Microcephaly-causing WDR81 mutation alters endosomal trafficking of EGFR and leads to reduced progenitor cell proliferation

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La mutation WDR81 causant la microcéphalie modifie le trafic endosomique d'EGFR et conduit à une prolifération des cellules progénitrices réduite

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ABSTRACT

The development of neocortex is a highly regulated process whereby each cell type must be correctly specified and positioned. Alterations in this process can lead to a variety of cortical malformations, including microcephaly (small brain). This latter pathology has been recently related to mutations in the WD repeat domain 81 (*WDR81*) gene. WDR81 is a poorly characterized transmembrane protein that has been involved in endosomal maturation as well as aggrephagy. It remains unknown how mutations in WDR81 alter the growth of the neocortex. To address this question, I have generated a CRISPR/Cas9 WDR81 knock-out mouse. Mutant animals for WDR81 die perinatally and recapitulate the human microcephaly phenotype. I demonstrate that the reduced brain size is not due to increased apoptosis or altered cell fate, but to reduced neural stem cell proliferation rates. Patient-derived fibroblasts also display reduced proliferation, indicating that this may be a general feature of WDR81 loss of function. In this system I identified alterations in the MAP kinase signaling pathway, which shows reduced ERK phosphorylation in mutant cells following EGF stimulation. I further demonstrate that this defect is due to a strong reduction of the EGR receptor (EGFR) expression levels. EGFR levels can be rescued following EGF starvation, indicating that this downregulation is due to altered EGFR downstream signaling. Indeed, I observe EGFR intracellular clearance delays following its internalization after EGF binding. I demonstrate that EGFR accumulates in aberrant swollen early endosomes in WDR81 patient mutant cells, which we also observe in mutant mouse neural stem cells in vivo. Together, this work shows that WDR81 regulates endosomal trafficking of EGFR, and that loss of function leads to reduced MAP kinase pathway activation causing reduced neural stem cell proliferation and WDR81-linked microcephaly. However, these proliferation defects can be rescued *in vivo* by the expression of megalencephaly-causing mutated Cyclin D2 demonstrating that microcephaly and megalencephaly can be due to opposite effects on the proliferation rate of radial glial progenitors.

PREFACE

The development of the central nervous system implicates an extremely complex process, composed by an initial phase of severe expansion of the progenitors' pool, followed by the production of a plethora of neuronal and glial cell types that have to be generated at the right timing and in the correct amount. Defect in this process would lead to neurodevelopmental disorders: one such example is microlissencephaly.

In the introduction of this thesis I will describe the main steps of cortical development, focusing then on the main type of cells that constitute it and act as the main organizers of the developing neocortex. I will then describe the possible pathologies that could arise from anomalies during the formation of the cortex giving particular emphasis to microcephaly (core of this study). I will after introduce endosomal trafficking and connect it with microcephaly explaining how the protein WDR81 regulates it the developing neocortex.

INDEX

INTRODUCTION

1. THE NERVOUS SYSTEM

In all animals, the ability to receive and elaborate stimuli from the environment or from the organism itself depends on a very complex and highly connected organ system called the nervous system (Figure 1). In vertebrates, it consists of two main parts, the Central Nervous System (CNS) and the Peripheral Nervous System (PNS). The CNS consists mainly of the brain and the spinal cord, while the PNS is composed mainly of nerves. Nerves are enclosed bundles of axons, long nervous fibers originating from neurons transmitting electric signals from the PSN to the CNS and vice versa. Nerves can be classified in two main categories: 1) motor or efferent nerves that transmit signals from the CNS to the PNS; 2) sensory or afferent nerves that recollect and transmit information from the peripheral parts of the body and the environment to the CNS(Karemaker, 2017; Sousa et al., 2017).

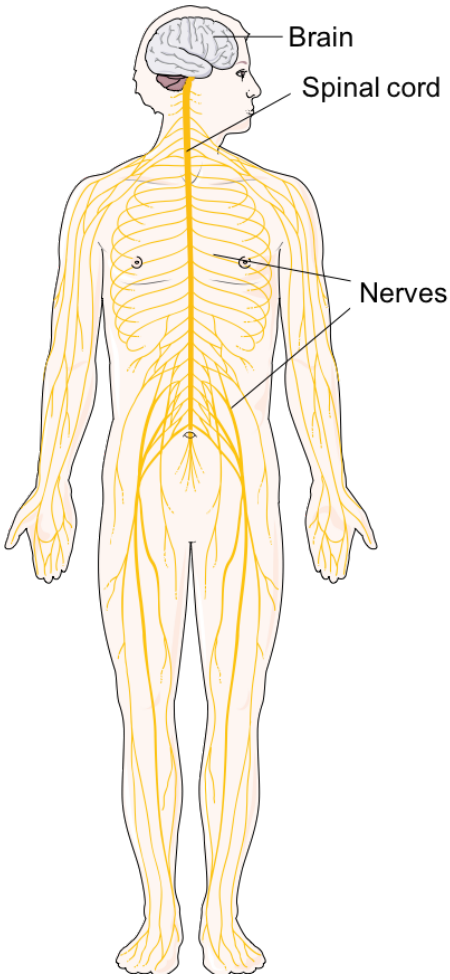


Figure 1 *Schematic representation of the human nervous system*

The PNS can be divided into three separate subunits: somatic, autonomic and enteric systems. The somatic system is specialized in mediating voluntary movements; The autonomic nervous system supplies smooth muscles and glands to influence the activity and regulation of internal organs. The enteric system controls the gastrointestinal apparatus. Both autonomic and enteric, are involuntary and are not controlled by somatic perception(Karemaker, 2017).

Most multicellular organisms display a nervous system, with a high variation in complexity. The only multicellular animals that do not possess a nervous system are sponges, placozoans and mesozoans. Radially symmetric organisms as ctenophores and cnidarians like jellyfish and anemones have a simple nervous system composed of a diffuse nerve net. All other animal species have a nervous system composed of a brain, a central cord, and nerves radiating from the brain and central cord. However, even though the basic functions of the nervous system are mostly conserved throughout the animal kingdom, its complexity gradually increased with the specification process (Sousa et al., 2017). Indeed, the more complex the organism is the more we can identify:

1. An increase of the total number of neurons
2. Formation of groups of neurons that will form ganglia and nerves
3. Specialization of function of a single neuron or a group of neurons
4. Increase of interneurons, connecting specialized neurons
5. Cephalization (formation of the head); a process where all the organs that are in charge of perception are concentrated in the anterior part of the body close to a highly elaborated group of neurons, center of the whole nervous system: the brain.

