh every 2 days.

(e) human differentiative medium: NeurocultTM NS-A Differentiation kit (StemCell Technologies #05752), supplemented with 1x Pen/Strept (Invitrogen #15140122).

- **Immunofluorescence**

As for neural cultures used for clonal assay **(Fig. 1,2,3,4,5, App2)**, proliferative assay **(Fig. 6, App2)**, differentiative assay **(Fig. App1)** or acutely dissociated preparations **(Fig6)**, cells were generally fixed by 4% PFA for 20 min at 4 °C and washed 3 times in 1× PBS and subsequently treated with blocking mix (1X PBS; 10% FBS; 1mg/ml BSA; 0.1% Triton X100) for at least 1 hour at RT. After that, incubation with primary antibody was performed in blocking mix, overnight at 4°C. The day after, samples were washed in "1X PBS-0.1% Triton X-100" 3 times for 5 minutes and then incubated with a secondary antibody in blocking mix, for 2 hours at RT. Samples were finally washed in 1X PBS for 5 minutes, 3 times and subsequently counterstained with DAPI (4',6'-diamidino-2- phenylindole) and mounted in Vectashield Mounting Medium (Vector).

The following primary antibodies were used:

- anti-βtubulin, mouse monoclonal (Covance MMS-435P), at 1:1000
- anti-GFAP rabbit polyclonal (DAKO #Z0334), at 1:500
- anti-Ki67, mouse monoclonal (BD Pharmingen #550609), at 1:50
- anti-EGFP, chicken polyclonal (Abcam #137970), at 1:800

The following secondary antibodies were used:

- Alexa488 Goat Anti-Rabbit 1:500;
- Alexa594 Goat Anti-Mouse 1:500;
- Alexa488 Goat Anti-Chicken 1:800;

Immunofluorescences were photographed on a Nikon C1 apparatus, equipped with a Hamamatsu C4742-95 camera. Images were generally acquired in ordinary modality. A 20x in air objective was used (ordinary pictures, to evaluate immunofluorescent cell and clone frequency in vitro).

As for clonal assays, a single clone is identified, blind of βTub and Gfap signal, as a group of DAPI+ cells whose reciprocal distance is less than one cell diameter. Neuronal clones include only βTub+ cells, astroglial clones only GFAP+ cells, while mixed clones include both βTub+ and GFAP+ cells. All in vitro assays where set starting from pools of neural precursors originating from littermate embryos. *n* biological replicates (i.e. independently transduced and cultured cell samples) were evaluated for each condition. >100 clones or >400 cells (collected from about 10 and 5 randomly assorted photographic fields, respectively) were scored per each biological replicate (**Fig. 1B,D, 2B,C**, **3B,C,D,E, 4,E,F 5D, App. 2B,E,G** and **6B,C, App.1B,D, App.2D,F** respectively). In all cases, sample randomization and cell countings were performed by an operator blind of sample identity. Immunoreactive cell frequencies were averaged, s.e.m.'s were calculated and in case of **(Fig. 6B, App.1 B,D, App.2D,F)** results were normalized against controls. Their statistical significance was evaluated by the *t-test* (one-tail; unpaired) or *t-test* (one-tail; paired **Fig. 6B,C**)

- **Quantitative RT-PCR**

Cells **(Fig 4C,D, 5B, App.1F)** or acutely dissociated brain tissue **(Fig 4G)** were processed for RNA extraction by Trizol™ Reagent (ThermoFisher) according to manufacturer's instructions. In case of Fig 4G, cortex and hippocampus were micro-dissected from E13.5 mouse pups and separately processed. RNA preparations were treated by TURBO™ DNase (2U/µl)(Ambion™) 1 hour at 37 °C. At least 0.75 µg of genomic DNA-free total RNA from each sample was retro-transcribed by SuperScriptIII™ (Invitrogen) in the presence of random hexamers, according to manufacturer's instructions. 1/100 of the resulting cDNA was used as substrate of any qPCR reaction. Limited to intronless amplicons,

negative control amplifications were run on RT(-) RNA preparations. PCR reactions were performed by SsoAdvanced SYBR Green Supermix^{TM} (Biorad), according to manufacturer's instructions. Per each transcript under examination and each sample, cDNA was PCR-analyzed at least in technical triplicate and results averaged. When not otherwise specified, averages were double normalized against *Gapdh* and controls. Experiments were performed at least in biological triplicates and analyzed by Student's t test.

The following oligonucleotides have been employed in this study:

- Couptf1/F: 5'CTTCATGGACCACATCCGCATCTTTCAGGAACAG 3' ;
- Couptf1/R: 5' TCACATACTCCTCCAGGGCACACTGTGATTTCTC 3' ;
- EGFP/F: 5' CAAGCAGAAGAACGGCATCAA 3' ;
- EGFP/R: 5' GGTGCTCAGGTAGTGGTTGTC 3' ;
- Emx2/F : 5' CACAAGTCCCGAGAGTTTCCTTTTGCACAA 3' ;
- Emx2/R : 5' ACCTGAGTTTCCGTAAGACTGAGACTGTGA 3' ;
- Foxg1/F: 5' CGACCCTGCCCTGTGAGTCTTTAAG 3' ;
- Foxg1/R: 5' GGGTTGGAAGAAGACCCCTGATTTTGATG 3' ;
- Gapdh/F: 5' ATCTTCTTGTGCAGTGCCAGCCTCGTC 3' ;
- Gapdh/R: 5' GAACATGTAGACCATGTAGTTGAGGTCAATGAAGG 3' ;
- Gp130/F: 5' ACAGAACCACGTCCAGTGTCACGTT 3' ;
- Gp130/R: 5' TCTCCCTTCCGGGGTCCCACT 3' ;
- hsa-Nfia/AF: 5' GAGGTTGGACCTTGTTATGGTGATT 3' ;
- hsa-NfIa/AR: 5' GGGTTGGACACAGAGCCCTGGATTA 3' ;
- hsa-Couptf1/AF: 5' CGCGTCGTGGCCTTCATGGACCACATC 3' ;
- hsa-Couptf1/AR: 5' GCGTCTGACGTGAACAGCACGATGGCT 3' ;
- hsa-Sox9/AF: 5' GACGTCATCTCCAACATCGAGACCTTC 3' ;
- hsa-Sox9/AR: 5' CTGATGCCGTAGCTGCCCGTGTAG 3' ;
- hsa-Zbtb20/AF: 5' CCTGGAACGCAACGAATCCGAGGAGT 3' ;

- hsa-Zbtb20/AR: 5' CAGGCCCAAACTGCTGCTCCACCGA 3' ;
- Jak2/F: 5’ TGGCGGCATGATTTTGTTCACGGATGG 3' ;
- Jak2/R: 5’ GGATCTTCGCTCGAACGCACTTTGG 3' ;
- mCherry/F1: 5' CCGACATCCCCGACTACTTGAAG 3' ;
- mCherry/R1: 5' CTTGTAGATGAACTCGCCGTCCTGCA 3' ;
- Nfia/F: 5’ TTGGACCTCGTCATGGTGATC 3' ;
- Nfia/R: 5’ TGGACACAGAGCCCTGGATTA 3' ;
- Sox9/F: 5’ CCAACATTGAGACCTTCGACGT 3' ;
- Sox9/R: 5’ ATGCCGTAACTGCCAGTGTAGG 3' ;
- Zbtb20/F: 5’ AACGCAATGAATCCGAGGAGT 3' ;
- Zbtb20/R: 5’ CCCAAACTGTTGCTCCACTGA 3' ;
- ZsGreen/F: 5' TGCATGTACCACGAGTCCAAGTTCTAC 3’;
- ZsGreen/R 5' CTTCAGCAGCAGGTACATGCTCACGT 3' .

In all cases, the assays were performed on derivatives of pooled neural precursors originating from littermate embryos. *n* is the number of biological replicates (i.e. independently transduced and cultured samples of at least 300,000 neural cells).

- **Statistical evaluation of results.**

It was performed according to standard procedures (full details reported in Legends to Figures). When not otherwise stated, p-values are indicated as follows: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; ***** $p<0.00001$; ****** $p<0.000001$.

4. RESULTS

4.1 *Emx2* and *Foxg1* master the differential regional commitment of early pallial NSCs to astroglial fate.

Astrocyte generation rates within the rodent neonatal pallium are the result of two concomitant, finely regulated processes: the commitment of neural stem cells (NSCs) to macroglial fates and the subsequent tuning of astroblasts proliferation/ differentiation. It has been shown that both processes are differentially regulated in time (Miller, Gauthier 2007, Falcone et al., 2019). We wondered if they are also differentially regulated in distinct regions of the pallial field.

To asses if NSCs deriving from distinct pallial subfields are differentially committed to generate astrocytes, we microdissected rostro-lateral (RL) and caudo-medial (CM) pallium from E13.5 mouse brains, just prior to the "opening" of astroglial gene chromatin (Takizawa *et al*. 2001; Hatada *et al*., 2008), and we processed them for clonal assay analysis. Specifically, we cultured neural precursors originating from their dissociation as floating neurospheres for two days, and then we transferred their single cell derivatives onto poly-D-lysine coated coverslips, at clonal density under serum-containing medium. Finally, four more days later, upon pre-terminal leukemia inhibiting factor (LIF) stimulation, we scored the resulting cultures for frequencies of different clone types **(Fig.1A, Fig. S1.1)**. Fractions of pure neuronal, mixed and pure astroglial clones were 0.367±0.036 vs 0.655±0.042, 0.376±0.025 vs 0.269±0.024 and 0.264±0.025 vs 0.076±0.021 in CM vs RL cultures, respectively (with $p<0.001$, $p<0.009$, $p<0.001$ and $n= 5,4$) **(Fig. 1B, Fig. S1.2)**. To corroborate these results and get insight into temporal articulation of regional fate commitment, we repeated this assay starting from E11.5 pallial precursors **(Fig. 1C)**. In this case, fractions of pure neuronal, mixed

and pure astroglial clones were 0.380±0.059 vs 0.535±0.057, 0.265±0.056 vs 0.255±0.062 and 0.358±0.069 vs 0.202±0.027 in CM vs RL cultures, respectively (with p<0.042, p<0.455, p< 0.025 and n= 6,7) **(Fig. 1D, Fig. S1.2)**. In a few words, well before the activation of gliogenesis, CM and RL pallial subfields are more prone to astrocytogensis and neuronogensis, respectively, and this histogenetic bias is already encoded as many as 3 or 4 days before the "opening" of astroglial gene chromatin .

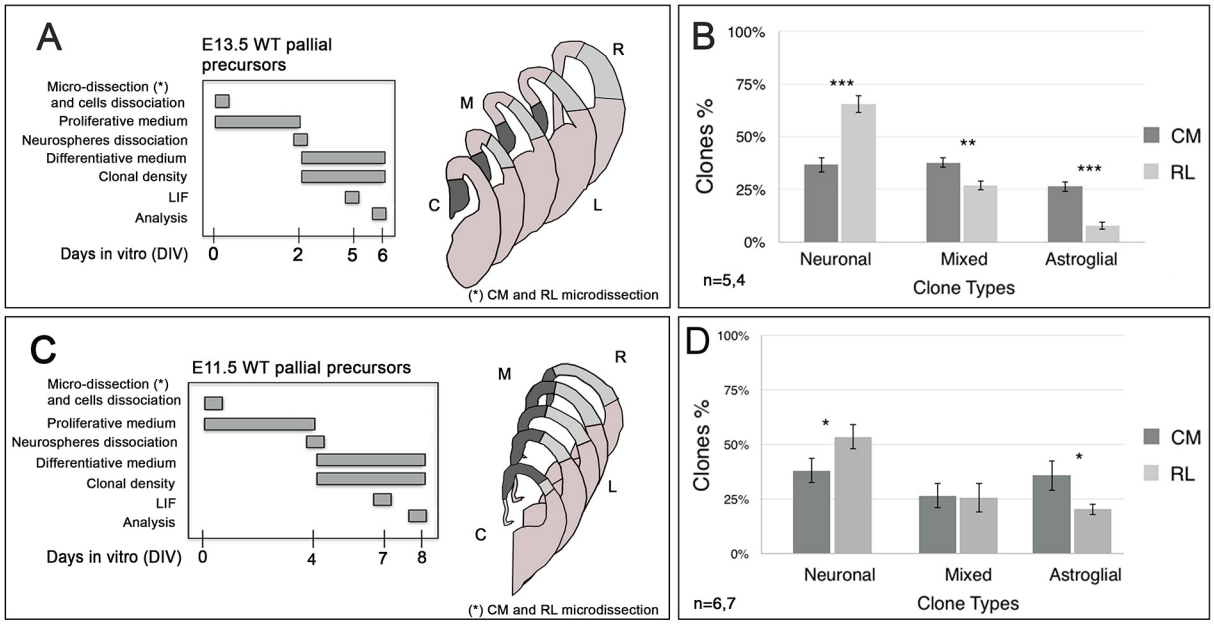

Fig. 1. Differential astrogenic commitment of NSCs along the medio-caudal / rostro-lateral axis of the pallial field. (A,C) Protocols (to left) with microdissection details (to right). **(B,D)** Absolutes frequencies of neuronal, mixed and astroglial clones generated by derivatives of NSCs taken from the caudal-medial (CM) and rostro-lateral (RL), E13.5 (B) and E11.5 (D) pallial subfields. Statistical significance of results evaluated by t-test (1-tail, unpaired). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. *n* is the number of biological replicates, i.e aliquots of pre-pooled, independently cultured neural cells. Scalebars represent s.e.m's.

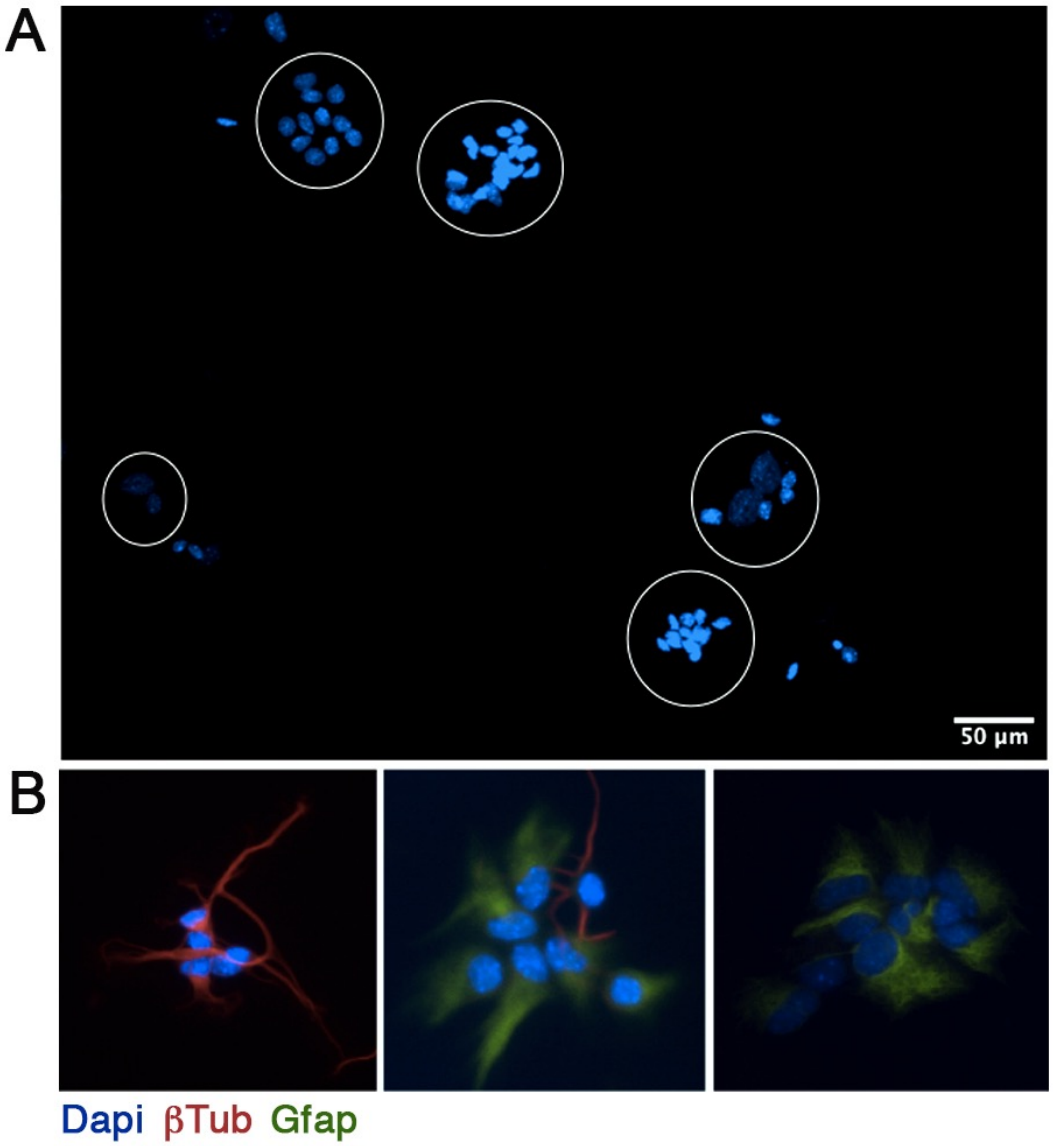

Fig. S1.1 Example of clones identification and characterization**.** (A) A single clone is identified, in blind of βTub and Gfap signal, as a group of DAPI$^+$ cells whose distance is less than one cell nucleus diameter. (B) Neuronal clones include only βTub$^+$ cells, astroglial clones only GFAP$^+$ cells, while mixed clones include both βTub$^+$ and GFAP$^+$ cells. Magnification of pure neuronal (left), mixed (middle), and pure astroglial (right) clones.

