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Julien Pigeon

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Sorbonne Université

Ecole doctorale 158

# **The role of NEUROG2 T149 phosphorylation site in the developing human neocortex**

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Thèse de doctorat de Neurosciences

Dirigée par Pr. Bassem Hassan

Présentée et soutenue publiquement le 03/05/2024

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*Je dédie cette thèse à mon grand-père, Georges, qui n'en aura pas vu le commencement et à  
ma grand-mère, Charlise, qui n'en aura pas vu la fin.*

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# List of abbreviations

aRGCs: apical radial glial cells

Asc : achaete scute complex

ASDs: autism spectrum disorders

Ato : atonal

bRGCs: basal radial glial cells

Cdk: cyclins dependant kinases

CGE: cadal ganglionic eminence

CSF: cerebrospinal fluid

DLX1: distal-less shomeobox 1

DLX2: distal-less shomeobox 2

EFG: Epidermal growth factor

EOMES: eomesodermin

FGF: Fibroblast growth factor

GE: Ganglionic eminences

HOPX: HOP homeobox

IGF: Insulin-like growth factor

IP: intermediate progenitors

iPSCs: induced pluripotent stem cells

LGE: lateral ganglionic eminence

MARK1: Microtubule affinity regulating kinase 1

MGE: medial ganglionic eminence

NEC: Neuroepithelial cells

NEUROD2: Neuronal differentiation 2

NEUROD4: Neuronal differentiation 4

NEUROD6: Neuronal differentiation 6

NEUROG1: Neurogenin1

NEUROG2: Neurogenin2

NPCs : neural progenitor cells

PAX6: Paired-box transcription factor 6

PLK1: Polo like kinase 1

RGC: radial glial cells

SHH: Sonic Hedgehog

SOX2: SRY box transcription factor 2

SP: serine-proline

TBR1: T-box brain transcription 1

TBR2: T-box brain transcription 2

WNT: wingless

BBS : Bardet-Biedl syndrome

NPHP : nephronophthisis

SNLS : Senior-Loken syndrome

ALMS: Alstrom syndrome

MKS: Meckel syndrome

JBTS: Joubert syndrome

OFD1: Oral-facial-digital Type I

PKD: polycystic kidney diseases

JATD: Jeune asphyxiating thoracic dystrophy

EVC: Ellis van Creveld

LCA; Leber congenital amaurosis



## Thesis abstract

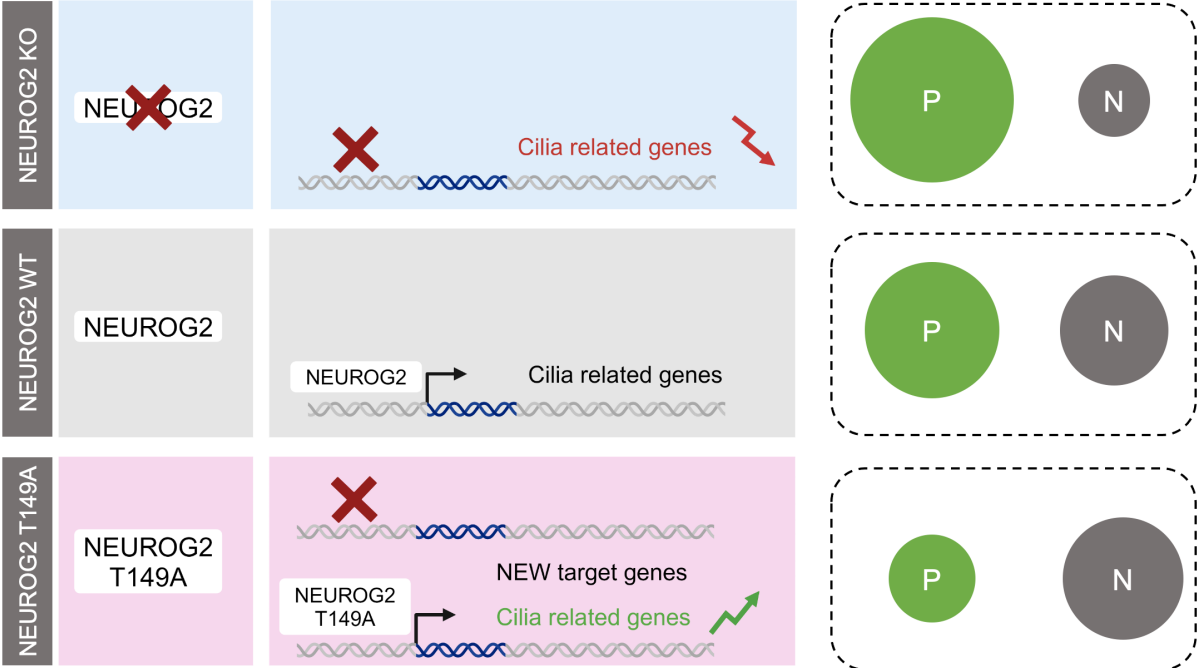
Neocortical expansion throughout evolution has been responsible for higher-order cognitive abilities and relies on the increased proliferative capacities of cortical progenitors to increase neuronal production. Therefore, in gyrencephalic species such as humans and other primates, where the neurogenic period is protracted, the regulation of the balance between progenitor maintenance and differentiation is of key importance for proper neuronal production. The control of this balance in the dorsal telencephalon, which gives rise to the neocortex, is mediated by feedback regulation between Notch signaling and the proneural transcription factor Neurogenin2 (NEUROG2). As the expression of NEUROG2 alone is sufficient to induce neurogenesis in the neocortex, its regulation at the transcriptional level has been extensively studied in mice. However, recent findings highlight that regulation at the protein level through post-translational modifications can profoundly influence protein activity and stability. Indeed, the modulation of the conserved NEUROG2 T149 phosphorylation site by overexpression in the developing mouse neocortex results in an altered pool of progenitors and number of neurons in the deep and upper layers. Nevertheless, it is not known how such post-translation modification regulates NEUROG2 activity in the development of the human neocortex under endogenous levels and its contribution to the development of the neocortex.

We hypothesize that modulation of the activity of the transcription factor NEUROG2 through this T149 phosphorylation site may regulate the pace of the temporal advance of human cortical progenitors down the differentiation landscape.

To test this hypothesis in humans, we used 3D cortical organoids derived from CRISPR/Cas9 engineered iPSCs lines to study cortical neurogenesis. Before diving into the role of post-translational modifications regulating NEUROG2 activity, we started by confirming, for the first time in humans, that Neurogenin2 is indeed the gateway gene of neuronal differentiation. In differentiated iPSCs NEUROG2 KO clones, we observed reduced proportions of neurons after 70 and 140 days in vitro at both the mid and late stages of cortical organoid development. This phenotype is accompanied by a ventralization of these dorsal forebrain organoids with a downregulation of the genes encoding for the dorsal forebrain identity and an upregulation of the genes encoding for the ventral forebrain identity. Knowing that Neurogenin2 is required for cortical neurogenesis, we next studied how the loss of NEUROG2 phosphorylation site T149 by its replacement with an Alanine (T149A) at endogenous levels alters neuronal production.

To this end we combined live imaging of radial glial clones, immunohistochemistry for key cell fate markers, machine-learning based cell type quantification, transcriptional activation and stem cell reprogramming assays, RNA sequencing and chromatin immunoprecipitation to analyze cortical neurogenesis. We found, on the one hand, the TA/TA mutant does not change the pattern of NEUROG2 expression in both radial glial cells and intermediate progenitors, nor its ability to bind and activate target genes or reprogram human stem cells to neurons. However, the TA/TA mutant radial glia switch their division mode from proliferative to neurogenic and generate more neurons at both the mid and late stages of cortical development in organoids. Mechanistically, we found that this phenotype is accompanied by an upregulation of the genes encoding the organization and the movements of the primary cilium of radial glial cells, which are downregulated in the NEUROG2 KO clones. These results suggest a strong link between the primary cilium, Neurogenin2, and its phosphorylation profile with the regulation of neurogenesis in human cortical organoids.

Graphical abstract and current working model:



## General introduction

Centuries ago, long before the dawn of our modern era, our Greek predecessors were already trying to explain the greatest phenomena of nature, by building a mythology that would become one of the most important legacies of our occidental culture. In fact, they were looking for answers to the same questions that scientists have been asking themselves since the dawn of times: where do we, humans, come from, and how did we become “masters and possessors of nature” as the renowned French philosopher René Descartes wrote in the *Discours de la méthode* ?

Anthropologists, archaeologists and historians agreed over the first milestone of building civilizations, the mastery of fire. According to the Greek mythology, it is the Titan Prometheus that gifted fire to humans after being banished from Mount Olympus by Zeus which symbolically captures a transformative moment in human history. As a matter of fact, the domestication of fire is the forerunner sign of greater abilities that we specifically developed, i.e., the capacity to analyze, to reason, to learn from our environment, and to transmit this knowledge through generations to keep on enriching it. These outstanding capacities are known as cognitive functions of *Homo sapiens sapiens* and find their biological substrate in the most complex and mysterious organ of the human body, the brain.

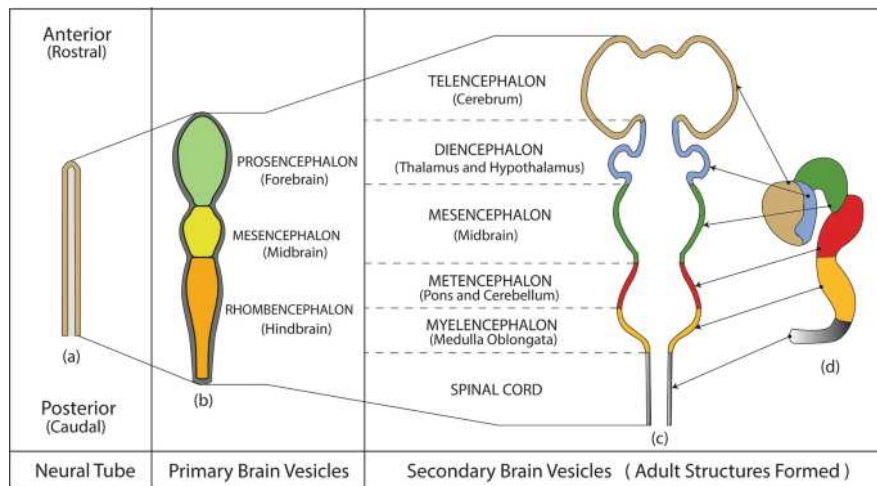
The brain is to living animals what a central processing unit is to computers, the heart of computation. In a broad sense, computation is the action of mathematical calculation, logical operations, and data manipulation. When applied to biological systems, computation encompasses the assimilation and analysis of environmental inputs, processing them through complex neural networks of different subtypes of neurons interconnected and supported by glial cells to generate appropriate responses and actions. This biological computation enables organisms to perceive their surroundings, learn from experiences, make decisions, and interact with their environment. Therefore, understanding the development of the computational organ of living organisms will shed light on the biological foundations of cognition and decision-making, which make us stand out from all the other living species. Thus, from this basic observation emerges a self-exploratory and crucial question for Homo Sapiens: what are the biological and molecular grounds for these capacities that emerged across evolution?

# Introduction

## I. The development of the central nervous system

### A. The neural tube

During early mammalian embryonic development, the nervous system emerges from a single layer of neuroectodermal cells that form the neural plate. This plate then folds on itself to create the neural tube (Figure 1.A). As development progresses, this tube enlarges at its rostral part to create three primary brain vesicles: the prosencephalon (forebrain), the mesencephalon (midbrain), and the rhombencephalon (hindbrain) (Figure 1.B) (Bayer & Altman, 2007; O’Rahilly & Muller, 2006; Yamada et al., 2010; Silbereis et al., 2016).



**Figure 1 : Schematic representation of the central nervous system development.**

- Representation of the neural tube.
- Development of the three brain vesicles.
- Subdivision of the five brain vesicles.

The three primary vesicles will give rise to five secondary vesicles. The prosencephalon folds on itself and expands to develop the telencephalon, which gives rise to the cerebral cortex (neocortex) and the diencephalon. Additionally, the mesencephalon remains a single vesicle that serves as a sensory information relay and coordinator of motor responses. Lastly, the rhombencephalon divides into the metencephalon, giving rise to the pons and cerebellum, essential structures for motor coordination, and the myelencephalon, crucial for autonomic functions such as breathing and heart rate regulation. Finally, the caudal part of the neural tube will give the spinal cord an essential relay of sensory and motor signals.

## **B. The telencephalon**

Among the various structures arising from the CNS, it is in the telencephalon that higher cognitive abilities are thought to reside. The telencephalon is further divided along the dorsoventral axis by several morphogens among which Gli3, a zinc-finger transcription factor that will promote the dorsalization of the telencephalon, while Sonic Hedgehog (Shh) will promote its ventralization (Hébert & Fishell, 2008). The ventral telencephalon will give rise to the basal ganglia, which includes the Medial Ganglionic Eminence (MGE), the Lateral Ganglionic Eminence (LGE), and the Caudal Ganglionic Eminence (CGE), while the dorsal telencephalon will give rise to the hippocampus, the olfactory cortex, and the most important of all, the neocortex (Campbell, 2003; Rakic, 2009).

## **C. Neocortical function and structure**

### **1. Common neocortical features**

#### **a) Horizontal subdivision of the neocortex**

From an evolutionary perspective, the neocortex is the last and largest brain structure to appear in mammalian species, serving as the biological foundation for cognitive functions (Molnár et al., 2006; Kaas, 2019). It is a mantle of grey matter covering both cerebral hemispheres, enclosing the underlying white matter composed of a variety of cell types, including interconnected excitatory and inhibitory neurons supported by glial cells, astrocytes, and oligodendrocytes with microglial cells and the vasculature (Rakic, 2009). Interestingly, the neocortex is not a homogenous structure. Horizontally, it is subdivided into highly specialized regions known as Brodmann areas, which are responsible for sensory perception, visual processing, motor response, and memory (Brodmann, 1905; Rakic, 1988; Zilles, 2018).

#### **b) Vertical subdivision of the neocortex**

Vertically, it is subdivided into six layers of glutamatergic projecting neurons with a stereotypical morphology: a pyramidal shape, a single projecting axon, and apical dendrites facing the neocortical surface (Marín-Padilla, 1992). The excitatory neurons are born locally in the ventricular zone (VZ) and subventricular zone (SVZ) from many different progenitors that we will describe later in this thesis. When born, these neurons will migrate to their final destination, which correlates with their time of birth during development.

Indeed, the neocortex develops in an inside-out manner where the early-born neurons will establish the deep layers of the cortex while the late-born neurons will have to migrate through the deep layers to reach the upper layers of the cortex (Angevine & Sidman, 1961; Caviness, 1982; Rakic & Lombroso, 1998).

### **c) Cellular composition of the neocortex**

#### **(1) Excitatory neurons**

Neocortical deep layers, which include layers 6 and 5, consist mainly of corticofugal projection neurons that target subcortical structures. Neurons of the sixth layer project mainly to the thalamus and express well-known markers used for their identification, such as TBR1 and FOXP2, whereas neurons of the fifth layer express FEZF2 and CTIP2 as markers and project mainly to the midbrain, hindbrain, and spinal cord (Dennis et al., 2017; Molnár & Cheung, 2006). Neocortical upper layers, which include layers 2 and 3, consist of corticocortical neurons projecting their axon to ipsilateral and contralateral cortical areas and express the markers SATB2, CUX1/2, and BRN2 (Molyneaux et al., 2007). Finally, layer 4 is the most variable layer due to the input it receives from the thalamus to relay sensory information from the periphery (Balaram et al., 2014).

#### **(2) Inhibitory neurons**

These cortical layers also comprise interneurons that regulate the excitatory neuronal circuits. However, unlike pyramidal neurons, GABAergic inhibitory neurons are produced in the ventral telencephalon by the ganglionic eminences (GEs) before migrating tangentially to the neocortex (Parnavelas et al., 2000; Anderson et al., 2001; Marín & Rubenstein, 2001). The majority of interneuron subtypes are characterized by the expression of parvalbumin (PV), somatostatin (SST) or calretinin (CalR). PV<sup>+</sup> and SST<sup>+</sup> interneurons are generated in the MGE, whereas CalR<sup>+</sup> interneurons are generated in the CGE (Wonders & Anderson, 2006; Rudy et al. 2011).

#### **(3) Glial cells**

In parallel, in the ventral telencephalon, the progenitors will also give rise to another population of glial cells after neurogenesis in the GEs: the oligodendrocytes, which will migrate along the same routes as the inhibitory neurons (Spassky et al., 2001). In the neocortex, cortical progenitors will engage in gliogenesis to produce astrocytes right after neurogenesis (Nieto et al., 2001; Tabata, 2015).

## **2. Human-specific neocortical features**

The overall neocortical architecture is conserved across mammals, but the neocortex significantly expanded in primates and particularly in humans, which is thought to have contributed to the development of advanced cognitive abilities such as language, abstract thinking, and complex problem-solving (Kaas, 2019). The neocortical expansion is accompanied by a major increase in neuron production permitted by the folding of its surface in the limited space provided by the skull, classifying mammalian species into two subgroups: the lissencephalic species with a smooth neocortical surface like most rodents, and gyrencephalic species with a convoluted neocortical surface as seen in primates and humans (Kelava et al., 2013; Lewitus et al., 2014). For instance, the neocortex of a mouse and a rat contains 14 and 31 million neurons, respectively, while the rhesus macaque, an old-world monkey, has 1.7 billion neurons in its neocortex. In primates, this number increases even further, with our closest relatives, the Chimpanzee, having 6 billion neurons and humans having an astounding 16 billion neurons within their neocortex (Herculano-Houzel, 2009, 2012; Herculano-Houzel et al., 2015).

This increased neuronal production has affected all the neuronal subtypes: the primate neocortex has 5 to 10% more locally projecting interneurons that account for 25 to 30% of the total cortical neurons, while in rodents, they account for approximately 20% of all cortical neurons (Bakken et al., 2021; Yu et al., 2021). However, the highest increase concerns the upper-layer neurons. In the rodent neocortex, they represent 25% of cortical neurons, while in humans, they account for 45% (Balaram et al., 2014; Hutsler et al., 2005). Furthermore, beyond absolute numbers and proportions of neurons, these cortical neurons of the upper layers gained diversity with their classification in 5 subgroups in primates compared to 3 subgroups in rodents (Vanderhaeghen & Polleux, 2023).

### **D. From stem cells (NECs) to radial glial cells (RGCs)**

To understand the underlying mechanism behind this increased neuronal production, we must explore how neurons are generated from various progenitor subtypes. However, before delving into a mechanistic approach to neocortical expansion, it is important to establish a comprehensive understanding of the different progenitor subtypes.

## **1. Neuroepithelial cells (NECs)**

We have seen earlier that the neocortex emerges from the dorsal telencephalon, which is initially composed of a single layer of cells highly polarized named the neuroepithelial cells (NECs) (Bishop et al., 2000; Sur & Rubenstein, 2005). From their apical pole emerges an apical process that extends to the ventricular surface, while on their basal pole is also found a basal process that reaches the cortical primordium surface (Sidman & Rakic, 1973). Other specific features of NECs are their tight and adherent junctions at their apical surface (Taverna et al., 2014) that play a key role in their symmetric divisions to self-amplify, producing two NECs after each round. During the cell cycle, their nucleus will undergo specific movements known as Inter Kinetic Movement (INM), where the soma will move up towards the basal axis for the G1 and S phases and will move down to the apical surface for the G2 phase, and the mitosis (Hinds & Ruffett, 1971; Sidman & Rakic, 1973; T. Takahashi et al., 1993, 1995). NECs perform INM asynchronously, giving the neuroepithelium its characteristic pseudostratified appearance. These cells are highly connected to each other and display a single primary cilium that protrudes in the ventricles and acts as a microscopic sensory antenna to gather information contained in the cerebrospinal fluid (CSF) (Louvi & Grove, 2011).

## **2. Apical radial glial cells (aRGCs)**

In the cortical primordium, after several rounds of self-amplifying divisions, NECs will lose their tight junctions but retain their apical adherent junctions to become apical Radial Glial Cells (aRGCs) that express the paired-box transcription factor 6 (PAX6) and other glial markers (Taverna et al., 2014). aRGCs are highly conserved across phylogeny (Cheung et al., 2010) and retain some NEC features, such as an apical process that extends to the ventricle surface with the primary cilium and a basal process that anchors at the pial surface and will undergo INM, but the amplitude of nuclear movements is more limited toward the apical surface near the ventricle. The location of aRGCs defines the primary germinal layer of the cortical primordium, the Ventricular Zone (VZ), that is, as its name suggests, right on top of the cerebral ventricles. Thus, the apical belt formed by the adherent junctions at the apical process ensures isolation from the cerebrospinal fluid (CSF) and prevents hydrocephaly but also guarantees the integrity of the VZ (Villalba et al., 2021). The transition from NECs to aRGCs marks the initiation of cortical neurogenesis as they will produce neurons of the deep layer and the upper layers of the neocortex.



### 3. Basal radial glial cells (bRGCs)

These aRGCs will also give rise to another subtype of RGCs, the basal radial glial cells (bRGCs), also known as the outer radial glial cells (Fietz et al., 2010; Hansen et al., 2010). When produced, these bRGCs will migrate out of the VZ to establish the secondary germinal layer of the cortical primordium, the subventricular zone (SVZ), that is, as its name suggests, right above the VZ.

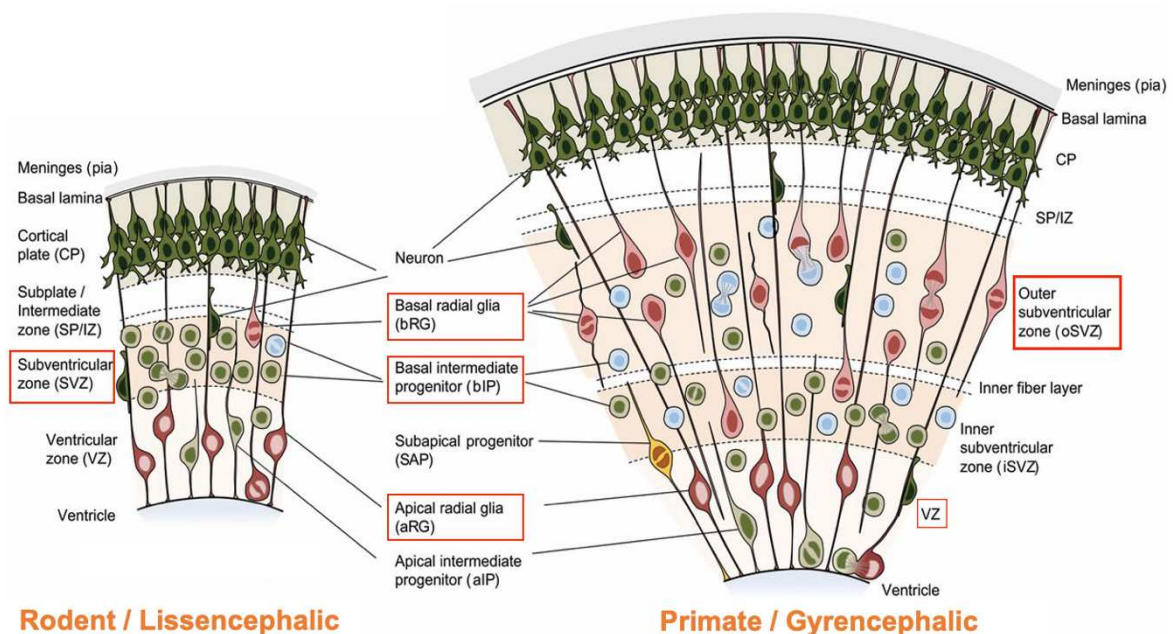
bRGCs are characterized by:

- 1) Their location in the SVZ in rodents, where they are very rare but largely expanded in primates, which led to the subdivision of the SVZ into an inner part, the iSVZ, and an outer part, the oSVZ (Smart et al., 2002; Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011).
- 2) Their morphology with an elongated basal process and the loss of their apical processes and, thus, their connection to the ventricular surface. However, bRGCs have been reported to have morphologic diversity in which certain subtypes have conserved an apical process that does not reach the ventricle (Betizeau et al., 2013).
- 3) Their behavior: before dividing, bRGCs undergo a stereotypic migration known as mitotic somal translocation (MST), in which the soma rapidly translocates toward the cortical plate immediately before cytokinesis (Ostrem et al., 2014).
- 4) Their specific transcriptomic profile with key marker genes such as HOPX, TNC, MOXD1 (Pollen et al., 2015; Florio et al., 2015; Nowakowski et al., 2017). However, at the protein level, the transcriptomic marker HOPX has been reported to label some aRGCs that are located in the VZ with two processes (see Figure 1E in the result section), which highlights the difficulties and challenges of correlating marker genes with a single marker of cell identity at the protein level.

As these progenitors are generated from aRGCs, they will naturally come sometime after the initiation of cortical neurogenesis but are thought to contribute significantly to the production of neurons from both deep and upper layers of the neocortex (Betizeau et al., 2013; Dehay et al., 2015).

#### 4. Intermediate progenitors (IPs)

Intermediate progenitors (IPs) arise from both aRGCs and bRGCs and are found in the SVZ (Taverna et al., 2014). Morphologically, IPs are less elongated compared to RGCs, and don't have the characteristic radial fibers that extend from the ventricular surface to the cortical surface. Nevertheless, they don't have a stereotypic morphology but retain some polarity and can be found as unipolar, bipolar or even multipolar cells. Unlike bRGCs, transcriptomic analyses have revealed that these cells express a unique set of genes used for their identification, among which the *Tbr2* (Englund et al., 2005) particularly stands out due to its specificity at the protein level. Its expression is induced by the transcription factor *Neurog2*, known to promote neuronal differentiation when stabilized (Bertrand et al., 2002). Interestingly, this population of progenitors is thought of as transient amplifying cells already committed to neurogenesis with limited proliferative capacities, 1 or 2 divisions before the terminal symmetric neurogenic divisions to generate 2 or 4 neurons (Taverna et al., 2014). In gyrencephalic species, IPs play a major role in cortical neuron production, as most of it is indirect (Villalba et al., 2021).



**Figure 2: schematic representation of the developing neocortex of lissencephalic and gyrencephalic species.**

- A. Representation of the germinal layers of lissencephalic species such as rodents and the specific cell subtype found in the VZ and SVZ.
- B. Representation of the germinal layers of gyrencephalic species such as primates and the specific cell subtype found in the VZ and SVZ.

Adapted from: Marta Florio, and Wieland B. Huttner Development 2014;141:2182-2194

Huttner Development 2014;141:2182-2194

For simplicity, aRGCs, bRGCs and IPs will be referred to hereafter as progenitors, even if this term does not reflect the stemness of all the cells described in Taverna et al., 2014.

## **5. Timing of neurogenesis in mice vs humans**

Another aspect to consider when studying neocortical expansion is its temporal aspect. Indeed, so far, we have focused on the spatial aspect of this expansion, but timing also plays a major role in neocortical development differences among mammals.

The first major difference is the increased gestation period observed in primates and humans compared to rodents, which is accompanied by a protraction of the neurogenic period. In mice, neurogenesis lasts for approximately 7 to 8 days from E10.5 to E18 or E18.5, while in humans, it lasts for approximately 3 to 4 months from GW8 to GW30 (Silbereis et al., 2016; Libé-Philippot & Vanderhaeghen, 2021). NECs' self-amplifying period is also prolonged in primates compared to mice: up to 2 weeks in primates compared to 1 day in mice (Silbereis et al., 2016). Thus, lengthening the primate neurogenic period by a factor of 10 where progenitors self-amplified for a longer period of time constitutes a key substrate for neocortical expansion (Vanderhaeghen & Polleux, 2023). Therefore, is time the only key to the origin of higher cognitive capacities in primates? If this was true, then the neocortical expansion would be purely proportional to time, and thus, we should see approximately a 10-fold increase in the number of cortical neurons in humans compared to mice. However, we are closer to a 1000-fold difference in the number of neurons in the human neocortex than in mice despite the increased duration of primates' cell cycle length. Interestingly, when a primate brain is compared to any other brain of the same size and weight, it always has more neurons (Herculano-Houzel, 2012). These differences in terms of number of neurons constrained in an inextensible space constitute the “primate advantage” (Herculano-Houzel, 2012) that from a cellular perspective might be due to the appearance of increased proportions of bRGCs (Dehay et al., 2015) and increased connectivity reviewed in (Vanderhaeghen & Polleux, 2023).