1.1 Establishment of the primordial nervous system in mammals

In mammals, nervous system formation and development starts with a process called gastrulation where the monolayered blastula starts to fold (Muhr & Ackerman, 2020; Williams & Solnica-Krezel, 2017). After that, three different layers of cells will differentiate giving rise to an upper layer called ectoderm, an intermediate layer called mesoderm, and a bottom layer called endoderm. The first step for the development of the nervous system is the formation of the notochord that originates from cells at the center of the mesoderm. The ectoderm will

fold in a region right above the notochord called the neural plate. The neural plate will then fold inward and fuse to give rise to a cylindric structure known as the neural tube. This structure will then give rise to the brain and then to the spinal cord (Le roux et al. 2003, Purves 2016). The neural tube is composed of neural stem cells that proliferate to expand their population. These cells later differentiate into neurons, astrocytes and oligodendrocytes creating the bases for a fully developed and mature nervous system. The floorplate plays a critical role in the definition of the dorsoventral polarity of the neural tube, through the releases of specific factors (figure 2) (Muhr & Ackerman, 2020; Williams & Solnica-Krezel, 2017). These factors include retinoic acid (RA), fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor (TGF), sonic hedgehog (SHH), and members of the wingless (Wnt) family, which are released in a fine-tuned spatial and temporal manner (Purves 2016). The precise function of each of these factors will be detailed later in the following sections.

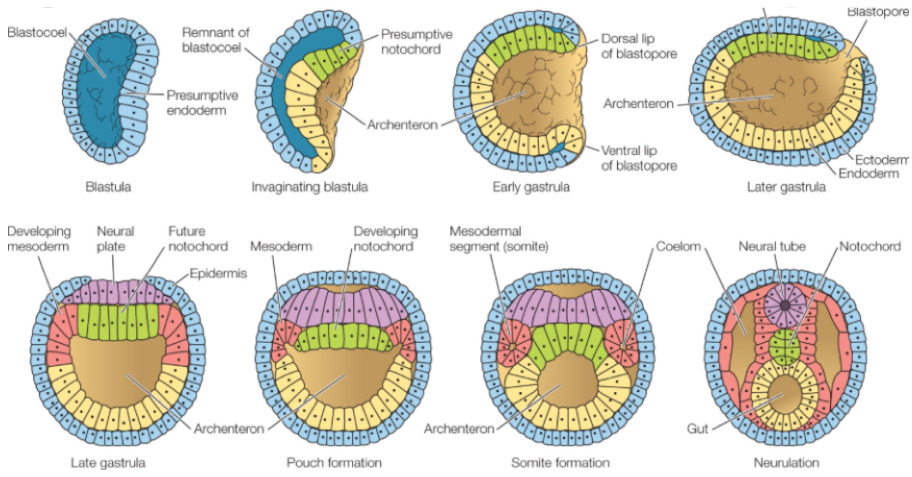


Figure 2 *schematic representation of developmental stages from gastrulation to neurulation (adapted by macmillanhighered.com). The blastula will start to fold inward creating the lumen of the gastrula. Once the gastrula is formed, the different layers will start to specify to end up in neurulation where the neural tube and notochord will form.*

2. CONSTITUTION OF THE BRAIN

Following closure and the generation of a lumen, the anterior part of the neural tube will fold and swell, shaping what will in the future become the brain. At this stage, different zones can begin to emerge: the prosencephalon, mesencephalon, rhombencephalon, telencephalon and, posteriorly, what will be the precursor of the spinal cord (Fig 3) (Darnell & Gilbert, 2017; Prakash & Wurst, 2006). The neural tube will fold forming a crook shaped structure characterized by two main bending sites: the cephalic flexure between the mesencephalon and the rhombencephalon, and the cervical flexure between the rhombencephalon and the spinal cord. The lumen will follow this morphological change and, at the end of the process, give rise to the ventricles and the vertebral channel (Darnell & Gilbert, 2017; Prakash & Wurst, 2006).

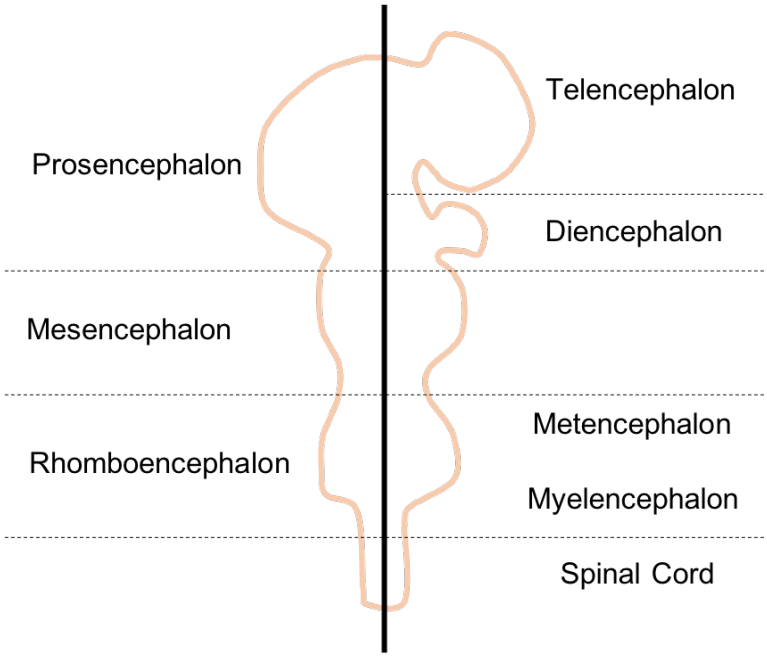


Figure 3 *Schematic representation of embryonic forebrain derivates*

As development progresses the different regions of the brain will continue maturing and partitioning. The prosencephalon will divide in two areas: the rostral part that will give rise to the telencephalon and the caudal part that will give rise to the diencephalon. The telencephalon is further divided in two more areas: a dorsal and a ventral one. The dorsal area will give rise to cerebral cortex and hippocampus, the ventral area will generate the basal

ganglia, the basal forebrain nuclei and the olfactory bulb. The diencephalon will continue to fold bilaterally, forming two optic cups from which the retina will form (Darnell & Gilbert, 2017; Prakash & Wurst, 2006). In addition to that, the diencephalon will give rise to the thalamus and hypothalamus. The mesencephalon, similarly to the telencephalon, can be divided in a dorsal and a ventral territory. The dorsal area will generate the superior and inferior colliculi, meanwhile the ventral part will give rise to the tegmentum. Finally, the rhombencephalon will give rise to the cerebellum, the pons and the medulla oblongata.