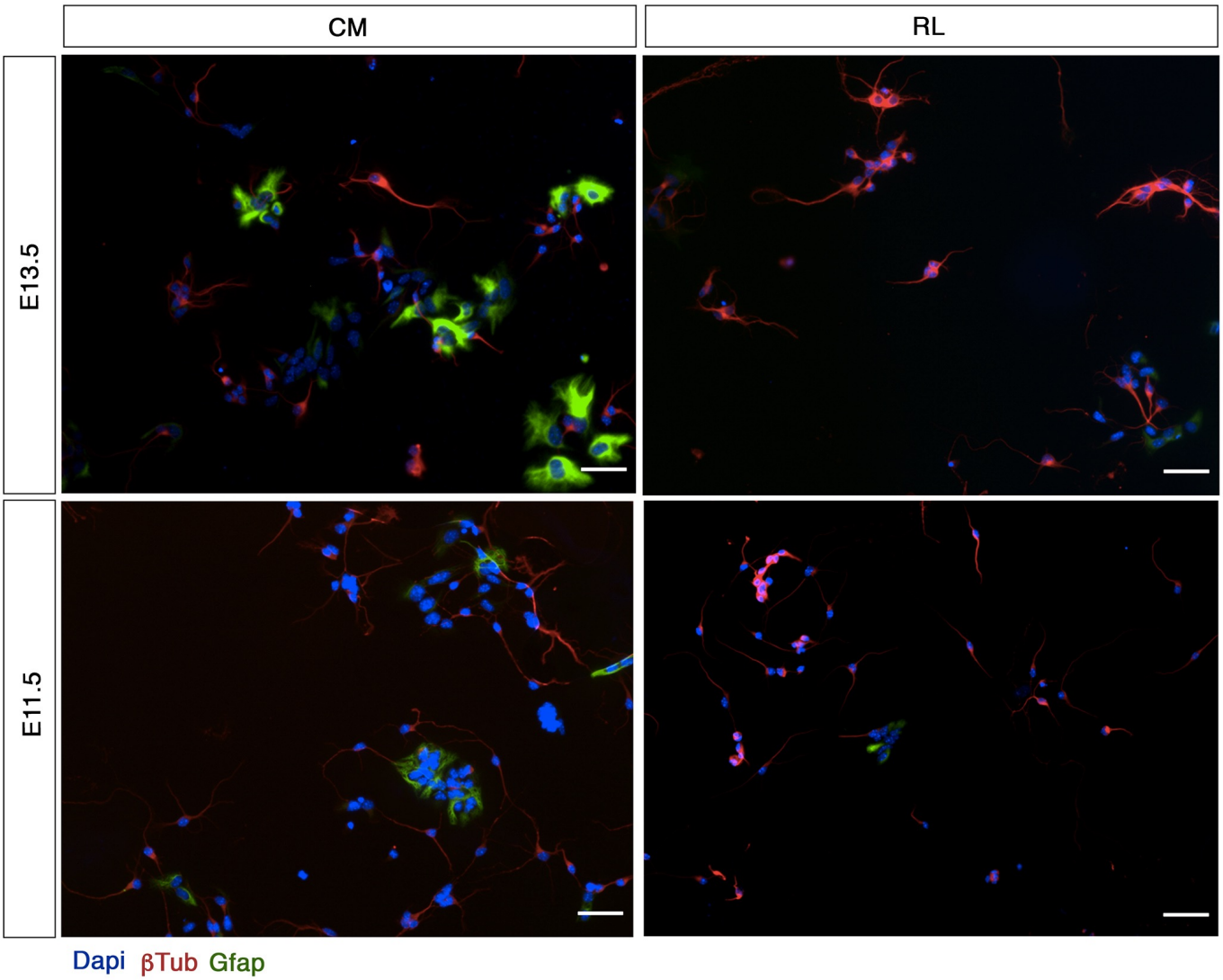

Fig. S1.2. **Primary data referred to in Fig. 1B,D.** Example of clonal assay read-out from E13.5 (upper) and E11.5 (lower) cortical precursors deriving from CM and RL field. Scale bar = 50μm.

We hypothesized that transcription factor (TF) genes allotting cortical neurons to different areal programs could also control differential fate choice within distinct subfields of the early cortical primordium. Actually, *Foxg1*, a TF gene highly expressed in the RL pallial field, silent in the hippocampal anlage and promoting paleo/neo-pallial programs at the expense of archipallial ones (Muzio, Mallamaci 2005), has been recently shown to sustain the generation of neuronal clones at the expense of astroglial ones (Falcone et al.2019). We speculated that other TFs implicated in early pallial regionalization might also control precursors fate

choice. To address this issue, we evaluated the impact on this process by *Emx2*, a key TF gene expressed along a CM^{high}-to-RL^{low} gradient (Simeone et al., 1992; Gulisano et al., 1996, Mallamaci et al 1998), crucial to hippocampus and visual cortex development (Bishop et al 2000). We manipulated its expression levels, upwards or downwards, in E11.5 pallial stem cells (SCs), by conditional, somatic lentiviral transgenesis, and we assayed histogenetic properties of their derivatives by a clonal assay **(Fig. 2A)**. *Emx2* up-regulation halved neuronal clones while increasing more than 5 folds astroglial ones (fractions of pure neuronal, mixed and pure astroglial clones were 0.250±0.048 vs 0.556±0.085, 0.418±0.014 vs 0.391±0.076 and 0.332±0.036 vs 0.053±0.015 in *Emx2*-gain-of-function (*Emx2*-GOF) vs control cultures, respectively, with $p<0.019$, $p<0.373$, $p<0.001$ and n= 3,3) **(Fig. 2B, Fig. S2)**. Conversely, *Emx2* down-regulation elicited a slight increase of neuronal clones and a moderate decrease of mixed ones (fractions of pure neuronal, mixed and pure astroglial clones were 0.431±0.020 vs 0.341±0.042, 0.472±0.019 vs 0.564±0.005 and 0.093±0.012 vs 0.093±0.039 in *Emx2*-LOF vs control cultures, respectively, with $p<0.035$, $p<0.006$, $p< 0.490$ and n= 5,3) **(Fig. 2C, Fig. S2)**. All that indicates that *Emx2* preferentially commits NSCs towards astrogenesis, ruling out the occurrence of dominant negative effects upon GOF manipulation. Moreover, it suggests that even moderate changes of *Emx2* expression levels may sensibly impact on NSCs fate choice.

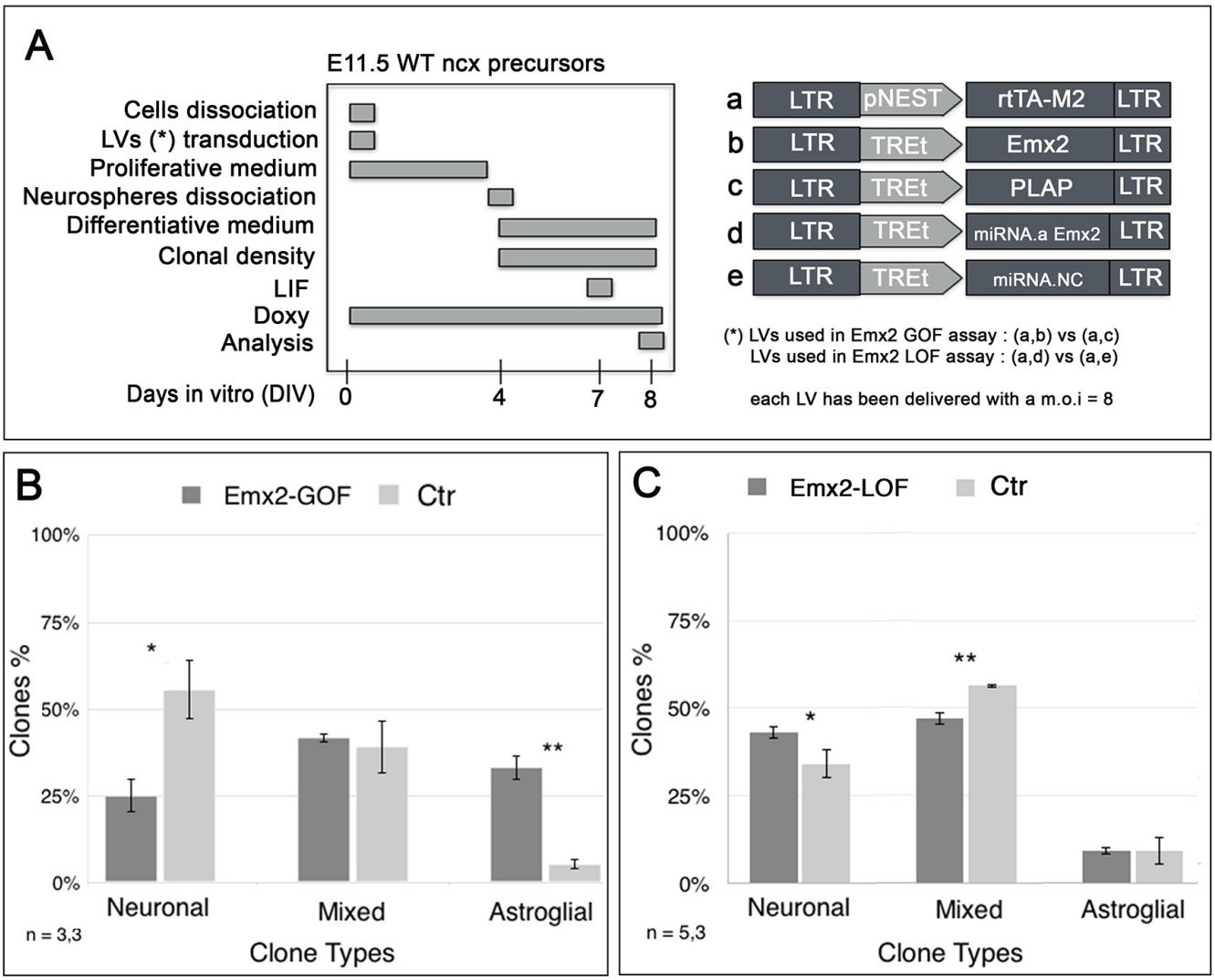

Fig. 2. *Emx2* control of NSCs fate choice within the E11.5 pallial field (A) Protocol and lentiviral vectors employed. **(B,C)** Absolutes frequencies of neuronal, mixed and astroglial clones generated by derivatives of E11 pallial NSCs, upon gain-of-function (GOF) and loss-of-function (LOF) manipulation, respectively. Statistical significance of results evaluated by t-test (1-tail, unpaired). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. *n* is the number of biological replicates, i.e aliquots of pre-pooled, independently lentivirus-transduced and cultured neural cells. Scalebars represent s.e.m's.

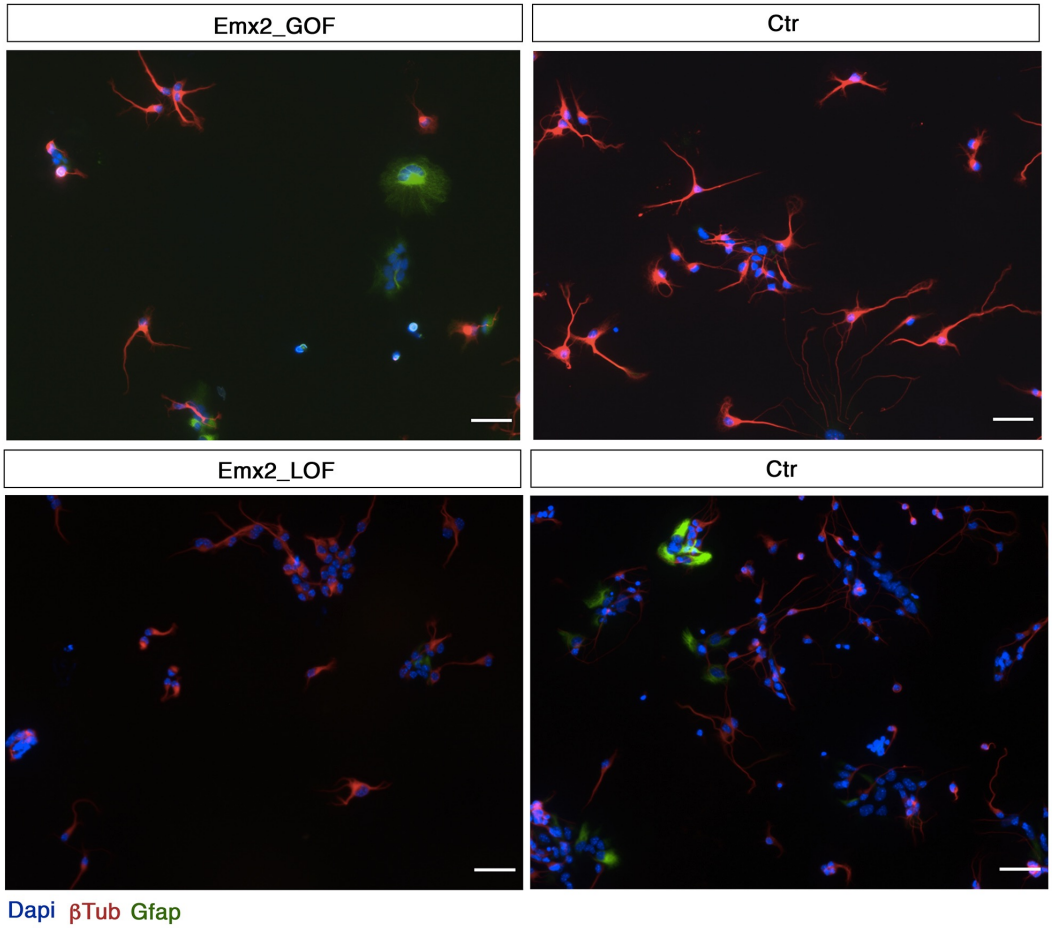

Fig. S2. Primary data referred to in Fig. 2B,C. Example of clonal assay read-out upon Emx2_GOF vs Ctr (upper part) and Emx2_LOF vs Ctr (lower part) manipulations of E11.5 pallial precursors. Scale bar = 50μm.

Then, to asses functional relevance of *Emx2* and *Foxg1* to differential fate choices peculiar to distinct pallial subfields, we systematically perturbed expression levels of these genes, both upwards and downwards, either in CM- and RL-NSCs and scored derivatives of these cells by our standard clonal assay **(Fig. 3A)**. We found that, compared to mock-transduced controls, up-regulation of *Emx2* in RL-NSCs resulted in a significant decrease of neuronal clones (0.291±0.082 vs 0.590±0.043, with $p<0.009$, and n=4,3) and an increase of astroglial ones (0.271±0.072 vs 0.093±0.012 with $p<0.018$)**(Fig. 3B, Fig. S3)**. Conversely, down-regulation of the same gene in CM-NSCs led to an increase of neuronal clones (0.229±0.020 vs 0.131±0.013 with $p<0.007$, and n=3,3) and a slight decrease of astroglial ones (0.310±0.066 vs 0.478±0.065 with

p<0.074); mixed clones were unaffected **(Fig. 3C)**. Intriguingly, even artificial *Emx2* upregulation in CM-NSCs decreased neuronal clones (0.131±0.015 vs 0.461±0.038 with p<0.001, and n=3,3), while increasing mixed ones (0.663±0.040 vs 0.333±0.023 with p<0.001) **(Fig. 3B).** All this suggests that the *Emx2* expression gradient is instrumental to differential declination of the astrogenic-vs-neuronogenic bias along the CM-to-RL axis of the pallial field. It further points to a possible, *Emx2*-dependent hystogenetic heterogeneity of the CM field itself. As for *Foxg1*, its upregulation in CM-NSCs resulted in a decrease of astroglial clones (0.166±0.004 vs 0.227±0.010 with p<0.003, and n=3,3) while not affecting neuronal and mixed ones **(Fig. 3D, Fig. S3)**. Other *Foxg1* manipulations were apparently ineffective **(Fig. 3D,E)**, except for *Foxg1* upregulation in RL-NSCs, decreasing frequency of pure neuronal clones (0.218±0.054 vs 0.402±0.037 with p<0.025, and n=3,3)**(Fig. 3D)**. Albeit consistent with the previously reported negative Foxg1 impact on astroglial commitment (Falcone et al. 2019), these results point to a more marginal involvement of this gene in differential allocation of CM- and RL-NSCs to alternative fate choices. Of note, reduction of neuronal clones among derivatives of Foxg1-GOF RL-NSCs was unexpected. Apparently at odds with Falcone et al. (2019), it likely originated from terminal pulsing of cultures by LIF, resulting into a generalized up-regulation of mixed clones at the expense of neuronal ones.