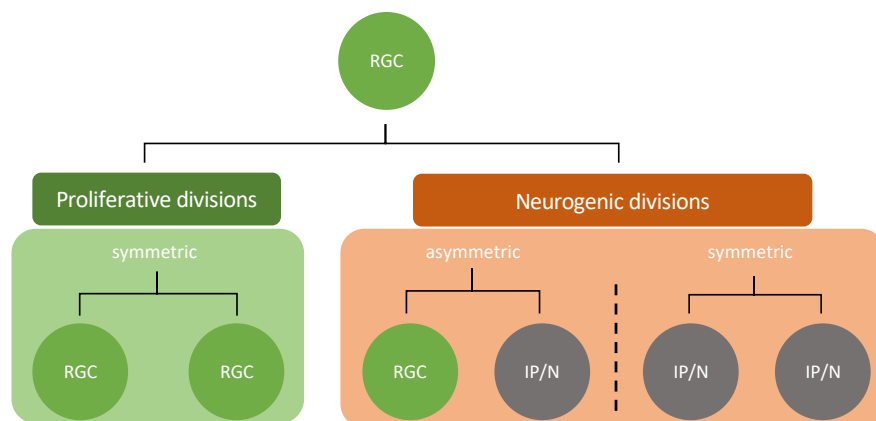
Nevertheless, to guarantee the neocortical expansion and increased cellular diversity, there are two requirements to be met: 1) these cells have to engage first in self-amplifying divisions for a prolonged period of time so neurogenesis can start from increased proportions of progenitors, and 2) they have to differentiate into neurons sequentially and not at the same time to avoid progenitor depletion and thus early stop of neurogenesis that could cause microcephaly and intellectual disabilities.

## II. Proliferative and neurogenic divisions of progenitors

### A. Proliferative versus neurogenic divisions: a matter of symmetry

It is particularly important to regulate the type of divisions progenitors make to generate the right number of neurons at the right time. A simplified view of progenitors is that they can make only two choices regarding their divisions (Figure3):

- 1) Either they will perform proliferative divisions where the daughter cells are identical to their mother cell and, by definition, symmetric.
- 2) Or they will perform neurogenic divisions in which at least one of the daughter cells is more specified into the neurogenic path than the mother cell, such as a neuron or an IP. This would be defined as asymmetric neurogenic division, while symmetric neurogenic division would cause terminal differentiation of the RGCs into either two neurons or two IPs that are already committed to neurogenesis.



**Figure 3: simplified schematic of division types that a RGC can undergo.**

- Proliferative divisions: a RGC give rise to two RGCs
- Neurogenic divisions: a RGC giving rise to one or two daughter cells committed to neurogenesis
- Asymmetric neurogenic divisions: a RGC giving rise to one RGC and one IP or Neuron
- Symmetric neurogenic divisions: a RGC giving rise to either two IPs or two neurons

During embryonic development, the local environment of progenitors located in the VZ and SVZ will play an important role in the regulation of the balance regulating proliferative divisions versus neurogenic divisions. Indeed, progenitors are not impermeable to their environment and are highly connected to each other through the different junctions we described earlier but also through their elongated processes and their primary cilium sensing the environment and its variations.

Throughout cortical development, this local environment will change due to the variety of cell subtypes emerging that will use different signaling pathways, resulting in the secretion of many different molecules that will stimulate the neighboring cells. Furthermore, organizing centers such as the cortical hem or the anti hem will contribute to reshaping the molecular environment of progenitors through the secreted factors that will diffuse all over the neocortical primordium through the CSF, the vasculature to influence progenitors' types of divisions directly.

Thus, understanding the signals that affect the balance between proliferative divisions and neurogenic divisions is crucial for producing the right number of neurons and ensuring neocortical expansion. But what signals are at work in regulating this balance, and how do they dynamically influence the choice of every progenitor?

## **B. Signaling pathways**

### **1. Sonic Hedgehog**

Shh signaling is an evolutionarily conserved pathway with broad implications in brain development, among which the ventralization of the telencephalon has been the most studied (Aoto et al., 2002; Rallu et al., 2002; Hébert & Fishell, 2008). Shh is a ligand of the transmembrane receptor Patched (Ptch) and coreceptors Cdon, Boc and Gas1 that releases Smoothed (Smo), which initiates the activation of the zinc-finger transcription factor Gli and its nuclear translocation for regulating Shh target genes (Yabut & Pleasure, 2018).

Additionally, Shh signaling may play a role in cortical neurogenesis. In the developing neocortex, Shh is secreted by different sources, such as the tangentially migrating interneurons from the GEs to the cortex and the Cajal-Retzius cells (Komada et al., 2008). It is also found in the CSF from which aRGCs are in direct contact through their apical process and primary cilium. A constitutively active form of Smo mimicking a constitutive activation of Shh signaling in mouse cortical progenitors at mid-corticogenesis promotes the expansion of a very rare pool of cells, the bRGCs with an increased proliferation of IPs leading to the amplification of both cell types through an unknown mechanism that involves the primary cilium (L. Wang et al., 2016). Ultimately, this increase in the proportions of bRGCs and IP leads to greater production of upper-layer neurons and thus to neocortical growth and even the folding of the cingulate cortex (L. Wang et al., 2016). Conversely, the deletion of Smo leads to a completely reversed phenotype with a reduction in the pool of IP and almost a complete loss of bRGCs

accompanied by increased proportions of deep-layer neurons and decreased proportions of upper-layer neurons (L. Wang et al., 2016). However, these effects are timing-dependent, as the loss of *Ptch1* at early stages results in the increased proliferative divisions of aRGCs and the loss of IPs (Shikata et al., 2011). In human cortical progenitors, it is unknown what the role of Shh signaling in cortical neurogenesis is, but the disruption of Shh signaling through a missense mutation or duplication of *PTCH1* causes holoprosencephaly (HPE), a genetically heterogeneous malformation of forebrain development accompanied by microcephaly and mild intellectual disabilities (Heussler et al., 2002; Derwińska et al., 2009; Izumi et al., 2011).

## 2. Intrinsic program: Notch signaling pathway

Another key pathway used as the cellular communication tool of neighbouring progenitors is the Notch signaling pathway, which contributes to all types of divisions, starting from proliferative to neurogenic and, ultimately, gliogenic divisions (Kageyama et al., 2009; Taylor et al., 2007). Controlling progenitor fate commitment by proneural genes can be viewed as a two-step process: an initial phase where proneural proteins oscillate to initiate Notch signaling while retaining their proneural activity and the terminal phase where the proneural proteins are sustained to induce neuronal differentiation (Shimojo et al., 2008).

The proneural genes, in vertebrates, activate the expression of *Delta* and *Jagged*, ligands of the *Notch* receptor, which all are transmembrane proteins with large extracellular domains that consist primarily of epidermal growth factor (EGF)-like repeats. The ligand binding promotes proteolytic cleavages in the Notch receptor to release the Notch Intracellular Domain (NICD) that will translocate to form a transactivation complex with the DNA-binding protein CSL (named after CBF1, Su(H) and LAG-1) and its co-activator Mastermind (Mam). This complex will then transactivate proneural repressor genes of the basic-Helix-Loop-Helix family, *hairy* and *enhancer of split* (*Hes1* and *Hes5*) (Kageyama et al., 2007, 2009). However, their expression oscillates in cycles of 2 to 3 hours due to their ability to also bind the Nboxes sequence of their own promoter (Shimojo et al., 2008). Thus, *Hes* genes are direct drivers of their dynamic expression and indirect drivers of the oscillatory expression of the proneural genes that they repress. As a consequence, *Hes* and proneural genes are always expressed out-of-phase in the same progenitor, and their transient nature defines their “salt and pepper” expression pattern in the tissue when looking at a specific time point (Kageyama et al., 2008; Oproescu et al., 2021). From a molecular point of view, these short cycles of expression of both *Hes* and proneural genes are permitted by the short half-life of their proteins which are rapidly

degraded (Nguyen et al., 2006; Kovach et al., 2013) thereby inhibiting neuronal differentiation to promote progenitor proliferation. Blocking the Notch receptor leads to premature neuronal differentiation (Nelson et al., 2007), while its overexpression causes the failure of the progenitors to differentiate into neurons and will eventually cause its differentiation primarily into astrocytes (Vetter and Moore, 2001). The overexpression of either Hes1 or Hes5 leads to an increased pool of progenitors and bRGCs-like cells in the mice SVZ (Ohtsuka & Kageyama, 2021) suggesting Notch as a potential mechanism for progenitors diversity.

In conclusion, the Hes genes and the Notch receptor are necessary for maintaining the progenitor state and potentially for engineering new subtypes of progenitors, while Neurogenin2 is important for dividing these progenitors into neurons. Thus, to weigh on cortical neurogenesis with this pathway, you have three significant ways: the Notch receptor itself, Hes, or Neurogenin2 in the developing neocortex.

### **3. Wnt signaling pathway**

Another key and conserved pathway that promotes RGCs proliferation at early stages of neocortical development is the canonical Wnt/ B catenin signaling. In the absence of Wnt ligand, a complex constituted of AXIN, adenomatous polyposis (APC) and the Glycogen Synthase Kinase-3 (GSK3) phosphorylate B-catenin leading to its ubiquitinylation and destruction by the proteasome. However, upon the binding of a Wnt ligand to a receptor complex composed of Frizzled (Fzd) and its coreceptors Low-density lipoprotein receptor related protein (LRP), AXIN is recruited at the membrane with GSK3 and thus the degradation complex is disrupted leading to the accumulation of B-catenin in the cytoplasm before translocating into the nucleus, where it functions as a co-activator of Wnt target gene transcription by binding to T-cell factor/lymphoid enhancer factor (TCF/LEF) family of DNA-binding proteins (Chenn, 2008).

Overexpression of B-catenin in the developing mouse neocortex results in the progenitor amplification, leading to neocortical expansion and even folding resembling sulci and gyri observed in gyrencephalic species but also repress neuronal differentiation (Chenn & Walsh, 2002; Stocker & Chenn, 2015; Villalba et al., 2021; Woodhead et al., 2006). Conversely, the inactivation of Wnt/ $\beta$ -catenin before the onset of cortical neurogenesis in mice neocortex results in a loss of dorsal identity of progenitors (Backman et al., 2005; Chenn, 2008) while its inactivation at mid-corticogenesis leads to a cell cycle exits and premature production of IPs

and neurons (Mutch et al., 2010; Woodhead et al., 2006). Furthermore, the role of Wnt in RGCs proliferation or differentiation largely depends on the type of Wnt ligands as the loss of Wnt7a at later stages disrupt neurogenesis (Hirabayashi et al., 2004; Stocker & Chenn, 2015; Villalba et al., 2021).

In conclusion, all these different signaling pathways play different roles in promoting proliferative divisions and neurogenic divisions highlighting the complex interplay of each of these signals and the computation that progenitors have to make during cortical development. Furthermore, there is now more and more evidence demonstrating other classical biological mechanisms at work during neurogenesis that bring exciting perspectives.

#### **4. Other influences on proliferative vs neurogenic divisions**

##### **a) Metabolism**

Through differentiation and maturation, cells must remodel their metabolic state to meet new demands for energy and biomolecules. During neocortical development, two metabolic pathways are used by cortical progenitors and neurons: glycolysis and the tricarboxylic cycle (TCA) with oxidative phosphorylation (OXPHOS) in mitochondria (Khacho et al., 2019). Generally, glycolysis is used to produce two adenosine triphosphates (ATP), an immediate form of energy combined to 2 reduced nicotinamide adenine dinucleotides (NADH) used in the electron transport chain in mitochondria to produce more ATPs. However, the final product, pyruvate, can be used depending on oxygen levels: in aerobic conditions where there is oxygen, pyruvate will be used in mitochondria by Krebs's cycle. In anaerobic conditions, where there is no oxygen, pyruvate will be converted into lactate (Khacho et al., 2019; Rumpf et al., 2023; Wallace & Pollen, 2024).

In general, in aerobic conditions, progenitors preferentially use glycolysis to produce ATP during cycling while switching to OXPHOS during neuronal differentiation for its higher yield in terms of ATP, crucial for energy-consuming action potential firing (Miyazawa & Aulehla, 2018; Wallace & Pollen, 2024). Shortly after progenitor division, cells destined to self-renewing will undergo mitochondrial fusion, whereas those retaining high levels of mitochondria fission become neurons, suggesting short-fate plasticity after divisions (Iwata et al., 2020).

Furthermore, manipulations to enhance mitochondria oxidative metabolism lead to accelerated



maturation of both mouse and human neurons, highlighting mitochondrial metabolism as a key player of species-specific differences in the developmental tempo (Iwata et al., 2023). However, it is not known how mitochondrial metabolism is linked with all the different signaling pathways described above, especially the proneural genes thought to drive neurogenesis. These results bring exciting and challenging perspectives in correlating proliferative and neurogenic cues with metabolic changes necessary to neuronal firing functions.

### **b) Primary cilium**

Another cellular signaling system that plays an important role in brain development and function is the primary cilium, a microscopic sensory antenna that cells use to gather information about their environment through growth factors, morphogens, neuromodulators, and neurotransmitters (Liu et al., 2021). It is an organelle found on the cell surface of most mammalian cells, including NECs, all the different progenitors, neurons and astrocytes (Arellano et al., 2012; Sengupta, 2017). For example, during embryonic development, primary cilia are important mediators of Shh, a critical regulator of the dorsoventral patterning, and seem to be involved in cortical neurogenesis as we have seen (L. Wang et al., 2016; Y. Wang et al., 2009).

In the early stage of neurogenesis, the primary cilium and mother centriole of aRGCs located at their apical process are endocytosed and asymmetrically inherited by one of the two daughter cells upon cellular division, which would be immediately ready to continue receiving proliferative signals from the CSF, thus potentiating the aRGC fate (Paridaen et al., 2013a). However, inhibiting this reabsorption, or blockade of asymmetric inheritance, drives cell cycle exit and neuronal differentiation in human iPSCs derived cerebral organoids (Gabriel et al., 2016). However, in the case of symmetric divisions, being proliferative or neurogenic, little is known about the role of the primary cilium.

Nevertheless, it is well known that disrupting the physiological functions of primary cilia in humans results in a broad range of multi-organ disease phenotypes such as obesity, anosmia, congenital heart defects, and retinal degeneration (Liu et al., 2021). In the CNS, primary cilia malfunctions result in neurological syndromes, including intellectual disabilities, autism spectrum disorders, epilepsy with structural brain anomalies with microcephaly, neuronal heterotopia, and disrupted neuronal layer formation (Guo et al., 2015; Liu et al., 2021; Novarino et al., 2011; Valente et al., 2014). The neurological disorders resulting from aberrant primary

cilia function include Bardet-Biedl syndrome (BBS), nephronophthisis (NPHP), Senior-Loken syndrome (SNLS), Alstrom syndrome (ALMS), Meckel syndrome (MKS), Joubert syndrome (JBTS), Oral-facial-digital Type I (OFD 1), polycystic kidney diseases (PKD), Jeune asphyxiating thoracic dystrophy (JATD), Ellis van Creveld (EVC), and Leber congenital amaurosis (LCA) reviewed in (Liu et al., 2021; Reiter & Leroux, 2017; Ringers et al., 2019).

The wide range of primary cilia-related developmental brain malformations emphasizes the importance of this sensory organelle for the proper development and differentiation of cortical progenitors, neurons, and glia.

### **c) Epigenomic regulation**

As the neocortex contains a wide variety of cells located at different places, transcriptomic studies have demonstrated the transcriptional heterogeneity of each cell type (Eze et al., 2021; Nowakowski et al., 2017; Pollen et al., 2015). However, gene expression is influenced by epigenetic regulations such as DNA methylation, chromatin accessibility, histone modifications, and the 3D organization of the genome. Indeed, more and more evidence demonstrates that chromatin loops, by bringing enhancer and promoter elements closer to each other, play a major role in neurogenesis (Aboelnour & Bonev, 2021; Bonev et al., 2017). In the developing mouse neocortex, it appears that the proneural protein Neurog2 directly mediates enhancer activity, DNA demethylation, and increased accessibility of the chromatin and its looping in vivo, providing more evidence of the pioneering effect of proneural genes (Noack et al., 2022). However, little is known about such regulations in the developing human neocortex.

## **C. Mechanisms of neurogenic divisions**

After all the signals described above and their cross-regulation have been transmitted to the progenitors, they will divide according to the computational result between proliferative and neurogenic cues. However, these division types don't require the same "effort" from the signaling pathways to remodel their identity. Indeed, classical analyses using immunohistochemistry and, more recently, single-cell transcriptomics have revealed that distinct cellular states are associated with specific gene expression patterns. Therefore, the acquisition of a specific cellular identity requires a remodeling of these gene regulatory networks (GRNs) that underlie cell state-specific expression patterns. Therefore, proliferative divisions don't require any major remodeling of the cellular GRN, while the specification

of a progenitor into a neuron requires a complete remodification of the transcriptomic landscape by turning many genes on and off through transcription factors such as the proneural proteins.

## 1. Proneural genes:

### a) From invertebrate to vertebrate

Initially, proneural genes were discovered in *Drosophila Melanogaster* mutants and precisely identified at the end of the twentieth century as two families, *achaete-scute* complex (*asc*) and *atonal* (*ato*) with *cato* and *amos* reviewed in (García-Bellido, 1979; Villares & Cabrera, 1987; Ghysen & Dambly-Chaudière, 1988; Jarman et al., 1994; Bertrand et al., 2002).

These genes share similar features that classify them as proneural:

- 1) They all have the basic-Helix-Loop-Helix domain used for homo or heterodimerization and DNA binding.
- 2) They are both required and sufficient to promote neural differentiation.
- 3) They are expressed in cells before any sign of their neural differentiation to confer both panneuronal properties and neuronal subtype-specific identities (Jan & Jan, 1994).
- 4) They are specific to regions of the nervous system and, hence, are expressed in specific subtypes of progenitors throughout the central and peripheral nervous systems, which correlates with the production of specific neuronal types, highlighting their role in neuronal specification.

Proneural proteins are transcription factors that will bind to the promoter of many genes with E-boxes, DNA sequences composed of the core hexanucleotide motif, CANNTG. However, to be active, they must form heterodimers with ubiquitous bHLH proteins known as E-proteins encoded in flies by the gene *da*, or *E2A* (and its two forms: E12 and E47), *HEB* and *E2-2* (Cabrera & Alonso, 1991; Johnson et al., 1992; Massari & Murre, 2000). They can also form homodimers, which appear to change the E-box specificity, representing numerous dimerization opportunities for combinatorial control of bHLH transcriptional activity during development (Oproescu et al., 2021). However, precise and clear preferred binding motifs for certain hetero/homodimers is not yet known

Across evolution, the proneural genes, like any other gene, have been submitted to selective

pressure and have been multiplied in vertebrates. They can be classified into two main groups based on their homology with their *Drosophila* counterparts: the *atonal* subgroup now also includes the *Neurogenin* proneural genes (*Neurog1*, *Neurog2*, *Neurog3*), the neurogenic differentiation genes (*NeuroD1*, *NeuroD2*, *NeuroD4*, *NeuroD6*) and Olig differentiation genes (*Olig1*, *Olig2*, *Olig3*). Interestingly, the only homolog of the *asc* family is *Achaete scute-like 1* (*Ascl1*) (Bertrand et al., 2002). Proneural genes in both vertebrates and invertebrates share several functional characteristics; for example, their ability to transactivate Notch ligands and initiate Notch signaling as well as transactivation of neuronal differentiation genes (Huang et al., 2014).

## **2. The telencephalic proneural genes**

In vertebrates, proneural genes are also expressed in specific regions throughout the neural tube, determining unique neuronal identities (Bertrand et al., 2002; Parras et al., 2002). In the telencephalon, the highest levels of *Ascl1* expression are found in the ventral part, and the ganglionic eminences more specifically, suggesting a significant role in the specification of ventrally born GABAergic interneurons. In contrast, *Neurog1* and *Neurog2* are expressed exclusively in the dorsal part of the telencephalon where they play an important role for the production of excitatory cortical neurons (Bertrand et al., 2002; Fode et al., 2000; Parras et al., 2002).

### **a) The neurogenins**

In 1996, three *ato* orthologs were discovered in vertebrates, *Neurog1*, *Neurog2* and *Neurog3* as novel bHLH genes with potential proneural functions in both the CNS and PNS of vertebrates (Gradwohl et al., 1996; Q. Ma et al., 1996; Sommer et al., 1996).

#### **(1) Neurogenin3**

Neurogenin3 is a key gene in specifying the endocrine fate of pluripotent pancreatic progenitors in both mice and humans (Pelling et al., 2011; Yong et al., 2021). In the mouse brain, *Neurog3* is found in some mitotic cells of the hypothalamus and is required for the proper development of the neuronal subtypes (Pelling et al., 2011). Furthermore, Neurogenin3 is implicated in gliogenesis in the mouse spinal cord to specify both astrocytes and oligodendrocytes (J. Lee et al., 2003). However, little is known about its role in the human hypothalamus and spinal cord.

#### **(2) Neurogenin 1 and Neurogenin2**

In the developing mouse neocortex, both Neurog1 and Neurog2 can be found at the beginning of neurogenesis at E10.5 in the VZ but also in the SVZ later at E15.5, suggesting that it can be found in both aRGCs and IPs (Britz et al., 2006). Indeed, Neurog2 induces the expression of Tbr2 and thus the transition from aRGCs to IPs. Neurog2 is the main driver of neurogenesis in the developing mouse neocortex as Neurog2 is required and sufficient to specify a glutamatergic neuronal identity (Fode et al., 2000; Mattar et al., 2008; Oproescu et al., 2021; Schuurmans et al., 2004) while Neurog1 does it at a slower rate and suppresses Neurog2 neurogenic activity through homodimerization (Han et al., 2018). Therefore, for the remainder of this introduction, we will focus only on Neurog2.

#### **(a) Neurogenin2 KO in mice**

To better understand what Neurog2 is capable of, researchers started by inducing the loss of Neurog2 during mouse embryonic development, and to a further extent, loss of both Neurog2 and Neurog1 resulted in the down-regulation of many transcription factors expressed in cortical neurons such as Neurod1, Neurod2, Neurod6, Tbr1, Tbr2, vGlut1 and vGlut2 and thus to the loss of a subset of projection neurons in the neocortical deep layers. In contrast, the generation of later-born neurons of the upper layers of the neocortex seems unaffected by the losses of the NGNs (Schuurmans et al., 2004; Kovach et al., 2013). These data suggest that Ngn2 play a central role in initiating the glutamatergic programme in cortical progenitors but not in the generation of neurons of the upper layers which appears to depend on other transcription factors such as Pax6 and Tlx (Schuurmans et al., 2004). Furthermore, the loss of Ngn2 resulted in a surprising overexpression of Ascl1 by the cortical progenitors as well as their subsequent ectopic expression of ventral markers, including Dlx1, Dlx2, Dlx5, and the GABA biosynthetic enzymes Gad1 and Gad2 (Fode et al., 2000). Therefore, Ngn2 not only specifies cortical progenitors into excitatory neurons but also suppresses an alternative basal ganglia differentiation program that would specify cortical progenitors in inhibitory neurons (Mattar et al., 2008).

#### **b) Neurogenin2 gain of function/overexpression**

Overexpression in mice cortical progenitors at E12.5 results in a depletion of the progenitor pool and an increased production of neurons from the deep layers at the expense of the upper layer neurons of the neocortex (Quan et al., 2016).

Furthermore, overexpression of *Neurog2* in human iPSCs leads to direct and rapid differentiation of iPSCs in neurons with high efficiency and bypassing the progenitor stage (Zhang et al., 2013). However, neuronal fate highly depends on the levels and duration of *Neurogenin2* forced expression (Lin et al., 2021), suggesting the possibility of generating specific subtypes of neurons when controlling these two parameters.

These results demonstrate that *Neurog2* is sufficient to drive cortical neurogenesis from cortical progenitors but also from human stem cells, iPSCs. Thus, in order to understand how neurons are generated and potentially how the increased neuronal diversity observed in primates and humans more specifically, we need to understand how *Neurog2* is regulated at both the gene and protein levels.

### **3. *Neurog2* regulation**

The expression of *Neurog2* in the dorsal telencephalon is controlled by the regional patterning transcription factor *Pax6*, which has been shown to bind and activate the *Neurog2* enhancer (Fode et al., 2000; Scardigli et al., 2001, 2003).

We saw in a previous section that proneural genes are key players in Notch signaling by inducing the expression of Notch ligands to transactivate the *Hes1* and *Hes5* genes in neighbouring progenitors to repress *Neurog2*. Thus, *Neurog2* expression oscillates as a consequence of *Hes* oscillations. However, when stabilized, *Neurog2* will induce the neuronal differentiation of progenitors by activating its downstream targets, such as *Neurod1* (Bertrand et al., 2002). Nevertheless, it is still not clear how *Neurog2* is stabilized or potentiated, but several studies suggest that post-translational modifications could also regulate *Neurog2* proneural activity as developed below (F. Ali et al., 2011; Quan et al., 2016).

#### **D. Post-translational modifications of *Neurog2***

In the early 2000s, the mechanism through which phosphorylation of transcription factors alters the DNA binding and hence their transcriptional activity was discovered (Cowley & Graves, 2000; Pufall et al., 2005) which when applied to proneural proteins brings new perspectives as phosphorylation is very dynamic and highly context-specific (F. Ali et al., 2011; F. R. Ali et al., 2014; Quan et al., 2016; Hardwick & Philpott, 2018). Thus, proneural proteins and thus proneural activity could be dynamically regulated based on the presence or absence of kinases and phosphatases during neocortical development.

## **1. The rheostat model**

During the cell cycle, Neurogenin2 appears to be phosphorylated by the cyclin-dependent-kinases (Cdk) on nine serine-proline (SP) sites to regulate its proneural activity. Indeed, in dividing progenitors, the levels of Cdk will rise increasing the phosphorylation of Neurog2 and inhibiting the transactivation of the downstream genes it regulates (F. Ali et al., 2011). Therefore, the phosphorylated Neurogenin2 loses its ability to transactivate Neurod1, a key player in neuronal differentiation, but can still transactivate the Notch ligand, Dll1, and thus engage in progenitor maintenance. Importantly, the SP pairs can be phosphorylated by other kinases, such as GSK3, which we have seen as an important kinase in Wnt signaling but also in Shh signaling and the MAP kinases implicated in ERK signaling and JNK signaling. This suggests that phosphoregulation of Neurogenin2 proneural activity might integrate more cellular signaling events (Hindley et al., 2012).

## **2. Phosphorylation of Neurog2 Serine 231 and 234**

Indeed, the Serine/Threonine kinase GSK3 has been shown to phosphorylate two other phosphorylation sites located in the Neurog2 C-terminal, the S231 and S234 that play an important role in the interaction with the Lim homeodomain for motor neurons generation in the spinal cord. Replacing these residues with an Alanine, a neutral amino acid, prevents the binding between Neurog2 and the Lim homeodomain composed of Lhx3/Isl1 and thus the differentiation of spinal cord progenitors into motor neurons (Y.-C. Ma et al., 2008). Interestingly, several signaling pathways regulate the GSK3 kinase, such as Wnt and the SHH signaling pathway (Jia et al., 2002), highlighting the cross-talk between these pathways and suggesting new mechanisms regulating Neurog2 proneural activity.