3. THE CEREBRAL CORTEX

3.1 Structure and Function

The cerebral cortex is the area of the brain that can be reported as a hallmark of humans and other mammals. It is the part of the brain that lies under the meninges and covers the area of the two hemispheres. It mostly consists of the six-layered neocortex and the four-layered allocortex (a thin area of the cortex composed mostly by the olfactory system and the hippocampus). The cerebral cortex exhibits a remarkable and peculiar shape with a mix of bulges known as gyri and sulci. The combination of these two creates multiple folds that allow for a dramatic increase in brain size without the need for an enlarged cranial space. As a consequence, the cerebral cortex will become the largest site of neural integration in the central nervous system (Saladin et al. 2011). It controls higher brain functions such as speech development, attention, perception, memory and consciousness. In human, the cerebral cortex is composed of 10 to 12 billion cortical neurons, for a total average weight of 1500 g. It is divided into the right and the left hemisphere, which take on different roles and functions.

For example, the left hemisphere is dominant for language, initiation of movement and artistic abilities. It has indeed been demonstrated that strokes that affect this hemisphere lead to language and movement loss or partial invalidation (Ghaleh et al., 2018).

The cerebral cortex can be divided in four different lobes that display different functions: frontal, temporal, parietal and occipital lobes (fig 4). The *frontal lobe* is involved in controlling motor commands for voluntary movement. It is the most rostral area of the brain and it is separated from the parietal lobe by the central sulcus (Pirau & Lui, 2020). The *temporal lobe* is involved in speech understanding and emotions. It is located behind the frontal lobe, just under the parietal lobe and it is separated from them by the lateral (or Sylvian) sulcus (R. L. Jackson et al., 2018; Kamm et al., 2018). The *parietal lobe* plays a role in perception of the surrounding environment. It lies posterior to the frontal lobe and is separated from it by the central sulcus (Ghoneim et al., 2018). The *occipital lobe* is the most caudal, located just behind the parietal lobe and the temporal lobe, and is involved in the elaboration of visual stimuli (Flores, 2002).

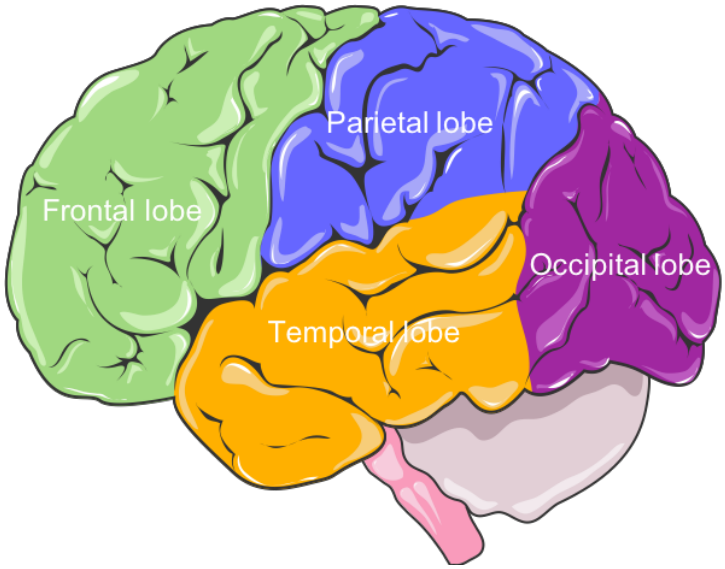


Figure 4 *Schematic representation of the cerebral lobe organization of a human brain*

3.2 Neocortical areas

Apart from the partition into lobes, the neocortex is divided into different zones or areas based on their cytoarchitecture or, in other words, based on differences in cellular density, size, shape and synaptic inputs/outputs. These features are critical for the functional diversify of each area (Amunts & Zilles, 2001).

The neocortex can be divided in two main groups: sensory cortices and motor cortices. The first of the sensory cortices is the Somatosensory cortex, located in the parietal lobe, is involved in recollecting and associating sensory information from the afferent nerves (Kropf et al., 2019).

The second sensory cortex is the visual cortex and it is located in the occipital lobe. It is responsible for the interpretation of the visual signals and recognition of shapes and forms(Murray et al., 2016).

Auditory cortex is situated in the temporal lobe and is involved in the interpretation of sound, responsible for understanding of language(Pickles, 2015).

Within the motor cortices group we can find:

The primary motor cortex is located in the frontal lobe. From here most of the corticospinal tract nerves and cranial nerves are originated. This area is well connected with the thalamus and the sensory cortical areas (Svoboda & Li, 2018).

The motor association cortex (or premotor cortex). It receives inputs from the sensory association cortex and the thalamus allowing the connection between the sensory and motor cortices enabling the motor response (Papale & Hooks, 2018).

The prefrontal cortex is the most developed in humans and indeed, defines human behavior acting on attention, memory and judgement, and personality definition.

This cortex is well connected to all other cortical regions via association fibers and receives inputs as well from the zones that control emotions and sleep like amygdala and limbic system(Dixon et al., 2017).

3.3 Arealization of the cortex

3.3.1 Patterning centers

Early specification of cortical territories and their arealization is controlled by intricated interactions between different extracellular signals such as Sonic Hedgehog (Shh), Fibroblast Growth Factors (FGFs), Retinoic Acid (RA), Bone morphogenetic Proteins (BMPs), the WNT family proteins and Epidermal Growth Factors (EGFs) (Puelles, 2011; Sansom & Livesey, 2009). Most of these molecules start to be expressed from early stages of development (E8.5-E9.0 in mouse and GW 4-5 in human). They are produced and released by specific areas called patterning centers, and act in a dose and time dependent manner to regulate proliferation, differentiation and migration.