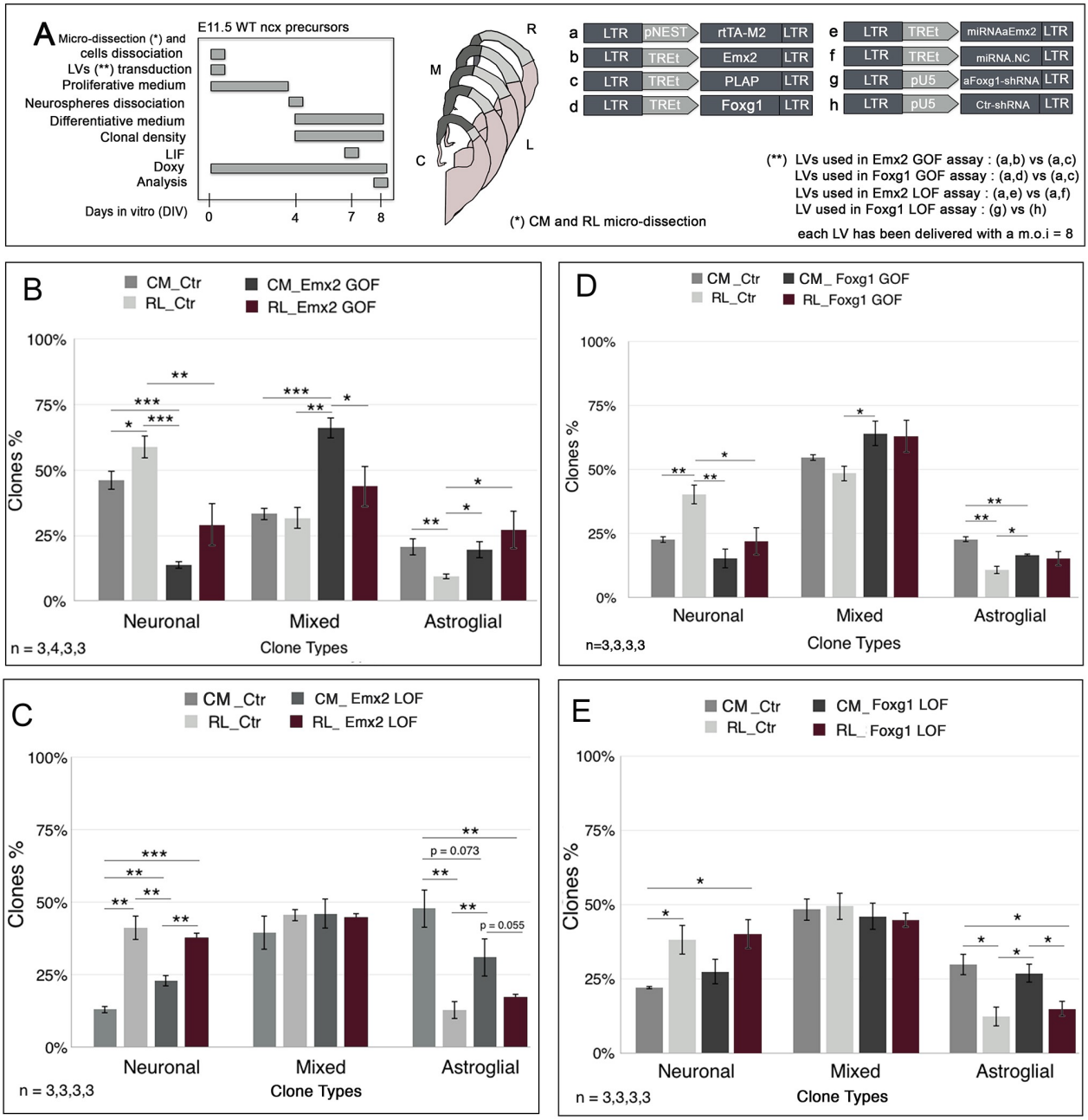

Fig. 3. *Emx2* and *Foxg1* control of astrogenic NSCs commitment along the medio-caudal / rostro-lateral axis of the E11.5 pallial field. (A) Protocol followed for the assessment of gene control over regional fate choice, with lentiviruses employed. **(B-E)** Absolutes frequencies of neuronal, mixed and astroglial clones generated by derivatives of E11 NSCs taken from the MC and RL pallial subfields, upon *Emx2*-GOF (B), *Emx2*-LOF (C), *Foxg1*-GOF (D) and *Foxg1*-LOF (E) manipulation. Statistical significance of results evaluated by t-test (1-tail, unpaired). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. *n* is the number of biological replicates, i.e aliquots of pre-pooled, independently lentivirus-transduced and cultured neural cells. Scalebars represent s.e.m's.

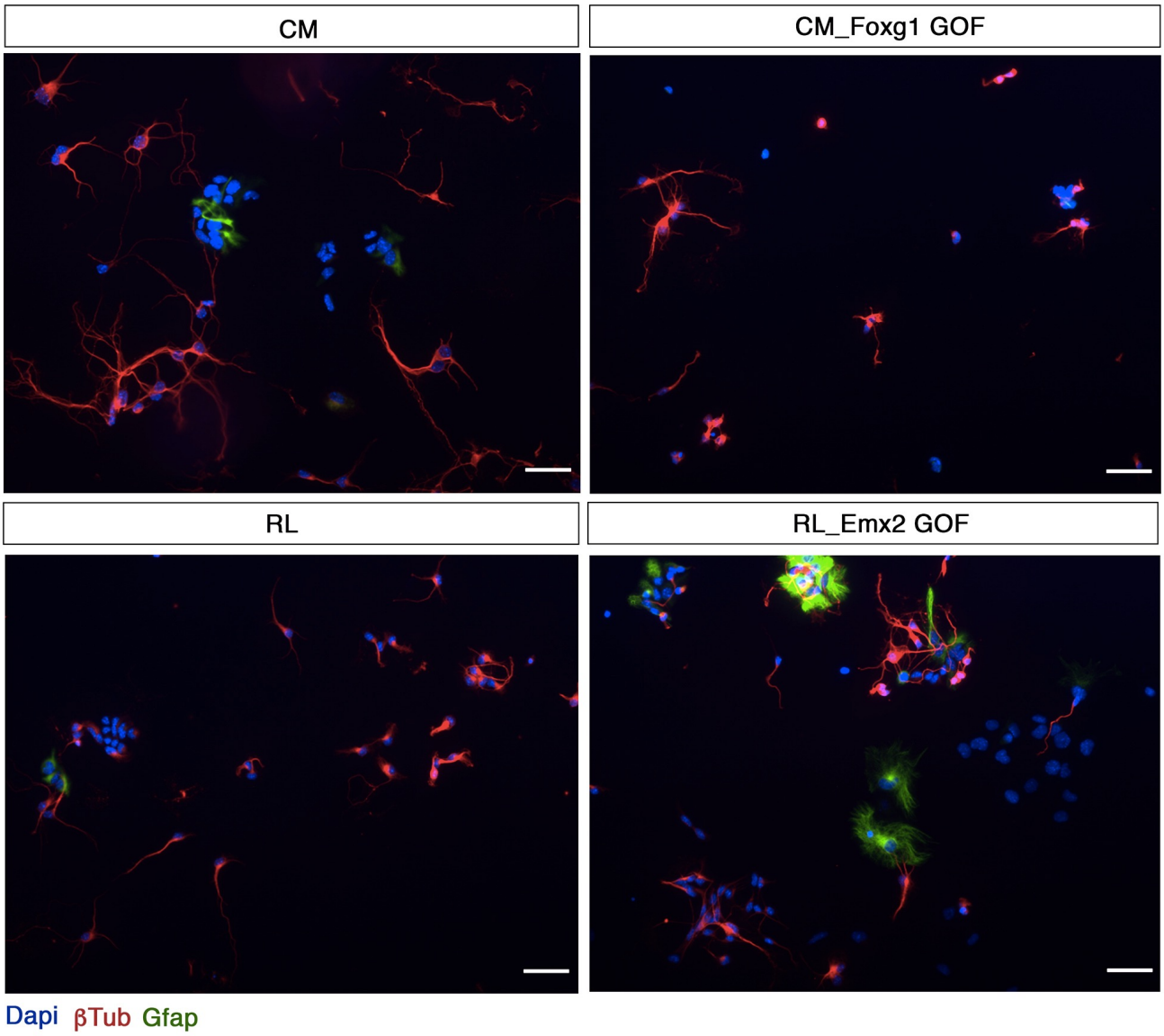

Fig. S3. Primary data referred to in Fig. 3B,D. Example of clonal assay read-out of Foxg1_GOF derivatives of CM_Ctr pallial field (upper part) and of Emx2_GOF in derivatives of RL_Ctr pallial field (lower part). Scale bar = 50μm.

4.2 Molecular mediators of Emx2 control over regional, pallial astrogenic commitment.

To get insight into molecular mechanisms mediating *Emx2* impact on fate choice, firstly we scored expression levels of a cohort of genes mastering NSCs progression to astrogenesis (*Zbtb20, Sox9, Nfia, Couptf1, Jak2, Gp130*) in neural cultures alternatively made GOF or LOF for *Emx2* by somatic lentiviral transgenesis **(Fig. 4A)**. We found that, compared to control, *Emx2* up-regulation increased *Sox9*- (1.239±0.099 vs 1±0.049 with $p<0.030$ and n=6,6) and *Jak2*-mRNA levels (1.1820±0.029 vs 1±0.068 with $p<0.028$ and n=5,5) **(Fig. 4C)**, whereas *Emx2* down-regulation decreased both *Nfia* (0.842±0.041 vs 1±0.058 with $p<0.047$ and n=3,4) and *Couptf1* (0.616±0.088 vs 1±0.102 with $p<0.015$ and n=4,4) **(Fig. 4D)**. To asses functional relevance of these mis-regulated genes to the phenotype displayed by *Emx2* "mutant" cultures, we performed ad-hoc rescue experiments. Specifically, we over-expressed *Nfia* and *Couptf1* in an *Emx2*-LOF environment and we monitored the histogenetic outcome of the resulting engineered cultures via clonal analysis **(Fig. 4B).** As expected, compared to control cultures, *Emx2*-LOF samples showed an increase in neuronal clones (0.457±0.041 vs 0.265±0.028 with $p<0.035$ and n=3,3) and a decrease in astroglial ones (0.158±0.005 vs 0.333±0.024 with $p<0.017$). Concomitant up-regulation of *Nfia* antagonized changes elicited by *Emx2* downregulation. Specifically, neuronal clones were decreased (0.125±0.009 vs 0.457±0.041 with $p<0.001$ and n=3,3) and astroglial ones were increased (0.450±0.057 vs 0.158±0.005 with $p<0.004$)**(Fig. 4E, Fig. S4)**. When over-expressed in control cultures, *Nfia* also reduced the former (0.104±0.022 vs 0.265±0.028 with $p<0.023$ and n=3,4) and increased the latter (0.563±0.042 vs 0.333±0.024 with $p<0.009$), respectively **(Fig. 4E)**. A similar rescue of neuronal (0.242±0.029 vs 0.457±0.041 with $p<0.003$ and

n=3,4) and astroglial clones frequencies (0.289±0.039 vs 0.158±0.005 with p<0.019) was also elicited by *Couptf1* over-expression in *Emx2*-LOF cultures **(Fig. 4F, Fig. S4)** The same manipulation was conversely ineffective in control cultures **(Fig. 4F)**. These data confirm *Nfia* and *Couptf1* mediation of *Emx2* impact on fate choice. Poor responsivity of control cultures to *Couptf1* further corroborates this inference, ruling out that over-compensatory mechanisms could artifactually contribute to the observed rescue. To complete the functional validation of molecular mediators, we are planning to repeat these experiments down-regulating *Sox9*-mRNA and pharmacologically inhibiting Jak2 kinase in an *Emx2*-GOF environment.

To further asses *Emx2* and *Foxg1* implication in regional fate choice, we evaluated if molecular candidates sensitive to change in their expression levels, are also differentially expressed in CM and RL subfields of E13.5 pallium. In particular, we focused on *Zbtb20, Sox9, Nfia, Couptf1 and Jak2,* all responding to *Emx2* and/or *Foxg1* GOF/LOF manipulations (Fig4 C,D; Falcone et al 2019). *Zbtb20, Sox9* and *Couptf1* were higher expressed in CM compared to RL samples (2.13±0.152 vs 1±0.111 with p<0.001 and n=7,7, 1,48±0.136 vs 1±0.075 with p<0.014, 1,98±0.127 vs 1±0.134 with p<0.001, respectively), *Nfia and Jak2* levels did not display regional differences. As expected, *Emx2* and *Foxg1* mRNAs were more abundant in CM and RL fields, respectively (2,24±0.235 vs 1±0.049 with p< 0.001 and 0.56±0.048 vs 1±0.094 with p<0.002) **(Fig. 4G)**.

Based on these results, among candidates scored above *Couptf1* is the only genuine mediator of *Emx2* impact on regional fate choice. In fact: (a) its expression is positively correlated with *Emx2* levels **(Fig. 4D)**, (b) its over-expression neatly rescues the hypo-astrogenic bias of *Emx2*-LOF NSCs **(Fig. 4F)**, (c) levels of its mRNA are doubled in CM compared to RL samples **(Fig. 4G)**. *Nfia* impact on fate choice is terrific **(Fig. 4E),** however likely not relevant to its differential regional articulation. *Nfia* expression, in

fact, is flattened along the CM-to-RL axis **(Fig. 4G)**, possibly as a consequence of its moderate sensitivity to *Emx2* levels **(Fig 4C,D)**. Finally, while not responding to *Emx2* manipulation, *Zbtb20* is much more abundant in CM compared to RL field, pointing to a likely implication of it in differential regional articulation of fate choice. Intriguingly, *Zbtb20* expression is anti-correlated with *Foxg1* levels and the former rescues the hypo-astrogenic phenotype elicited by the latter (Falcone et al. 2019). As such, *Zbtb20* might be the key mediator of *Foxg1* inhibition of astrogenesis in CM field **(Fig. 3D)**.

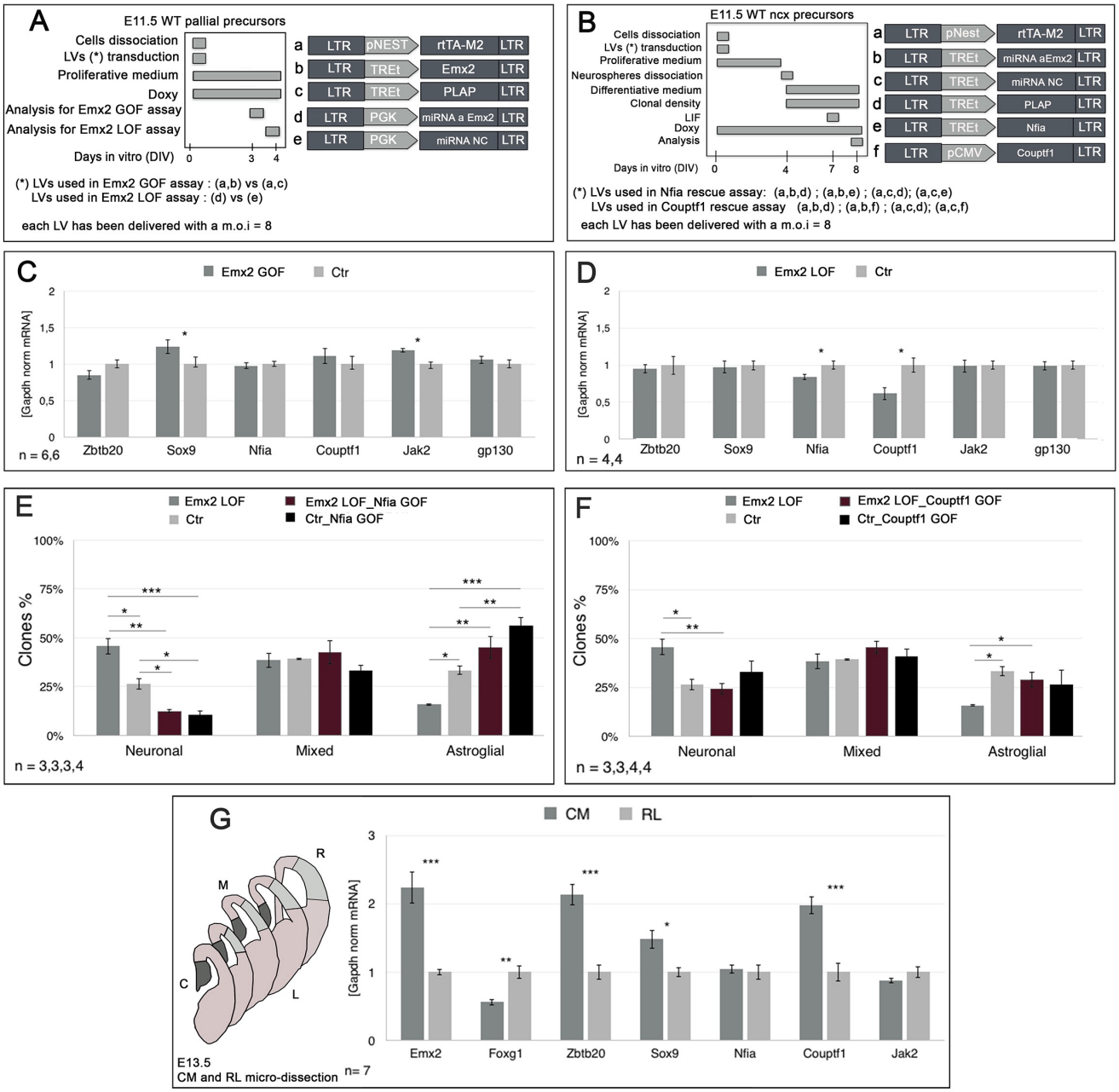

Fig. 4. Master regulators of fate choice: their sensitivity to *Emx2* levels (A,C,D), functional involvement in *Emx2* control of fate choice (B,E,F), and differential expression in MC/RL pallial subfields (G). (A) Protocol and lentiviral vectors employed for evaluation of gene expression. **(C,D)** mRNA levels of master fate choice regulators upon *Emx2*-GOF (C) and *Emx2*-LOF (D) manipulation. Results double-normalized against *Gapdh* and controls. **(B)** Protocol followed for the evaluation of functional involvement of *Nfia* and *Couptf1* in Emx2 control of regional fate choice, with lentiviruses employed. **(E,F)** Fractions of neuronal, mixed and astroglial clones generated by derivatives of E11.5 pallial NSCs, upon upwards or downwards co-manipulation of *Emx2, Nfia* and *Couptf1*. **(G)** mRNA levels of master fate choice regulators in different pallial subfields. Results double-normalized against *Gapdh* and RL pallial subfield. Statistical significance of results evaluated by t-test (1-tail, unpaired)

(E,F) or (1-tail, paired) (Z). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n is the number of biological replicates. These are aliquots of pre-pooled neural cells, independently (lentivirus-transduced and) cultured (E,F) or acutely microdissected pallial subfields, originating from single E13.5 pups (Z). Scalebars represent s.e.m's.