## **3. Phosphorylation of Neurog2 Tyrosine 241**

Furthermore, the phosphorylation of a single Neurog2 residue located outside the bHLH domain, the Tyrosine 241 is of particular importance to specify the dendritic morphology of pyramidal neurons and to initiate the radial migration (Hand et al., 2005). However, the phosphorylation of Neurog2 Y241 is not required for the transactivation of Neurod1 and thus for Neurog2 proneural activity. Even if the precise kinases involved in this mechanism have not been identified, this work highlights the remodeling of Neurog2 activity after inducing neurogenesis through the modulation of a single amino acid.

#### **4. Phosphorylation of Neurog2 Threonine 149**

So far, we have seen that phosphorylation sites outside the bHLH domains can modulate the different aspects of Neurog2 activity. In 2016, Quan et al., identified the role of the threonine 149 (T149), a phosphorylation site within the bHLH domain, as a binary modulator of Neurog2 proneural activity (Quan et al., 2016). Located at the junction between the loop and the second helix (L-H2), its phosphorylation induces a repulsion of charges due to the close proximity of the added phosphate group with the DNA backbone composed of phosphates and, thus, a loss of neurogenic functions. The ablation of this phosphorylation site through the substitution of Neurog2 T149 by an alanine, Neurog2 T149A, displayed a gain of functions when overexpressed in mouse cortical progenitor similarly as the overexpression of Neurog2 WT. However, the ectopic overexpression of the Neurog2 T149D mutant where the Threonine is substituted by an Aspartate to mimic a phosphorylation results in a loss of function as its overexpression does not increase the proportions of neurons.

Interestingly, the sequence surrounding this phosphorylation site does not match classical kinase sequences, suggesting a restriction in the potential kinases that could phosphorylate the T149, resulting in an increased specificity of regulating neurogenesis to key kinases. Among the potential kinases involved in the phosphorylation of this site are Plk1, implicated in neurogenesis (Sakai et al., 2012), and Mark1, a kinase associated with autism spectrum disorders (ASD) (Maussion et al., 2008) and the regulation of dendritic spine morphogenesis in the mice hippocampus (Kelly-Castro et al., 2024).

Overall, these results suggest that modulating the phosphorylation site all the phosphorylation sites described above through overexpression experiments, directly impacts the proneural activity of Neurog2 in the developing mouse neocortex and thus alters both the progenitor pool and the neurons they produce. However, it is not known how this phosphor-regulations and the modulation of NEUROG2 T149 could regulate the temporal aspect of human cortical neurogenesis under endogenous levels.



## **E. Human iPSCs, cortical organoids, and CRISPR/Cas9 to study the human brain development**

### **1. iPSCs**

Scientific progress on the knowledge of the human brain has always been hindered by the difficulty to reach it, for obvious ethical reasons. A major turning point in this quest has been achieved over the last decade, by the dedifferentiation of fibroblast cells into induced pluripotent stem cells (iPSCs) that can be differentiated in any cell types of the three major embryonic tissue, the ectoderm, the endoderm, and the mesoderm (K. Takahashi et al., 2007; K. Takahashi & Yamanaka, 2006). Thus, iPSCs represent a revolutionary advancement in the field of regenerative medicine and biological research and opened new horizons in personalized medicine, drug discovery, and the understanding of complex diseases. Their development was rewarded by the 2012 Nobel Prize of Physiology or Medicine to Pr. Shinya Yamanaka and Pr. John B. Gurdon. Furthermore, the understanding of their differentiation through their incubation in specific culturing media supplemented by molecules and cytokines led to their broad use in laboratories all over the world, turning them into a reference model for the study of brain development.

### **2. Cerebral organoids**

Initially, iPSCs were differentiated in 2D cultures of neurons and astrocytes to study the physiological and pathological development of the brain. Later, the discovery of the self-assembly and organization of iPSCs during differentiation paved the way for the development of 3D culturing conditions, pioneered by the work of Pr. Sasai in Japan has led to 3D organoids as we know and use them now (Eiraku et al., 2008; Lancaster et al., 2013; Lancaster & Knoblich, 2014; Paşca et al., 2022; Paşca, 2018; Sasai, 2013; Watanabe et al., 2005).

Overall, neural organoids can be categorized into two categories based on the level of guidance provided by supplementation with specific biomolecules. The first kind is composed of unguided organoids, composed of a high diversity of neural cells, typically representing different regions of the nervous system in different proportions. The main issue presented by these organoids is their lack of reproducibility as the proportions of the different brain structure may vary from one organoid to another. To overcome this major obstacle, guided protocols were developed to differentiate iPSCs into specific brain regions such as midbrain, ventral

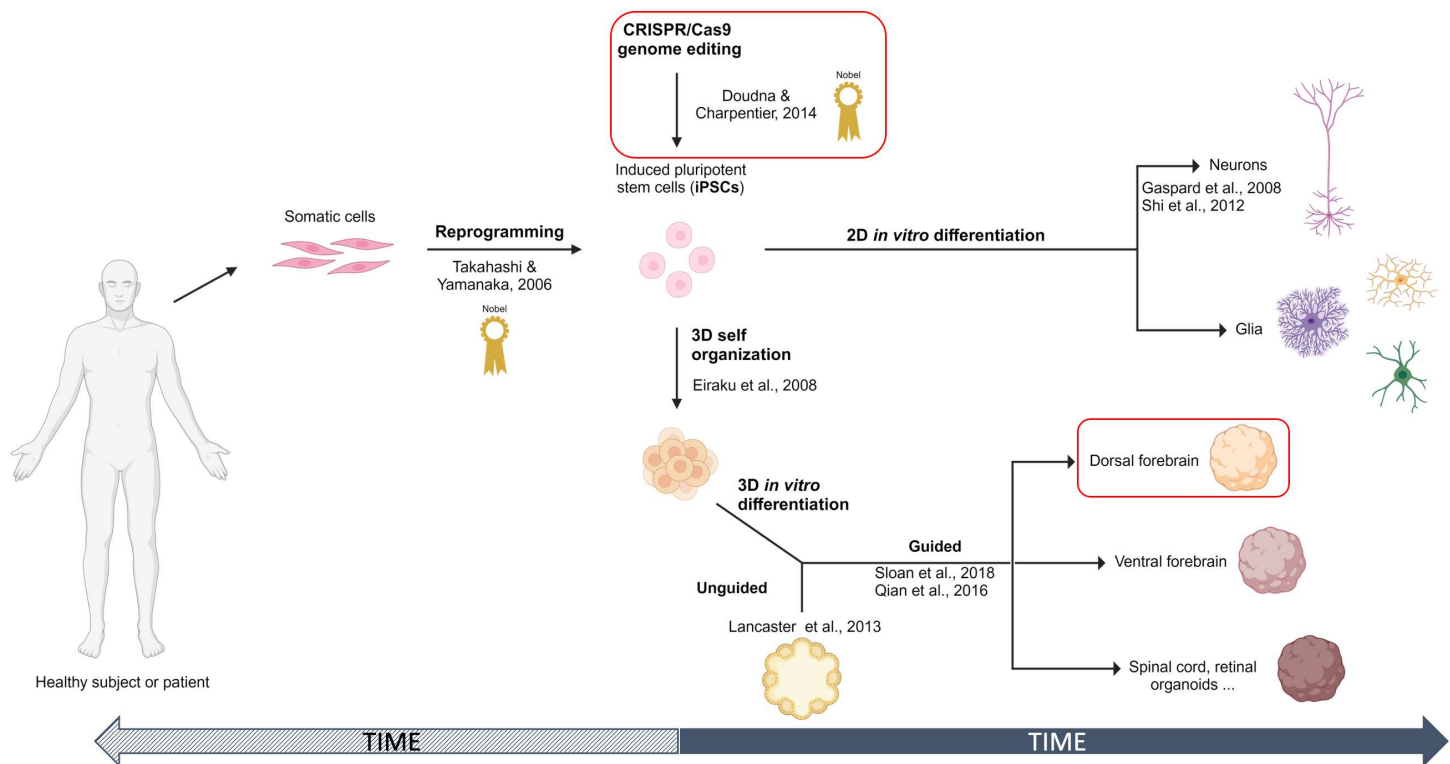
forebrain, and dorsal forebrain organoids named cerebral cortical organoids, which are the model of interest in this thesis.

In short, the development of cerebral cortical organoids, hereafter referred to as cortical organoids, has brought the possibility to study the development of the human neocortex and its alteration found in many diseases. The high reproducibility of their development and their close proximity to the developing human fetal cortex, from a transcriptomics perspective, has made cortical organoids a model of reference for the study of neocortical development (Uzquiano et al., 2022; Velasco et al., 2019; Yoon et al., 2019). However, great challenges remain to be addressed to unlock the full potential of this relatively new model, which brings exciting opportunities to young scientists.

### **3. CRISPR/Cas9, the most famous scissor**

A pivotal landmark in the understanding of our own brain is the discovery of the CRISPR/Cas9 system. Originating in bacteria as a defense mechanism to cleave invading viral DNA, this molecular apparatus has been ingeniously adapted by scientists for genome editing. The mechanism entails the CRISPR/Cas9 complex navigating to a specific location within the double helix of DNA, guided by a synthesized RNA sequence that matches the target DNA sequence. Upon location, the Cas9 cuts across both strands of the DNA that will be repaired and that can be manipulated to insert any specific mutations and thus precise gene editing. Its use has now become a standard tool in research and has been rewarded by the Nobel prize of Chemistry in 2020 to Pr Emmanuelle Charpentier and Pr. Jennifer A. Doudna.

Altogether, 3D models of the human neocortex and CRISPR/Cas9 genetic remodification hold great promises and adventures for future discoveries on human brain development and treating its alterations.



**Figure 4: major scientific breakthrough that made the study of the developing human brain possible**

- iPSCs cells are made from human skin biopsies.
- CRISPR/Cas9 allow to genetically modify the iPSCs DNA.
- 2D differentiation protocols of iPSCs allows for the production of specific brain cell subtypes (neurons and glial cells).
- Differentiating iPSCs tends to self-organize in 3D which gave rise to 3D organoids that can be guided or not towards specific brain structures.

## **F. Summary of the results**

Thus, to study human neurogenesis in the neocortex, we first generated two different iPSC lines where Neurogenin2 is knocked out to demonstrate for the first time its role in human cortical neurogenesis using 3D cortical organoid differentiation protocols. We discovered that NEUROG2 KO induces a loss of glutamatergic neurons at both mid and late stages of cortical organoid development, respectively after 70 and 140 days in culture that is accompanied by a ventralization of cortical progenitors with a downregulation of the genes encoding for the dorsal forebrain identity and an upregulation of the genes encoding for the ventral forebrain identity.

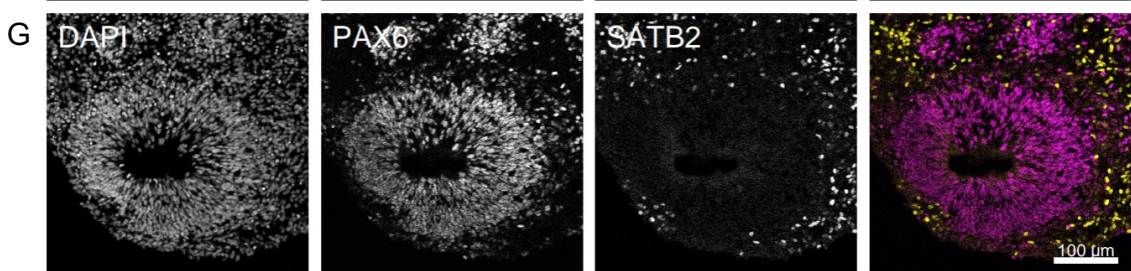
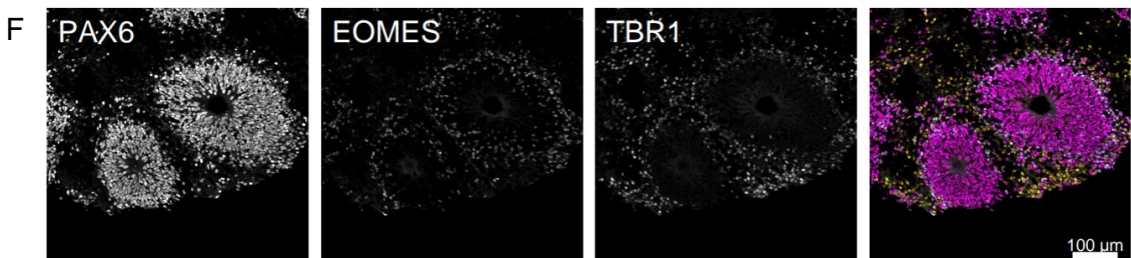
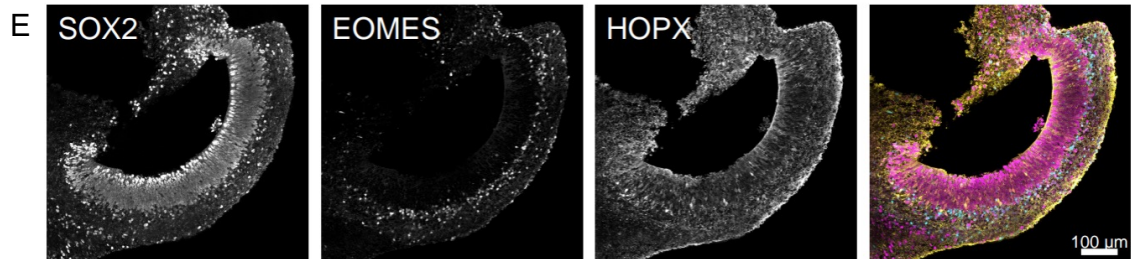
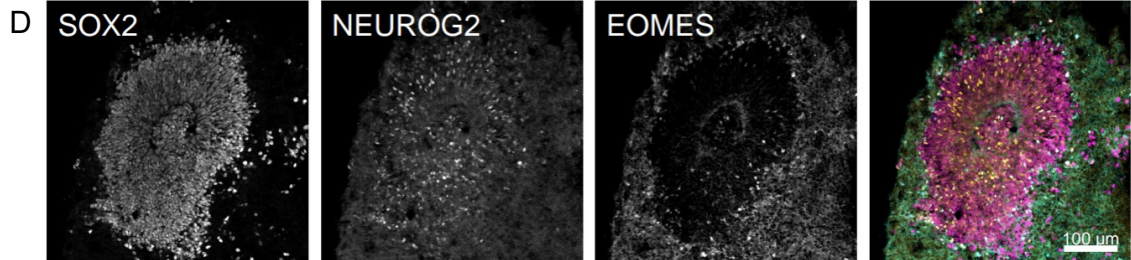
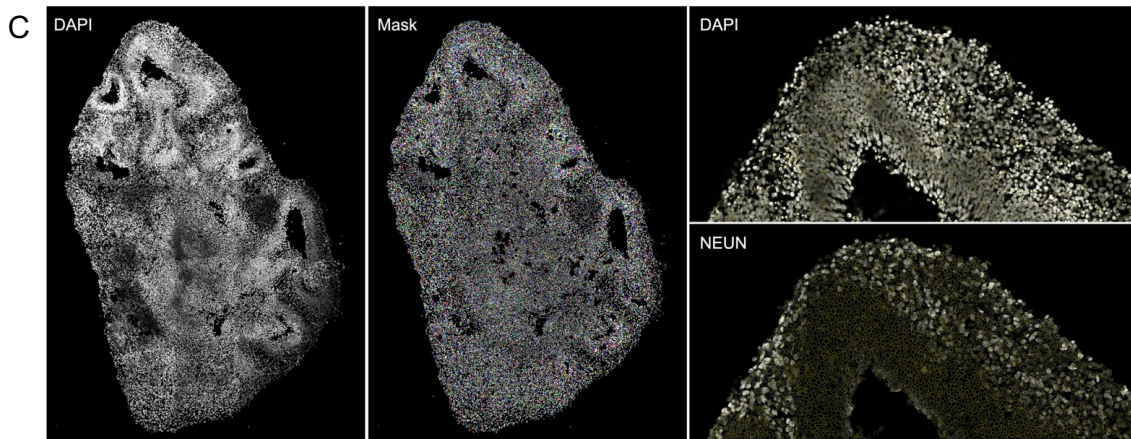
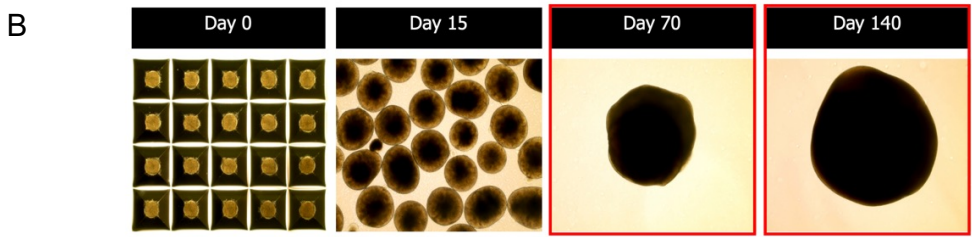
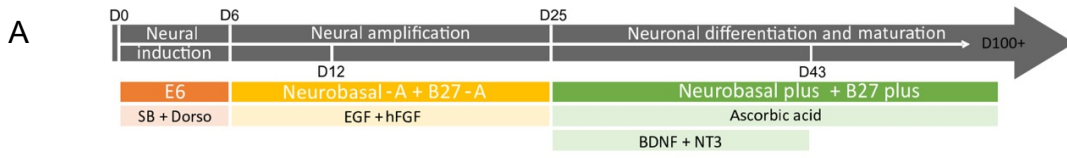
Knowing that Neurogenin2 is required for human cortical neurogenesis, we next studied how the loss of NEUROG2 phosphorylation site T149 by its replacement with an Alanine (T149A) at endogenous levels alters neuronal production. To this end we combined live imaging of radial glial clones, immunohistochemistry for key cell fate markers, machine-learning based cell type quantification to have precise quantifications of neuronal production over time. We completed our analyses with experiments on transcriptional activation and stem cell reprogramming assays, RNA sequencing and chromatin immunoprecipitation to characterize the intrinsic properties of our NEUROG2 T149A mutant and potential molecular mechanisms in action.

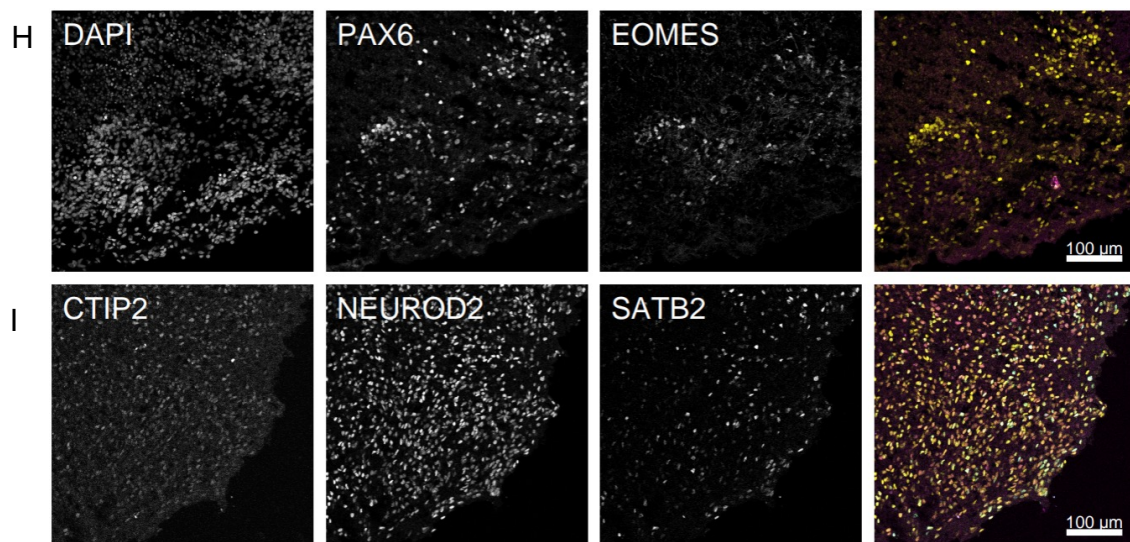
We found, on the one hand, the NEUROG2 T149A homozygote mutant does not change the pattern of NEUROG2 expression in both RGCs and IPs nor its ability to bind and activate well known target genes or reprogram human stem cells into neurons. However, the TA/TA mutant radial glia cells switch their division mode from proliferative to neurogenic which induces an increased proportions of neurons per organoid at both mid and late stages of cortical development in organoids. Mechanistically, we found that this phenotype is accompanied by an upregulation of the genes encoding the organization and the movements of the primary cilium of RGCs, which are downregulated in the NEUROG2 KO clones. These results suggest a strong link between Neurogenin2, and its phosphorylation profile with the regulation of neurogenesis in human cortical organoids and the primary cilium, a structure well known for its implication in neurodevelopmental disorders and intellectual disabilities.

# Results

## **Validation of the generation of cortical organoids from iPSCs clones.**

To study neurogenesis in human cortical organoids, we first validated the 3D differentiation protocol adapted from (Sloan et al., 2018) (Figure 1A) and the timing of NEUROG2 expression. We found that day 70 represents a good time point as most progenitor subtypes, the aRGCs (EOMES-, HOPX-), the IPs (EOMES+) (Figure 1E & 1F), and the bRGCs (HOPX+) (Figure 1E), are found in the rosettes, ventricle-like structures surrounded by two germinal layers, the VZ and SVZ. Furthermore, we found that most neurons had deep layer identity (TBR1+) at this stage (Figure 1F) with some upper layer neurons that start to appear (Figure 1G). After 140 days in culture, we found that organoids still contain RGCs (PAX6+, EOMES-) and IPs (EOMES+) (Figure 1H), and excitatory neurons from the deep (CTIP2+) and upper layer (SATB2+) (Figure 1I). Overall, these images show that organoids are not homogenous structures, which biases the quantifications of specific regions of interest defined by the experimenter. To remediate this major problem, a fine-tuned deep learning algorithm named StarDist (Schmidt et al., 2018; Weigert et al., 2020) was used for instance segmentation, to identify each nucleus as a single object based on the DAPI channel on the whole organoid slices taken at different depth of the 3D structure (Figure 1C). Next, a machine learning algorithm based on random forest encoded in the open-source software ilastik was used to classify each of these objects into specific categories defined by the user based on the different markers used for the immunostainings (Figure 1C). Thus, we established a robust semi-automated image analysis pipeline based on artificial intelligence to detect and classify millions of nuclei spread across thousands of images.





**Figure 1: protocol of 3D differentiation of iPSCs into cortical organoids**

(A) Schematic of the protocol pipeline modified from Sloan et al., 2018; (B) Brightfield images of cultured organoids; (C) Image analysis pipeline of whole sliced images from cortical organoids with segmentation of the DAPI channel resulting in a mask that allows for the classification of cells with object classifiers; (D) Immunostaining for SOX2, NEUROG2 and EOMES at day 70; (E) Immunostaining for SOX2, EOMES and HOPX at day 70; (F) Immunostaining for PAX6, TBR1, EOMES at day 70; (G) Immunostaining for DAPI, SOX2 and SATB2 at day 70; (H) Immunostaining for DAPI, PAX6 and EOMES at day 140; (I) Immunostaining for CTIP2, NEUROD2 and SATB2 at day 140.

## **Neurog2 is the proneural gene responsible for neuronal differentiation in human cortical organoids.**

In the developing mouse neocortex, Neurog2 is considered the main driver of cortical neurogenesis and plays a critical role in specifying a glutamatergic neuron identity, especially for the deep-layer neurons. However, little is known about its implications in the developing human neocortex. To validate the role of NEUROG2 in neurogenesis, we used CRISPR/Cas9 to generate NEUROG2-KO iPSC clones. We selected two KO clones, KO1 and KO2, with early stop codons and a knockout score of 100% and 96%, respectively (Figure 2A & 2C), and one control clone, which was not modified by CRISPR/Cas9 treatment. All these clones were differentiated into 3D cortical organoids cultured for up to 140 days using an adapted protocol from Sloan et al., 2018 (Figure 1A).



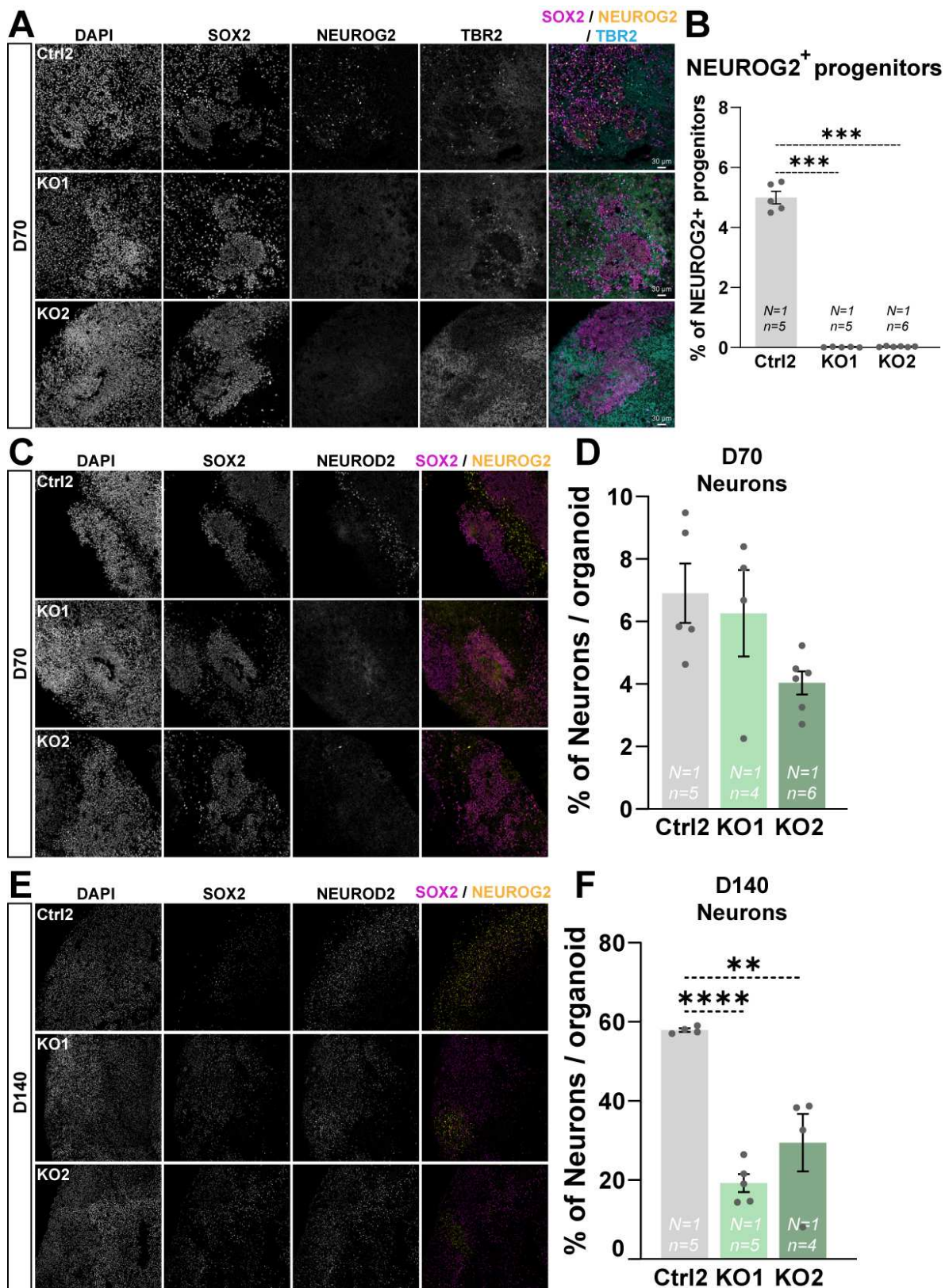
**Figure 2: Validation of NEUROG2 KO in iPSCs clones.**

(A) Screening of sequencing results with Synthego to validate the loss of *NEUROG2* expression with a Knockout score of 100; (B) Sequencing of *NEUROG2* in CRISPR/Cas9 remodified iPSCs clone 1 showing a deletion of 13 bp on both *NEUROG2* alleles resulting in a stop codon in position 111; (C) Screening of sequencing results with Synthego to validate the loss of *NEUROG2* expression with a Knockout score of 100; (D) Sequencing of *NEUROG2* in CRISPR/Cas9 remodified iPSCs clone 2 showing a deletion of 10 bp on one *NEUROG2* allele resulting in a stop codon in position 112 while there is a 1bp insertion on the other *NEUROG2* allele resulting in a stop codon in position 87.