In mouse, there are five main telencephalic patterning centers: the anterior neural ridge, the cortical hem, the antihem, the nasal placode and the ventral area of the telencephalon. The anterior neural ridge (ANR), a region located at the anterior margin of the folding neural tube, begins to release Fgf8 together with Fgf15, 17 and 18 in between E8-E8.5 covers the role of inducing proliferative commitment of the newly formed neuroepithelium (Puelles, 2011). This region will also express Noggin and Chordin with the effect of inhibiting Bmp in the closest zone around the ANR, stimulating the neuroectoderm fate (Anderson et al., 2002). The hem is located next to what will give rise to the hippocampus; its cells produce and secrete Wnt and Bmp responsible for neuronal patterning (Assimacopoulos et al., 2003). The antihem gets his name due to its opposite position with respect to the hem and to the

production of Sfrp2 which is a potent antagonist of Wnt signaling produced in the hem, acting as a negative regulator of migration and apoptosis (Kim et al., 2001). It also produces several other trophic factors, including TGFα, Neuregulin 1, Neuregulin 3 and Fgf7. The nasal placode (the olfactory epithelium of the nasal cavity) releases high levels of RA inducing neurogenesis along a rostrolateral-high to mediocaudal-low gradient (LaMantia et al., 1993). Finally, the ventral telencephalon secretes Sonic Hedgheog (SHH), inducing ventral forebrain identity, as well as the development of the hypothalamus (Sagai et al., 2019).

3.3.2 Patterning genes

In response to these morphogenic factors, a high number of genes are expressed by progenitors all along the dorsolateral area of the developing forebrain, fine-tuning neurogenesis. In mouse for instance, Lhx2 represses the dorsal fates in the early phases of development. Emx2 and COUP-TF1 induce caudal corticogenesis, and Pax6 and SP8 trigger the establishment of the dorsal neocortical progenitor domain and guide the development of most of the anterior areas (Hamasaki et al., 2004; Molyneaux et al., 2007). *Emx2* and *COUP-TF1* are normally expressed in progenitors and act in a concentration-dependent manner to specify the size and positioning of the primary cortical areas. If the gradient of Emx2 or COUP-TF1 is modified along the length of the developing neocortex, there will be changes in the size of certain areas of the adult brain (Hamasaki et al., 2004). For instance, a loss of function of Emx2 or COUP-TF1 will reduce caudal cortical area (Mallamaci et al., 2000), whereas a gain of function of Emx2 will result in their expansion (Hamasaki et al., 2004). *Pax6* is expressed mainly in the dorsal part of a developing neocortex, more specifically in progenitors localized in the VZ and SVZ. Its absence will result in the repression of certain factors, such as *Mash1* or

Gsh1, which will cause the production of an unbalanced mixture of neurons (Muzio & Mallamaci, 2005).

3.3 Neuronal Layering

The grey matter is the major component of the central nervous system, mainly consisting of neuronal cell bodies that are interconnected to form a network of glutamatergic (inhibiting) and GABAergic (stimulating) neurons, supported by glia and blood vessels (Florio & Huttner, 2014). The different neuronal cell types will be described later on. However, for the correct function of the brain another major group of cells is required: the glial cells. Oligodendrocytes are responsible for axon myelinization, while astrocytes maintain the microarchitecture of the brain parenchyma, and are essential for brain homeostasis, providing nutrients and sustaining synaptogenesis and synaptic efficiency (Nayak et al., 2014; Y. Wu et al., 2015). In addition to that, microglia are the resident immune phagocytes of the brain that act against damage and diseases.

Neurons are arranged in columns and work modularly in interconnected microunits (Bystron et al., 2008). They are organized in six layers from I to VI with I being the uppermost and VI the deepest. Within each layer, neurons tend to share similar cell type identities and connectivity patterns(Douglas & Martin, 2004). Each layer develops from the same pool of neural stem cells located at the ventricular zone of the developing neocortex, in a process known as corticogenesis (Lui et al., 2011). During neurodevelopment each newly produced neurons will migrate on top of the previous ones, in an “Inside out process”, reaching the typical stratified organization of an adult cortex. Going apico-basally (from the ventricle to the meninges), layers will be organized from VI to I (Agirman et al., 2017; Cooper, 2008; Marín et al., 2010).

Layer VI, or the fusiform layer, is composed of multipolar neurons of various sizes. It is characterized by the presence of excitatory and inhibitory connections with the thalamus (Proulx et al., 2014). Layer V, or internal pyramidal layer, contains large excitatory neurons and connects with basal ganglia, corpus callosum and thalamus (Palomero-Gallagher & Zilles, 2019; Rockland & DeFelipe, 2018a, 2018b). Layer IV, or internal granular layer, is enriched with stellate GABAergic inhibitory neurons; here the neural fibers connect the two hemispheres (Palomero-Gallagher & Zilles, 2019; Rockland & DeFelipe, 2018a, 2018b). Layer III, or external laminar layer, holds small and medium size pyramidal neurons. It contains mainly afferent corticotectal connections (Palomero-Gallagher & Zilles, 2019; Rockland & DeFelipe, 2018a, 2018b). Layer II, also called external granulate layer, includes small pyramidal excitatory neurons and a high number of stellate inhibitory neurons (Palomero-Gallagher & Zilles, 2019; Rockland & DeFelipe, 2018a, 2018b). It contains callosal projections. Finally, layer I is the molecular, and consists of dendritic extensions of the pyramidal neurons standing underneath, horizontally oriented axons, as well as glial cells. However, it is worth mentioning that layer I is also extremely important for cortical development, even though this process does not happen in the adult brain; indeed, it is the first to rise and differentiate from the ventricular zone. It contains highly specialized cells called Cajal-Retzius cells (C-R). These cells will produce and release reelin, a strong chemoattractant that will regulate migration and positioning of neurons (Bielle et al., 2005; Marín et al., 2010).