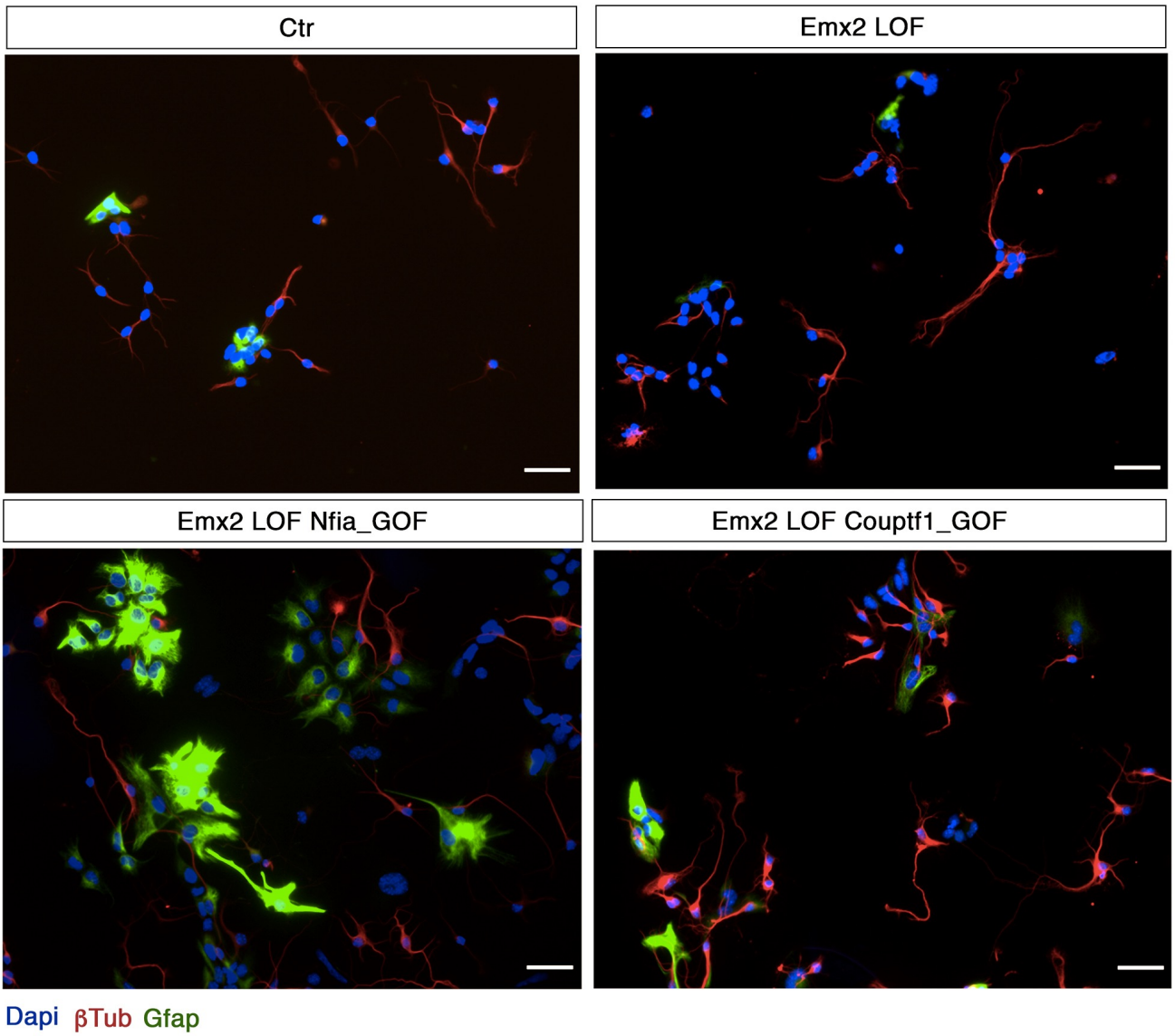

Fig. S4. Primary data referred to in Fig. 4E,F Example of clonal assay read-out of Emx2_LOF vs Ctr (upper part) E11.5 pallial precursors. As, above, upon further Nfia- and Couptf1-GOF manipulation (lower part). Scale bar = 50μm.

4.3 Functional Emx2/Foxg1 interplay in pallial astrogenic commitment.

We have shown that *Emx2* and *Foxg1* antithetically modulate the astrogenic bias of CM- and RL-NSCs: what is their reciprocal interaction in such context, if any?.

To address this issue, first we interrogated primary cultures originating from E11.5 precursors for possible reciprocal regulation of these effectors in NSCs **(Fig. 5A)**. As expected *Emx2* upregulation decreased Foxg1 expression by about 1/5 (0.78±0.032 vs 1±0.067 with $p<0.009$ and n=6,6), *Emx2* levels were significantly reduced in a Foxg1-GOF context (0.69±0.048 vs 1±.0.081 with $p<0.017$ and n=3,4) **(Fig. 5B)**. Next, we overexpressed both genes in E11.5 pallial NSCs by combined lentiviral transgenesis and we scored the impact of this manipulation on the clonal outcome of cultures **(Fig. 5C)**. Intriguingly, frequencies of neuronal and mixed clones were not affected, astroglial clones were only slightly increased (0.32±0.01 vs 0.28±0.01 with $p<0.028$ and n=5,5) **(Fig. 5D)**.

These data suggest that, albeit distinct, molecular machineries mastered by *Emx2* and *Foxg1,* relevant to regional fate choice, antagonize each other at different hierarchical levels of the functional cascade.

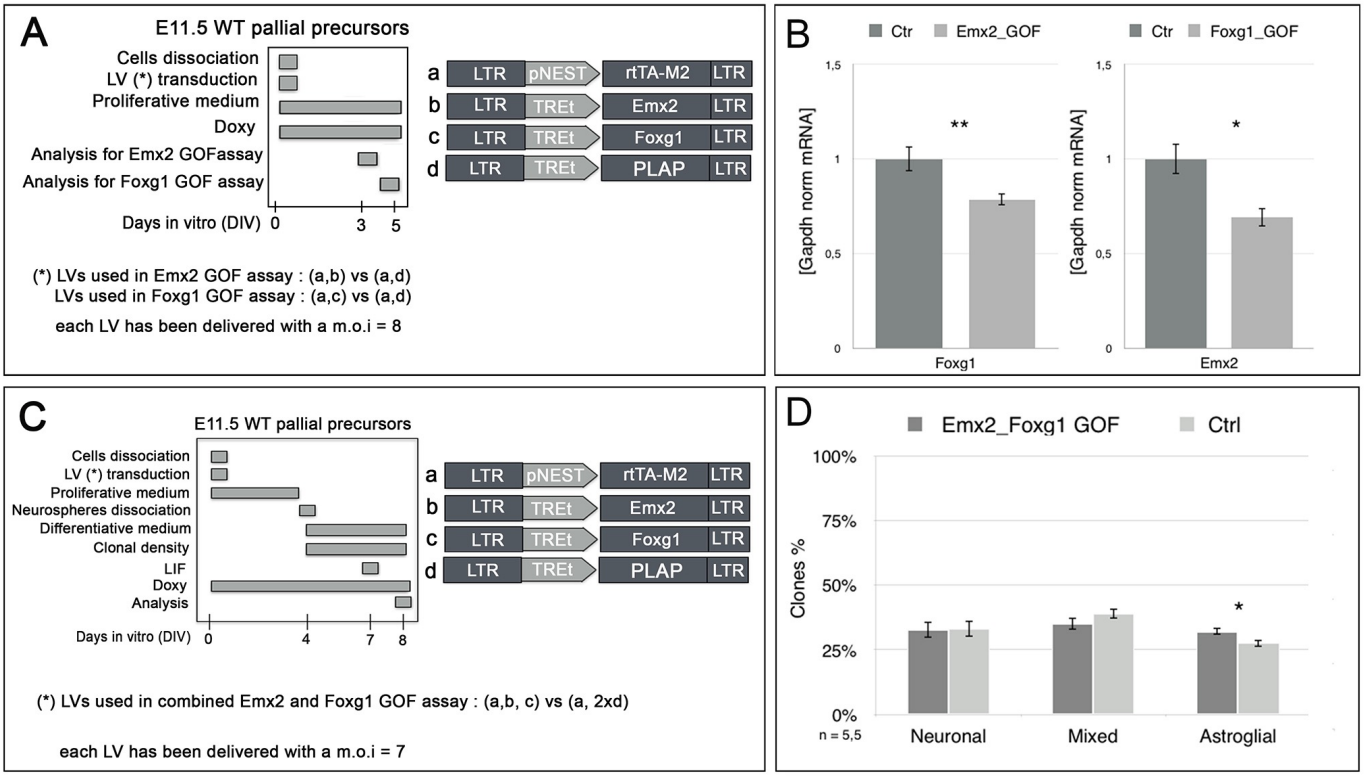

Fig. 5 Co-regulation of cell fate choice by Emx2 and Foxg1. (A,B) Reciprocal modulation of Emx2 and Foxg1 mRNA levels upon GOF manipulation of NSCs : protocol with lentiviruses employed (A) and results (B). Data double-normalized against *Gapdh* and controls. **(C,D)** Absolutes frequencies of neuronal, mixed and astroglial clones generated by derivatives of E11 pallial precursors upon combined *Emx2* and *Foxg1* overexpression in NSCs. Statistical significance of results evaluated by t-test (1-tail, unpaired). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. *n* is the number of biological replicates, i.e aliquots of pre-pooled neural cells, independently lentivirus-transduced and cultured. Scalebars represent s.e.m's.

3.4 Differential regional progression of pallial astroblasts to mature astrocytes.

Once precursor cells have been instructed toward astroglial lineage, the proliferative behavior of committed progenitors will substantially impact on the final astroglial output (Ge et al. 2012). We speculated that the exposure of astrocyte-commited progenitors to high *Emx2* or *Foxg1* levels could evoke different proliferation profiles in these cells. In this respect,

Emx2 effect on these progenitors has been already investigated in vitro and in vivo (Falcone et al 2015). In particular, it has been shown that *Emx2* overexpression inhibits astroblasts proliferation. Moreover, it has been documented that such effect is due to downregulation of *EgfR* and Fgf9, two key promoters of astroblasts proliferation, (Viti et al. 2003; Lum et al. 2009; Mayer et al. 2009) via *Bmp* upregulation and *Sox2* repression. Inspired by the opposite impact that *Emx2* and *Foxg1* have on commitment, we investigated if the overexpression of latter could enhance astroblasts proliferation. To this aim, we dissected E12.5 cortices and we kept neurosphere cultures originating from them in proliferative conditions for ten days, thus overcoming the temporal window in which they usually give birth to neurons. At DIV 10 we transduced cells with a lentiviral mix enabling the pPgk/Tet ON-driven, inducible expression of a Foxg1-transgene or a control. We transferred them into differentiative medium supplemented with doxycicline and, after one week, we immunoprofiled them for astroglial (Gfap) and proliferative (Ki67) markers **(Fig. 6A)**. We found that the fraction of proliferating astrocytes ($ki67^{+}Gfap^{+}/Gfap^{+}$) was increased in Foxg1-GOF samples compared to controls (1.291±0.045 vs 1±0.104 with n=3,3 and $p<0.032$) **(Fig 6B, Fig. S6.1)**. These results indicate that, even in case of differentiative progression, *Emx2*- and *Foxg1*- overexpression give rise to opposite phenotypes. Together with different expression levels displayed by endogenous *Emx2* and *Foxg1* in CM-and RL- pallial fields, they suggested us that astroglial progenitors lying in these fields might be characterized by distinct differentiation biases. To test this hypothesis we micro-dissected lateral neo-cortex and hippocampus from P0 and P4 Aldh1l1-EGFP mice, we dissociated them to single cells, exposed cells to differentiative medium for two hours and finally, we co-immunoprofiled such cells for pan-astroglial (Aldh1l1-EGFP) and mature-astroglial (Gfap) markers (Neymeyer et al., 1997; Eng et al., 2000) **(Fig. 6C)**. We found that the fraction of differentiated astrocytes

(Aldh1l1^{+}Gfap^{+}/Aldh1l1^{+}) was almost doubled in P0 hippocampus respect to P0 neocortex (0.745±0.091 vs 0.375±0.091 with n=3 and p<0.0001). Even if still detectable, this difference is attenuated at P4 (0.715±0.036 vs 0.550±0.020 with n=4 and p<0.0015) , due to an increase in the fraction of differentiated astrocytes in neocortex at this stage **(Fig. 6D, Fig. S6.2)**. Together with the differential astrogenic bias of CM and RL NSCs, these results support the hypothesis that different patterns of progression characterize astrogenesis of specific pallial regions and suggest that hippocampal astrocytes might reach a mature profile before neo-cortical ones. Intriguingly, compared to lateral neocortex, the P0 hippocampus displays a higher absolute prevalence of mature Aldh1l1^{+}Gfap^{+} astrocytes (0.268±0.061 vs 0.126±0.065 with n=3 and p<0.019) not anymore detectable at P4 **(Fig. S6.3)**.