First, we started by confirming the loss of NEUROG2 protein in our cortical organoids after 70 days of culture, representing a mid-stage of development through immunostaining for SOX2, a marker of all progenitors that should reveal all RGCs and IPs at this stage, as well as NEUROG2 and TBR2, a marker of IPs only (Figure 3A). Our CRISPR/Cas9 remodifications led to a complete loss of the NEUROG2 protein (KO1: 0.01% ± 0,005%; KO2: 0.01% ± 0,006% NEUROG2+ cells). Additionally, we observed that among all progenitors, RGCs and IPs, 5% ± 0,2% expressed NEUROG2 (Figure 3B) at this stage.



Second, we assessed the proportions of progenitors and neurons per cortical organoids after 70 and 140 days of *In Vitro* culturing through immunostaining for SOX2 to identify all progenitors and NEUROD2 for all excitatory neurons (Figure 3C and 3E). We found, at day 70, a non-statistically significant decrease in the percentages of neurons per organoid in both KO clones (KO1:  $6,2\% \pm 1,4\%$ ; KO2:  $4\% \pm 0,37\%$ ) but a tendency with the KO2 clone (Figure 3D). However, after 140 days in cultures, we found a significant 2,4-fold decrease in the percentages of neurons per organoid in the two KO clones (KO1:  $19,2 \pm 2,3\%$ ; KO2:  $29,4 \pm 7,2\%$ ) compared to their control (Ctrl2:  $57,9\% \pm 0,4\%$ ) (Figure 3F). These results suggest that the loss of NEUROG2 does not alter the neuronal production at day 70 but a strong 2,4-fold decrease of neurons at later stages of cortical organoids development when upper layer neurons are mostly produced which demonstrate the importance of NEUROG2 in human cortical neurogenesis.



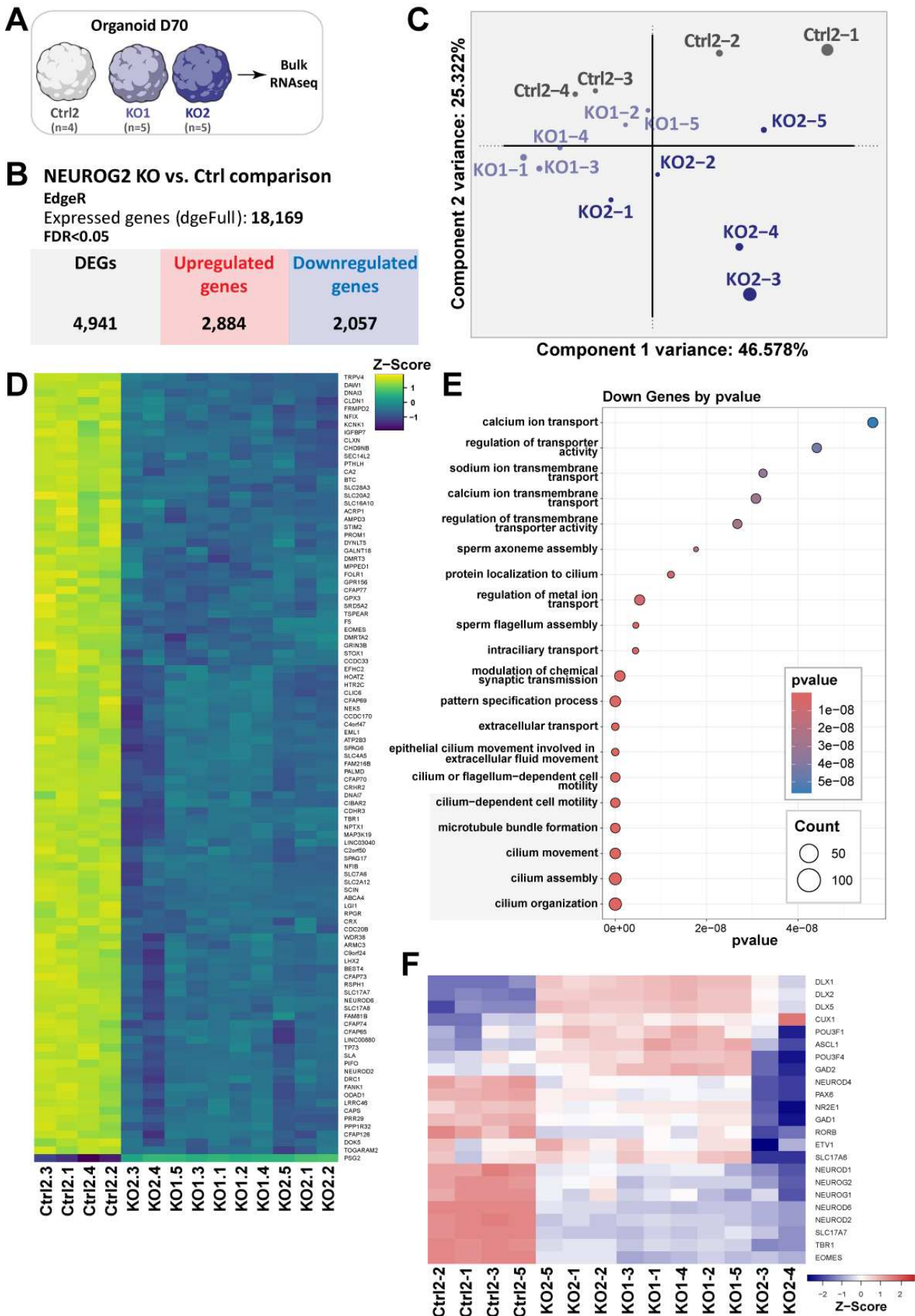
**Figure 3: NEUROG2 is the proneural protein for progenitor differentiation into excitatory neurons.**

(A) Immunostaining for SOX2, NEUROG2 and TBR2 on day 70 cortical organoids from the control and two KO clones; (B) Quantification of all NEUROG2 positive cells in all the organoids across control and KO clones; (C) Immunostaining for SOX2 and NEUROD2 at day 70; (D) Quantification of the proportion of neurons (NEUROD2+) among each organoids across the control and the two KO clones; (E) Immunostaining for SOX2 and NEUROD2 at day 140; (F) Quantification of the

**The loss of NEUROG2 induces a ventralization of cortical organoids and downregulates genes related to the primary cilium organization and functions.**

We further dived into the disturbances of the loss of NEUROG2 on the global transcriptome of the organoids at day 70 through bulk RNA sequencing analyses on both KO clones and control. We obtained 4,941 differentially expressed genes (2,884 upregulated and 2,057 downregulated, including *NEUROG2* as expected, see Figure 4F) between the control and the two KO clones resulting in the clustering of the KO clones and their technical repeats separately from the cluster composed of Ctrl2 and its technical repeats (Figure 4B & 4C). Among the 100 most differentially expressed genes, we found that almost all of them, 99 genes are downregulated, including neuronal markers such as *NEUROD2*, *NEUROD6*, and *TBRI*, a specific marker of deep layer neurons and *EOMES*, the marker of IPs in both of the KO clones compared to their control.

To gain further insight into the disturbances caused by the loss of NEUROG2, we employed Gene Ontology analysis of all the downregulated genes and found that among all of the GO terms, many highlighted the primary cilium as the most affected structure with genes involved in its organization, assembly, movement, and intraciliary transports (Figure 4E). Further analysis of the genes expressed in the telencephalon shows a downregulation of both *NEUROG2* and *NEUROG1* combined with an upregulation of several ventral telencephalic transcription factors such as *ASCL1*, and the markers of inhibitory progenitors, *DLX1*, *DLX2*, *DLX5* and the biosynthetic enzymes for GABA, *GADI*, *GAD2* inhibitory almost all the general markers of excitatory neurons, *NEUROD1*, *NEUROD2*, *NEUROD4* and *NEUROD6* suggesting that all components of a subcortical, GABAergic differentiation program in the NEUROG2 KO clones compared to their control (Figure 4F).



**Figure 4: NEUROG2 loss induces a ventralization of cortical progenitors at day 70, accompanied by a downregulation of genes involved in primary cilium assembly and motility.**

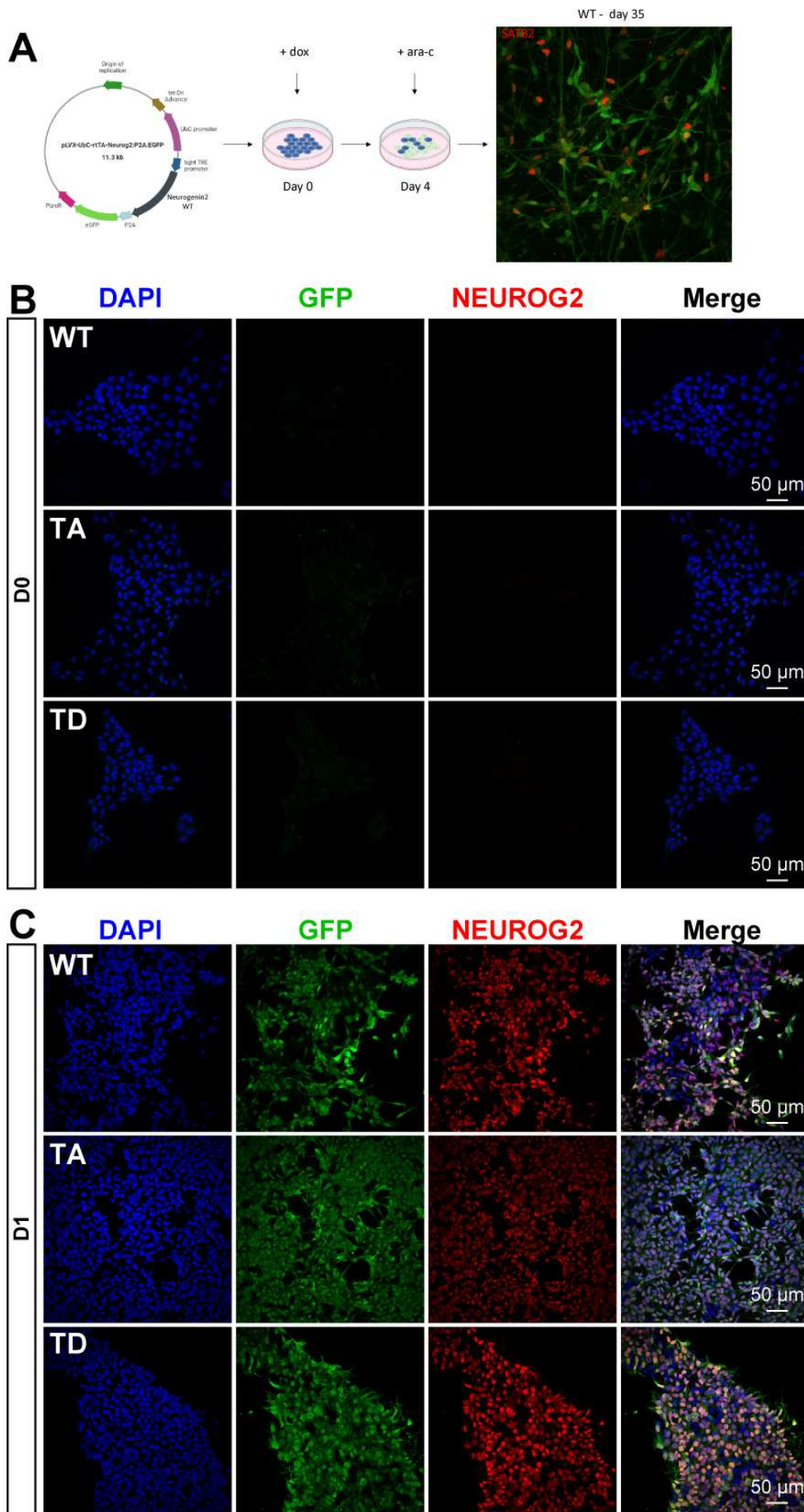
(A) Schematic representation of the bulk RNA sequencing and the number of replicates per clones done at day 70 of cortical organoids development; (B) Differentially expressed genes found in the two KO clones compared to the control; (C) PCA analysis of all clones and their corresponding replicates; (D) heatmap of the most 100 genes differentially expressed; (E) Gene ontology of all the downregulated genes in the two KO clones; (F) Heatmap of manually selected genes known for their implication in the ventral and dorsal telencephalic.

## **Transactivation properties and iPSCs reprogramming capacities are lost for NEUROG2 T149D and reduced for NEUROG2 T149A compared to NEUROG2 WT.**

Now that we have shown that NEUROG2 is responsible for neuronal production in human cortical organoids, we can dive into the role of phosphoregulation of NEUROG2 T149 and its role in the developing human neocortex. Thus, we started replicating the results from Neurog2 overexpression Quan et al., 2016 which demonstrated that Neurog2 T149A is an active proneural protein while Neurog2 T149D results in a loss of function. In order to characterize the effects of the T149A and T149D mutations on the human NEUROG2 properties, we used a well-known protocol to reprogram iPSCs into neurons within just a few days by overexpressing NEUROG2, as initially described in (Zhang et al., 2013). We used an all-in-one plasmid containing the rtTA-inducible expression of the human NEUROG2 linked to eGFP (see Figure 5A). The control iPSC line was transduced with three versions of this plasmid encapsulated in a lentiviral vector for genetic insertions: NEUROG2 WT, NEUROG2 T149A, and NEUROG2 T149D. The transduced cells were purified using puromycin in the culturing medium. On day 0, the medium was replaced with a doxycycline-containing medium to induce the expression of NEUROG2 that was detected after one day (Figure 5B & 5C). However, even after selecting the transduced iPSCs using puromycin resistance selection pressure, some non-transduced iPSCs survived and proliferated in neuronal cultures. To address their proliferation, we applied cytarabine (ara-c), an inhibitor of DNA synthesis, to the culturing medium for 3 days followed by a thorough wash to avoid any toxicity. After 6 days of overexpression, we quantified the proportions of MAP2-positive cells, a general marker for neurons, among all the transduced cells stained with GFP that are expected to express NEUROG2 (Figure 6A). Our analysis revealed a remarkable efficiency of 99.6% conversion of iPSCs into MAP2+ neurons when NEUROG2 WT is expressed; however, there was a significant decrease in iPSCs' conversion with NEUROG2 T149A resulting in only 62.3% iPSCs' transformation into neurons. Moreover, NEUROG2 T149AD completely failed to convert iPSCs into neurons at 0% efficiency (Figure 6B).

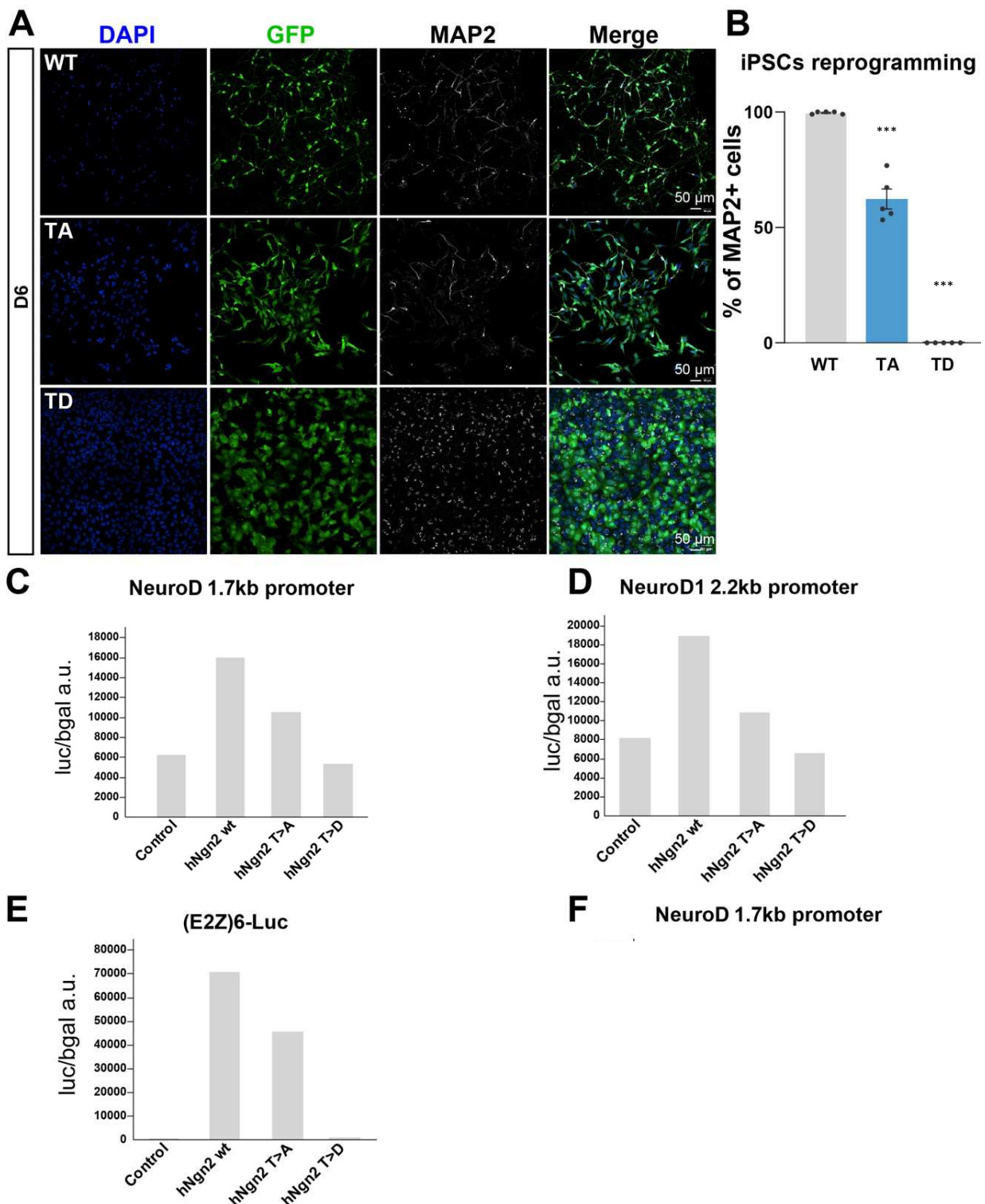
Surprisingly, these results demonstrate that the NEUROG2 T149A is less efficient in driving iPSCs conversion into neurons compared to NEUROG2 WT, while its overexpression in mice cortical progenitors results in a depletion of the pool of progenitors and an increased production of neurons similar to an overexpression of NGNG2 WT. Thus, to elucidate the potential mechanisms behind this reduced efficiency, we studied the transactivation properties of our mutants with transcriptional reporter assays for *Neurod1* (Figure 6C & 6D) and a multimerized version of *Ngn2* E-boxes from the *Dll1* gene promoter named (E2Z)6-luc (Figure 6E). The different mutants and the control NEUROG2 were expressed using the same construct used in the iPSCs reprogramming experiment in P19 cells which do not express any endogenous NEUROG2. These cells were transfected with plasmids containing two versions of the *Neurod1* promoter: a short 1.7kb version and a longer one with 2.2kb and an artificial promoter of *Dll1*. We found that NEUROG2 T149D is unable to induce the expression of any of the promoters, suggesting a complete loss of transcriptional activation for the well-known target genes, whereas the NEUROG T149A mutant still transactivates its target genes *Neurod1* and *Dll1*, but to a much lesser extent compared to NEUROG2 WT (Figure 6C & 6D & 6E).

These results are consistent with the results found by Quan et al., 2016 that T149 phosphorylation leads to a loss of Neurog2 function at the protein level upon overexpression in mouse cortical progenitors. Surprisingly, however, the NEUROG2 T149A mutant is less efficient in driving iPSCs conversion into neurons through potentially decreased transactivation capacities of *Neurod1*, suggesting that the human NEUROG2 T149A mutation results may also result in a loss of function.



**Figure 5: Protocol of iPSCs conversion into neurons through NEUROG2 overexpression.**

(A) Schematic representation of the protocol resulting in the production of neurons of the upper layer of the neocortex (SATB2+) after 35 days in culture; (B) Immunostaining for DAPI, GFP and NEUROG2 on transduced iPSCs before the induced expression of NEUROG2 construct; (C) Immunostaining for DAPI, GFP and NEUROG2 after 1 day post induction.



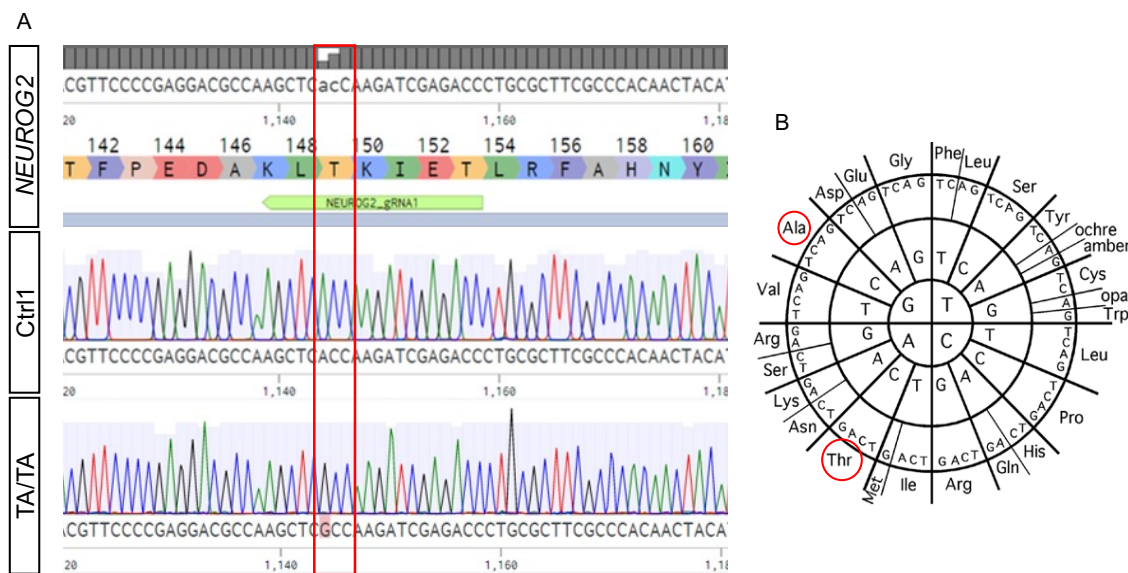
**Figure 6: NEUROG2 mutants present lower abilities to convert iPSCs into neurons and are less potent in the transactivation of their target genes.**

(A) Immunostaining for DAPI, GFP and MAP2 6 days post induction of NEUROG2 expression; (B) Quantification of the proportions of iPSCs converted to neurons; (C) Schematic of the transcriptional reporter assay; (D) Transcriptional reporter assay with the long version of the Neurod1 promoter; (E) Quantifications of the reporter assay with the artificial promoter; (F) Quantifications of the reporter assay with the short version of the Neurod1 promoter.



## The loss of NEUROG2 phosphorylation site T149 by its replacement with an alanine result in a gain of function

To explore the potential impact of phosphoregulation on NEUROG2 and its role in neurogenesis in the developing human neocortex under endogenous levels, we generated an iPSC clone with a single nucleotide substitution in the Threonine 149 codon to replace it with an Alanine with CRISPR/Cas9 in order to prevent phosphorylation of NEUROG2 at this site. First, we sequenced *NEUROG2* in iPSCs clones to verify the mutation T149A at both DNA levels (Figure 7A) and RNA levels in our cortical organoids at day 70 (data not shown) and found the proper mutation. We further analyzed the modified clone and its corresponding control to validate the absence of CRISPR/Cas9 related insertion and deletion in the genome with Bionano and its optical genome mapping technology (data not shown).



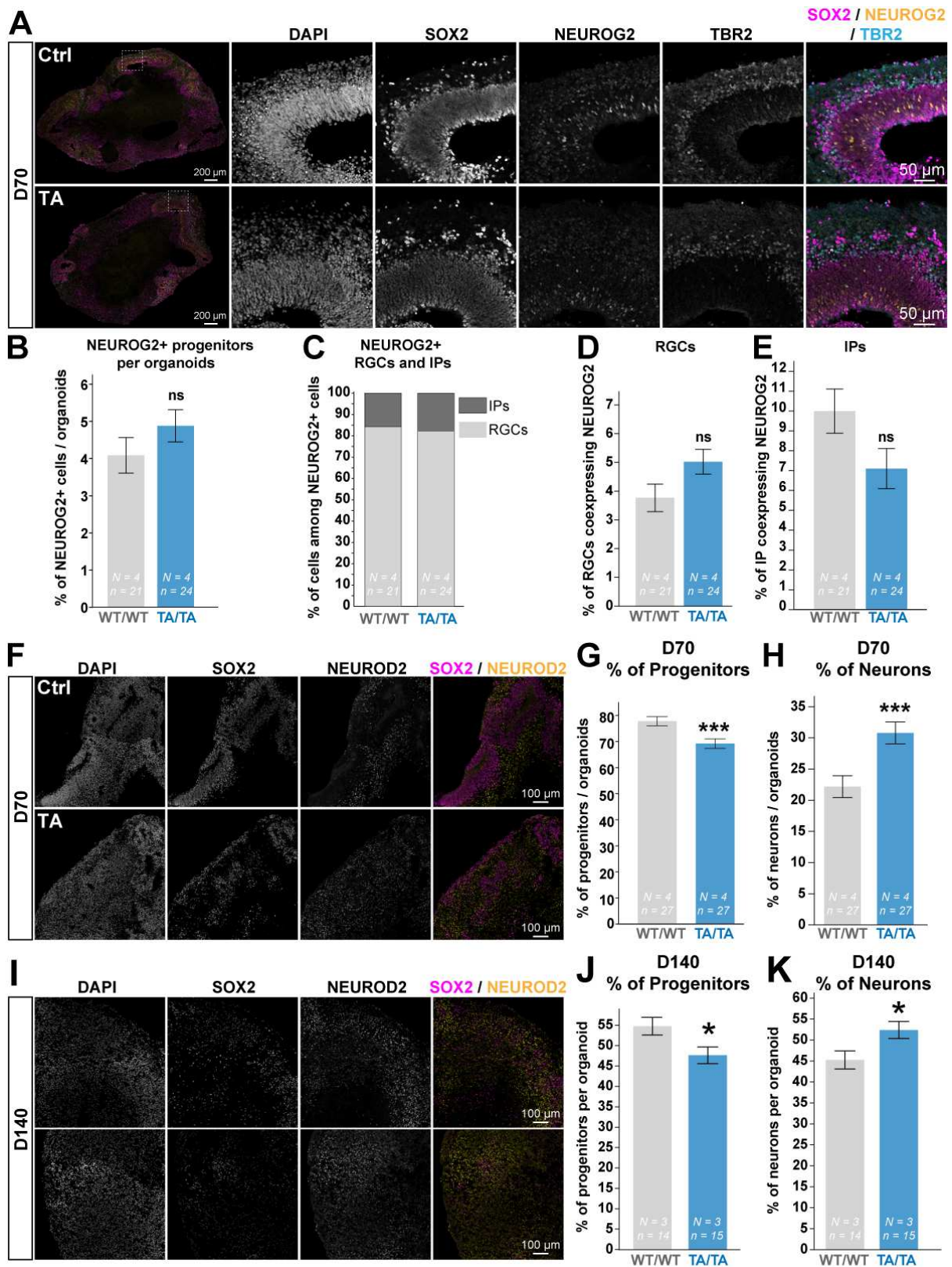
**Figure 7: *NEUROG2* sequencing after CRISPR/Cas9 remodification in human iPSCs.**

(A) Alignment of the sequencing results of the control clone and the phosphomutant; (B) Amino acids code.