4. DEVELOPMENT OF THE CEREBRAL CORTEX

The cerebral cortex develops from neuroepithelial cells that massively proliferate at Embryonical developmental day 11-12 (E11-E12) in mice and Gestational Week 5-6 (GW5-6) in human, to form brain vesicles(Bystron et al., 2008). During this period, a ventricular zone (VZ) and a sub-ventricular zone (SVZ), located just upon the VZ, are established. These areas of the brain are composed almost exclusively of neural progenitors, at least in the early stages of development (Noctor et al., 2004b). The first generated neurons are the Cajal-Retzius cells and subplate cells (SP) from E10 in mouse and GW5-7 in human. These cells are transient, quite similar in morphology and constitute what will be called the preplate. SP and C-R cells guide the migration and positioning of newly born neurons through the release of reelin, a protein that acts as a chemo attractant (Lakatosova & Ostatnikova, 2012). As the development continues the Intermediate Zone (IZ) arises. This area is a transition area characterized by the presence of multipolar and bipolar neurons that are migrating to form the cortical plate. The cortical plate will form between E13 to E17 in mice and GW7-20 in human. At the very beginning of neurogenesis (E13-E14 in mouse), neurons are believed to be directly generated

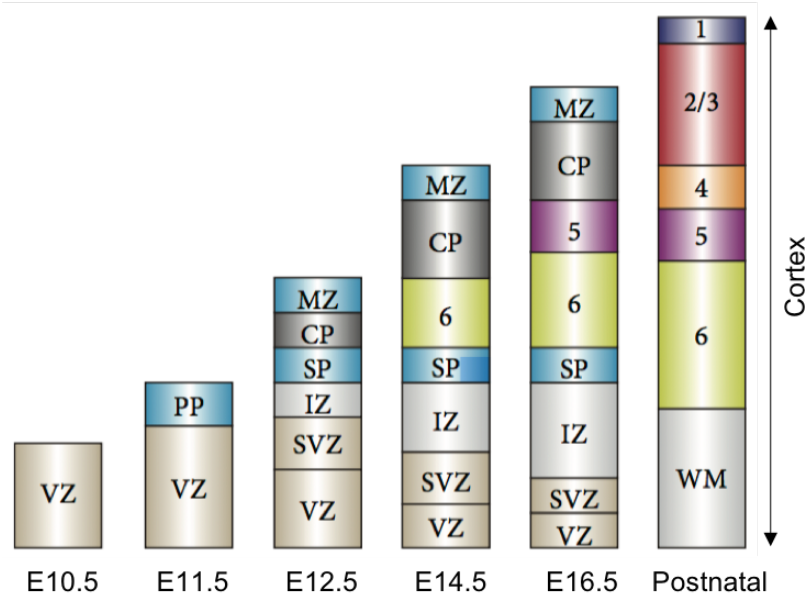


Figure 5 *schematic representation of cortical developmental stages in mouse. MZ and 1 : cajal-retzius cells ; CP : newly generated nd migrated neurons ; 2-4 : upper layers neurons ; 5-6 : deep layer neurons ; SP : subplate neurons ; SVZ : basal progenitor cells ; VZ : RG cells*

from radial glial cells (RGC), whereas later (E15-E17) they mainly originate from intermediate progenitor cells (IPC) or basal progenitors deriving form RGC cells (Corbin et al., 2008; Noctor et al., 2004). In humans, and more generally in primate, cortical neurogenesis is much more complex than in rodents and relies on an additional germinal zone, the outer SVZ (oSVZ). This zone contains another type of radial glial cells, the outer RG or basal RG (oRG or bRG). bRG cells are responsible for the expanded production of neurons, especially in the second half of human corticogenesis (GW16-22). These cells are also major players in the gyrification of the neocortex, and therefore play a pivotal role in cortical expansion (Astick & Vanderhaeghen, 2018).

4.1 Progenitors of the developing neocortex

A variety of neural progenitor types have been identified, which can be classified into two main classes: apical progenitors and basal progenitors (Fig 6 and 7).

4.1.1 Apical progenitors

Three types of Apical Progenitors (APs) have been described so far: neuroepithelial cells (NECs), apical radial glia (aRG) cells, and apical intermediate progenitors (aIPs).

Neuroepithelial cells (NECs) are the cells that from the neural plate at the very early stages of development and later on, form the epithelium that covers the neural tube(Huttner & Brand, 1997). At early developmental stages NECs undergo symmetric proliferative divisions to expand their population (Rakic & Caviness, 1995), resulting in a double effect on the growth of the neocortex: the expansion in lateral dimension and growth in radial dimension leading to an increase in the overall thickness of the neuroepithelium. NEC cells have a typical and

easily recognizable shape: they are highly polarized cells exhibiting an apico-basal axis and span the entire width of the neuroepithelium. Their basal plasma membrane is attached to the basal lamina, while their apical domain connects the lumen of the neural tube(Götz & Huttner, 2005a). These connections are held in place by the presence of occludin-positive tight junctions (Aaku-Saraste et al., 1996).

On their apical domain, NECs extend a primary cilium into the ventricles that function as an antenna for sensing signaling molecules present in the cerebrospinal fluid(Taverna et al., 2014). As NECs proceed in cell cycle, their nucleus oscillates along the apico-basal axis of the cell within the neuroepithelium, entering mitosis when reaching the ventricular surface where the centrosome is located (Bertipaglia et al., 2018). This process is called interkinetic nuclear migration (INM) (Taverna & Huttner, 2010) and is a typical feature of neural progenitors.

Later in neurodevelopment, in between E10-E12 in mouse (Matsuzaki & Shitamukai, 2015), NECs will switch from symmetric proliferative to asymmetric proliferative and differentiative cell division, turning from a purely proliferative to a neurogenic state (Götz & Huttner, 2005a). This is mirrored by their differentiation into apical radial glial (aRG) progenitors, that become the predominant neuronal progenitor cell type in the developing neocortex (Malatesta et al., 2008; Noctor et al., 2004a). The transition from NECs to aRGs is critical for determining the size of the initial pool of progenitors available to generate neurons and therefore crucial for the definition of the final neurons output and brain size (Fernández et al., 2016).