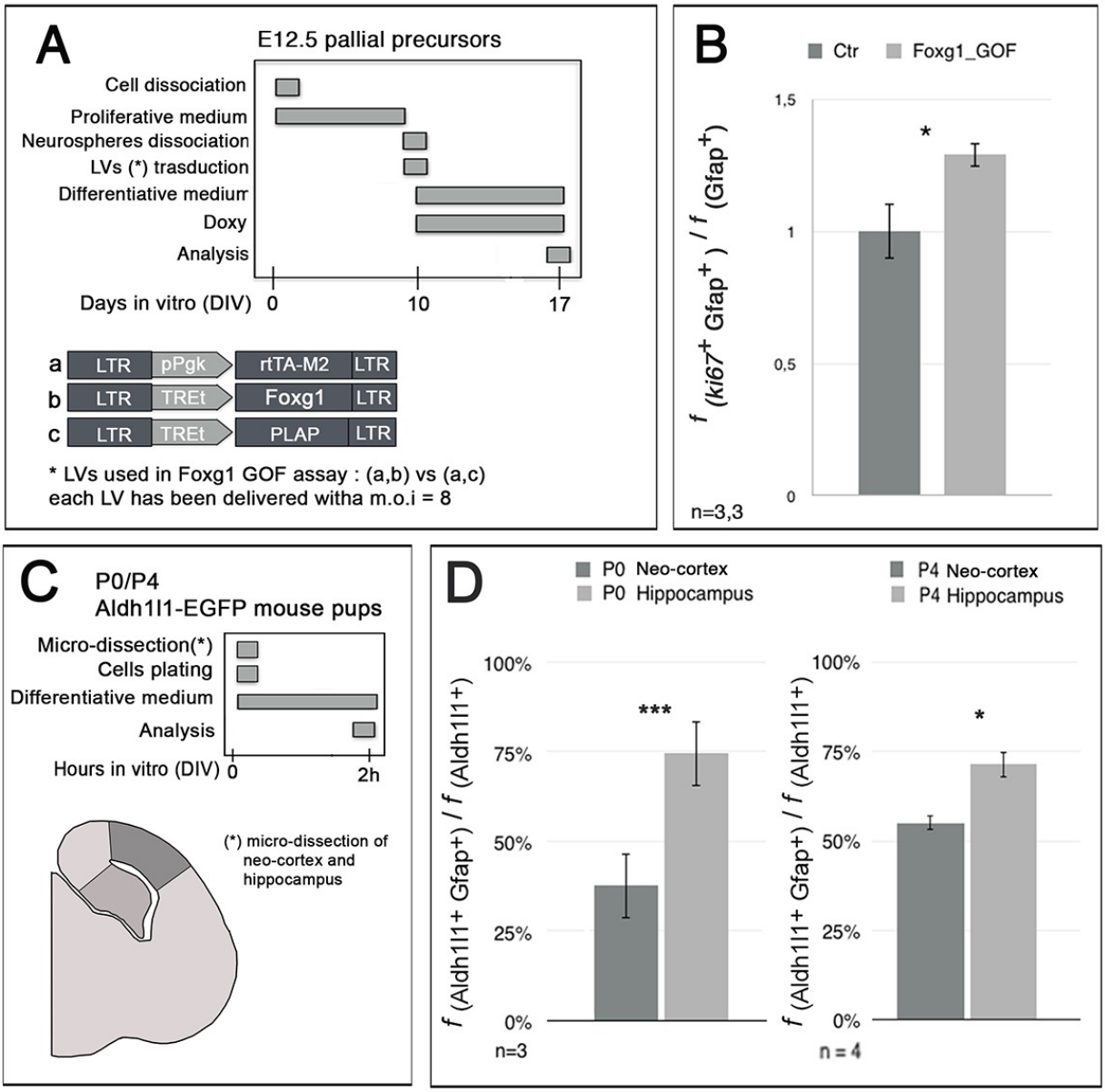

Fig. 6. Effect of Foxg1 overexpression on astroblast proliferation and regional progression of pallial astroblasts to mature astrocytes. (A) Protocol and lentiviral vectors employed. **(B)** Control-normalized frequencies of ki67^{+}Gfap^{+}/Gfap^{+} generated by derivatives of E12.5 pallial NSCs, upon Foxg1 gain-of-function (GOF) manipulation. **(C)** Temporal protocol and schematic representation of micro-dissection of cortex and hippocampus from Aldh1l1-EGFP mouse pups **(D)** Absolute frequencies of (Aldh1l1^{+}Gfap^{+}/Aldh1l1^{+}) in P0 (left) or P4 (right) cortex and hippocampus. Statistical significance of results evaluated by t-test (1-tail, unpaired (B) or paired (D). * p<0.05, ** p<0.01, *** p<0.001. n is the number of biological replicates, i.e aliquots of pre-pooled, (independently lentivirus-transduced) and cultured neural cells. Scalebars represent s.e.m's.

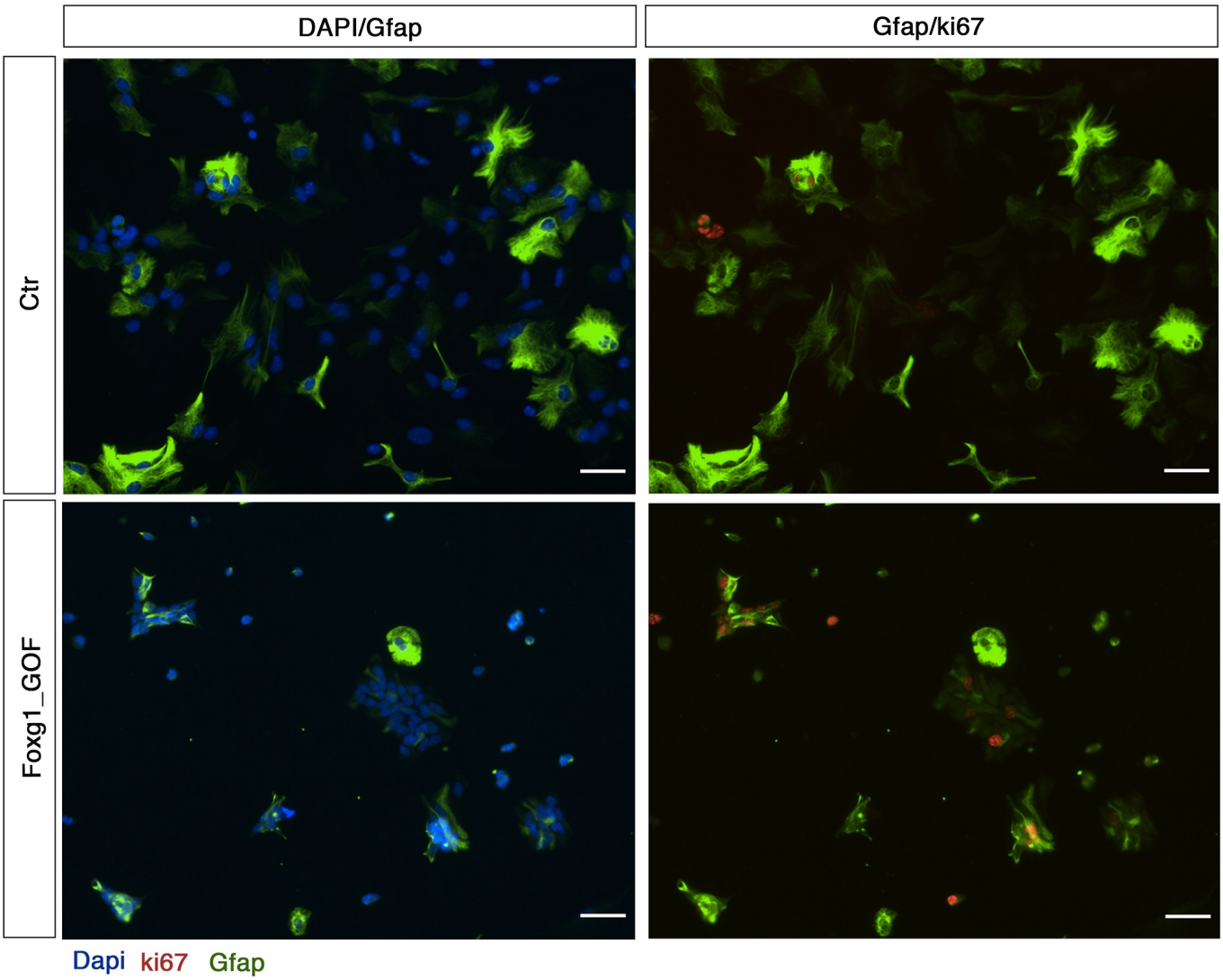

Fig. S6.1. Primary data referred to in Fig. 6.B. Example of astrocytes proliferation assay read-out in Foxg1-GOF vs Ctr. samples. Scale bar = 50μm.

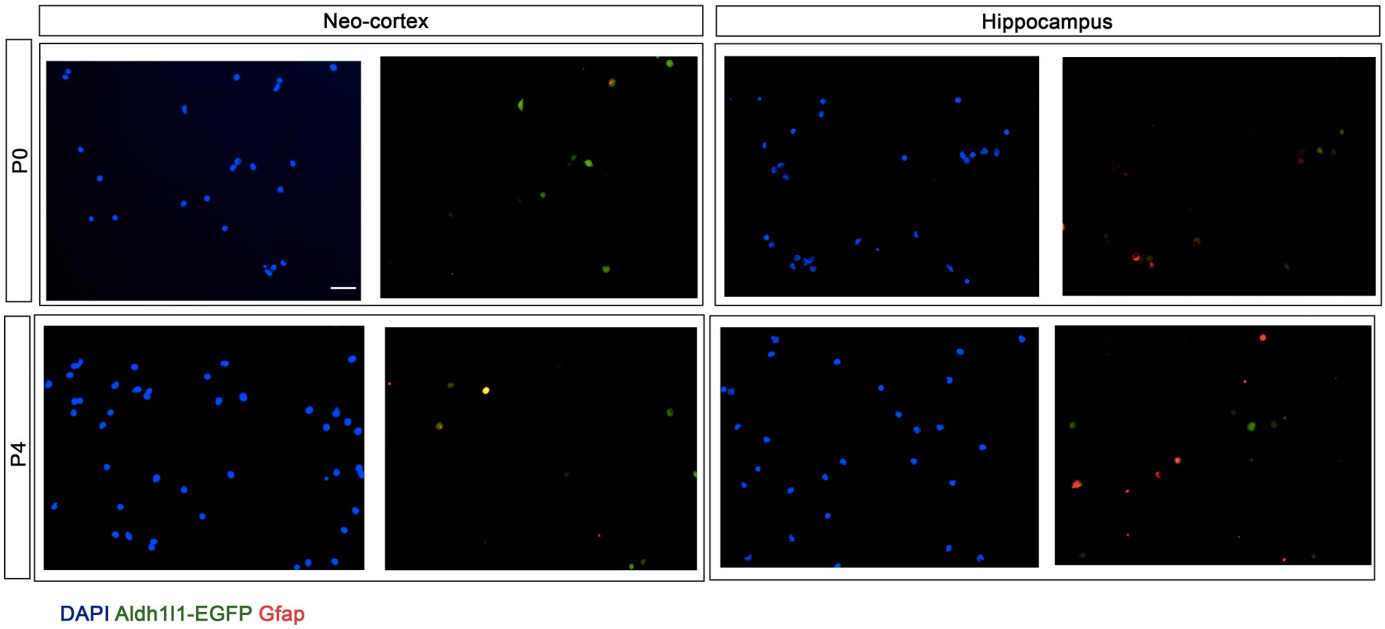

Fig. S6.2. Primary data referred to in Fig. 6.D. Example of acutely dissociated cells from P0 (upper) or P4 (lower) neo-cortex and hippocampus from Aldh1l1-EGFP co-stained with DAPI and Gfap. Scale bar = 50μm.

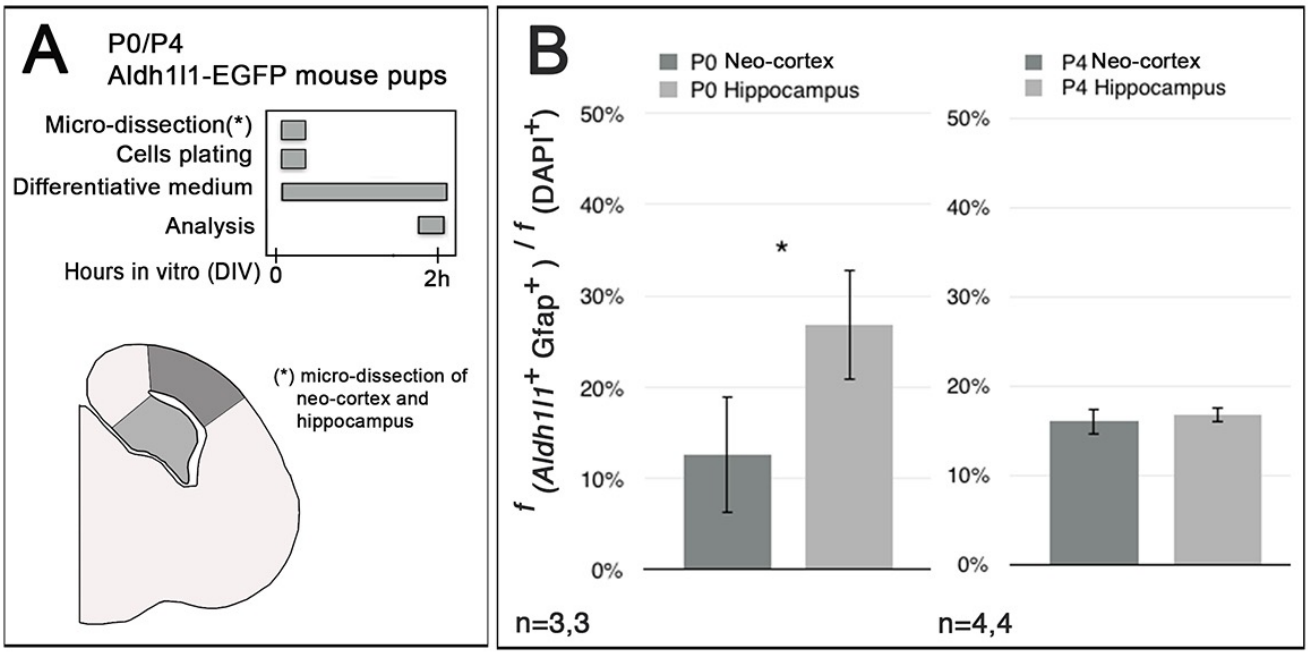

Fig. S6.3 (A) Temporal protocol and schematic representation of micro-dissection of cortex and hippocampus from Aldh1l1-EGFP mouse pups. **(B)** Frequency of Aldh1l1$^+$Gfap$^+$ normalized against DAPI$^+$ cells in in P0 (left) or P4 (right) cortex and hippocampus. Statistical significance of results evaluated by t-test (1-tail, paired).

* $p<0.05$, ** $p<0.01$, *** $p<0.001$. n is the number of biological replicates, i.e aliquots of independently cultured neural cells. Scalebars represent s.e.m's.

5. DISCUSSION

Here we investigated regional articulation of pallial astrogenesis progression and molecular mechanisms controlling it. We have found that NCSs originating from CM and RL pallial fields display higher and lower astrogenic bias **(Fig. 1)**. We showed that *Emx2,* already known for its implication in cortical arealization as a promoter of hippocampal and occipital fates*,* enhances astrogenic NSCs commitment **(Fig. 2)**. Next, we demonstrated that *Emx2* is largely responsible for differential regional astrogenic bias of the cortical field, while *Foxg1*, known to inhibit archipallial programs and to antagonize astrogenesis, plays only a more marginal role this context **(Fig. 3)**. Then, we found that a subset of genes mastering astrogenesis activation (*Sox9, Ztbtb20* and *Couptf1)* are differentially expressed along the CM-RL pallial axis, consistently with regional articulation of astrogenic commitment, and two of them, *Couptf1* and *Zbt20,* mediate *Emx2* and *Foxg1* impact on such articulation, respectively **(Fig. 4)**. Moreover we found that functional cascades controlling regional fate choice, driven by *Emx2* and *Foxg1,* antagonize each other **(Fig. 5)**. Finally, we found that the fraction of perinatal astrocyte-lineage cells differentiated as astrocytes is doubled in the hippocampus compared to the neocortex **(Fig. 6)**, likely reflecting pro-differentiative (Falcone et al., 2015) and pro-proliferative **(Fig. 6)** effects exerted by *Emx2* and *Foxg1* within this lineage, respectively.

The overall resulting scenario leads us to hypothesize “a more astrogenesis-biased NSCs pool, generating differentiation-prone astroblasts” in hippocampus, versus “a less astrogenesis-oriented NSCs pool, giving rise to proliferation-keen astroblasts” in more lateral cortex. Although novel, such scenario is reminiscent of cerebellum, where vermian astrocytes seem to originate starting from a relatively large number of NSCs, via committed progenitors endowed with reduced

proliferative potential (Cerrato,Parmigiani et al. 2018). As for the cortex, this model might reflect geometrical constraints and/or functional needs peculiar to different regions of the cortical primordium. Specifically, whereas hippocampus and neocortex originate from a tangentially extended and relatively mid-sized apical proliferative layer, respectively, the tiny apical compartment of latero-ventral pallium is, alone, in charge of generating the entire paleocortex, the claustrum and part of the amygdala (Yun et al., 200; Puelles et al 2000). Next, increased early astroglial commitment of medial NSCs might anticipate special metabolic needs of neonatal hippocampus. Characterized by far higher levels of NMDA- and AMPA- receptors compared to age-matched neocortical pyramids (Pickard et al. 2000), in fact, neonatal hippocampal neurons might require an enhanced K^+- and glutamate-buffering activity. High astroglial densities already available in neonatal hippocampus, as a consequence of its specific histogenetic timetable (Fig. 6; see also Ogata et al. 2002 and Zhu et al., 2012) might just help to fulfill such requirement. Of note, such need to finely tune local density of mature astrocytes might be particularly stringent, due to autochthonous features of these cells. As shown by adenoviral tracing, in fact, pallial astrocytes typically retain tangential locations peculiar to their NSCs ancestors (Tsai et al. 2012). Some tangential dispersion actually occurs at about P4-P7, however it predominantly does not exceed 150 μm (Clavreul et al. 2019).

Emx2 impact on pallial precursors fate choice has been already addressed in a series of previous studies. According to Heins et al. (2001), *Emx2* overexpression in embryonic precursors increases the frequency of mixed, i.e. neuronal/glial, clones at the expense of pure neuronal ones. That was related to *Emx2* capability to promote self-renewal and multipotency of pallial precursor cells (i.e. their stemness). No change in glial clones frequency was detected. The apparent

discrepancy between this result and ours might reflect distinct cell clustering criteria adopted in such study for clone identification. Furthermore, It might be exacerbated by aggregate counting of distinct glial types. In a subsequent study, by Galli et al. (2002), *Emx2* overexpression in derivatives of neonatal subventricular zone was conversely reported to promote their differentiation at the expense of proliferation, while not altering at all their ultimate glial-to-neuronal output ratio. This last finding could be due to generalized *Emx2* manipulation and aggregate evaluation of glial progenies, blurring *Emx2* impact on NSCs fate choice. In fact, when *Emx2* overexpression was limited to NSCs and quantification of glial cells was restricted to immediate NSCs derivatives (Brancaccio et al. 2010), this resulted in a large excess of astroglial committed progenitors upon normalization against their NSC ancestors, consistently with the take-home message of the present study.