Second, we characterized if this mutation affected NEUROG2 T149A expression among all progenitors present in cortical organoids at day 70 using immunostaining for SOX2 (all progenitors), NEUROG2, and TBR2 (IPs) (Figure 8A). The analysis revealed that  $4\% \pm 0.5\%$  of all the progenitors expressed NEUROG2 in the control organoids, while  $4.9\% \pm 0.4\%$  of them expressed it in the phospho-mutant TA organoids, with no significant differences observed between them (Figure 8B). Among these NEUROG2<sup>+</sup> cells, we found in the control

that  $84.2\% \pm 2\%$  are RGCs, and the remaining  $15.8 \pm 2\%$  are IPs. Similarly, we found that among the NEUROG2 T149A+ cells, we find  $82.3 \pm 1.9\%$  of RGCs, and the remaining  $17.7\% \pm 1.9\%$  are IPs, suggesting that the phospho-mutation does not change the proportion of progenitors expressing NEUROG2. Importantly, these data show that, in human cortical organoids, most cells expressing NEUROG2 are RGCs. Additionally, we observed that on day 70 in the whole organoids, an average of only  $3.8\% \pm 0.5\%$  of all RGCs (SOX2+/TBR2-) coexpress NEUROG2 in the control compared to  $5\% \pm 0.4\%$  in the phosphomutant (Figure 8D). In contrast, the proportion of IPs expressing endogenous NEUROG2 represents  $10\% \pm 1.1\%$ , while it's at  $7.1\% \pm 1\%$  in the phospho-mutant organoids (Figure 8E) suggesting that the NEUROG2 T149A mutation does not change the proportions of progenitors expressing it.

After validating that the pattern of NEUROG2 expression was not altered after the phospho-mutation, we then assessed the proneural activity of the phosphomutant by quantifying the proportions of all progenitor subtypes (SOX2+) versus all excitatory neurons (NEUROD2+) through immunostaining at both mid and late stage of cortical organoids development (corresponding to days 70 and 140) (Figure 8F & 8I). Across four separate 3D differentiations of control and phosphor mutant iPSC clones, our analysis revealed significant differences in cellular composition. At day 70, control organoids predominantly consisted of progenitor cells, accounting for  $77.8\% \pm 1.7\%$  of the total cell population, with neurons comprising the remaining  $22.2\% \pm 1.7\%$ . In contrast, the phospho-mutant organoids exhibited a statistically significant reduction in progenitor cell proportions to  $69.2\% \pm 1.7\%$ , whereas neuronal populations increased to  $30.8\% \pm 1.7\%$ . This statistically significant increased neuronal proportion at the expense of progenitor cells was also observed at the later development stage, at day 140, with control organoids containing  $57.8\% \pm 2.1\%$  progenitor cells compared to  $47.6\% \pm 2\%$  in the mutant organoids. Conversely, neuronal populations were more prevalent in the phospho-mutant organoids, representing  $52.4\% \pm 2\%$ , as opposed to  $44.2\% \pm 2.1\%$  in the control group. These findings suggest that the NEUROG2 phospho-mutation exerts a significant influence on the differentiation trajectory of cortical progenitors favoring neuronal over progenitor cell fate.

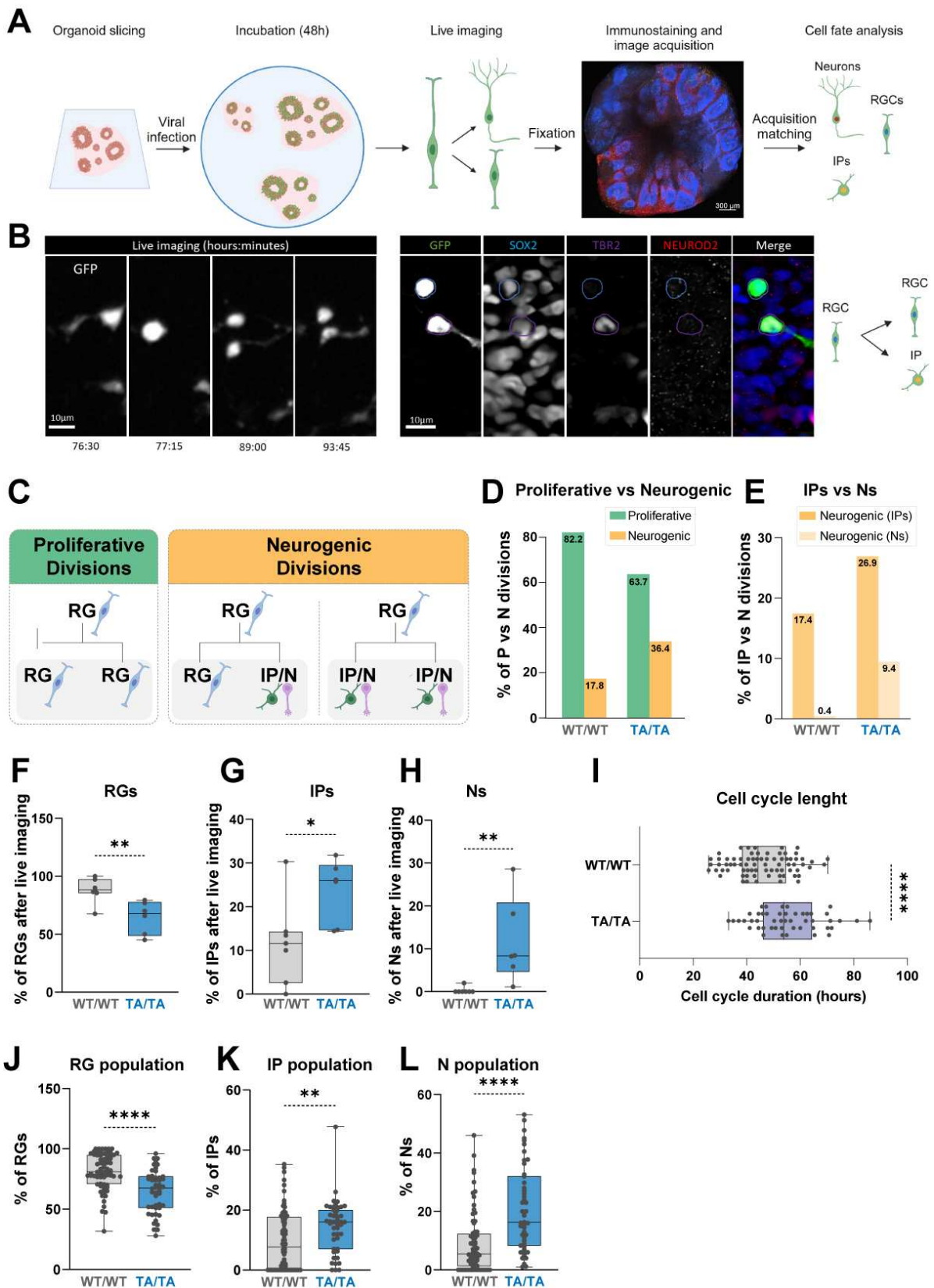


**Figure 8: NEUROG2 T149A is a gain of function leading to increased proportions of neurons at both mid and late stages of cortical organoids development.**

(A) Immunostaining for SOX2, NEUROG2 and EOMES on day 70 whole slices of cortical organoids illustrated with a zoom onto the rosettes; (B) Quantification of the proportions of NEUROG2+ cells per organoids at day 70 in both control and phosphor-mutant; (C) Proportions of RGCs and IPs among the NEUROG2+ cells found in cortical organoids at day 70; (D) Quantification of the proportion of RGCs coexpressing NEUROG2 among all RGCs present in the organoids at day 70; (E) Quantification of the proportion of IPs coexpressing NEUROG2 among all IPs present in the organoids at day 70; (F) Immunostaining for SOX2, NEUROD2 on day 70 cortical organoids; (G) Quantifications of the proportions of progenitors found in these stained cortical organoids at day 70 in both control and mutant clones; (H) Quantifications of the proportions of neurons found in these stained cortical organoids at day 70 in both control and mutant clones; (I) Immunostainings for SOX2 and NEUROD2 on day 140 cortical organoids; (J) Quantifications of the proportions of progenitors found in these stained cortical organoids at day 140 in both control and mutant clones; (K) Quantifications of the proportions of neurons found in these stained cortical organoids at day 140 in both control and mutant clones.

## **NEUROG2 T149A favors neurogenic divisions of progenitors**

To more directly measure the effect of NEUROG2 T149A mutation on the division pattern of progenitors, we used a method developed by (Coquand et al., 2024a) to identify the fate acquired by daughter cells following the progenitor divisions in week 7 to 9 cortical organoids. Briefly, cortical organoids were sliced before their transduction with a retrovirus expressing GFP to transfect all the dividing cells, such as aRGCs, bRGCs or IPs. These divisions were then followed through live imaging for four days for week 7 slices also to measure the cell cycle length, while week 9 slices were imaged for two days. Next, the transduced slices were fixed and stained for the cell fate markers, SOX2, EOMES, and NEUROD2, before whole slice imaging. During these processes, the live imaged positions on the cortical organoid slices were lost. Thus, a semi-automatic program realigned the movies to the corresponding slices mounted on the microscope slides to correlate the types of divisions with the cell fate of the daughter cells (Figure 9B). Before diving into the division maps of progenitors, it's important to note that using retrovirus labeling made differentiating between RGCs and IPs challenging as they are purely based on two imprecise criteria: their morphology and their location in the cortical organoids. To partially circumvent this problem, we examined the expression of HOPX in our rosettes and found very few HOPX<sup>+</sup> cells (as shown in Figure 1E), suggesting that most RGCs at this stage are aRGCs. Second, we deduced the fate of the dividing progenitor based on the fate of its daughters: by definition, any division resulting in at least one RGC daughter cell must have occurred in an RGC mother cell, while any division producing two differentiated daughters (IPs or neurons) is almost certainly performed by an IP. These two criteria allowed us to quantify the patterns of division of the labelled progenitors we traced (Figure 9C).



**Figure 9: NEUROG2 T149A changes the pattern of RGCs divisions from proliferative to neurogenic divisions.**

(A) Schematic representation of the cortical organoids treatment for live imaging and the correlated immunostaining; (B) Live images and its correlated immunostaining for the cell fate markers SOX2, EOMES and NEUROD2 illustrating a asymmetric neurogenic divisions producing an IP and a RGCs; (C) Potential types of proliferative and neurogenic divisions; (D) Quantifications of divisions types among all the observations of live imaging; (E) Quantifications of the divisions responsible for the production of IPs and neurons among all the neurogenic divisions; (F) (G) (H) quantifications of the proportions of RGCs, IPs and neurons among all the cells followed during the live imaging; (I) Quantification of the duration of the cell cycle length of RGCs among all the cells followed by live imaging in both control and phosphor mutant cortical organoids at week 7, corresponding to approximately day 50; (J) (K) (L) Quantifications of the proportions of RGCs, IPs and neurons among all the GFP cells selected randomly all across the cortical organoid slices at both week 7 and 9.

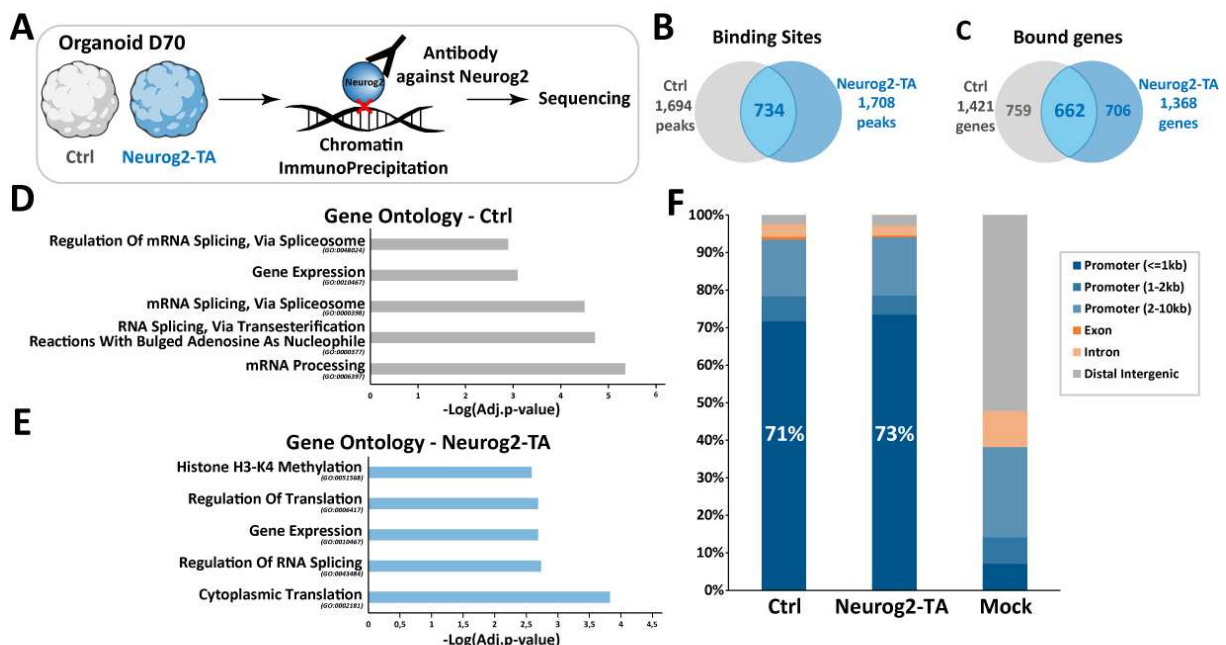
stage of cortical organoid development, the majority of divisions made by RGCs were proliferative and self-amplifying, accounting for 82.2% of divisions, while the remaining 17.8% were neurogenic divisions in the control clone. In contrast, phospho-mutant cortical organoids exhibited a significant shift towards neurogenic divisions, which accounted for 36.4% of all divisions at the expense of proliferative divisions by RGCs, representing 63.7% of all divisions (Figure 9D). Further examination of neurogenic divisions reveals that in the control clone, out of the 17.8%, 17.4% are dedicated to IP production and only 0.4% to neuronal production. In the phospho-mutants organoids, out of the 36.4% of neurogenic divisions found, 26.9% account for IP production and 9.4% for neuron production. The phospho mutant not only switches the pattern of RGCs divisions toward neurogenic behavior but also increases the proportion of neurogenic divisions producing IPs and neurons compared to the control, suggesting a strong bias toward indirect neurogenesis. Furthermore, there is a correlation between the commitment to the neurogenic division of a progenitor and its cell cycle length. A longer cell cycle correlates with neurogenic division (Dehay & Kennedy, 2007). Thus, we measured the cell cycle length of RGCs across several rounds of proliferative divisions and found that, on average, RGCs in control organoids divide every 44 hours while RGCs from the phospho-mutant organoids divide every 55 hours, accounting for a significant increase of 11 hours in the cell cycle duration.

We then looked at the proportions of cell types after live imaging in the movies that we collected and found that among all the GFP positive cells in the control organoids, 88% were RGCs while they represent 65.6% of the GFP+ cells in the phospho mutant (Figure 9F). We also found an increased proportions of both IPs in the mutant with 23.6% compared to 12% in the control (Figure 9G) and neurons with 11.8% of neurons compared to 0.4% in the control (Figure 9I). In order to confirm these results, we manually selected random small regions of interest all over the different slices of both control and phospho-mutant cortical organoids that were used for the live imaging. We observed the exact same pattern: a depletion of RGCs representing 69% of the GFP+ cells in the mutant compared to 85% in the control (Figure 9J), an increased proportions of both IPs representing 15% of GFP+ cell sin the TA compared to 9% in the control (Figure 9K) and neurons accounting for 16% in the mutant compared to 6% in the control (Figure 9L).

Thus, we observe that the NEUROG2 T149A mutation results in a clear gain of function with a shift of RGCs to undergo neurogenic divisions favoring indirect neurogenesis through the production of more IPs resulting in a higher production of neurons.

## The primary cilium, a potential organelle for NEUROG2 T149A gain of function

To further understand the action mechanisms employed by the phospho-mutant to enhance neuronal production from progenitors during mid-stages of cortical organoid development, we used Chromatin Immunoprecipitation to identify the binding sites of NEUROG2 and NEUROG2 T149A at day 70 in cortical organoids. Sequencing of DNA fragments bound by NEUROG2 resulted in 1,694 peaks, with more than 90% located in promoter regions (Figure 10B & 10F). In contrast, sequencing of DNA fragments bound by NEUROG2 T149A yielded slightly more peaks, with over 90% also found in promoter regions (Figure 10B & 10F). The endogenous NEUROG2 was found to bind to 1,421 genes, while the phosphor-mutant bound slightly fewer genes with 1,368 (Figure 10C). Among these genes, both endogenous NEUROG2 and NEUROG2 T149A were shown to bind 662 genes in common, corresponding to approximately half of all identified genes. Further gene ontologies highlighted gene regulation processes for both types, such as gene expression and regulation of RNA splicing, without major changes in their roles or functions (Figure 10D & 10E). These results suggest that the mutant NEUROG2 T149A presents a distinct binding pattern of genes compared to the endogenous NEUROG2 in cortical organoids at day 70, which are involved in very general biological processes such as gene regulation.



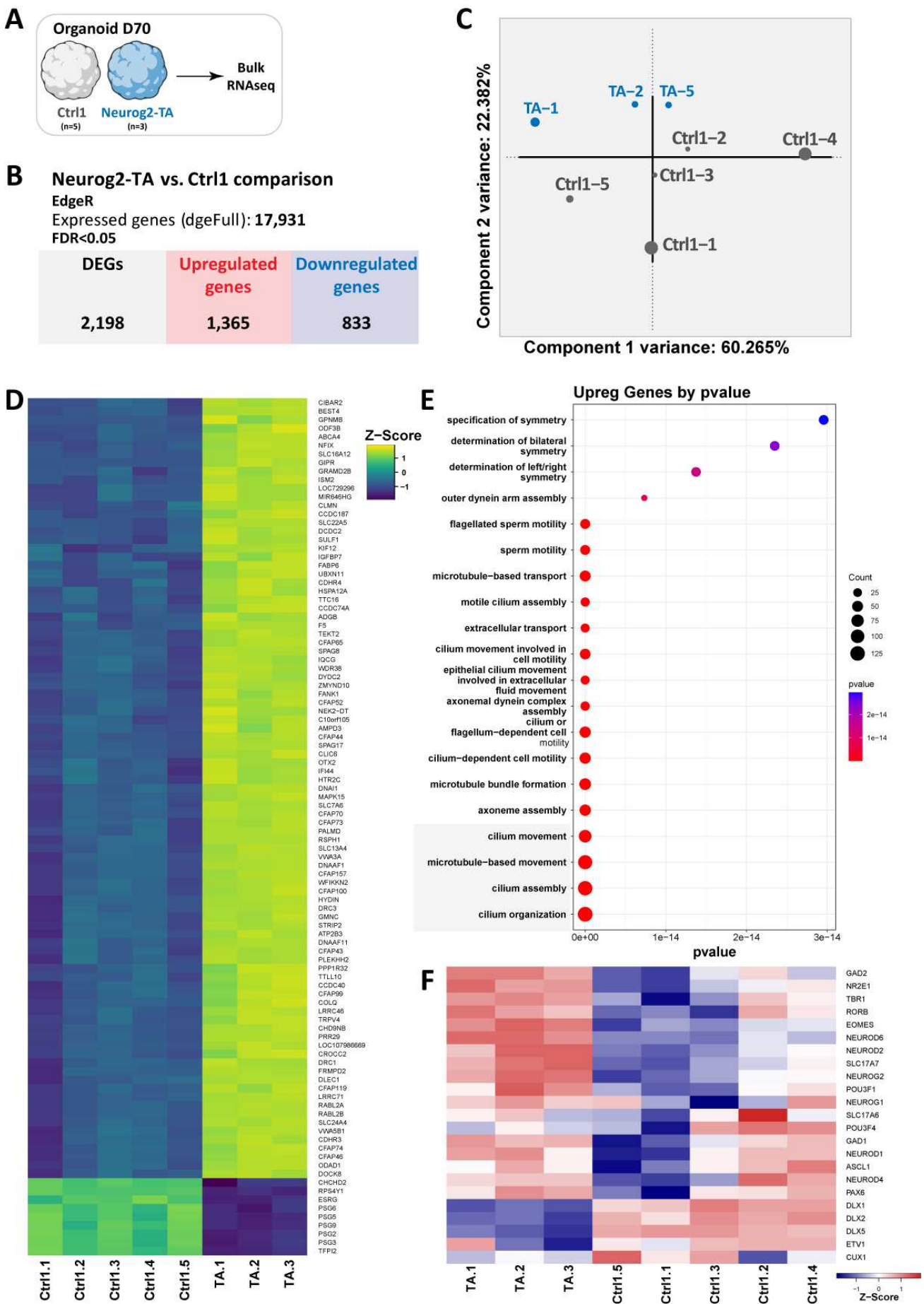
**Figure 10: Binding properties of NEUROG2 T149A compared to the WT NEUROG2 in cortical organoids at day 70.**

(A) Schematic representation of the protocol followed for ChIP on day 70 cortical organoids of both control and phosphomutant clones; (B) Venn diagram of the binding sites, representing peaks; (C) Venn diagram of the bound genes; (D) (E) Gene ontologies of all the genes bound by the WT NEUROG2 and the NEUROG T149A mutant; (F) Quantification of the location of binding sites in the human genome.

However, to measure the potential impact of the change in the binding pattern on the biological properties of cortical progenitors, we performed bulk RNA sequencing on both control and mutant cortical organoids at the same time point as the ChIP experiment, day 70 (Figure 11A). We found that 2,198 genes were differentially expressed, with 1,365 genes upregulated and 833 genes downregulated by the phospho mutation of NEUROG2 T149A (Figure 11B). These differences resulted in PCA plots in a clear separation of the control samples and the TA mutants suggesting potential differences (Figure 11C). Among the 100 genes, the most differentially expressed, we found that 91 were upregulated in the NEUROG2 T149A mutant suggesting that this mutation induces a stronger expression of many genes, including many genes important for the primary cilium such as KIF12 or CFAP70. Thus, we analyzed the gene ontologies of all the upregulated genes and found 10 GO terms associated with primary cilium organization and function (Figure 11E), similar to the GO terms found in the downregulated genes in the NEUROG2 KO clones (Figure 4E). We then combined both RNA bulk sequencing datasets obtained from NEUROG2 KO clones with NEUROG2 T149A and their corresponding controls to focus on the primary cilia genes. We found in this combined dataset that out of 160 genes related to primary cilium, 121 of them were commonly differentially expressed in both KO clones and TA mutant. Indeed, all the genes were downregulated in the KO clones and upregulated in the NEUROG2 T149A mutant (Figure 12A & 12B), suggesting a strong link between the primary cilium, NEUROG2, and its phosphorylation status.

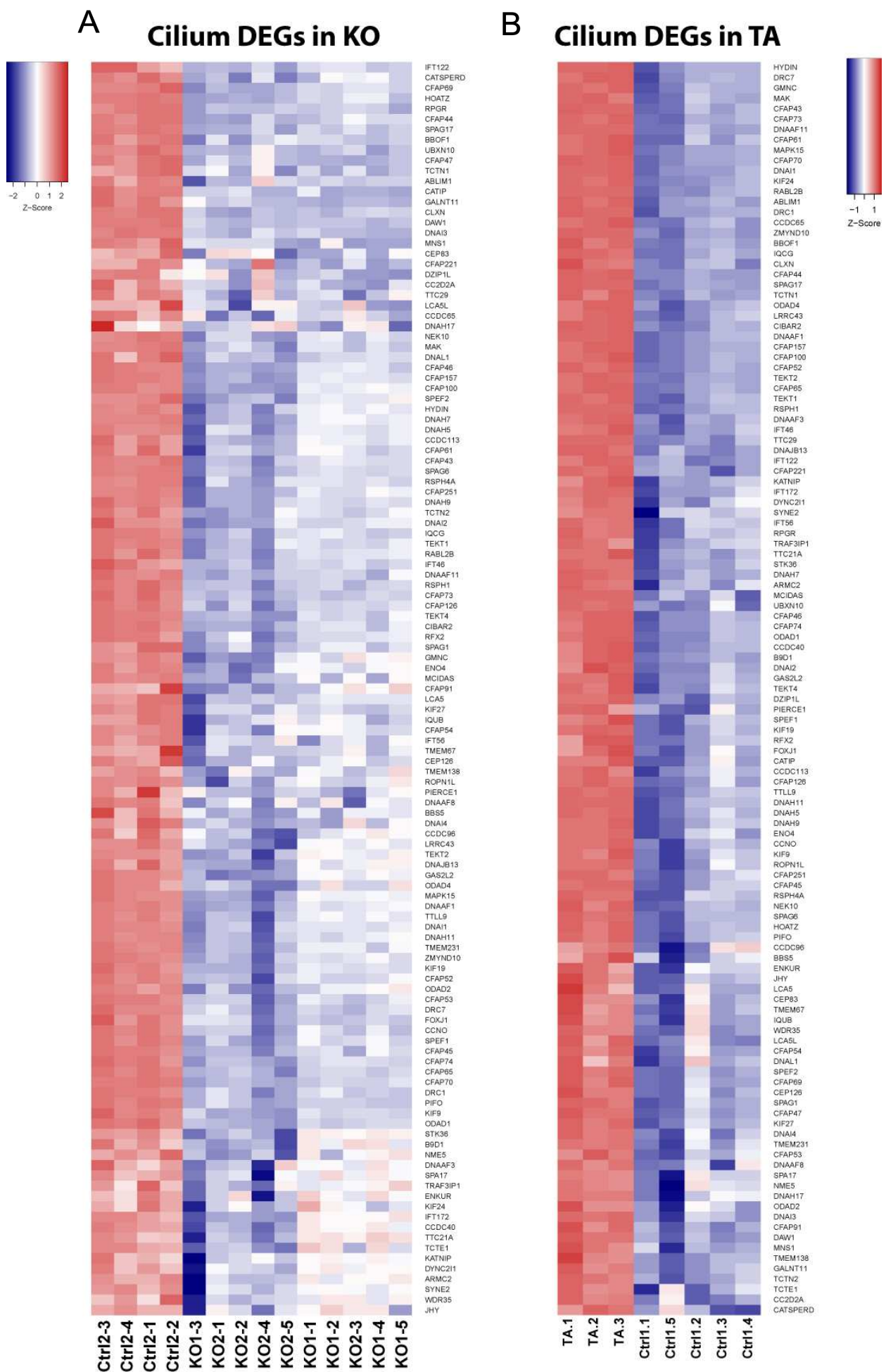
These data suggest that the increased neurogenic drive observed in the NEUROG2 T149A mutant is not due to a stronger proneural activity per se, as shown by the decreased transactivation of *NEUROD1* (Figure 6C & 6D), but rather through a change in the specificity of target gene regulation involving genes that regulate the primary cilium.