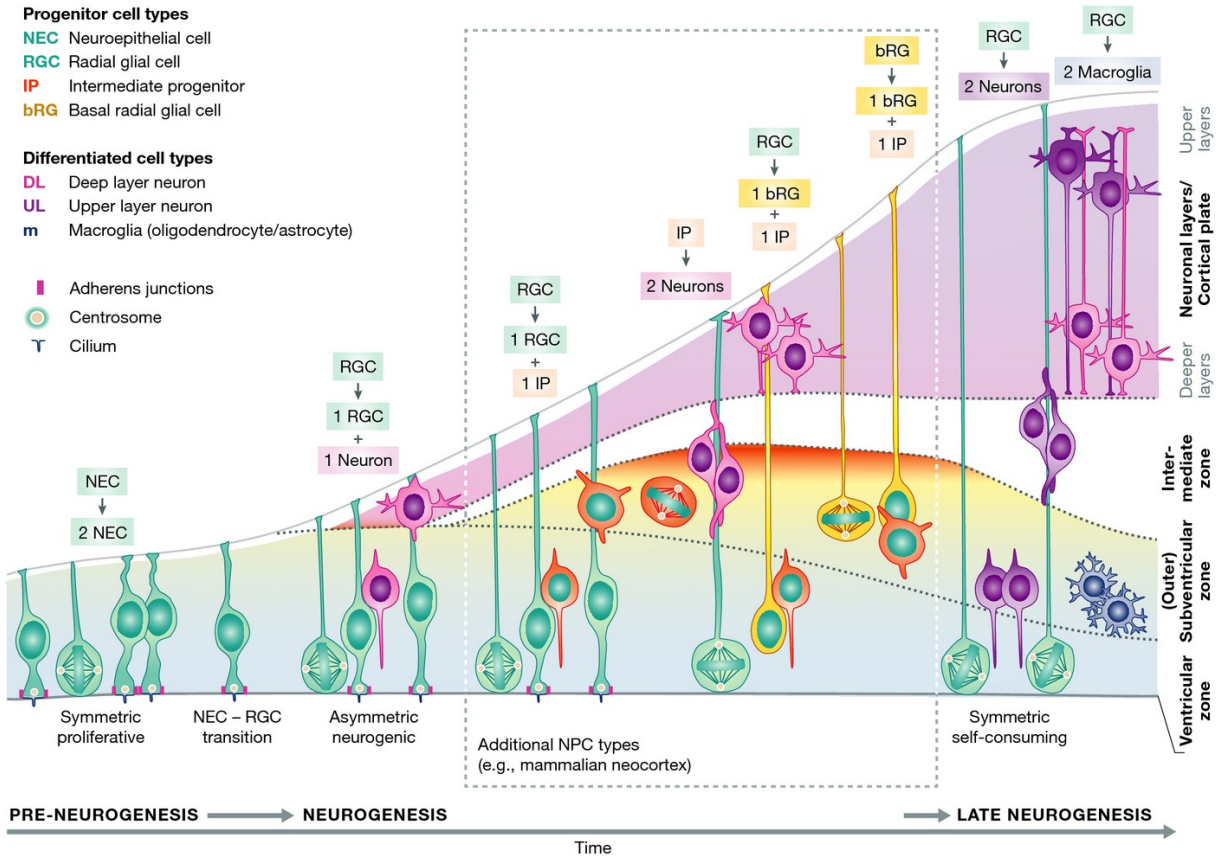


Figure 6 *Schematic overview of neurogenesis in the embryonic vertebrate CNS (adapted from Paridaen and Huttner 2014). In pre-neurogenesis NEC undergo symmetric proliferative divisions to enhance their population. In neurogenesis NEC will become RGC and undergo un asymmetric differentiative divisions producing one RGC and an intermediate progenitor. The IP will then divide to produce two neurons that will use the basal process of the RGC to reach the apex of the cortex.*

Apical radial glial cells (aRGs) originate from the transformation of NECs to a new type of neurogenic progenitor that has more restricted proliferative potential, dividing mainly asymmetrically and representing the core of proper neurogenesis. aRGs indeed give rise to the totality of neurons and glial cells of the neocortex. Several factors involved in the transition from NECs to aRGs are also key for maintaining aRG identity. Notably, FGF triggers expression of aRG markers in NECs (Sahara & O'Leary, 2009) and also supports an aRG fate by inhibiting their transition towards a more committed neuronal progenitor. The Notch signaling pathway has also been described to be important for the transition from NECs to aRGs (Gaiano et al., 2000; Hatakeyama et al., 2004; Martynoga et al., 2012).

Whilst they lose tight junctions and develop the apical junction (AJ) belt, aRGs acquire astroglial features(Florio & Huttner, 2014; Götz et al., 2015) and become positive for a set of astroglial markers, such as the astrocyte-specific glutamate transporter and others. They also express key neurogenic transcription factors (TF), such as *Pax6* (Götz & Huttner, 2005). As NECs, aRG cells are highly polarized and are connected to the basal and apical surface with respectively a basal and an apical process. aRGs also undergo INM process, but the nuclear oscillation is restricted to the VZ where their soma resides (Götz & Huttner, 2005). As already mentioned, they undergo asymmetric divisions, thereby self-renewing while producing post-mitotic neurons (direct neurogenesis) or, in the majority of cases, neurogenic progenitors (indirect neurogenesis). During indirect neurogenesis, an aRG cell will produce an *intermediate progenitor* than will divide again to produce neuros, increasing the final overall neuronal output.

Apical intermediate progenitors (aIPs) are a minor cell population in the developing brain. They derive from asymmetrical divisions of aRGs, keeping some of their features such as maintenance of contacts with the ventricular surface and integration into the apical junction belt (Tyler & Haydar, 2013). These cells upregulate specific transcription factors like *Pax6* and *Tbr2* and divide apically after a single INM. aIPs most of the times do not possess a basal process and are therefore not in contact with the basal lamina. They perform INM and mitosis only once, thus rapidly self-consuming to produce two neurons (Tyler & Haydar, 2013).

Subapical progenitors (SAPs) are the most recently discovered progenitors in the brain. They are very rare in the brain and therefore so far considered a minor class of progenitors(Pilz et al., 2013). They possess an apical process and are located in a specific area of the ventricular

and subventricular zone. They divide within the ventricular zone but far from the apical surface (Pilz et al., 2013). So far very little is known about their features. Their transcriptional phenotype has just started to be investigated; so far it is known that these cells express *Pax6* and/or *Tbr2* (Betizeau et al., 2013; Lamonica et al., 2013).