As for *Foxg1,* we have recently shown that its expression levels in pallial NSCs is anticorrelated with their commitment to astrogenesis and functionally contributes to its temporal articulation (Falcone et al. 2019). Given *Foxg1* RL^{high}-to-CM^{low} expression gradient, we hypothesized that this gene might also contribute to differential regional allocation of NSCs to astroglial fate. This prediction turned out to be correct, however *Foxg1* role in this context was marginal compared to *Emx2.* On the other side, albeit co-correlated with astrogenic NSCs commitment rates, *Emx2* expression levels progressively decline in the embryonic mouse pallium. This rules out that *Emx2* dictates temporal progression of NSCs commitment towards astrogenesis.

All that prefigures a scenario, where different *Emx2* levels latently set the astrogenic bias of early pallial SCs and distinct *Foxg1* levels shape the temporal activation profile of the astrogenic program. An issue affecting this model is the absence of astrocytes in the early CM field, despite high *Emx2* and low *Foxg1* levels. This suggests that another effector with a

complementary spatial distribution (e.g. its *Foxj1* paralogue) might vicariate *Foxg1* in the early CM field. Alternatively, generation of committed astroblasts, not yet expressing mature astrocytes markers, might initiate in this territory well in advance compared with more lateral ones. This issue will be subject of future investigations.

The involvement of *Zbtb20*, *Sox9*, *Nfia and Coupft1* in timed astrogenesis activation, via an articulated impact on the epigenetic state of astroglial genes, has been thoroughly documented (Naka et al. 2008; Namihira et al. 2009; Kang et al. 2012; Nagao et al. 2016). Moreover, all four genes have been shown to be inhibited by *Foxg1*, so accounting for temporal relevance of its anti-astrogenic activity (Falcone et al 2019). Here we showed that three of these genes (all except *Zbtb20*), are stimulated by *Emx2.* Intriguingly, all except *Nfia* are also differentially expressed in CM vs RL field, likely contributing to diversified astrogenic biases of such fields. In this context, we propose that higher expression levels of *Couptf1* and *Zbtb20* within the CM territory can be relevant to *Emx2* and *Foxg1* control of the astrogenic bias of such territory, respectively.

As for molecular mechanisms mediating *Foxg1* and *Emx2* impact on proliferative/differentiative behaviour of committed neural progenitors, they have been already addressed in previous dedicated studies. In particular, it has been show that *Foxg1* prevents cell cycle exit by inhibiting transcription of *Cip1^{p21}* (Seoane et al., 2004), a gene specifically expressed in CM pallial marginal layer (Mallamaci et al., 2000). As for *Emx2,* it is implicated in a positive feedback loop with pro-differentiative (Nakashima et al., 1999) Bmp-signalling (Theil et al., 2002, Shimogori et al 2004, Falcone et al., 2015), mainly active around the pallial cortical hem, as well as in a mutually negative loop with pro-proliferative Fgf signalling (Garel et al., 2003; Storm et al., 2006, Fukuchi-Shimogori and Grove, 2003, Falcone et al., 2015, Falcone et al., 2016), which is

prevalently active within the rostral pallial field (Borello et al., 2008; Cholfin and Rubenstein, 2008). Intriguingly, the spatial articulation of proliferative/differentiative activities depicted by this literature nicely fits the scenario emerging from the present study, thus corroborating its take-home message.

To sum up, we found that different regions of the early pallium are endowed with distinct astrogenic potentials, we identified genes mastering this phenomenon, we showed that astrocyte committed progenitors lying within specific cortical regions display distinct differentiative biases, and we provided clues to molecular control of this aspect. Finally, we showed that all this leads to a transient, high availability of astrocytes in neonatal hippocampus, a phenomenon possibly reflecting special metabolic needs of this structure.

6. APPENDIX

6.1 Introduction

6.2 Aim

6.3 Results

6.4 Discussion

6.1 Introduction

• Evolutionary conservation of Foxg1 features and functions

As previously described, the transcription factor Foxg1 plays different and essential roles in sequential steps of brain development. Its functions, ranging from telencephalon specification, cell cycle control, to neuronal differentiation, are extensively shared in vertebrates. Consistent with that, Foxg1 coding sequence and protein product are highly conserved. The gene, located on chromosome 12 in mouse and on chromosome 14 in human, contains a single exon encoding for 50KDa protein, typically acting as a transcriptional repressor. From N- to C-term, this protein encompasses 4 main domains (Fig.25) (Wiese et al., 1995; Bredenkamp et al., 2007; Florian et al., 2011):

- an amino-terminal domain, including histidine, proline and glutamine-repeats,
- a forkhead DNA-binding domain (FHD), highly conserved across all members of the FOX family,
- a KDM5B (formerly JARID1B) -binding domain (JBD), recruiting a histone demethylase,
- a Groucho-binding domain (GBD), recruiting Groucho co-repressor proteins

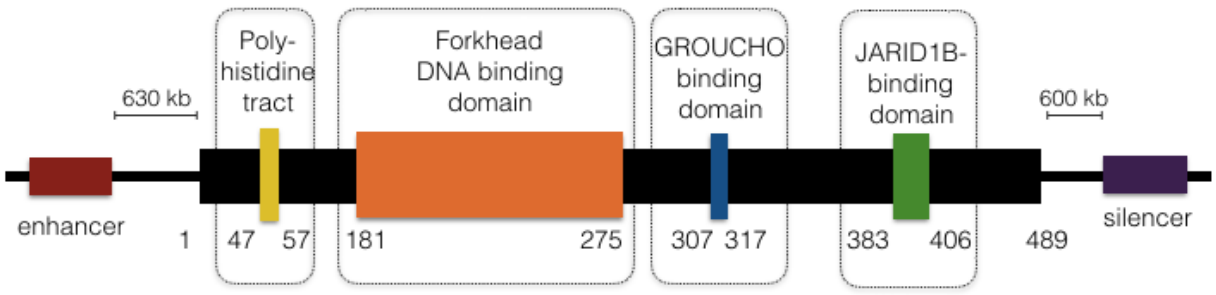

Fig. 25 Schematic representation of human FOXG1 gene. Shaded regions indicate the three functional domains of the protein: DNA-binding forkhead domain (FHD) (amino

acids 181–275), Gro- binding domain (GBD) (amino acids 307–317) and KDM5B (formerly JARID1B) binding domain (JBD) (amino acids 383–406 (Adapted from Foxg1 research foundation).

The most peculiar and conserved aspect of *Foxg1* is its role as telencephalic hallmark (Toresson et al. 1998). The expression profile of this gene in different vertebrates is consistent with its involvement in early telencephalic specification (Toresson et al. 1998, Roth et al. 2010). In developing human embryos, FOXG1 is first expressed in early telencephalic neuroepithelial progenitors. At intermediate corticogenesis stages, it is strongly expressed in the ventricular-subventricular zone and in the cortical plate (Onorati et al. 2014), thus showing a pattern similar to its mouse ortholog. Interestingly, even in invertebrates, Foxg1 expression marks the anterior-most central nervous system, suggesting a conserved evolutionary involvement of this gene in specifying rostral identity (Pani et al. 2012, Grossniklaus et al. 1994). The involvement of Foxg1 in the telencephalon's axial identity is another persistent feature peculiar to different vertebrates as studies in fish have demonstrated that signalling pathways coordinated by Foxg1, and involved in the dorso-ventral and medio-lateral specification, are highly conserved (Pottin et al. 2011).

Foxg1 pro-proliferative feature and its control over of cell cycle have also been documented in different vertebrates. Overexpression of Foxg1 in chick forebrain, for example, induced thickening of the neuroepithelium and an increase in cell number, confirming a conserved role for Foxg1 in expanding the telencephalic progenitor pool (Xuan et al. 1995; Ahlgren et al. 2003). Similarly, mouse models lacking Foxg1 are characterized by neural progenitor which exit the cell cycle prematurely and differentiate into neurons (Hanashima et al., 2002). Interestingly, evidence from clinical studies suggests that the importance of Foxg1 in controlling progenitors-pool's size and fate might be conserved up to humans. In this respect, micro-deletion of this gene, have been associated with microcephaly

(Kortum et al. 2011). Similarly, Foxg1 upregulation is detectable in multiple cancer cell lines and cancer patients' tissue (Chan et al. 2009; Verginelli et al. 2013), confirming its ability to sustain cells proliferation.

- **FOXG1-associated pathology.**

Strengthening the essential role played by Foxg1 during development, and throughout evolution, different reports from clinical studies have associated structural alteration in its locus to severe neurodevelopmental disorders. Depending on different genetic landscapes, symptoms may be very heterogeneous. They include seizures, inability to control body movements, and lack of speech. (Kortum et al. 2011). At the moment, 486 patients have been diagnosed for "FOXG1 syndrome" (from International Foxg1 Foundation, https://foxg1.org/), i.e. a rare neurodevelopmental disorder associated with heterozygous variants in the forkhead box G1 (FOXG1) gene (Mitter at al. 2018). Among these patients, single nucleotide mutations, deletions or duplications, in the human chromosome 14 FOXG1 locus have so far been described, and each of them is specifically associated to a peculiar constellation of clinical manifestations.

Acutally, Foxg1 syndrome patients were originally classified as affected by a specific "congenital variant of Rett syndrome" (Ariani et al. 2008; Philippe et al. 2010). This is a severe neurological disorder caused by mutations in the X-linked gene methyl-CpG-binding protein 2 (MECP2), a ubiquitously expressed transcriptional regulator. Rett syndrome (RT) is characterized by an initially normal neurological and physical development during the first months of life, followed by rapid regression and motor deterioration after 12-18 months of life (Kyle at al. 2018). The identification of patients with intragenic FOXG1 mutations and not in the MECP2 gene, however, suggested that, despite the substantial overlap of phenotypes, two distinct syndromes, with peculiar patterns of progression,

might arise according to the genetic background. Consistent with that, patients carrying deletions or inactivating mutations of FOXG1 are characterized by symptoms showimg an earlier onset in manifestation and interesting very early stages of post-natal life, usually not affected in classical RT patients (Kortum et al. 2011). Initially, a certain degree of heterogeneity in RT syndrome clinical manifestations was attributed to the effect of random X-inactivation (Mari et al. 2005). However, the evidence of male patients with typical RT features, strengthened the idea that genes different from MECP2 might account for the same clinical manifestation (Le Guen et al.2011).

Microduplications in the locus of Foxg1 have been reported in individuals with developmental delay, epilepsy and infantile spasms (Brunetti-Pierri et al., 2011; Striano et al., 2011). This phenotype is different from that showed by patients with 14q12 deletions, associated with RT syndrome, and suggested that the phenotypic spectrum of *FOXG1*-related disorders can also resemble West Syndrome (Striano et al. 2011). West syndrome (WS), in fact, is characterized by infantile-spasms, aberrant EEG pattern and impaired psychomotor development. In most cases, WS develops as a consequence of metabolic disorders or brain lesions, however, the etiology is often unknown (Pal et al., 2010, Striano et al., 2011). Interestingly, as described for Rett-like patient, even in the case of West-like phenotype, symptoms appear very early if patient's genetic alterations include *FOXG1* (Striano et al. 2011).

Foxg1 variants have also been associated with the manifestation of severe symptoms, and these include single nucleotide deletion or duplication, as well as missense mutations, all potentially leading to frameshift or to the appearance of stop codon signal (Ariani et al. 2008; Mencarelli et al. 2010; Bahi-Buisson et al. 2010; Philippe et al. 2010; Le Guen et al. 2011). Interestingly, according to which FOXG1 protein domain is involved, specific "genotype–phenotype" associations arise.

Consistent with its highly conserved structure, for example, the most severe phenotypes are generally associated with loss of DNA binding forkhead domain generally due to stop codon mutations leading to the production of a truncated FOXG1 protein (Ariani et al. 2008; Mitter et al. 2018). Missense variants in this domain, as well as truncating variants affecting the C-terminal domain, in contrast, were found in children with milder phenotypes possibly due to the persistence of a residual function of the protein (Mitter et al. 2018). In addition, single nucleotide deletions interesting JBD and GBD have also been reported, and they potentially lead to an impairment of interaction between FOXG1 and its important co-repressors (Ariani et al. 2008). All these clinical reports strengthen the evidence that the preservation of FOXG1 functional protein, and its correct allele dosage, are necessary for a normal embryonal and post-natal development and open the possibility of new potential targets for a therapeutical treatment of severe neurodevelopmental disorders.

The types of symptoms and anatomical alterations typical of FOXG1 patients perfectly match the role played by this gene in different steps of brain development. Interestingly, more and more pieces of evidence from literature further characterize the involvement of this gene in specific histogenetic contexts and shed light on the complex pathophysiology of FOXG1 syndrome. So far, the production and/or functionality of the three main cellular subtypes which colonize the mature cortex, i.e. neurons, astrocytes and oligodendrocytes, were found to be sensitive to altered *Foxg1* levels. As for neurons, *Foxg1* over-expression is sufficient to stimulate, in vitro as well as in vivo, dendrite elongation, via Hes1/pCreb1 activation and Syt/Ndr1 inhibition (Chiola et al 2019). Furthermore, a substantial increase in neuronal activity and hyper-synchronization of calcium-evoked events are also detectable in neuronal cultures where *Foxg1* overexpression is restricted to neurons (Tigani et al 2020). Consistent with that, mouse model overexpressing *Foxg1* within deep

neocortical pyramids showed an abnormal EEG and resulted to be more prone to kainic acid-evoked limbic motor seizures. A significant interneuron depletion was also detectable in this Foxg1-GOF mouse model, confirming the possible involvement of different cell types in epileptic-like behavior associated with higher Foxg1 allele dosage (Tigani et al. 2020). In this respect, a dampened interneuronal function has been suggested to contribute to seizures occurring in patients with structural FOXG1 mutations (Mitter et al. 2018; Vegas et al. 2018). In fact, ablation of one Foxg1 allele dampen the migration of interneuron from the basal forebrain ultimately leading to a decrease in their number (Shen et al. 2019), and also evokes interneurons neurite hypotrophy (Shen et al. 2019), collapse of Gad2 expression (Patriarchi et al. 2016), and reduction of interneuron electrical activity (Zhu et al. 2019). Interestingly, an up-regulation of Foxg1 and a concomitant over-production of GABAegic cells and their neurite overgrowth has been reported to occur in autism spectrum disorder (ASD) patient-derived neuro-organoids (Mariani et al. 2015). Here, the abnormal proportion of interneurons results from a Foxg1-dependent increased proliferation of early interneuron progenitor cells. These results further support the positive impact of FOXG1 in expanding progenitors pool and strongly support the evidence that both its overexpression or downregulation eventually lead to an unbalanced production of specific neuronal subtypes peculiar to different neuropathological contexts.

As for glial lineage, different reports confirmed that, in mouse, Foxg1 is positively correlated with the persistence of progenitors cells in proliferative state and to a delayed profile of differentiation of both astro- and oligo- committed progenitors (Brancaccio et al. 2010; Falcone et al. 2019; Dong et al. 2020).

Considering all the different functions exerted by Foxg1 in specific developmental contexts, and taking into account the heterogeneous

landscape of mutations characterizing Foxg1 syndromes, it is very challenging to shed light on the pathophysiology of this disease. A good knowledge of the normal physiology of this gene in the formation and maturation of cortical network is undoubtedly essential to understand which specific moment of development might be more susceptible to Foxg1 structural alterations. Furthermore, the study of the conservation between Foxg1 function in animal models and humans is essential to focus translational research to specific aspects of the phenotype.

6.2 Aim

Aim of this supplementary work was to address :

- conservation of selected mechanisms controlling astrogenesis between rodents and humans
- misregulation of such control associated to specific neuropathogenic mutations.

6.3 Results

6.3.1 Foxg1 antiastrogenic activity is conserved in human pallial precursors

As detailed in " Introduction: Role of Foxg1 during astrogenesis " we have recently shed light on Foxg1 involvement in controlling NSCs fate. In particular, we found that Foxg1 levels in mouse peri-ventricular layer of the developing cortex undergoes a strong decline as we approach the onset of gliogenesis and we proved that this is instrumental to have the proper activation of the astroglial program. Foxg1 over-expression in murine NSCs, in fact, antagonizes the generation of astrocytes through different concurrent mechanisms including : (a) inhibition of transcription factors essential to astrogenesis onset (Nfia, Ztb20, Coputf1 and Sox9), (b) direct trans-repression of genes active in mature astrocytes (S100b and Gfap), (c) tuning of pathways involved in promotion/inhibition of astrogenesis progression (IL6/Jak2/ Stat1,3; Bmp/Smad1,5,8; Nrg1/ ErbB4^{ICD}-NCoR; Dll1/Notch1^{ICD}).