**Figure 11: NEUROG2 T149A increases the expression of genes related to primary cilium organization and function.**

(A) Schematic representation of control and phosphomutant cortical organoids at day 70 for bulk RNA sequencing; (B) Table illustrating all the differentially expressed genes among the phosphomutant compared to the control; (C) PCA analysis of the control and phosphomutant clone with their respective replicates; (D) Heatmap of the 100 genes the most differentially expressed; (E) Gene ontology of all the upregulated genes in the phosphomutant cortical organoids; (F) Heatmap of selected genes involve in the dorsal and ventral telencephalon.



**Figure 12: Genes related to primary cilium organization and function are downregulated in NEUROG2 KO clones and upregulated in the NEUROG2 T149A clone in cortical organoids at day 70.**

(A) Heatmap of cilia genes in the two NEUROG2 KO clones compared to their respective control; (B) Heatmap of cilia genes expression in the phosphor mutant clone compared to its respective control.

# Discussion

## **The loss of NEUROG2 in human cortical progenitors results in the loss of neurons in cortical organoids**

Neocortical expansion observed in primate brains relies on the ability of cortical progenitor cells to produce more neurons, with approximately a 1000-fold increase in the number of neurons present in the human neocortex with 16 billion neurons compared to the mouse neocortex with 14 million (Herculano-Houzel, 2012). However, it is unclear how such proportions of neurons can be produced from human cortical progenitors with a cell cycle length significantly longer than mice cortical progenitors combined with only a 10-fold increase in the duration of neurogenesis. It is thought that this is due to the amplification of all the different progenitor subtypes of the developing dorsal telencephalon, starting from the self-amplification of NECs, aRGCs, followed by a rare progenitor subtype in the mouse neocortex but very abundant in the human neocortex, the bRGCs. Thus, the regulation of the type of division these progenitors undergo is of crucial importance for the generation of the proper amount of neurons in the developing neocortex. As we have seen, the balance between progenitor self-amplification and differentiation is mediated by feedback regulation of the Notch signaling pathway and the proneural protein, NEUROG2 that can be influenced by the different molecules and morphogens found in the local environment of the progenitors in which they are expressed. Furthermore, new evidence demonstrates that post-translational modifications such as phosphorylation could modulate the proneural activity of Neurog2 in mouse developing neocortex, potentially in a context-dependent manner with the requirement of specific kinases. Nevertheless, very little is known about the role of NEUROG2 in the developing human neocortex and how such post-translational modifications could regulate its activity under endogenous levels.

We demonstrated for the first time in human cortical organoids that the loss of NEUROG2 in CRISPR/Cas9-engineered iPSCs clones results in a 2.5-fold decrease of neurons at day 140 corresponding to late stages of cortical organoid development where mostly upper layer neurons are produced. At mid stages of cortical organoid development, we observe a relative decrease in the percentages of neurons per organoid that isn't statistically significant but suggests a trend that we will have to investigate further. Complementary to these results, we found through bulk RNA sequencing at day 70 a strong downregulation of the genes found in

cortical IPs (*EOMES*) and cortical neurons (*NEUROD2*, *NEUROD4*, *NEUROD6*) and more specifically of deep layer neurons (*TBR1* positive cells) with a relative increase in the genes involved in the ventral telencephalon identity with *ASCL1*, *DLX1*, *DLX5*, *GAD1*, *GAD2* in the *NEUROG2* KO cortical organoids compared to control. This suggests that the loss of *NEUROG2* in developing human cortical organoids results in a shift of identity from the dorsal telencephalon to the ventral telencephalon and a potential loss of cortical neurons in the deep layers. Thus, *NEUROG2* not only activates a dorsal fate with cortical-specific pathways but also represses at the same time ventral telencephalic programs which is consistent with what is known about the mouse *Neurog2* functions in the developing mouse neocortex (Schuurmans et al., 2004). Interestingly, we observed a downregulation of *NEUROG1* in *NEUROG2* KO clones, indicating that *NEUROG1* does not compensate the void left by *NEUROG2*'s absence in cortical progenitors. This suggests a degree of functional specificity among the *NEUROG* family members in cortical development, with *NEUROG1* potentially playing a non-redundant role in specifying dorsal telencephalic fate. On the other hand, the upregulation of *ASCL1* hints at a compensatory mechanism that switches the cellular fate of the cortical progenitors toward a ventral telencephalic identity encompassing inhibitory neurons as suggested by the *GAD1* and *GAD2* upregulation but also a potential oligodendrogenesis fate at later stages. The regulation of *ASCL1* and its contribution to ventral fate adoption in the absence of *NEUROG2* highlight, on the one hand, a complex interplay of proneural genes in determining the identity and fate of cortical progenitors and, on the other hand, a remarkable plasticity of progenitors during neocortical development.

Furthermore, we are currently characterizing the identity of the excitatory neurons produced at both the mid and late stages of cortical development of the organoids. These analyses are particularly important because mouse *Neurog2* is thought to drive mainly the production of deep-layer neurons and not the production of upper-layer neurons that rely mostly on *Pax6* and *Tlx* (Schuurmans et al., 2004). Thus, the outcome of these quantifications at both days 70 and 140 will determine if the mouse *Neurog2* and the human *NEUROG2* play the same role in excitatory specification of progenitors during cortical development. If *Neurog2* and *NEUROG2* share, similar activities in the neuronal specification identity in the neocortex implies that in *NEUROG2* KO cortical organoids at day 70, we should observe a significant decrease in the proportions of neurons of the deep layers (*TBR1* and/or *CTIP2* positive cells) whereas, at day 140, we should see an overall decrease in cortical neurons number (*NEUROD2*<sup>+</sup>) due to the major loss of deep layer neurons but conserved proportions of upper layer neurons (*SATB2* or

BRN2 positives cells). However, if this prediction is not true, it would suggest that Neurog2 and NEUROG2 might have different roles in the neuronal identity specification, highlighting potential species-specific activities.

## **NEUROG2 and the production of upper-layer neurons**

It is thought that the main drivers of neurogenesis of upper-layer neurons in the developing mouse neocortex are both Pax6 and Tlx, as the loss of Neurog2 at E15.5 does not alter the production of upper-layer neurons (Schuurmans et al., 2004). However, can NEUROG2 induce the specification of upper-layer neurons from cortical progenitors? Overall, in the protocol of direct generation of neurons from mouse ESCs and human iPSCs, among the excitatory glutamatergic neurons generated, most cortical neurons have an upper layer identity (SATB2+ corresponding to layer 2/3 of the neocortex), and very few express markers of the deep layers (TBR1 or CTIP2). As a matter of fact, to generate deep layers neurons from NEUROG2 overexpression, iPSCs have to be pretreated with specific molecules such as SB431542 and LDN, generally used in guiding iPSCs differentiation into a dorsal forebrain identity. Therefore, these results demonstrate the capacities of NEUROG2 to differentiate pluripotent stem cells into upper-layer neurons by skipping the multipotent progenitor phase, suggesting that this can be done in RGCs and IPs in the developing human neocortex. However, it remains unknown whether NEUROG2 is the main driver of upper-layer neurons in the human neocortex, which will be investigated with our NEUROG2 KO clones.

## **NEUROG2 T149A shows reduced transactivating properties under overexpression experiments**

After demonstrating that NEUROG2 plays a major role in cortical neurogenesis in human cortical organoids, we addressed the regulation of its proneural activity through the Threonine 149 phosphorylation site. The results from Quan et al., 2016 already demonstrated that the Neurog2 T149D mutation results in a loss of proneural function when overexpressed in mouse cortical progenitors while the Neurog2 T149A, like Neurog2 overexpression results in a gain of function with a depletion of the progenitor pool and an increased production of the neurons of the deep layer. We found through iPSCs conversion into neurons through overexpression of NEUROG2 that the NEUROG2 T149D results in a complete loss of function with no iPSCs

conversion into neurons and no transactivation of *Neurod1*. However, as the data shown in Quan et al., 2016 suggested, the human NEUROG2 T149A is not converting iPSCs as efficiently as the NEUROG2 WT and even present lower transactivation properties of *Neurod1* on both long and short promoters. Thus, it seems that under overexpression conditions, NEUROG2 T149A shows a partial loss of function. It would be of great value to repeat these experiments in cortical progenitors as iPSCs are not purposed to produce neurons.

### **NEUROG2 T149A, a gain of function?**

However, under endogenous levels NEUROG2 T149A does not alter the pattern of NEUROG2 expression among RGCs and IPs but induces a switch from proliferative to neurogenic divisions by RGCs with a tendency to produce more IPs and a relatively increased proportion of neurons. Thus, it appears that the unphosphorylated NEUROG2 on the Threonine 149 drives the transition from RGCs to IPs that results in increased production of neurons of 8% compared to the control, demonstrating a gain of function. These results in perspective of the partial loss of function of NEUROG2 to transactivate *NEUROD1* and *DLL1* are

However, is this mutation causing a gain of NEUROG2 global activity? To answer this basic question, we first need to define what the activity of a transcription factor is. A TF is active when it binds the promoters of its target genes and induces or represses their expression. Neurog2 being mostly an activator of gene expression in the dorsal telencephalon (Kovach et al., 2013), let's explore what could be a gain of activity:

- 1) A more active TF could increase the expression of its target genes and/or accelerate this expression.
- 2) It could also bind more target genes, thus becoming a more active transcription factor by inducing the expression of more genes.

ChIP analyses show that the NEUROG2 TA mutant does not bind more genes than the control NEUROG2. It actually binds fewer genes, 1,368 genes bound by NEUROG2 T149A compared to 1,421 genes bound by NEUROG2 WT. Thus, condition 2 is not met. If we now look at well-known target genes such as *NEUROD1* or *DLL1* with the transcriptional reporter assay, we find a reduced capacity of NEUROG2 T149A to transactivate these genes compared to the WT NEUROG2. Thus, the gain of function phenotype described above is not due to an increased

proneural activity but might be due to the antenna of the cells, the primary cilium. Indeed, bulk RNA sequencing showed an upregulation of 121 out of 160 genes involved in the primary cilium assembling, organization and motility, which all the 121 genes are downregulated in the NEUROG2 KO cortical organoids at day 70 compared to their respective controls. These results suggest a strong link between NEUROG2 and the primary cilia genes and, thus, an additional correlation between internal and external cues influencing cortical progenitors' fate. Indeed, the primary cilium, in aRGCs is found at the apical surface facing the CSF and is sensing the different morphogens and growth factors present such as Shh, Wnt3a, Wnt5a, fgf2 and IGF, which are key players in dorso ventral patterning and have a strong influence on the proliferative and neurogenic divisions undergone by the cortical progenitors (Lehtinen et al., 2011; Lehtinen & Walsh, 2011). Interestingly, in bRGCs and IPs, that are found in the SVZ, have a primary cilium located on their basal pole of (Paridaen et al., 2013b; Wilsch-Bräuninger et al., 2016; Wilsch-Bräuninger & Huttner, 2021) suggesting that aRGCs and bRGCs/IPs might not be influenced similarly to the various morphogens and factors. This distinction in cilium positioning and, by extension, in signal reception between aRGCs and bRGCs/IPs hints at a nuanced mechanism by which NEUROG2 might influence the developmental trajectory of cortical progenitors down the differentiation landscape through primary cilia-mediated pathways. Given the critical role of primary cilia in neural development, disruptions in their function, known as ciliopathies, can lead to profound neurodevelopmental disorders, highlighting the importance of further investigating the relationship between NEUROG2, primary cilium functionality, and cortical development.

### **The primary cilium, NEUROG2 and its phosphorylation state**

This is the first time that a study on proneural genes shows a putative link between Neurogenin 2, its phospho-regulation, and its consequences on the primary cilia gene expression. The primary cilium is found in almost all vertebrate cells. Although it is known to play a role in the neurogenesis of the neocortex, all the mechanisms at work are not identified. Therefore, one of the goals of our work would be to establish the precise gene regulatory network implicated in this regulation and to study how it regulates neurogenesis. To this end, we would have to determine the direct target genes of NEUROG2 by first selecting the genes bound by it, using the ChIP experiment, and selecting among these the ones with a downregulated expression in the NEUROG2 KOs. Among the genes kept, we would have to investigate:

- 1) If there are any genes related to the cilia organization and function in the direct targets of NEUROG2.
- 2) Are there any transcription factors that regulate all the different cilia genes either directly or indirectly through different signaling pathways?

Following the identification of the potential GRN linking NEUROG2 to the primary cilium, we would have to inhibit and rescue the signaling pathways to demonstrate a direct and causal link. Thus, the identification of the link between NEUROG2 and the primary cilium would also allow us to investigate further the role of the NEUROG2 T149 phosphorylation site in regulating this GRN. So far, we have observed many genes responsible for the primary cilium's organization and functioning upregulated, but how that translates phenotypically remains to be elucidated. One hypothesis is that through regulating the organization and functioning of the primary cilia, NEUROG2 might regulate the primary cilium's functionality, thereby affecting the progenitors' responsiveness to external cues. Thus, beyond its proneural activity, NEUROG2 would also define windows of responsiveness of cortical progenitors potentially influencing proliferative versus neurogenic divisions. This regulatory mechanism would ensure that neuronal differentiation and the expansion of the progenitor pool are tightly coordinated with the developmental stages and the local environment of the developing neocortex.

Moreover, the phosphorylation state of NEUROG2 emerges as a pivotal factor in this potential regulation of progenitors' responsiveness through the primary cilium. Through the broad range of potential kinases expressed in cortical progenitors, it is proposed that specific cellular contexts might induce the phosphorylation of NEUROG2, which subsequently exerts differential effects on primary cilium assembly, structural organization, and possibly motility. One possible candidate that is important for cilia disassembly before mitosis (G. Wang et al., 2013) and seems to be partly regulated by Wnt signaling (K. H. Lee et al., 2012), is PLK1, which we know as a strong candidate for the phosphorylation of the Threonine 149 of the mouse Neurog2 (Quan et al., 2016).

This hypothesis underscores the multifaceted roles of NEUROG2 in neural progenitor cell fate decisions, highlighting the importance of post-translational modifications and signaling pathways in modulating the developmental trajectory of the neocortex potentially through the modulation of a major signaling hub, the primary cilium.



## **NEUROG2 T149A, a gain of function mutation causing an early stop of neurogenesis through progenitor depletion?**

These results highlight a game-changing question: is this gain of function causing a depletion of the progenitor pool? When overexpressed in the mouse cortical progenitors, the NGN T149A mutant induced a depletion of progenitors followed by a change in the fate of the neurons produced toward the deep layer of the neocortex. Under endogenous levels, in the live imaging experiment where we correlated the types of divisions observed with the fate of the daughter cells, carried out at both week 7 and week 9 (corresponding to day 55 and 70), we found that RGCs were performing two times more neurogenic divisions which produced more IPs and more neurons compared to the control. This change in the pattern of divisions results at the populational level in a consistent increase of 7% more excitatory neurons (NEUROD2+ cells) accompanied by a relative decrease of 7% of progenitors (SOX2+ cells) at day 70 in the NEUROG2 T149A mutant cortical organoids compared to the control. However, if these increased proportions of neurogenic divisions in the phospho-mutant cause a depletion of progenitors, we should see at later stages very low proportions of progenitors close to their complete loss, resulting in the presence of almost only neurons in the organoids. Surprisingly, this is not what we observed on day 140. On average, in the control organoids, we found that 65% of cells in these organoids are progenitors (SOX2+), and the remaining 45% are excitatory neurons (NEUROD2+), while in the phospho-mutant organoids, we found 46% of progenitors and 54% of neurons per organoid across all 3D differentiations. Therefore, we see an increased pool of neurons of 8% exactly as seen at day 70 in phospho-mutant organoids which only present a mild depletion of the progenitor pool. However, it is important to note that not all SOX2+ cells are RGCs. Indeed, oligodendrocyte progenitor cells (OPCs) express SOX2 and are produced around this stage of cortical organoid development. Thus, it would be important to use a more specific marker of cortical RGCs such as Pax6 with TBR2 to differentiate the pool of RGCs and IPs with the pool of neurons.

Nevertheless, these results suggest the possibility that unphosphorylated NEUROG2 on the T149 could be a possible way to produce more neurons by inducing only a mild depletion of the pool of progenitors. To test this hypothesis, we must reach terminal stages where the depletion of progenitors is almost complete, and organoids contain only neurons. Thus, we could quantify the neuronal densities per organoid and assess any potential decrease in the phospho-mutant organoids. However, the result of such quantifications might be biased due to

a limitation specific to the organoid model, which is called the necrotic core. Indeed, as the organoids grow, the oxygen that penetrates the organoids through passive diffusion will not reach the cells located in the center, leading to hypoxic conditions resulting in massive cell death of early born cells. Thus, it is highly possible that this necrotic core could target specifically deep-layer neurons as they reside in the lower layer of the neocortex, which is to some extent recapitulated in cortical organoids. Several approaches are on the way to cope with this issue, such as protocols including blood vessels allowing to diffuse only oxygen passively because these vessels are not connected to a cardiorespiratory system. Other protocols use air-liquid culturing interfaces where organoids would stay on a permeable insert placed above a medium contained in a well of a 6-well plate.

## **Perspectives**

To complete this work, we are currently further investigating:

1) The role of the primary cilium in neurogenesis: there is evidence suggesting that the primary cilium plays a role in neurogenesis (L. Wang et al., 2016). The inhibition of its reabsorption during mitotic divisions results in neurogenic divisions, indicating that NEUROG2 T149A mutants, which undergo more neurogenic divisions, may have impaired or slowed cilium reabsorption upon division. Therefore, it would be interesting to investigate the reabsorption and inheritance of primary cilia and correlate it with the types of divisions, although this is currently a challenging task. However, we can hypothesize that higher expression of genes involved in primary cilia organization and function provides more material for cilia construction, and thus NEUROG2 TA progenitors may have longer cilia that are not fully reabsorbed upon division, leading to neurogenic divisions. This hypothesis focuses on a mechanical approach but overlooks a crucial aspect of the primary cilium: its role as a cellular signaling hub. Another hypothesis is that the NEUROG2 mutation affects not only the structure of the primary cilium but also its signaling, which is critical for proliferative divisions and progenitor amplification before their differentiation into neurons. Therefore, it is possible that changes in primary cilium signaling, rather than just its structure, contribute to the observed effects of NEUROG2 mutations on neurogenesis. To test these two hypotheses, First, we could measure and compare the size of primary cilia in different cell subtypes found in cortical organoids. This would allow us to determine whether NEUROG2 mutations lead to changes in cilia length and whether these changes correlate with the types of divisions undergone by the progenitors. Second, we could examine the presence and localization of cell signaling receptors

at the cilia membrane in different cell types. This would provide insight into whether NEUROG2 mutations affect primary cilium signaling and whether changes in signaling contribute to the observed effects on neurogenesis. Additionally, we could use genetic manipulations or drug treatments to selectively disrupt primary cilium structure or signaling and assess the effects on neurogenesis in cortical organoids by using the image analysis pipeline developed. This would provide direct evidence for the role of primary cilia in neurogenesis and the contribution of NEUROG2 mutations to this process.

2) Stability: the stability of NEUROG2 is also a major point that we have not described nor studied. To engage in its proneural activity, the current understanding is that Neurog2 needs to be stabilized to break from the oscillations caused by Hes1 to induce the expression of the Neurod to induce neuronal differentiation of the progenitors (Shimojo et al., 2008). Therefore, the stability of the NEUROG2 mutant is crucial in determining the duration of proneural protein activity and its ability to induce neurogenesis. Proneural activity and protein stability are intimately linked, and it is necessary to validate the stability of NEUROG2 T149A to fully comprehend the complex mechanisms underlying its gain of function. Although we might expect a more stable protein to be detectable for a longer period and potentially result in a higher proportion of NEUROG2-expressing cells, our data do not support this hypothesis. Nonetheless, performing western blots of NEUROG2 at regular intervals after blocking both transcription and translation would be a valuable approach to test this hypothesis and gain further insights into the stability and activity of NEUROG2 T149A.

3) Phosphorylation pattern: Quan et al., 2016 started by showing that the mouse Neurog2 is phosphorylated on the T149 in vivo. In our work, we have taken this conclusion for granted while studying the human NEUROG2 with an in vitro model. Thus, it is important to prove that NEUROG2 is phosphorylated on its T149 in our cortical organoids under our culturing conditions. However, such experiments, even simple in theory were proven to be challenging in practice. Indeed, only 5% of the progenitors will express NEUROG2 at any given time points around day 70 in culture representing a minority of the cells and thus it is important to perform immunoprecipitations of the endogenous NEUROG2 and NEUROG2 T149A mutant followed by western blotting using anti-phosphothreonine antibodies to measure the relative different levels of NEUROG2 phosphorylations in both WT and mutant NEUROG2. Furthermore, it could be interesting to also perform Mass Spectrometry to get the precise pattern of phosphorylation of NEUROG2 WT and T149A and see how this T149 changes the

conformation of the protein and eventually impact the rest of the phosphorylation that we have seen important for proneural activity and neuronal maturation (F. Ali et al., 2011; Hand et al., 2005).

4) Change in the binding partners: More and more evidence demonstrate that the dimerization partners change binding affinity to certain motif CAXXTG and thus the expression of some genes. In the future, it could be worth studying how the phosphorylation of NEUROG2 modifies its binding partners and how that could regulate its interaction with the known target genes and the primary cilium-related genes.

5) Pattern of NEUROG2 expression in developing human neocortex: it has never been shown what the pattern of NEUROG2 expression is among RGCs and IPs in the developing human neocortex at the protein level. Indeed, several studies used scRNA sequencing to establish a transcriptomic profile of the developing human neocortex at different gestational weeks, but nothing is known about the protein. In this work, we found that in cortical organoids NEUROG2 is mostly expressed by RGCs and not by IPCs which is the opposite of what has been found in mice neocortex. Thus, it would be important to use the same image analysis pipeline developed in this work to investigate where NEUROG2 is expressed in the developing human neocortex and in which specific cell subtypes based on marker expression and location in the VZ and SVZ.

## Conclusion

In conclusion, this work validated for the first time the role of the human NEUROG2 in developing human cortical organoids by showing that NEUROG2 induces a loss of glutamatergic neurons at both mid and late stages of cortical organoid development, respectively after 70 and 140 days in culture. Furthermore, the loss of NEUROG2 in cortical organoids leads to a ventralization of cortical progenitors with a downregulation of the genes encoding for the dorsal forebrain identity and an upregulation of the genes encoding for the ventral forebrain identity. These results demonstrate that NEUROG2 is the main driver of cortical neurogenesis in human cortical organoids but also represses an alternative ventral differentiation program that would specify cortical progenitors in inhibitory neurons.

We further investigated the role of the NEUROG2 T149 phosphorylation site under endogenous levels in developing human cortical organoids and found that on the one hand, the NEUROG2 T149A homozygote mutant does not change the pattern of NEUROG2 expression in both RGCs and IPs nor its ability to bind and activate well-known target genes or reprogram human stem cells into neurons. However, the TA/TA mutant radial glial cells switch their division mode from proliferative to neurogenic, which induces increased proportions of neurons per organoid at both mid and late stages of cortical development in organoids which could be potentially the consequence of increased production of intermediate progenitors that are by definition prone to neurogenesis.

Mechanistically, we found that this phenotype is accompanied by an upregulation of the genes encoding the organization and the movements of the primary cilium, which are downregulated in the NEUROG2 KO clones. These results suggest a strong link between Neurogenin2, its phosphorylation profile and the regulation of neurogenesis in human cortical organoids and the primary cilium, a structure well known for its implication in neurodevelopmental disorders and intellectual disabilities.

# Materials & Methods

## **iPSCs culturing and maintenance:**

iPSC cell lines WTSIi008-A and GM25256\*E were purchased from the European Bank for Induced Pluripotent Stem Cells (EBISC) and the Coriell Institute, respectively. These iPSCs clones were sequenced and validated using Bionano. Results revealed a duplication of chromosome 18 in WTSIi008-A which was found in all the clones studied. The comparison of the entire DNA sequence of the clones showed no differences validating that the CRISPR/Cas9 experiment did not cause genetic recombination (data not shown).

iPS cells were cultured on Geltrex LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (1%, Thermo Fisher Scientific, A1413302) coated dishes (B6 or B10 dishes) in mTeSR™ Plus (STEMCELL Technologies, #100-0276) supplemented with Antibiotic-Antimycotic (0,1%, Thermo Fisher Scientific, 15240062). The medium was changed every other day, and the iPS cells were passaged when iPSCs reach 80% confluency with 300µL of cGMP ReLeSR™ (STEMCELL Technologies, #100-0483) at various dilutions depending on the needs for the different experiments.

## **Generation of cortical organoids from hiPSCs**

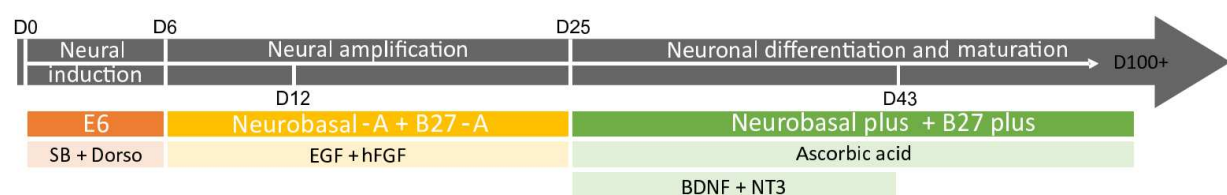
Cortical organoids were generated from human iPSCs using a previously reported protocol (Sloan et al., 2018) with modifications. hiPS cells were washed with PBS and incubated with 1mL of StemPro™ Accutase™ Cell Dissociation Reagent (Thermo Fisher Scientific, A1110501) for 5 min at 37 °C and dissociated into single cells. To obtain uniformly sized spheroids, approximately  $3 \times 10^6$  single cells were added per well in the AggreWell 800 plate (STEMCELL Technologies, 34815) with mTeSR™ Plus medium supplemented with Stemgent hES Cell Cloning & Recovery Supplement (1X, Ozyme, STE01-0014-500) and incubated at 37°C with 5% CO<sub>2</sub>. After 24 hours, spheroids from each microwell were collected by firmly pipetting medium in the well up and down and transferred into Corning® non-treated culture dishes (Merck, CLS430591-500EA) in TeSR™-E6 (StemCell Technologies, #05946) supplemented with two inhibitors of the SMAD signaling pathway, dorsomorphin (2.5 µM, STEMCELL Technologies, #72102) and SB-431542 (10 µM, STEMCELL Technologies, #72234). From day 2 to day 5, TeSR™-E6 supplemented with dorsomorphin and SB-431542 was changed daily.

On day 6, the medium was replaced by Neurobasal™-A (Thermo Fisher Scientific, 10888022), B-27™ Supplement minus vitamin A (50X) (1X, Thermo Fisher Scientific 12587010), supplemented with GlutaMAX™ (1%, Thermo Fisher Scientific, 35050038), 2-mercaptoethanol (0.1mM, Thermo Fisher Scientific, 31350010) and Antibiotic-Antimycotic (0.1%, Thermo Fisher Scientific, 15240062).

This medium was supplemented with 10 ng/mL Human Recombinant EGF, ACF (STEMCELL Technologies, #78136) and 10 ng/mL Human Recombinant bFGF, ACF (STEMCELL Technologies, #78134.1) and changed daily until day 12. From day 12 to day 24, the medium was changed every other day.

On day 25, the medium was replaced by Neurobasal™ Plus (Thermo Fisher Scientific, A3582901), supplemented with B-27™ Plus Supplement (50X) (1X, Thermo Fisher Scientific, A3582801), GlutaMAX™ (1%, Thermo Fisher Scientific, 35050038), 2-mercaptoethanol (0,1mM, Thermo Fisher Scientific, 31350010), L-Ascorbic acid (200µM, Sigma-Aldrich, A4403), and Antibiotic-Antimycotic (0,1%, Thermo Fisher Scientific, 15240062). From day 25 to day 43, this medium was supplemented with Human Recombinant BDNF, ACF (STEMCELL Technologies, #78133), and Human/Mouse Recombinant NT-3 (STEMCELL Technologies, # 78074) and changed twice a week.