4.1.2 Basal progenitors

Basal progenitor cells (BPs) derive from apical progenitors (NECs or aRGs) and represent two types of cells: intermediate progenitors (IPs) and basal radial glial (bRG) cells.

Intermediate Progenitors (IPs) are non-epithelial BPs: after their generation from aRG cells they delaminate from the apical junction belt; they retract their apical process and translocate into the SVZ. Subsequently, they begin expressing the transcription factor *Tbr2*(Cárdenas et al., 2018; Englund et al., 2005). In mouse, IPs divide symmetrically and rapidly self-consume to form two neurons, thereby doubling the neuronal output of aRG cells(Haubensak et al., 2004). In primates however IPs were shown to go through multiple rounds of symmetric proliferative divisions before terminal consumptive divisions(Betizeau et al., 2013; Noctor et al., 2004).

Basal radial glia (bRGs): bRGs have recently drawn a lot of attention since they are believed to be at the center of brain expansion and folding of gyrencephalic species. Indeed, they are more abundant in primates and ferrets than in lissencephalic species like mouse. They are produced from aRGs within early phases of neurodevelopment. Their soma is located in the SVZ with a basal process extending up to the basal surface. Importantly, and contrary to aRGs, they are not in contact with the apical surface and therefore are not epithelial cells. bRGs can

adopt various morphologies, including the presence of one basal process, one apical process (that does not reach the ventricle) or both processes (Betizeau et al., 2013).

bRGs have been shown to be produced following aRG cell division (Gertz et al., 2014; Lamonica et al., 2013), although direct delamination of aRGs has also been suggested (Gertz et al., 2014; Lamonica et al., 2013). However, bRGs share similarities with aRGs in terms of gene expression and cellular characteristics expressing *Pax6*, *nestin* and *vimentin*(Ostrem et al., 2017). In primate bRGS can divide multiple times, self-renewing while generating intermediate progenitors and neurons. However, despite all the similarities with aRGS, bRGs undergo a very specific form of nuclear migration called mitotic somal translocation (MST), during which the soma moves along the basal fiber just before cell division.

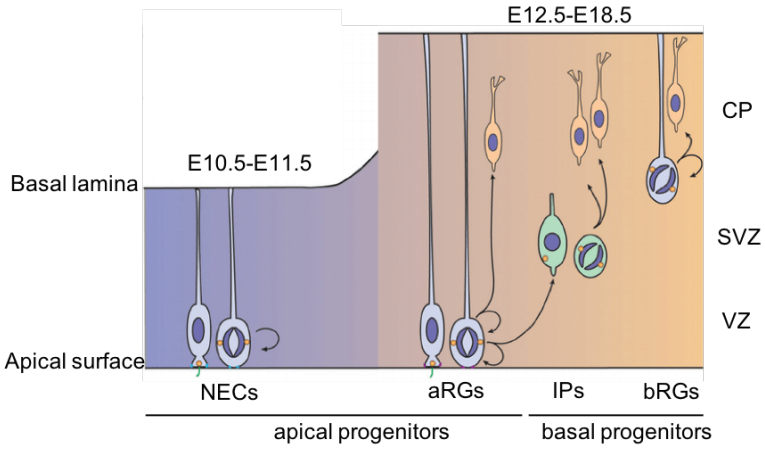


Figure 7 *Schematic representation of neural progenitors cells (adapted from Laguesse S 2014) showing the onset of apical progenitors NECs and aRGs at the early phases of neurodevelopment E10.5-E12.5 and basal progenitors in later phases.*

4.2 Cell cycle regulation of progenitor cells

Cell cycle length, in particular the length of the G1 phase has been associated with the balance between symmetric proliferative divisions (a hallmark of NECs), and asymmetric neurogenic divisions (a hallmark of aRG cells) (Calegari et al., 2005; Lange et al., 2009). It was observed that cell cycle duration is different according to the region of the brain and lengthens as development proceeds. In particular it has been observed that G1 length increases as development proceeds (Hara et al., 2005), and that artificially modifying its duration alters the proportion of neurogenic divisions (Calegari et al., 2005; Lange et al., 2009). Moreover, growth factors that influence cycle duration also affect cell fate. For example, enhancement of Fibroblast Growth Factor (FGF) or insulin like growth factor 1 (IGF-1) reduces G1 length and stimulates progenitor proliferative divisions (Mairet-Coello et al., 2009).

While these studies focused on G1 length, a recent study performed in ferret indicates that cell cycle differences between progenitors is mainly due to changes in S-phase, since little variation was observed in G1 when comparing different progenitor types (Turrero García et al., 2016). Moreover, Wong and co-workers (2016) reported a longer S-phase in bRG-like cells, suggesting that regulation of this phase is important for the cell cycle of bRGs.

Surprisingly, a recent study showed that upon cell cycle arrest of undifferentiated aRGs, these cells still turn on a transcriptional program associated with the neuronal output they are meant to produce (Okamoto et al., 2016). In this study, single-cell transcriptomics revealed a set of genes whose expression changes over time in undifferentiated aRGs at different developmental stages. In order to address if these transcriptional changes could be altered by defects in cell cycle progression, the authors induced cell cycle arrest of aRGs whilst maintaining them undifferentiated and performed the same type of single-cell transcriptomics analysis. Strikingly, the pattern of expression of the genes previously

identified was not altered, implying that differentiation is independent of cell cycle progression (Okamoto et al., 2016).

4.3 Factors influencing neuronal output

Although neural progenitors have homologous counterparts in most mammalian orders, not all types are equally abundant across species and they do not contribute equally to the final neuronal output (Fig 8). According to what is known so far, the most remarkable interspecies variation concerns the expansion of the BP pool in the SVZ. As stated above, in gyrencephalic species like primates, the SVZ is divided into an inner SVZ (iSVZ) and an outer SVZ (oSVZ). The iSVZ is reminiscent of the murine SVZ, while oSVZ is absent in most lissencephalic species (Reillo & Borrell, 2012). The oSVZ contains a highly abundant population of bRG cells, beleived to be critical for the gyrification of the cortex (Reillo & Borrell, 2012).