To assess if *FOXG1* antagonizes astrogenesis progression in humans like in rodents, we run ad hoc GOF and LOF assays in pallial precursors derived from a legal, human PCW10 abortion and pre-expanded in vitro over different times (Falcone et al., 2019). At the beginning of the procedure (DIV0), we transduced human PCW10 pre-expandend up to DIV 150, with 2 lentiviruses, expressing the rtTA^{M2} transactivator under the pNes promoter and Foxg1 (or a control) under the rtTA^{M2}/ doxycycline-responsive TREt promoter **(Fig. App.1A)**. We kept the engineered cells for 7 days in proliferation medium and 2 more days in differentiation medium, finally, we exposed the engineered cells to a terminal, 24 h pulse of LIF. Immunoprofiling of these cultures at DIV10 showed that GFAP^{+} cell frequency, analyzed as a proxy of the NSC astrogenic bias, was reduced by –27.66 ± 5.41% in Foxg1-GOF samples

compared with controls ($p < 0.003$, n = 4,4) **(Fig. App.1B, App.S1A)**. All this supports the hypothesis that Foxg1 may antagonize the NSC astrogenic progression in humans like in rodents.

Next, to corroborate these results, we interrogated a second preparation of the PCW10 neocortical precursors, pre-expanded in vitro over about 120 days, by a NSC-restricted FOXG1-LOF approach **(Fig.App.1C)**. To prevent any bias in results, possibly arising from a depletion of precursors pool in FOXG1-LOF environment, we decided to trace the "stem" population and eventually use it as normalizer for the evaluation of astrocytes frequency. For this purpose, we engineered two sister aliquots of precursors cells with a lentiviral mix allowing for pNes-driven expression of miR.aFOXG1.1690 or control, and both aliquots were further co-transduced with an inducible TRE-driven EGFP reporter. This resulted in two preparation where "green" NSCs were either FOXG1-LOF or not. Transduced cells were kept 7 days in proliferative medium plus 3 more days in a differentiative medium, and were terminally supplemented by LIF. Finally, cells were immuno-profiled for neurostem/astroglial markers. Frequency of $Egfp^{-}GFAP^{+}$ astrocytes was unaffected. Conversely, normalized against controls, NSCs frequency (identified as $Egfp^{+}GFAP^{\pm}$ since GFAP signal is specifically detectable in astroglial, but also neurostem human cells (Malatesta et al. 2008)) was reduced by $-26.97 \pm 7.82\%$ ($p < 0.025$, n = 3,3). All this points to a robust increase of the NSC-normalized $GFAP^{+}$ astroglial output (normalized against controls, $+58.59 \pm 3.83\%$, $p < 0.003$, n = 3,3) **(Fig. App.1D, App.S1B)**. It further excludes any possible dominant-negative effect originating from FOXG1-GOF assay.

Last, to assess possible conservation of mechanisms mediating Foxg1 impact on astrogenesis progression, we down-regulated *FOXG1* in human, PCW10+DIV120 neocortical precursors by a constitutively

expressed RNAi effector (aFoxg1-shRNA) and monitored the impact of this manipulation on human orthologs of murine mediators of this activity . In particular we focused our attention on *COUPTF1*-, *SOX9*-, *NFIA*-, *ZBTB20*-mRNA as well as on activity of p-STAT3 and p-SMAD1,5,8 (**Fig. App.1E)**. We found that, upon a –22.19 ±3.73% decline of FOXG1-mRNA ($p<0.049$, n = 3,3), *ZBTB20*-mRNA was upregulated by +26.48 ± 2.72% ($p< 0.005$, n = 3,3), and *COUPTF1*, *SOX9* and *NFIA* were unaffected. As a proxy of STAT and SMAD functional cascade, we analyzed the mRNAs of Egfp and ZsGreen reporters, driven by pStat1,3- and Bmp- responsive elements (REs) and co-delivered to neural cells by dedicated lentivectors with a Pgk1p-driven mCherry normalizer. We found that both reporters were also robustly upregulated, by +252.55 ± 105.72% ($p< 0.025$, n = 3,2) and +168.81 ± 71.90% ($p < 0.050$, n = 3,3), respectively **(Fig. App.1F)**. All that indicates that similar molecular mechanisms mediate *Foxg1* control of astrogenesis in mice and humans.

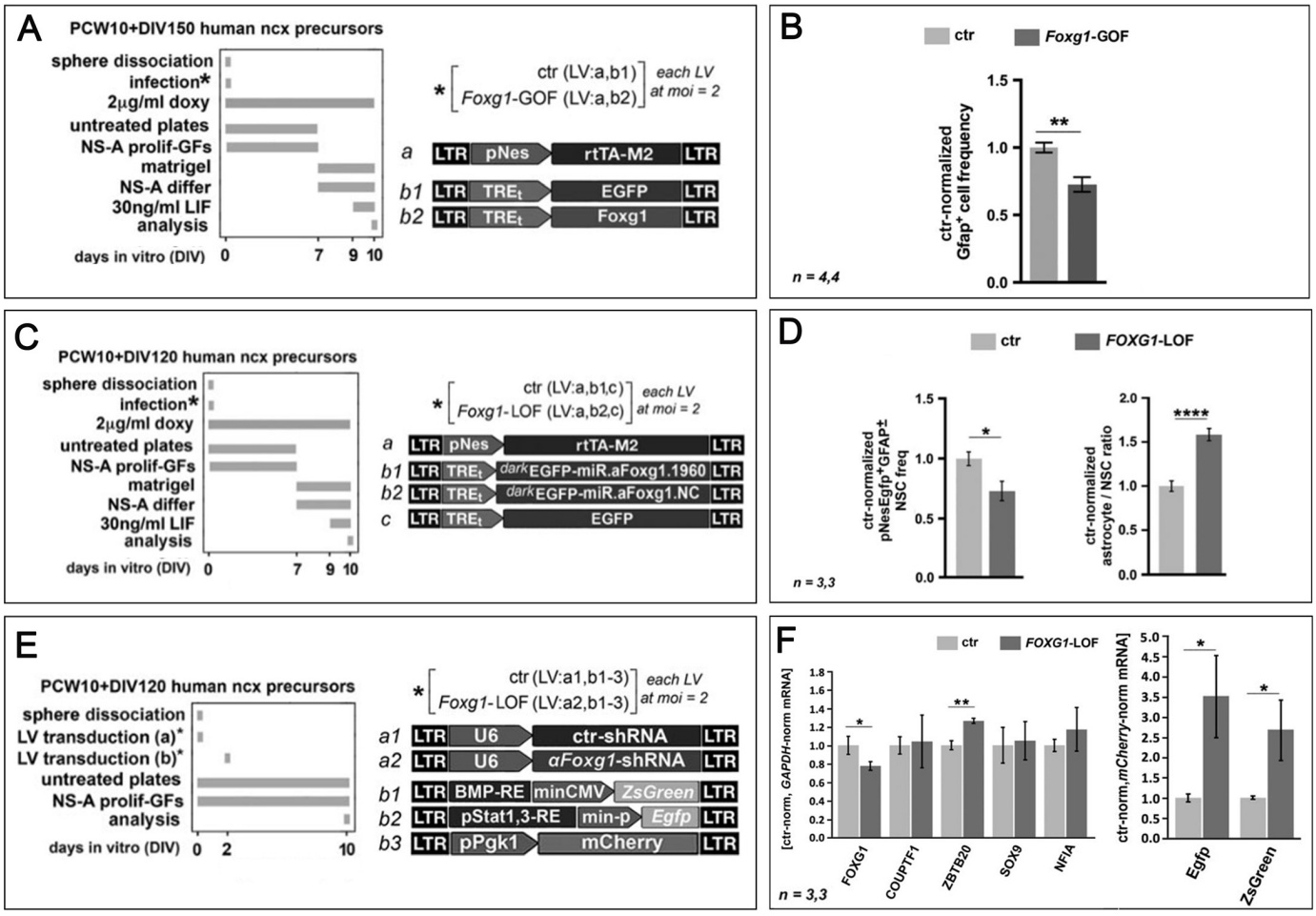

Fig. App.1. Foxg1 inhibits progression of human pallial precursors towards astrogenesis. (A–D) Impact of FOXG1 modulation on astrogenic outputs of engineered human neocortical precursors: temporal articulation of the histogenetic assays, lentiviral vectors employed, and results. The tests were run on human neocortical precursors, derived from PCW10 abortions and pre-expanded in vitro over 150 (A, B) or 120 (C,D) days. Astrocytic outputs were evaluated upon NSC-restricted (pNestin-rtTA^{M2}-driven) Foxg1-GOF and -LOF manipulations (A,B,C,D). Shown are control-normalized frequencies GFAP^{+} cells (B), pNesEgfp^{+}GFAP$^{\pm}$ NSCs (D), as well as control-normalized pNesEgfp-GFAP^{+}/ pNesEgfp^{+}GFAP$^{\pm}$ cell ratios (D). (E) Modulation of putative genes and pathways mediating the impact of FOXG1 downregulation on astrogenesis: protocols, lentiviruses employed and results. (F) Shown are control/ GADPH-double-normalized, FOXG1, COUPTF1, ZBTB20, SOX9, and NFIA mRNA levels, as well as control/mCherry-double-normalized, Egfp (pStat1,3-RE-Egfp) and ZsGreen (BMP-RE- ZsGreen) mRNA levels. Error bar = s.e.m. n is the number of biological replicates, that is, independently transduced neural cultures. P-values calculated by t-test (one- tail, unpaired).

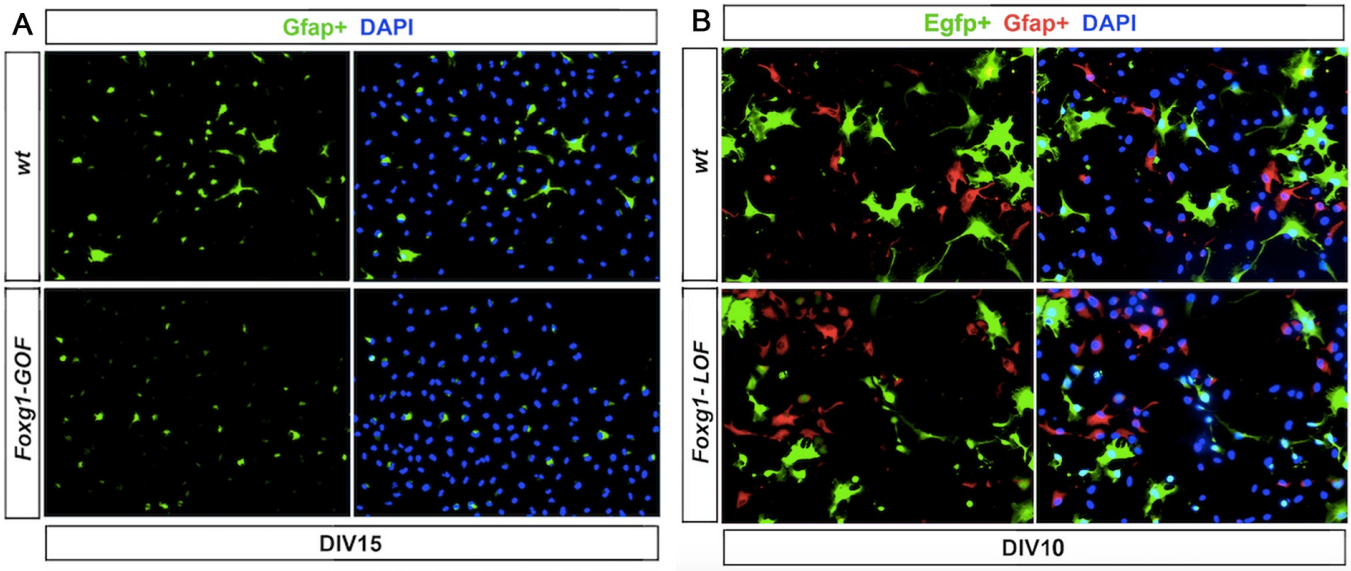

Fig. App.S1. Primary data referred to in Fig. App. 1B,D Example of Foxg1-GOF (right) and FOXG1-LOF(left) effect on differentiative potential of human pallial precursors.

6.3.2 Preliminary characterization of neuropathogenic *FOXG1^{G670A}* and *FOXG1^{G924A}* alleles.

General evolutionary conservation of Foxg1 functions prompted us to investigate if different human neuropathogenic alleles of this gene could be functionally characterized taking advantage of murine, primary neural cultures. This - in fact - would allow to rapidly model developmental anomalies peculiar to such alleles, thus helping to reconstruct neuropathogenic processes associated to them. We focused on two specific alleles, *FOXG1^{G670A}* (Cilio et al., personal communication) and *FOXG1^{G924A}* (Coriell), found in infant patients showing clinical symptoms of FOXG1 syndrome. The former encodes for the mutant G224S protein, harboring an altered DNA binding-domain, the latter for the prematurely truncated W308X protein, lacking both Groucho and Jarid1b binding-domains.

Our idea was to compare the capability of these alleles with performances of the human WT allele, in inhibiting NSCs-to-astrogenesis progression (Falcone et al. 2019) and enhancing astroblasts proliferation **(**see **Fig. 6)**, namely two activities well documented in case of murine Foxg1^{WT}. Prior to proceed, we checked that human FOXG1^{WT}-cds phenocopies its murine counterpart in both clonal and astroblasts proliferation assays **(Fig. App. 2A,C)**. In this respect, we confirmed that, compared to control, murine Foxg1^{WT}-cds induced an increase in neuronal clones (0.571±0.050 vs 0.431±0.036 with n=4,4 and $p<0.033$) and a decrease in astroglial ones (0.085±0.005 vs 0.127±0.019 with $p<0.039$). Similarly, we found that human FOXG1^{WT}-cds elicited a comparable decrease in the latter (0.082±0.014 vs 0.127±0.019 with n=4,4 and $p<0.051$) while slightly increasing the former **(Fig. App.2B, App.S2.1)**. Next**,** we also found that, compared to control, the over-expression of both WT alleles was sufficient to evoke a strong increase in the fraction of proliferating astrocytes, namely, ki67^{+}Gfap^{+}/Gfap^{+} (0.294±0.047 vs 0.135±0.009 with n=3,4 and

p<0.006, in mFoxg1 and Ctr samples, respectively; 0.270±0.053 vs 0.135±0.009 with n=4,4 and p<0.001, in hFoxg1 and Ctr ones, respectively). No difference in proliferative ratios was conversely detectable comparing mouse- to human- Foxg1-GOF samples. **(Fig. App.2D. App.S2.2)**.

Characterization of *FOXG1*^{G670A} and *FOXG1*^{G924A} in the pallial neuronogenic lineage, ongoing in our lab, had showed that they work as GOF and LOF alleles, respectively. To sharpen the capability of our clonal assay to rank anti-gliogenic activities of *FOXG1*^{G670A}-cds and its WT counterpart, we revised our original clonal assay protocol (Falcone et al., 2019), exposing differentiating cells to a supplemental, pre-terminal, 24h LIF treatment, thus over-biasing the culture towards astrogenesis. Following this protocol change, the overexpression of the WT human allele was not anymore sufficient to counteract the commitment of NSCs toward astrogenesis **(Fig. App.2E)**. Remarkably, *FOXG1*^{G670A}-cds, instead, elicited a pronounced decrease of pure astroglial clones compared to both control (0.100±0.020 vs 0.188±0.038 with n=3,3 and p<0.050) and hFOXG1-GOF samples (0.100±0.020 vs 0.193±0.003 with p<0.005). *FOXG1*^{G670A}-cds also increased the number of mixed clones compared to both control (0.566±0.009 vs 0.442±0.022 with n=3,3 and p<0.003) and WT human samples (0.566±0.009 vs 0.429±0.034 with n= 3,3 and p<0.009). Neuronal clones were generally unaffected **(Fig. App. 2E, App.S2.1)**. In a few words, *FOXG1*^{G670A} antagonizes NSCs-to-astroblast progression much better then its WT counterpart.

Next, we investigated *FOXG1*^{G670A} impact on astroblasts proliferation, by our standard proliferation assay **(Fig. App.2C)**. We found that it induced a significant increase in the fraction of proliferating astrocytes compared to both control (5.176±0.023 vs 1±0.015 with n=3,3 and p<0.0001) and FOXG1^{WT} (5.176±0.023 vs 2.912±0.024 with n=3,3 and p<0.001)**(Fig. App.2F)**, further confirming it generally outperforms the healthy allele.

As for *FOXG1*^{G924A} *,* evaluated by our standard clonal assay against the WT allele**,** it induced a strong decrease in neuronal clones (0.037±0.019 vs 0.487±0.055 with n=4,3 and p<0.039) and an increase of astroglial ones (0.143±0.027 vs 0.082±0.014 with p<0.040). Remarkably, overexpression of *FOXG1*^{G924A} further reduced the fraction of neuronal clones *compared to control*, pointing to a potential dominant negative effect of this allele **(Fig. App.2G, App.S2.1)**.