From day 43 until the last time points of interest, the same medium was changed twice a week without BDNF nor NT3.



**Figure 1: Schematic representation of the 3D differentiation protocol of iPSCs into cortical organoids.**

### **Cryopreservation of cortical organoids**

Organoids were collected in 2mL Eppendorf tubes, washed with PBS, and fixed in 4% PFA (Electron Microscopy Sciences, 15714) at RT for 6 hours in dark. Organoids were washed several times in PBS before the incubation in sucrose 30% for at least 24h. When organoids are at the bottom of the tubes, the organoids were collected and placed in the cryomolds for cryopreservation with EpreDia™ Neg-50™ Frozen Section Medium (Fisher Scientific, 12688086) and frozen on dry ice. Organoids were then transferred to -80°C upon slicing.

## Slicing of cortical organoids

For immunostaining, cortical organoids were sectioned at a thickness of 14  $\mu\text{m}$  using a Leica cryostat (Leica CM3050S) and collected on Superfrost Plus glass slides 25x75 (Fischer scientific, 11950657). The slides were stored at  $-80^{\circ}\text{C}$  upon immunostaining.

## Immunostainings

Slides were incubated at room temperature (RT) for 2 hours until completely dry. Slides were then washed with phosphate-buffered saline (PBS) to remove all cryoprotectant and blocked for 1 hour at RT with a solution containing 5% heat-inactivated horse serum (Thermo Fisher Scientific, 26050070), 3% bovine serum albumin (BSA) (Sigma-Aldrich, A9647-100G), and 0.3% Triton X-100 (Sigma Aldrich, X100-500mL). The sections were then incubated overnight at  $4^{\circ}\text{C}$  with primary antibodies diluted in the same blocking solution. After three washes with PBS for 5 minutes each, the sections were incubated with secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) (1:5000, Sigma-Aldrich, D9564) diluted in the same blocking solution for 1 hour at RT. Following three additional washes with PBS, slides were mounted with VECTASHIELD Vibrance Antifade Mounting Medium (Eurobio Scientific, H170010) and stored at  $4^{\circ}\text{C}$  until imaging. Nail polish was added after polymerization of the mounting medium to ensure airtight sealing for long-term storage.

All the antibodies used in this thesis were diluted in glycerol at 50%. All the dilutions in table 1 are based on antibodies already diluted 1:2 in glycerol (Sigma Aldrich, 49767).

| Antibody | Provider           | Reference   | Dilution |
|----------|--------------------|-------------|----------|
| SOX2     | Miltenyi Biotec    | 130-106-231 | 1/200    |
| NEUROG2  | Cell signaling     | 13144       | 1/200    |
| TBR2     | R&D systems        | AF6166      | 1/200    |
| NEUROD2  | Abcam              | ab104430    | 1/200    |
| HOPX     | Fischer scientific | 16858483    | 1/200    |
| PAX6     | Proteintech        | 12323-1-AP  | 1/200    |
| TBR1     | Synaptic Systems   | 328005      | 1/200    |
| SATB2    | Abcam              | ab51502     | 1/200    |

**Table 1: List of antibodies used for this project and their provider.**



## **Imaging of whole sliced organoids**

Slides were positioned in the 4-slide plate (inverted-face down) and left at RT to warm before imaging with an inverted Nikon confocal AXR microscope. Imaging is a very important step and all the images were taken with the following settings: an overview of the slides was done using a 4X objective with very low resolution to gain speed (mode: Resonant 1, 512x512 pixels, a large pinhole of 6, and intense gain to capture each section in the 405nm wavelength corresponding to DAPI). Then, with an air immersion 20X objective, the positions of every slice were set with Perfect System Focus to maintain the z position over time. Each slice was imaged in a mosaic with 15% overlap to capture the entire organoid slice with the following mode: Galvano1, dwell time of 0,8, pinhole 1, 2048x2048 pixels, with imaging channels starting from the 647, 555, 488 and 405 nm to avoid any cross excitation of the fluorophores. The size of the mosaic is fixed for all slices. Thus, its size was determined based on the largest slice and set to image all the others.

## **Image processing**

### **Image selection and cropping**

Images coming from Nikon or any proprietary microscope brand come with their specific format. For Nikon, images were automatically saved in “.nd2” but converted to tiff for optimal image processing (using the NIS software directly or ImageJ based on personal preference). Then all the images were opened in ImageJ and manually cropped to save storage space but also discarded if:

- The stainings were faint,
- The slice is not imaged entirely,
- The focus was lost on a part of the organoid slice.

### **Channel selection and 8bit saving of the images**

To perform the segmentation, the DAPI channel was isolated and saved in 8bits to reduce the image size and fasten the nuclear segmentation.

## **Fine-tuning of StarDist, a deep learning algorithm for nuclear segmentation**

### **Image annotations**

Images taken with the Nikon AXR confocal microscope were manually annotated using Labkit and label images were saved as tiff images.

### **Training**

StarDist is a state-of-the-art machine learning-based method for cell segmentation that employs a star-convex polygon shape model to accurately segment nuclei (Schmidt et al., 2018; Weigert et al., 2020). While the pre-trained version of StarDist yields impressive results, it is highly recommended to fine-tune the model on your images for optimal nuclear segmentation. This is because the initial models were not trained on your specific data. The authors of StarDist have conveniently provided Jupyter notebooks to facilitate the training process, which can be found on their GitHub repository (<https://github.com/stardist/stardist>).

One of StarDist's advantages is its ability to process images with varying spatial dimensions due to its use of a convolutional neural network (CNN). This means that crops of images do not need to have the same number of pixels along the x and y axes.

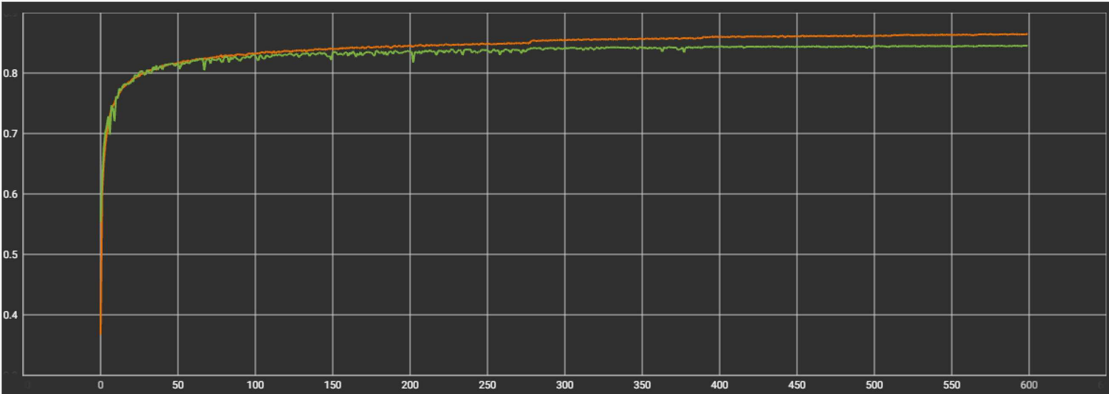
For the training of StarDist, a diverse and extensive dataset of 2,212 images and their corresponding masks were collected from various sources. These include:

- 64 manually annotated crops of images taken with the confocal microscope Nikon AXR at 20X and 40X objectives.
- 447 images downloaded from StarDist's GitHub corresponding to the stage1\_train images from the Kaggle Data Science Bowl available in full from the Broad Bioimage Benchmark Collection. The folder with the 447 images can be downloaded here:
  - o <https://github.com/stardist/stardist/releases/download/0.1.0/dsb2018.zip>
- A collection of 1,588 images, obtained from the TissueNet dataset from (Greenwald et al., 2022) can be found here: <https://datasets.deepcell.org/data>
- A collection of 113 images, downloaded from the Dynamic Nuclear Net Tracking from (Schwartz et al., 2023) can be found here: <https://datasets.deepcell.org/data>

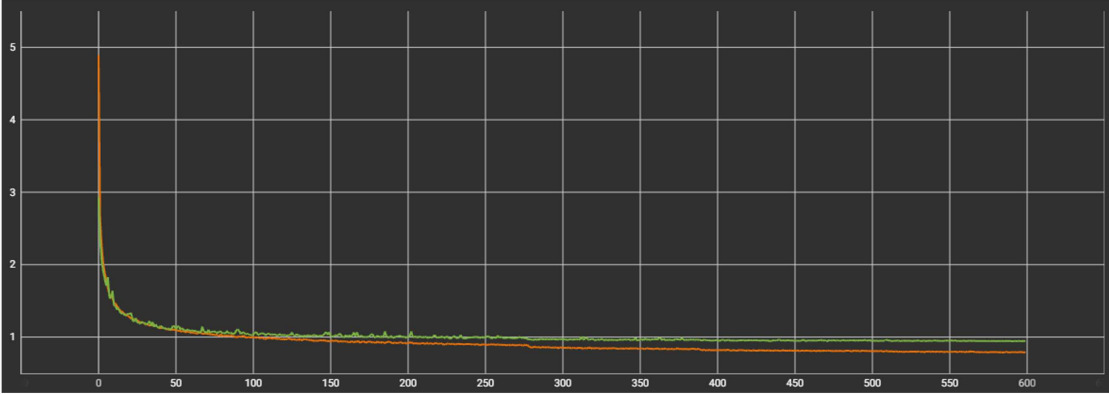
To enhance the training further, the 2,212 images were augmented with techniques such as flipping, rotations, random intensity changes, and Gaussian blur and 15% of these images were used for validation of the training, representing 332 images out of 2,212.

During the model’s training, epoch-wise IoU and loss curves exhibited consistent and expected behavior with no unusual or irregular fluctuations. The epoch dist IoU curve demonstrated a steady increase (Figure 2A), indicating that the model's ability to accurately predict objects was improving over time. Simultaneously, the epoch dist loss curve showed a gradual decrease, reflecting that the discrepancy between the model's predictions and the ground truth labels was diminishing.

**A - Epoch dist IoU metric**



**B - Epoch dist loss metric**



**Figure 2: Tensorboard monitoring of StarDist training**  
**(A)** Epoch-wise Intersection over Union (IoU) Metric: This panel illustrates the progression of the IoU metric across the training epochs. The IoU measures the overlap between the predicted bounding boxes and the ground truth bounding boxes. A higher IoU score indicates a better alignment of the model's predictions with the actual objects. The line graph depicts the mean IoU score (y-axis) for each epoch (x-axis); **(B)** Epoch-wise Loss Metric: This panel presents the evolution of the loss metric throughout the training epochs. The loss function quantifies the discrepancy between the model's predictions and the ground truth labels. A lower loss value signifies that the model's predictions are closer to the actual values. The line graph in this panel plots the mean loss value (y-axis) for each epoch (x-axis).

Here are all the parameters used for training the model:

```
Config2D(axes='YXC', backbone='unet', train_loss_weights=[1, 0.2],
grid=(2, 2), train_n_val_patches=None,
n_channel_in=1, train_patch_size=[256, 256],
n_channel_out=33, train_reduce_lr={'factor': 0.5, 'patience': 40,
n_classes=None, 'min_delta': 0},
n_dim=2, train_sample_cache=True,
n_rays=32, train_shape_completion=False,
net_conv_after_unet=128, train_steps_per_epoch=400,
net_input_shape=[None, None, 1], train_tensorboard=True,
net_mask_shape=[None, None, 1], unet_activation='relu',
train_background_reg=0.0001, unet_batch_norm=False,
train_batch_size=8, unet_dropout=0.0,
train_checkpoint='weights_best.h5', unet_kernel_size=[3, 3],
train_checkpoint_epoch='weights_now.h5', unet_last_activation='relu',
train_checkpoint_last='weights_last.h5', unet_n_conv_per_depth=2,
train_class_weights=[1, 1], unet_n_depth=3,
train_completion_crop=32, unet_n_filter_base=32,
train_dist_loss='mae', unet_pool=[2, 2],
train_epochs=600, unet_prefix="",
train_foreground_only=0.9, use_gpu=True
train_learning_rate=0.0003,
```

### **Testing the model**

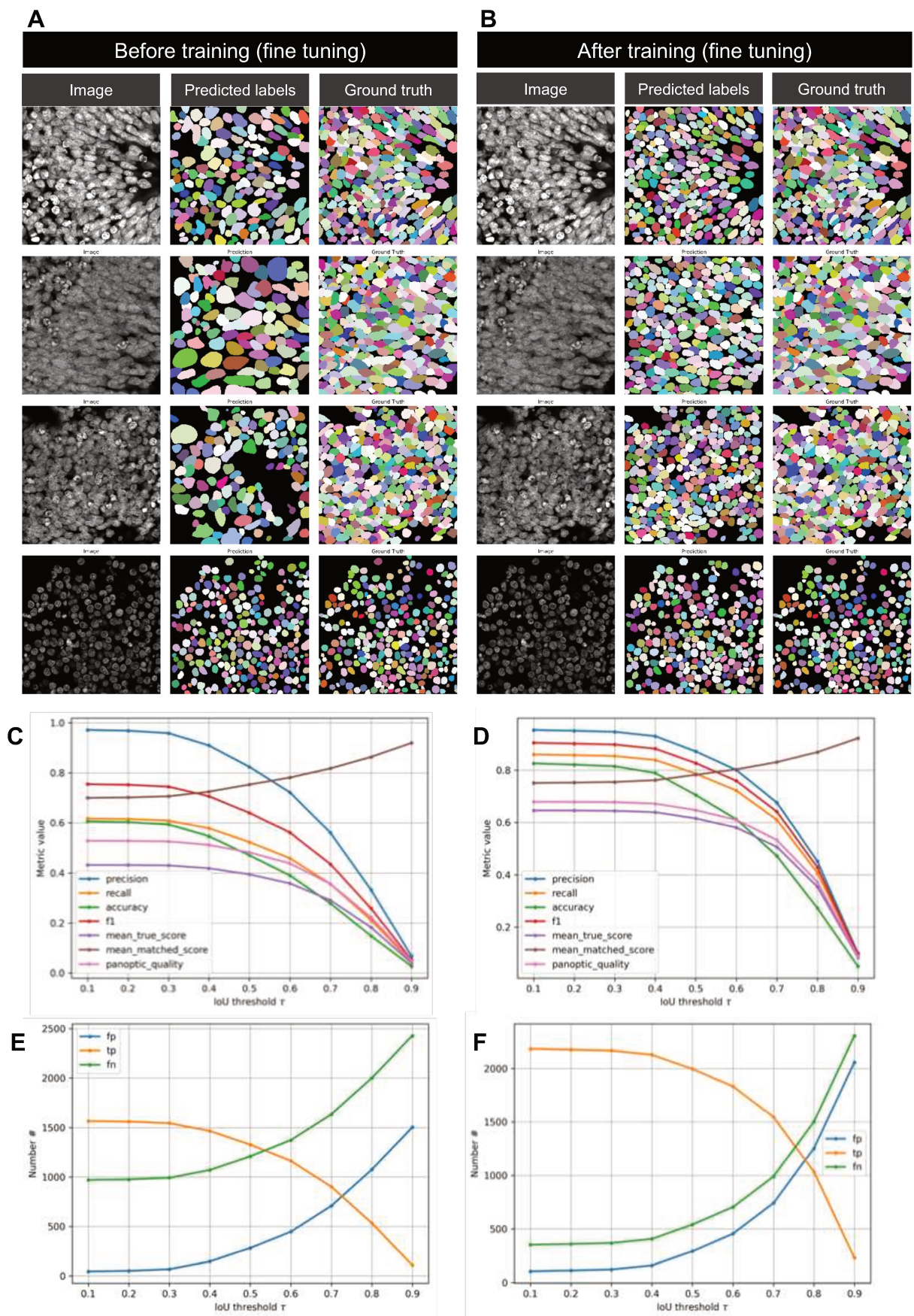
After training, the thresholds were optimized on 9 manually annotated crops of images taken with the confocal microscope Nikon AXR that were not used for training or validation. These images were further used to test the fine-tuned model `stardist_all4_u3` with the pretrained version `StarDist Versatile` (fluorescent nuclei).

The most used value for assessing the quality of the training is the f1 score; it is a combination of the precision, which represents the proportion of true positive predictions (TP) among all positive predictions made by the model (TP + FP). It measures the model's ability to avoid false alarms, i.e., predicting positive when the actual label is negative. Recall is the proportion of true positive predictions (TP) among all actual positive instances in the data (TP + FN). It measures the model's ability to detect all positive cases, i.e., minimizing the number of false negatives (FN). The closer the f1 score is to 1, the better the model's performance in terms of balancing precision and recall. An F1 score of 1 indicates that the model has perfect precision and recall, meaning it correctly identifies all positive instances in the data while minimizing both false positives and false negatives. Conversely, an F1 score closer to 0 suggests that the model struggles to balance precision and recall, and its overall performance is poor.

The pretrained version of StarDist Versatile (fluorescent nuclei) has an F1 score of 0.64, while the fine-tuned StarDist: stardit\_all4\_u3 has an F1 score of 0.83 (Table 2).

|                              | Before training    | After training   |
|------------------------------|--------------------|------------------|
| Data based on an IoU of 0.5: | Stardist_Versatile | stardist_all4_u3 |
| Precision                    | 0,824              | 0,872            |
| Recall                       | 0,523              | 0,787            |
| Accuracy                     | 0,471              | 0,705            |
| <b>f1</b>                    | <b>0,640</b>       | <b>0,827</b>     |
| n_true                       | 2536               | 2536             |
| n_pred                       | 1611               | 2288             |
| mean_true_score              | 0,394              | 0,616            |
| mean_matched_score           | 0,753              | 0,782            |
| panoptic_quality             | 0,482              | 0,647            |
| by_image                     | False              | False            |
| false positive               | 284                | 293              |
| total positive               | 1327               | 1995             |
| false negative               | 1209               | 541              |

**Table 2: Quantitative measurements of deep learning algorithm performance measured on 9 manually annotated images before and after training.**



**Figure 3: Performance of StarDist before and after training.**

(A) Image prediction of StarDist Versatile (fluorescent nuclei) pretrained model on 4 images compared to their ground truth annotations done manually ; (B) Image prediction of the fine-tuned StarDist named stardist\_all4\_u3 on the same 4 images compared to their ground truth annotations done manually ; (C) & (D) Plot of the different performance metrics before and after training at different IoU values; (E) & (F) Plot of the fp: false positive, tp: total positive, fn: false negative values before and after training at different IoU values.

## Nuclear segmentation

There are two main approaches that you can consider when segmenting nuclei in microscopy images based on your programming skills and the number of nuclei present in your images.

### With ImageJ

One user-friendly and straightforward option is to use the ImageJ plugin developed by the authors of StarDist. This plugin provides a simple interface for segmenting nuclei in your images and can even be called from a macro to automatically segment and save the corresponding masks for all your images. The plugin labels each nucleus with a unique pixel value, allowing you to easily identify and analyze individual nuclei. However, it's important to note that the plugin uses a 16-bit encoding for the label images, which limits the maximum number of nuclei that can be labeled to 65,536.

Confocal images of large cortical organoid slices have an average size of 500 Mb, which requires the use of GPU to segment the entire image. Here are the steps that I followed to connect ImageJ to the internal NVIDIA GPU of our Windows analysis computer.

- 1) Download the CSBDeep plugin to have the Tensorflow in the options from the edit tab of ImageJ: <https://imagej.net/imagej-wiki-static/TensorFlow-GPU>
- 2) Install the right CUDA and CuDNN that correspond to your TensorFlow version.
- 3) Add the path to your environment (Figure 4).

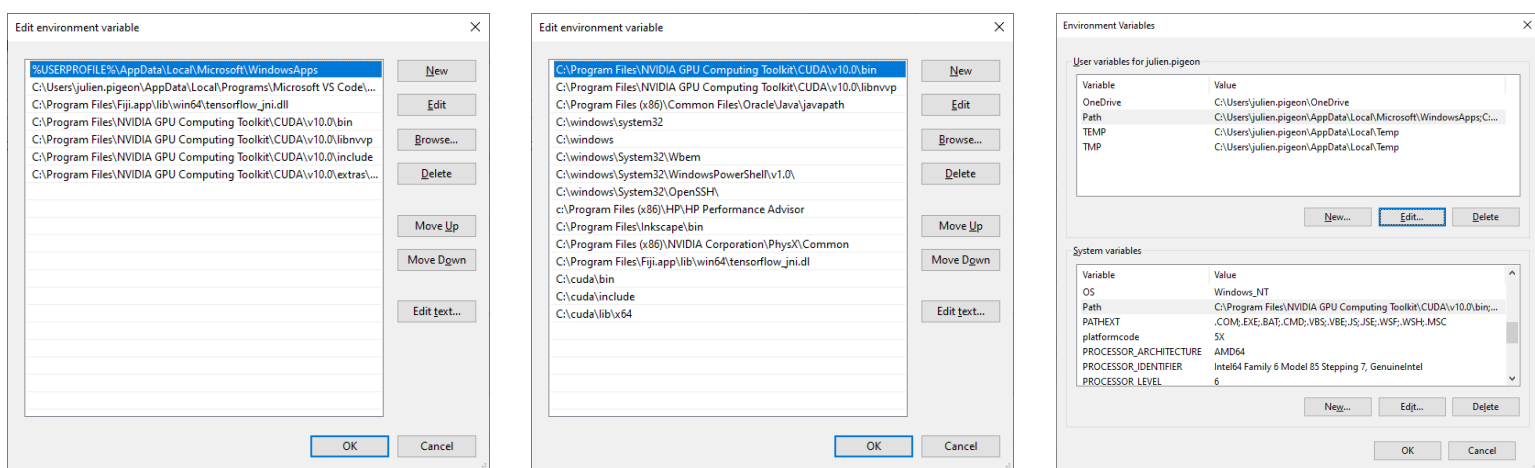


Figure 4: Screenshots of the system environment containing the precise path of the CUDA toolkit installation.

## **With Python**

However, if your images contain a larger number of nuclei, you can manually crop a part of the necrotic core and continue your quantifications or you can use a more advanced coding-based approach. This involves using a Python script to run the segmentation and save the label image as 32 bits, allowing for 4,294,967,296 possible pixel values and thus 4,294,967,296 possible nuclei in one image. The use of Python presents the advantage to also run the segmentation on any computer or laptop without necessitating a GPU and a quick segmentation with tiling of the image as developed by the authors.

All image segmentations were performed using a Python script that I developed, which will be made available upon publication of this work.

## **Image selection for training the object classifier and assessing its performance**

The mean intensity of the cellular markers of the different channels was measured. The average intensity of all cells has been calculated per image to plot the distribution of image intensities for a channel of interest across all images taken for the immunostaining of all the clones of the same 3D differentiation of iPSCs into organoids. Thus, images that were too dark or too bright were excluded from the analysis to avoid any bias in the training of the object classifier. In general, 2 images were selected per genotypes for the training of the object classifier, with overall high, mean, and low intensities for the markers of interest. This step was repeated to test the performance of the trained object classifier on small crops of 200x200 pixels, with 3 images of different average intensities per clone. Then, all these crops were manually quantified before training the object classifier for comparison between ground truth and the classifier quantification.

## **Object classification**

We used a free and user-friendly software, ilastik, to train object classifiers (Berg et al., 2019). Training images and their corresponding masks were loaded, and specific features were selected for training (such as the mean intensity, the total intensity, the skewness of intensities, and the object's area, along with many other parameters proposed by ilastik). Categories were created based on what type of cell population were stained. The training was done by assigning nuclei, and thus segmented objects, to each subcategory created in all the training images. The classifier measures all the previously selected features to establish a general profile of the cell that would fit both nuclei, which could have channel intensities too bright or too dark.



## **Validation of the trained object classifier**

Using the batch processing tab in Ilastik allows for the quantification of the test images by the classifier for comparison to the initial manual quantifications. The object classifier training was validated if the differences in cell counts per subcategory were no more than 5 to 7%.

## **Overall considerations of the quantifications**

We have seen that a fine-tuned deep learning algorithm for nuclei segmented is of crucial importance to detect all the objects present in the image. Nevertheless, reaching 100% segmentation is impossible due to 1) potential over annotations from the user and 2) the nature of the ground truth data annotated by the user might change over time with experience and time allocated to perform such manual annotations. Furthermore, the trained object classifier never matches perfectly the values of what has been manually quantified by the user because the data might be too variable.

Therefore, the power of such quantifications relies on the high number of observations made and thus on the number of 3D differentiations and the number of organoids collected at each time point of interest.

## **Correlative immunostaining**

The following technique is based on (Coquand et al., 2024b).

### **Viral infection with retroviruses**

Cortical organoids at both week 7 and week 9, corresponding to 55 and 63 days in culture, respectively, were collected and embedded in low-gelling agarose (XX) for subsequent slicing. A Leica VT1200S vibratome was used to slice the organoids into 250 $\mu$ m thick sections at a 200 $\mu$ m/sec speed, while maintaining the samples in cold DMEM/F-12 (Thermo Fisher Scientific, 10565018). Slices were transferred in a 24 wells plate for retroviral transduction (MSCV-IRES-GFP, Addgene plasmid #20672) diluted at 1/30 in DMEM/F-12. After 2 hours of incubation, slices were washed using PBS and incubated in DMEM/F-12 supplemented with B-27 (Thermo Fisher Scientific, 10565018), N2 (1X, Thermo Fisher Scientific, 17502048), 10ng/mL FGF2 (STEMCELL Technologies, #78133.1), 10ng/mL EGF (STEMCELL Technologies, #78136), 5% fetal bovine serum (1X, Thermo Fisher Scientific, A5670801) and 5% horse serum (Thermo Fisher Scientific, 26050070) for 2 days before live imaging.

## Live Imaging

In order to follow RGCs and IP divisions over several days, slices were transferred onto a permeable membrane (Millicell Cell Culture Insert, 30 mm, hydrophilic PTFE, 0.4  $\mu\text{m}$ , PICM0RG50, Merck) to create an air-liquid interface that was inserted onto a 35mm glass bottom dish (Fluorodish WPI, FD35-100) with 1mL of culture medium supplemented with EGF and FGF at a final concentration of 10ng/mL under the membrane. Based on the GFP signal, positions were chosen and imaged every 15 minutes in 100 $\mu\text{m}$  stacks to capture migrating progenitors. However, the z step for each stack depends on the number of positions you have to fit all imaging in 15 minutes, so you have a good temporal resolution of cell divisions and migration to correlate the types of divisions with the daughter cell fate. At the end of the live imaging, low magnification images were taken with a 4X objective for alignment of the movies with the fixed, stained and mounted slices of cortical organoids.

## Immunostaining and movie processing

Subsequently, the slices were fixed using 4% PFA, with 1 mL applied under the membrane and 1 mL on top, to ensure maximal penetration of PFA. The slices were then washed with PBS for three 10-minute intervals and distributed into the wells of a 24-well plate. They were incubated for 1 hour in a blocking buffer consisting of PBS supplemented with 0.3% Triton-X and 2% donkey serum. Primary antibodies were incubated in the same solution overnight at 4°C on a shaking plate. Following this, the slices were washed using PBS + 0.05% Tween-20 before **the incubation** with secondary antibodies (diluted 1:1000 from our stock) **in the same blocking buffer**. The slices were then washed and mounted on Superfrost slides using Aquapolymount (18606-20, PolySciences). The slides were left in the dark at room temperature for polymerization and subsequently stored at 4°C until use.

## Bulk RNA sequencing

Total RNAs were extracted from day 70 cortical organoids with NucleoSpin® RNA kit (Macherey-Nagel, 740955). An RNA-seq library was generated with the KAPA mRNA HyperPrep Kit (Roche, 8098115702) and sequenced by a NovaSeq X plus Illumina.

All RNA-seq data were analyzed using the Galaxy Project (<https://usegalaxy.org/>). Quality of reads was checked with fastqc tool and mapping was done using RNAStar onto hg38 human reference genome. Reads per annotated gene were counted using featureCounts (-p enabled; -Q 10). The edgeR R-package was used to perform both data normalization (TMM) and

differential expression analyses (quasi-likelihood F-test, Robust settings and P-Value Adjustment Method: Benjamini and Hochberg). Genes were considered regulated when  $FDR < 0.05$ . Gene Ontology (GO) analysis was performed using Enrichr (GO Biological Process 2021; <https://maayanlab.cloud/Enrichr>).

### **Chromatin immunoprecipitation sequencing (ChIP-seq)**

ChIP-seq assays were performed using iDeal ChIP-seq kit for Transcription Factors (Diagenode, C01010055). Briefly, cells were fixed in 1% formaldehyde (EMS, 15714) for 10 min at room temperature and reaction was quenched with 125 mM glycine (Sigma-Aldrich, G8898) for 5 min at room temperature. Lysate was sonicated with a Bioruptor Pico sonicator (Diagenode, total time 10 min) and 4 $\mu$ g of antibodies were added to sheared chromatin and incubated at 4°C overnight. Antibodies used were: anti-Neurog2 (Cell signaling, 13144), anti-H3K27Ac (Abcam, ab177178), anti-H3K27me3 (Cell Signaling, 9733) and anti-H3K4me1 (Abcam, ab176877), H3K4me3 (Active Motif, 39160). Mock (Rabbit IgG, from kit Diagenode, C01010055) was used as negative control. Chromatin-protein complexes were immunoprecipitated with protein A/G magnetic beads and washed sequentially. Input (non-immunoprecipitated chromatin) was used as control in each individual experiments.

The ChIP-seq libraries were prepared using MicroPlex Library Preparation Kit (Diagenode, C05010001) and Dual indexes for MicroPlex Kit (Diagenode, C05010004) and sequenced with Novaseq X platform.

### **ChIP-seq analysis**

All ChIP-seq analysis were done using the Galaxy Project (<https://usegalaxy.org/>). Reads were trimmed using Cutadapt (--max-n 4) and Trimmomatic (TRAILING 1; SLIDINGWINDOW 4 and cutoff 20; LEADING 20; MINLEN 50) and mapped using Bowtie2 onto hg38 human reference genome. PCR-derived duplicates were removed using PICARD MarkDuplicates. Bigwig files were generated with bamCoverage (binsize=1). To determine significant peaks in a condition, peak calling was performed using MACS2 callpeak with Input as control and with options: --qvalue 0.05; --nomodel; --keep-dup 1; --broad.

Bound genes were determined using CHIPseeker, with the TSS upstream/downstream region set at 10000 bases. Coverage and peaks were visualized using IGV <http://software.broadinstitute.org/software/igv/home> (Robinson et al., 2011).

### **iPSCs infection with the NEUROG2 lentiviruses**

The parental clone of a WT iPSCs line was infected with the three different lentiviruses containing the human Neurog2 mutated. On day 0, 60,000 iPSCs were incubated in a coated plate with 12 wells with the classical conditions as described earlier. On day 1, the cells were transduced with the lentiviruses at a MOI of 4 in mTeSR™ Plus (STEMCELL Technologies, #100-0276) supplemented with Stemgent hES Cell Cloning & Recovery Supplement (1X, Ozyme, STE01-0014-500) and 8µg/mL polybrene (Hexadimethrine bromide, Sigma-Aldrich, H9268-5G) for 3 days. The medium was then changed everyday with puromycine (Sigma-Aldrich, P9620-10ML) to start the selection process: two days at 2µg/mL followed by three days at 1µg/mL. iPSCs were then amplified and frozen in CryoStor CS10 (STEMCELL Technologies, 07930) upon their use.

### **iPSCs conversion into neurons**

On day -1, 60,000 iPSCs were incubated in a well of a 12 wells plate coated with Geltrex LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (1%, Thermo Fisher Scientific, A1413302) in mTeSR™ Plus supplemented with hES. On day 0, the the expression of NEUROG2 and NEUROG2 mutants were done by changing the medium with mTeSR™ Plus supplemented with 0.4µg/mL doxycycline (Sigma-Aldrich, D9891-5G). On day 1, the medium was replaced by Neurobasal Plus supplemented with BDNF and NT3 at a final concentration of 20 ng/mL with 0.4 µg/mL doxycycline. On day 3, the medium was replaced with the same medium as day1 with cytosine β-D-arabinofuranoside (Sigma-Aldrich, C6645) to remove all the potential dividing iPSCs that escaped the selection process. After two days, on day 5, the medium was replaced with the exact same medium prepared on day 1.

### **Induced neurons**

On day 6, neurons were fixed using PFA 4% for 10 minutes and washed three times very gently to prevent neuron detachment from the coverslips. Immunostaining using antibodies anti-GFP () and anti-MAP2 () was done to assess the conversion efficiency of each *NEUROG2* construct.

### **Luciferase assay**

P19 embryonic carcinoma cells (ATCC# CRL-1825) were maintained in Minimum Essential a Medium (GIBCO) supplemented with 10% fetal bovine serum and 50 units/mL penicillin-streptomycin (GIBCO). P19 cells were seeded into 24-well plates (Nalge Nunc) a day prior

transfection. All the three plasmids containing the different promoters (Neurod1 long and short version) and the multiplexed promoter of Dll1 were transfected using Lipofectamine 3000 reagent (Invitrogen #L3000015), following the manufacturer's protocol, with 0.5 mg total of expression vector. 24 h post-transfection, transfection media was replaced with fresh media. 24 h later, cells were harvested and firefly luciferase and Renilla activities were measured using the Dual-luciferase Reporter Assay System (Promega #E1910) following the manufacturer's instructions and using a TD 20/20 Luminometer (Turner Designs).

### **Statistical analyses**

The normality of all data presented here was tested using Shapiro-Wilk tests and then according to the results, the parametric or non parametric tests were run using GraphPad Prism and R studio for all the quantifications done on organoids sections.

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# Annex

## Timing neurogenesis: a clock or an algorithm?

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### Abstract

Emerging evidence supports the existence of dedicated molecular mechanisms under evolutionary selection to control time during neurogenesis. Here, we briefly review these mechanisms and discuss a potentially useful conceptual framework inspired by Computer Science to think about how these biological mechanisms operate during brain development and evolution.

### Main text

In a fundamental sense, time represents the continuous progression of existence and events. It unfolds moment by moment and permits a structured chronology where events occur in a specific sequence, not randomly or all at once. This framework allows events to be ordered from the past through the present into the future, which in turn helps us understand causal chains where causes precede effects.

In developmental biology, the concept of time is closely intertwined with the pace of embryonic development. This connection can be viewed in at least two ways: the specific sequence of events that occur in a particular chronological order as well as the total amount of time this sequence occupies. Both of these dimensions are found in one of the most studied and evolutionarily conserved biological mechanisms, neurogenesis, the generation of neurons from neural progenitor cells (NPCs). Not only are different neuronal subtypes generated at different time points in a specific order but the total amount of time dedicated to neurogenesis is itself a species-feature of brain development. Intriguingly, while the sequential stages of brain

development share ancestral blueprints, the temporal dynamic and duration can diverge considerably among species. The human brain, for example, undergoes a particularly extended period of neurogenesis compared to rodents and even other primates, allowing for a disproportional increase in the number of neurons, especially in the cerebral neocortex [1], [2]. Thus, there is a correlation between differences in timing and brain size, presumably intimately linked to differences in cognitive capabilities.

This review will discuss the temporal dynamic of neurogenesis and the factors that drive neural progenitor cells (NPCs) to their transition into post-mitotic neurons. We will explore a conceptual framework for understanding how and why changes in timing arose during evolution and what this framework predicts as possible future experimental avenues.

#### Time in cell state transitions and its control through intrinsic and extrinsic cues

Classical analyses using immunohistochemistry and, more recently, single-cell transcriptomics have revealed that distinct cellular states are associated with specific gene expression patterns. Therefore, the acquisition of a specific cellular identity requires a remodeling of these gene regulatory networks (GRNs) that underlie cell state-specific expression patterns. Studies across species and neural tissue types, from fruit flies to human *in vitro* models, have shown that the commitment of a NPC to neurogenesis is influenced by internal and external cellular cues that act on its GRN to determine whether it will continue to self-renew as a progenitor or differentiate into a neuron. One of the causal molecular mechanisms of this dynamic equilibrium between the progenitor and neuron states is mediated by feedback regulation between the Notch signaling pathway and a class of basic Helix-Loop-Helix (bHLH) transcription factors called proneural proteins [3]. When the proneural protein is maintained and stabilized in a NPC, it will overcome the anti-differentiation signal of the Notch pathway and promote neurogenesis ([3]–[5]). Additionally, in the local environment of these NPCs, many secreted morphogens by specific brain regions and the NPCs themselves with their neuronal progeny will weigh on this balance. For example, WNT, FGF, and Shh, play a significant role in maintaining progenitors' self-renewal through different processes to repress proneural genes (reviewed in [6]). In other words, controlling the temporal dynamics of the expression and activity of transcription factors by intrinsic and extrinsic cues can significantly impact the timing of the switch from progenitor self-renewal to differentiation and, thus, the time available for neurogenesis. In the case of the particularly slow pace of human cortical neurogenesis, it has been shown that a human-specific variant of the Notch protein contributes

to the developmental tempo by intrinsically promoting increased levels of Notch activity in progenitors, presumably inhibiting proneural protein expression [7\*\*]. On the other hand, the conserved Amyloid Precursor Protein involved in Alzheimer's disease is required in human cortical NPCs to mediate extrinsic WNT signaling to delay the NPC to neuron transition, and this correlates with reduced expression of the proneural protein Neurogenin2 [8\*\*]. Finally, evidence shows that the rate of degradation of transcription factors involved in neurogenesis is slower in humans than in mouse NPCs [9\*\*]. There is also evidence that more stable or longer-lived proneural proteins correlate with [10] and can cause [11\*] an increase in neuronal production. Emerging evidence also causally implicates mitochondrial metabolism in the NPC-neuron fate decision [12]. Whether this is mechanistically linked to changes in transcription factor activity or stability remains unclear. Interestingly, however, stem cell programming into neurons by Neurogenin2 overexpression results in large-scale remodeling of cellular organelles and proteomes, suggesting that a link may also exist during normal neurogenesis [13\*].

Overall, these examples support the view that the temporal dynamic of neurogenesis is controlled mainly by cell-intrinsic mechanisms that appear to converge on regulating the expression and activity levels of transcription factors and that these mechanisms operate on different time scales in different species. However, this only pushes the question from why the phenotypes (i.e. neurogenesis) emerge on different time scales to why the intrinsic cellular mechanisms operate on different time scales. This question is particularly intriguing because the operant mechanisms discovered so far are highly conserved across eukaryotes. Although mitochondrial homeostasis and metabolism or protein turnover seem to be slower in humans than in mice, for example, there is no evidence that the highly conserved enzymes that carry out these reactions have intrinsically slower substrate processing rates. Finally, given that all these mechanisms appear to converge on the activity of transcription factors that are also themselves highly conserved, the conundrum seems even more perplexing. One way to think about the problem from a broader perspective is to shift from a mechanistic view to a theoretical view in order to provide a general framework into which the currently understood mechanisms would fit. This would guide future hypotheses that could be tested with mechanistic approaches. What might such a theoretical framework look like?

#### Time from Computer Science to Biology

Perhaps our human conception of time as something to be measured with clocks misleads our understanding of developmental time. In Computer Science, time is a measure of computational

complexity. It describes the number of elementary operations an algorithm uses to process a set of input data. Interestingly, this measurement, known as time complexity, characterizes how the execution time of an algorithm increases as a function of the size of the input. The larger the input data set, the longer an algorithm will take to complete its processing.

To translate this concept to biology, one needs to begin by defining what constitutes an algorithm and what constitutes the source of data. We propose that an operationally useful way of thinking about this is to consider the sum of all cellular biological reactions as the algorithm. After all, during development, various proteins literally “process” input in the shape of other biomolecules or metabolites to produce a cell state transition as a phenotypic output. More importantly, the genome is the only source of biological data for which we have evidence supporting a causal role in generating evolutionary change. As we have argued above, and as decades of molecular genetic mechanistic studies have shown, the biochemistry of fundamental cellular processes, that is to say, the algorithm in this metaphor, is highly conserved. What is changing is the data in the shape of evolutionary innovations in the content of genomic information. A particularly striking example in the context of neurogenesis and neuronal differentiation comes from the transplantation of human cortical pyramidal neurons into the neonatal mouse brain, which develops much faster than the human brain. What these studies show is that the maturation of the human neurons in the mouse brain takes up to a year [14], [15] suggesting that the mechanisms underlying the developmental timing are cell intrinsic and encoded by the human genome. A last important angle to consider is the processing modality of the cellular algorithm. Most, if not all, cellular interaction networks are partially promiscuous. This is referred to as “many-to-many” interaction networks, and they are very prevalent in ligand-receptor and transcription factor dimerization networks, for example, including for key neurogenesis pathways such as WNT, Notch and the proneural proteins. It has recently been argued that such networks are both more robust and have greater computational power [16\*] than highly specific, one-to-one interaction networks, likely explaining why they have been favored by evolution.

In summary, a potentially helpful way to think about how the temporal dynamics of biological processes operate is to assume that a highly conserved algorithm of flexible biochemical interactions processes a variable amount of data encoded by the genome. In this conception, the differences in developmental timing would arise principally from differences in the amount of information encoded by the genome.

## Sources of differences in the amount of genomic information

One important source of increase in genomic information during evolution is gene duplication, a major evolutionary driving force [17]. Interestingly, in *Drosophila*, where proneural genes were discovered, there are 58 bHLH genes whereas there are 39 in *C. elegans* and 125 in humans [18], [19]. Moreover, these transcription factors induce the expression of Notch ligands such as Delta and Serrate in flies. Here again, we observe an evolutionary change in the number of proteins. In flies, there are two ligands of Notch, Delta and Serrate, while in vertebrates there are 5 known ligands: Delta like DLL1, DLL3, DLL4 and the Jagged family composed of Jagged1 and Jagged2. In addition, the number of Notch receptors has been multiplied. In flies, there is a single Notch receptor, while in humans, there are 4 (Notch1-4) [20]. These ligands and receptors are expressed in complex overlapping spatio-temporal patterns. Altogether, these duplications of many genes multiply the possible interactions between ligands and receptors. The increased complexity in these ligand-receptor interactions carrying both activation and inhibition signals can create delays in signal interpretation into a specific pattern of effector activation [21]. Furthermore, different ligands can transmit opposing patterning information [22] and thus their co-expression would also require more time for that information to be resolved into a fate decision. Finally, many molecular interactions, especially ligand-receptor interactions and gene activation by proneural proteins, are quantitative in that they depend on the absolute amount of active protein. Because levels of any biomolecule at any time are subject to variation due to both stochastic and random noise, the greater the number of proteins in an interaction network, the more noise in the network and, thus, the longer it may take for efficient signal transmission [23]. Having a single Notch receptor and a single ligand makes the signal transmission direct. As discussed above, gene duplication in humans appears to have been taken to a new level with entire families of rapidly evolving genes dedicated to the attenuation of neurogenic signals, as in the example of *Noch2NL* [24\*\*].

Other potential sources of increase in information are gene regulatory elements, notably highly conserved enhancer/repressor sequences that have been subject to variation only in the human lineage called Human Accelerated Regions (HARs). Interestingly, more than half of these regions tested in vitro displayed enhancer activity, specifically in NPCs [25]–[27] such as HAR5 that enhances WNT signaling through FZD8 to promote NPCs self-renewal [28]. Another source of quantitative regulation of protein levels is post-transcriptional control by noncoding RNAs, considered as another driver of brain evolution. They represent 10 to 15% of the human genome [29]. There is almost twice the number of miRNAs in humans than in mice



and six times that in *Drosophila* [30]. For example, the miR-2115, a great ape-specific miRNA, promotes NPCs proliferation by targeting the ancient gene *ORC4* mutations which cause the Meier-Gorlin microcephalic syndrome [31\*], [32]. Furthermore, a primate lncRNA mediates Notch signaling during neuronal development by sequestering miRNA, promoting NPCs self-renewal [33]. The increase in the number of quantitative post-transcriptional inhibitors of neurogenic proteins would be expected to delay the accumulation of these proteins to functional levels and thus delay cell state transitions that depend on these proteins.

In summary, the combination of gene duplication and increase in the number of regulators increases both the total amount of information the cellular algorithm must process as well as the amount of noise in this information. The combined effect is to lengthen processing time and thus delay the time interval of cell state transitions during neurogenesis.

#### Time, robustness, and size

Why would natural selection have favored these temporal delays over evolutionary time, in the primate and specifically human lineage for example? Needless to say, evolution does not have a plan to produce humans. It must, therefore, be that such a delay in neurogenesis confers a selective advantage for the fitness of the organism. One argument for which there is both experimental and theoretical evidence is that the increase in the number of genes, the promiscuity of protein interactions, and the stochastic noise in molecular processes all confer greater robustness on cell state transitions and developmental patterning [16\*], [23] in this scenario, the principal selection pressure is on robustness and the increase in neurogenic time is an unintended but inevitable consequence; an exaptation [34] in the “Gouldian” sense, rather than an adaptation. However, it can also be argued that increasing the amount of time a neural progenitor remains in a progenitor state increases the number of neurons it can generate, and this, in turn, results in increasing the brain’s adaptive cognitive capabilities. In this scenario, the random accident of gene duplication, for example, gave rise to time delay, which gave rise to an increase in brain size with increased cognitive capabilities, and it is this that confers the selective advantage [35]. The truth most probably is that both views are correct. Exaptation and adaptation are synergistic, and both processes can be seen at work at organismal, cellular, and subcellular levels in evolutionary history [36].

#### Conclusion

In this review, we hypothesized that the amount, rather than the type, of information encoded by the genome controls developmental time, with a focus on neurogenesis and its mechanisms as an example. Using a concept borrowed from computer science, we suggest that fundamental cellular metabolism, the *type* of information in this metaphor, constitutes a conserved algorithm that processes information at a conserved pace. In contrast, the total amount of molecules and their interactions constitute the data that this algorithm must process, and the *amount* of this data dictates the time necessary for a developmental process like neurogenesis to be completed. The emerging evidence that conserved processes and genes can have different effects on neurogenesis in different species [8\*\*], [9\*\*] by altering time is testament to the key importance of temporal regulation in brain evolution. If the genomic changes that drive brain evolution have the main effect of altering developmental time, it can be reasonably argued that temporal patterning is the main feature of the genetic code, and it is related to the total amount of “data” encoded by the genome. To test this idea, future experiments and modeling efforts should be directed towards greater accuracy in the measurement of time in molecular processes at high resolution during cell state transitions and deciphering the causal effects of quantitative changes in protein levels on the timing, size and complexity of neurogenic processes.

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#### Author contributions:

J.P. and B.A.H conceived of and wrote the manuscript. J.P. came up with the idea of comparing biological processes to an algorithm.

#### Competing interest:

The authors declare no competing interests.

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## Résumé de la thèse

Le développement des fonctions cognitives supérieures observée au cours de l'évolution des mammifères, repose sur la capacité des progéniteurs corticaux à augmenter leur production neuronale et ainsi étendre la surface du néocortex. Chez les mammifères dit gyrencéphaliques, où la période de production neuronale est allongée, la régulation du type de division, proliférative ou neurogénique, des progéniteurs corticaux est d'autant plus importante pour garantir l'accumulation de neurones. Dans le télencéphale dorsal, à l'origine du néocortex, c'est l'articulation de la voie de signalisation Notch et du gène proneural Neurogenin2 (NEUROG2) qui contrôle le choix de division. L'expression de NEUROG2 à elle seule étant suffisante pour induire la production de neurones dans le néocortex, sa régulation au niveau génique a déjà fait l'objet d'études approfondies chez la souris. Cependant, de nouveaux travaux démontrent qu'au niveau protéique, les modifications post-traductionnelles peuvent aussi influencer profondément l'activité et la stabilité des protéines. Ainsi, la modulation du site de phosphorylation T149 de NEUROG2 dans le néocortex murin perturbe les proportions de progéniteurs corticaux et les différents sous types de neurones des couches profondes et superficielles qu'ils produisent. Toutefois, il n'est pas connu comment ces régulations pourraient moduler l'activité de NEUROG2 sous des niveaux endogènes et comment cela pourrait affecter le développement du néocortex humain.

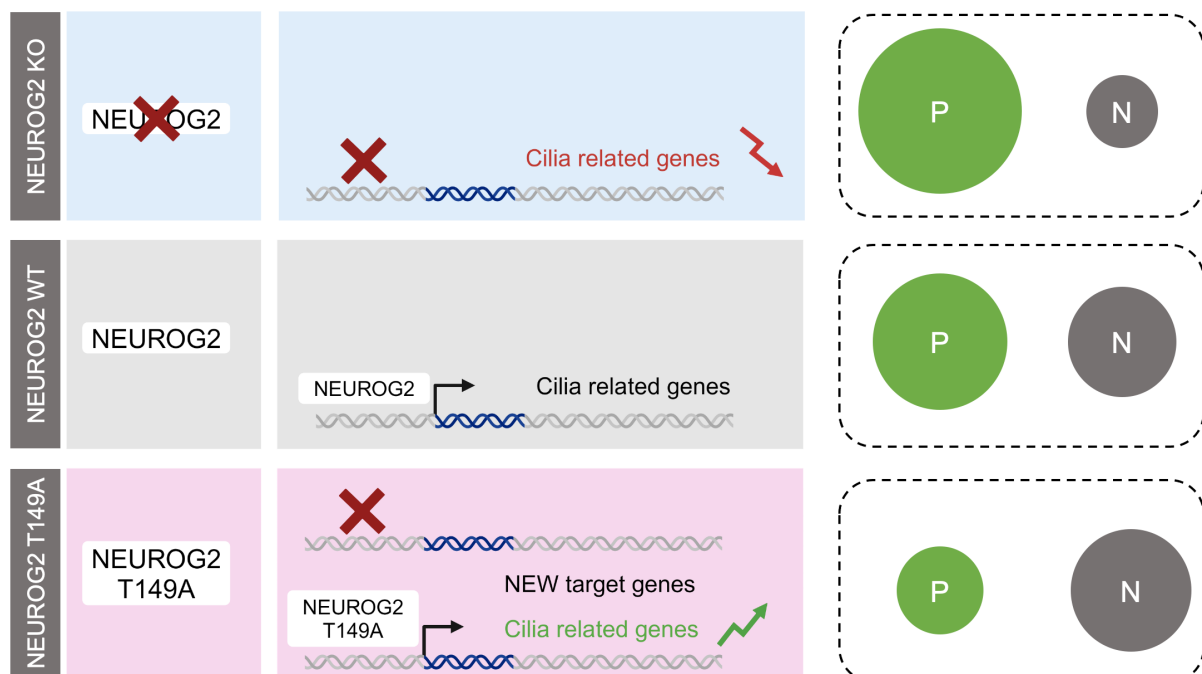
Nous avons donc supposé que la régulation de l'activité de NEUROG2 via la modulation du site de phosphorylation T149 pourrait réguler la différenciation des progéniteurs corticaux en neurones dans le développement cortical humain.

Afin de tester cette hypothèse, nous avons utilisé des organoïdes corticaux issus de la différenciation de cellules iPS génétiquement remodifiées. Nous avons commencé par étudier le rôle de NEUROG2 dans la différenciation neuronale des progéniteurs en induisant la perte d'expression de NEUROG2 grâce aux ciseaux moléculaires CRISPR/Cas9. Nous avons observé une diminution des proportions de neurones à des stades intermédiaire et avancé du développement des organoïdes corticaux. A cela s'ajoute une ventralisation des progéniteurs corticaux via la diminution de l'expression de gènes leur conférant une identité dorsale et une augmentation de ceux leur conférant une identité ventrale. Ainsi, grâce à la validation du rôle crucial de NEUROG2 dans la neurogénèse corticale chez l'humain, nous avons étudié comment la perte du site de phosphorylation T149 de NEUROG2 via son remplacement par une Alanine,

T149A affecte la production neuronale dans le néocortex humain.

Pour cela, nous avons combiné de l'imagerie sur cellules vivantes et fixées dont nous avons quantifiés les proportions avec des algorithmes d'apprentissage profond combinées à des techniques de reprogrammation cellulaire ainsi que du séquençage ARN et de la ChIP pour étudier les propriétés de notre NEUROG2 T149A mutant sur la neurogenese corticale. Nous avons observé que la mutation T149A homozygote ne change ni l'expression de NEUROG2 dans les cellules de la glie radiaire ni dans les progéniteurs intermédiaires, ni sa capacité à se lier à l'ADN et à activer l'expression de ses gènes cibles. Cependant, nous avons observé que les cellules de la glie radiaire effectuent plus de divisions neurogéniques, produisant donc plus de neurones, aux stades intermédiaire et avancé du développement des organoïdes corticaux. On note d'autre part que ce phénotype s'accompagne d'une augmentation de l'expression des gènes responsables de l'organisation structurale et fonctionnelle du cil des cellules de la glie radiaire. Or, ces gènes sont moins exprimés dans les mutants NEUROG2 KO suggérant un lien fort entre ce cil, NEUROG2, son profil de phosphorylation, et la régulation de la neurogène corticale chez l'humain ce qui pourrait donc constituer un potentiel mécanisme moléculaire.

Abstract graphique et modèle proposé :





## **Résumé de la thèse vulgarisé pour le grand public**

Les fonctions cognitives supérieures observées chez l'être humain sont le reflet de l'expansion d'une structure de son cerveau, le néocortex où l'on trouve la plus grande densité de neurones. Or la régulation du gène responsable de la production de neurones à partir de progéniteurs, Neurogenin2 (NEUROG2) est bien connue chez la souris mais encore peu explorée chez l'humain. Ainsi nos travaux démontrent pour la première fois que NEUROG2 est bien le gène responsable de la production de neurones dans le néocortex humain et que la substitution d'un de ses constituants, la Thréonine 149 par une Alanine conduit à un gain de fonctions. De fait, NEUROG2 T149A induit la production de plus de neurones à partir des progéniteurs potentiellement grâce à la régulation des gènes responsables de l'organisation et du fonctionnement de leur antenne, le cil primaire connu pour son rôle dans les maladies du neurodéveloppement suggérant ainsi, un nouveau mécanisme d'action de NEUROG2 sur les progéniteurs.

Mots clés : Neurogénèse, Neurogenin2, Organoides corticaux humains, Cil primaire

## **Thesis summary simplified for the general public**

Higher order cognitive abilities observed in humans is the reflect of neocortical expansion, a brain structure with the highest neuronal density compared to any other mammals. The regulation the gene responsible for neuronal differentiation of progenitors, Neurogenin2 (NEUROG2), is well characterized in mice neocortical development but not in humans. Thus, our work demonstrates for the first time that NEUROG2 is responsible for neuronal production in the human neocortex and that the substitution of one of its components, the Threonine 149 by an Alanine results in a gain of functions. Therefore, NEUROG2 T149A, produces more neurons from progenitors potentially through the regulation of the genes responsible for the organization and the function of the progenitor antennae, the primary cilium known for its implications in neurodevelopmental disorders. This study highlights a new regulatory mechanism of neurogenesis in human cortical development through NEUROG2 and the primary cilium.

Keywords: Neurogenesis, Neurogenin2, Human cortical organoids, Primary cilium