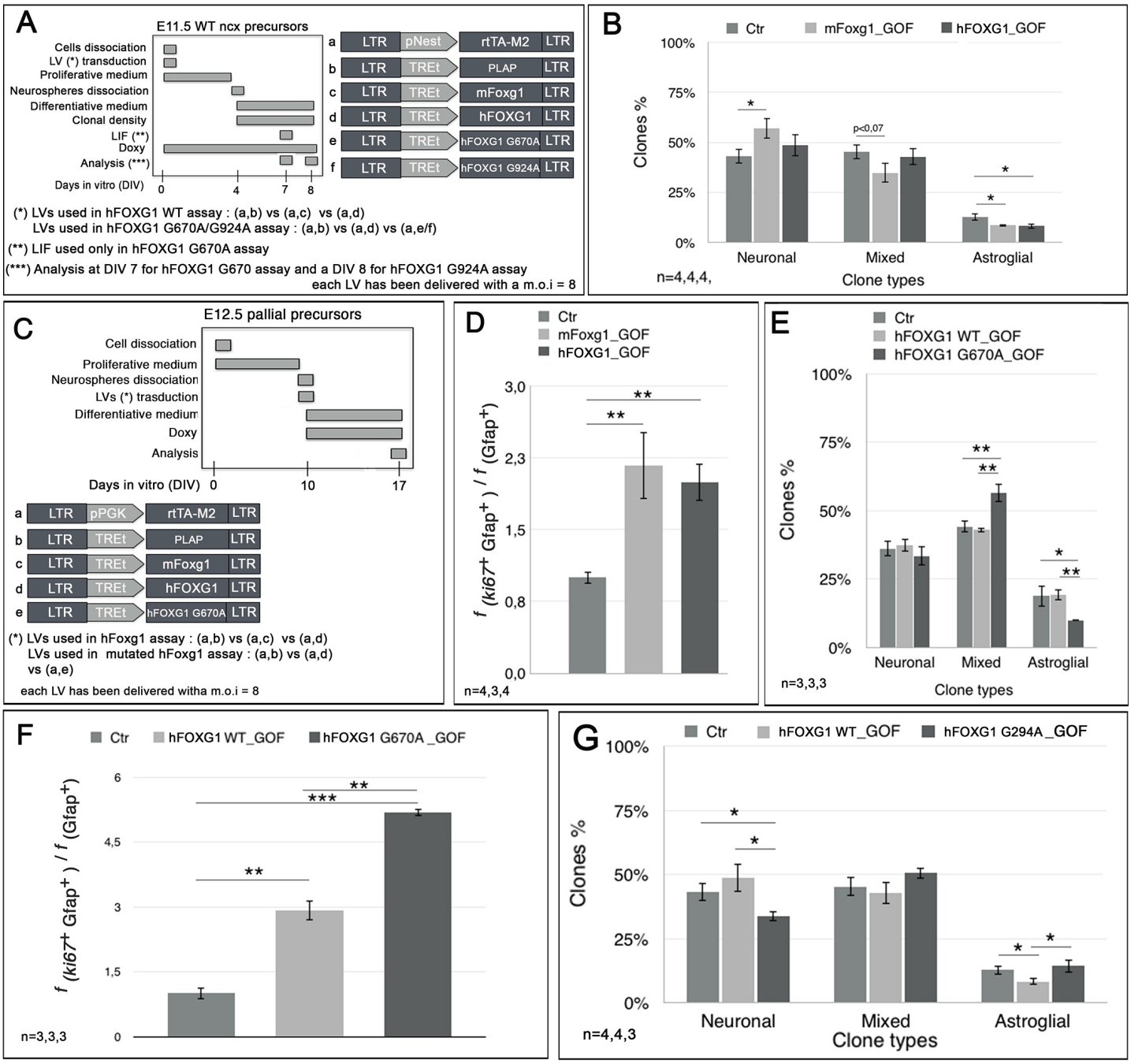

Fig. App.2. Preliminary characterization of neuropathogenic *FOXG1^{G670A}* and *FOXG1^{G924A}* alleles.

(A) Protocol and lentiviral vectors employed in clonal assay. **(B,E,G)** Absolutes frequencies of neuronal, mixed and astroglial clones generated by derivatives of E11 pallial NSCs, upon gain-of-function (GOF) of mouse and human WT Foxg1 allele (B) and *FOXG1^{G670A}* and *FOXG1^{G924A}* alleles (E,G)**. (C)** Protocol and lentiviral vectors employed in astrocyte-committed progenitors proliferation assay. **(D, F)** Frequencies of ki67^{+}Gfap^{+}/Gfap^{+} cells upon GOF of mouse and human WT Foxg1 allele (D) and *FOXG1^{G670A}* allele. Statistical significance of results evaluated by t-test (1-tail, unpaired). * p<0.05, ** p<0.01, *** p<0.001. n is the number of biological replicates, i.e aliquots of pre-pooled, independently lentivirus-transduced and cultured neural cells. Scalebars represent s.e.m's.

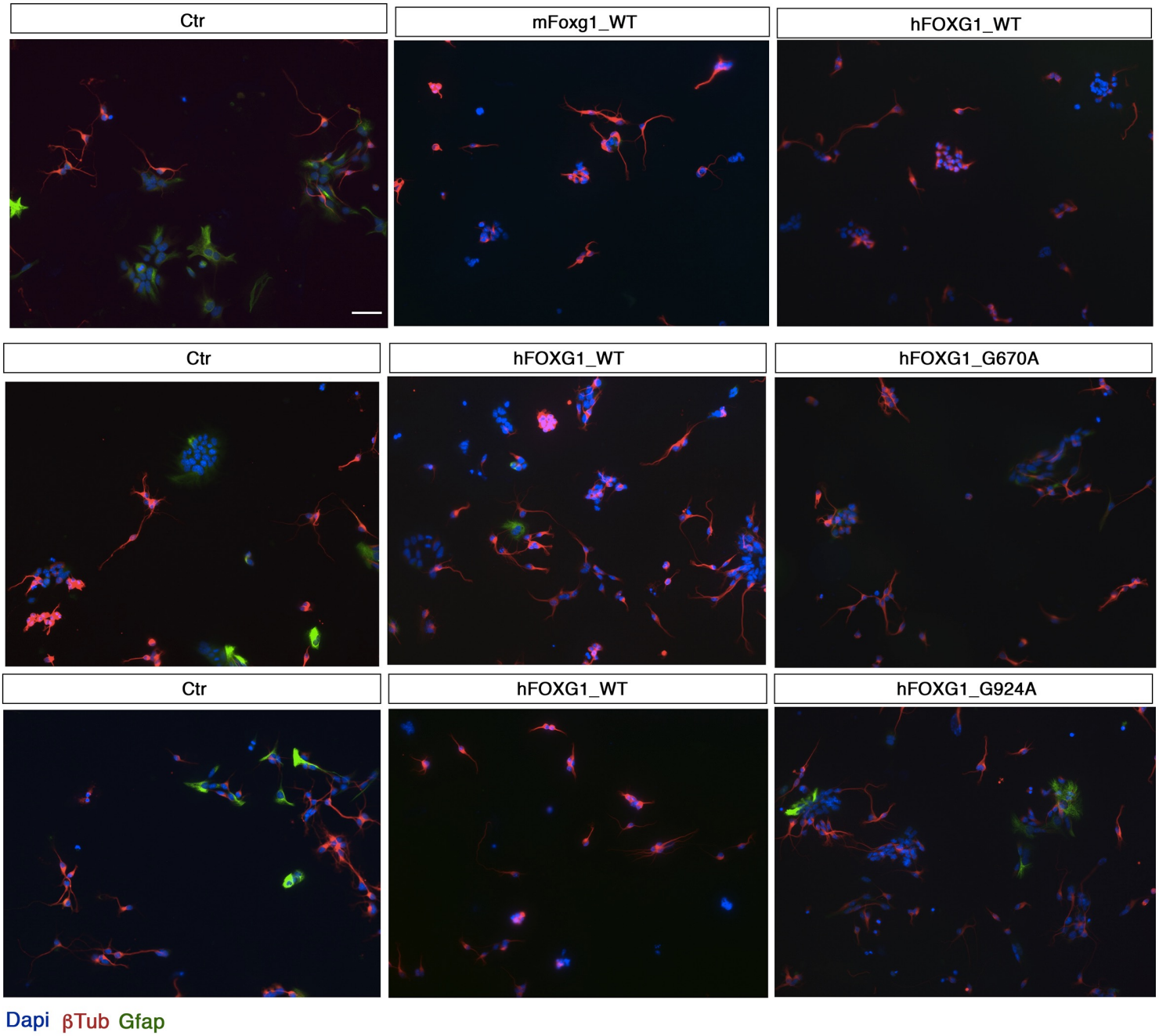

Fig. App.S2.1. Primary data referred to in Fig. App. 2B,E,G Example of clonal assay read-out on E11.5 pallial precursors upon murine and human Foxg1 WT- GOF vs Ctr (uppert), hFOXG1 WT- and hFOXG1_G670A- GOF vs Ctr (middle) and hFOXG1 WT and hFOXG1_G924A- GOF (lower). Scale bar = 50μm.

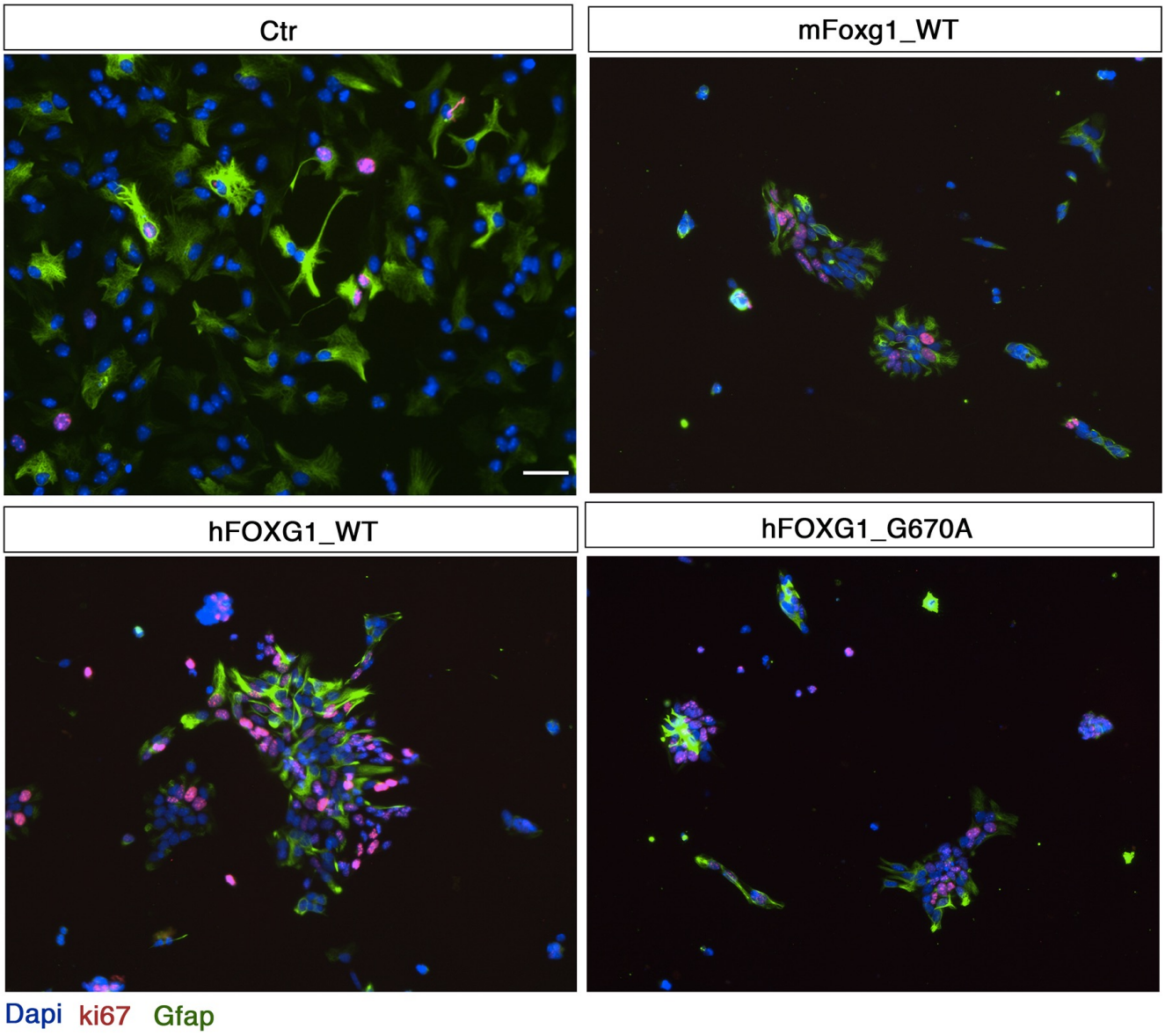

Fig. App.S2.2. Primary data referred to in Fig. App. 2D,F. Example of astrocytes proliferation assay read-out of Ctr , mFoxg1 WT- and hFOXG1 WT- and hFOXG1 G670A -GOF pallial precursors. Scale bar = 50μm.

6.4 Discussion

Foxg1 is a gene essential to proper brain development (Xuan et al ., 1995), it is involved in different steps of telencephalic histogenetic program (Hanashima et al., 2002; Hanashima et al 2007; Miyoshi and Fishell 2012; Cargnin et al., 2018), and its functions are largely shared among vertebrates (Toresson et al., 1998; Bredenkamp et al., 2007). Not surprisingly, a number of clinical studies correlate FOXG1 copy number variations and structural alterations with a large spectrum of neuroclinical presentations, now collectively referred to as “Foxg1-syndrome” (Hou, Ó hAilín, Vogel and Hanashima 2020).

In this appendix, we provide new evidence of evolutionary conservation of Foxg1 functions and its pathological implications.

First, we demonstrated that Foxg1 anti-astrogenic activity, as documented in our recent work by Falcone et al., (2019), is conserved between mice and humans, where it relies on similar cellular and molecular mechanisms **(Fig. App.1)**. In this respect, we showed that *Foxg1* up-regulation induced a reduction of the astroglial output **(Fig. App.1B)**. Vice-versa, its down-regulation resulted in an absolute shrinkage of the NSCs pool and, upon normalization against NSCs, a concomitant increase of the astroglial one **(Fig. App.1D)**.

Next, we functionally characterized mutant FOXG1 alleles taking advantage of fast, in vitro assays in primary murine cultures. By ranking the magnitude of histogenic anomalies evoked by the overexpression of WT- or mutated-FOXG1, in fact, it is possible to classify the latter as GOF or LOF. As a proof of principle, we profiled two neuropathogenic alleles, $FOXG1^{G670A}$ and $FOXG1^{G924A}$ **(Fig. App.2)**. We found that the former controls both NSCs-to-astroblast progression and astroblasts proliferation as a GOF allele, while the latter is apparently ineffective. Actually, *FOXG1* alleles peculiar to FOXG1-syndrome patients are highly diversified and

the genotype-phenotype correlation is often very challenging. Our fast characterization of these alleles in murine pallial precursors may contribute to fix this issue. Clarifying how FOXG1-mutations impact on distinct aspects of telencephalic development, it may further help defining a window of opportunity for future gene therapy intervention. Last, comparative assessment of *FOXG1*-alleles in different scenarios can unveil protein domains required for the corresponding FOXG1 neurodevelopmental functions. Related to that, it may help identifying FOXG1 co-factors needed for proper tuning of distinct histogenetic subroutines.

Actually, our results are consistent with primary molecular correlates of the two mutations. *FOXG1*^{G924A} leads to a truncated form of the protein, missing the Groucho and Jarid1b binding-domains. As such, it should likely imply a LOF scenario. Consistent with that, *FOXG1*^{G924A} overexpression did not antagonize the NSCs progression to the astroglial lineage **(Fig. App.2G)**. By contrast, characterized by a mutation in FBD, *FOXG1*^{G670A} displays an enhanced activity, compared to the WT allele, in both clonal- and astroblasts proliferation assays **(Fig. App.2E,F)**. This effect could originate from a stronger affinity of mutated FBD for its target sequence or from higher stability of FOXG1^{G670A} mRNA or its G224S mutant protein product.

It is important to stress that, based on our experimental design, some aspects of GOF and LOF phenotypes associated to FOXG1 clinical variants might have been underestimated. We over-expressed, in fact, the mutated form in a context already harboring two WT Foxg1 alleles. Future experiments, based on replacement of one endogenous Foxg1 allele with WT or mutant human FOXG1 alleles, will allow to fix this issue.

These results are an encouraging starting point for the systematic characterization of patient-specific FOXG1 variants in different developmental contexts. Combined with other histogenetic and functional

assays, in fact, they could represent a standardized methodology to rapidly assess the functional relevance of novel FOXG1 mutations. Fast characterization of clinical FOXG1 variants as effectors playing “defective” or “exaggerated” activity, can be extremely useful from a therapeutical perspective. In fact, *FOXG1* can be a potential target of pre-natal RNA interference or activation (Fimiani et al., 2016), allowing to prevent or, at least, counteract patient-specific symptoms. Furthermore, the possibility of generating patient-derived neural subtypes starting from hiPSCs, will offers more stringent experimental contexts to validate RNA-based gene-therapy, as a step propedeutic to future prenatal treatments of FOXG1 syndrome.

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