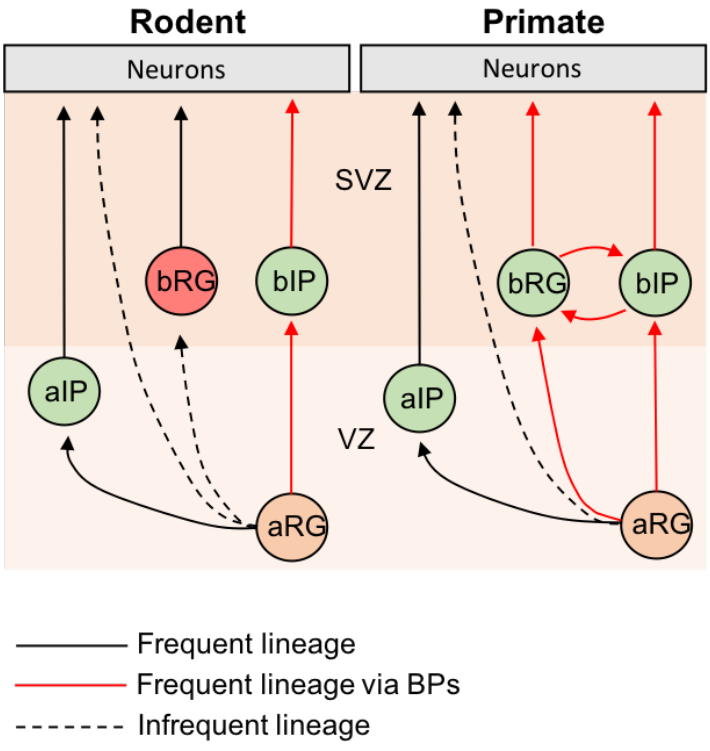


Figure 8 *schematic representation of lineage derivates in rodents and primates developing brain*

The proliferative potential of progenitor subtypes also contributes to interspecies divergence, generating differences in the final neuronal output. For example, in lissencephalic rodents the prevailing output of aRG differentiative divisions are neurogenic IPs which will then divide once symmetrically to generate two neurons(Haubensak et al., 2004; Shitamukai

et al., 2011). In gyrencephalic primates on the other hand, aRGs will produce bRGs, which will self-amplify before producing neurons, increasing exponentially the neuronal output(Betizeau et al., 2013; Lamonica et al., 2013).

The length of neurogenesis also varies between species and can explain the differences in the total neuronal output. More specifically, the main differences are: 1) the onset of neurogenesis, which is delayed in primates compared to rodents, allowing a greater increase in the pool of NECs in the earliest phases of neurogenesis; 2) neurogenesis duration, which is longer in primates than in rodents (Rakic & Caviness, 1995), allowing the expansion of NPCs (for example bRGs). Notably, a longer neurogenic period will lead to an increase of the number of neurons only if the cell cycle length stays the same. Indeed, a longer cell cycle would lead to a lower neuronal output merely because cells take more time to divide. Hence, since cell cycle lasts around 20-25 hours in rodents versus 40-50 hours in primates (Dehay & Kennedy, 2007) we would expect rodents to have a higher number of neurons. However, the longer time window in which neurodevelopment occurs in primates will largely compensate the difference in cell cycle length (Betizeau et al., 2013; Dehay & Kennedy, 2007).

5. CORTICAL MALFORMATIONS

As described above, cortical development is a fine-tuned and complex process during which many different types of cells develop and interact with each other. It appears clear that any alteration or mis-regulation of any of the processes involved in neurodevelopment could have dramatic consequences and lead to cortical malformations. These malformations can have environmental causes, such as infection of the mother during pregnancy. One such example is the Zika virus, which causes microcephaly. However, for the purpose of this thesis only the genetic causes of primary brain malformations will be described in the coming lines.

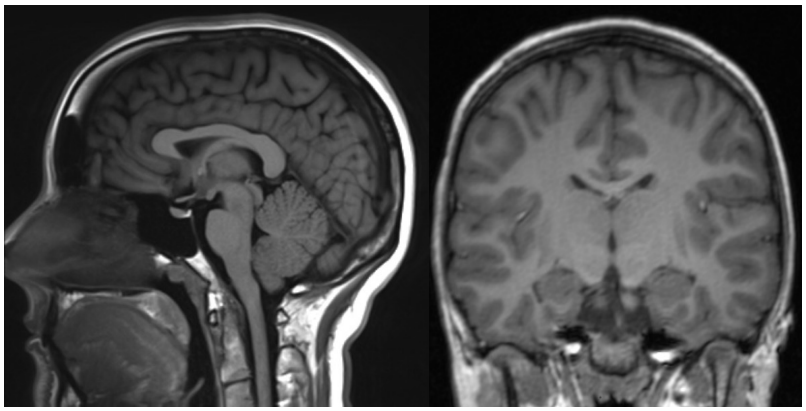


Figure 9 *MRI of a human adult brain. In the sagittal section sulci and gyri can be appreciated; in coronal section it can be appreciated how gyrification has a strong role in increasing of cortical surface area (adapted from Barkovich et al. 2012)*

In 2012, Barkovic (Barkovich et al., 2012) updated the classification scheme of cortical which is based on the types of predicted defects: cell proliferation, cell migration or cell organization.

1)Proliferation: as described previously, progenitor cells proliferate at early stages of cortical development before they differentiate. During this stage, malformations are caused either by altered proliferation or apoptosis (Barkovich et al., 2012) and can lead to microcephaly or megalencephaly.

Microcephaly: is described as a head circumference of 3 or more standard deviations below the normal for age and gender (Fig 10). It may be suspected or diagnosed clinically by measuring the head circumference or by computed tomography (CT) or magnetic resonance imaging (MRI). Images typically show small overall brain size with less gyri and abnormally shallow sulci. There is as strong reduction of white matter with preservation of normal myelinization. Corpus callosal agenesis and/or cerebellar hypoplasia may be present (Barkovich et al., 2012). In most cases it is characterized by the presence of normal to thin cortex. It is generally autosomal recessively inherited. A detailed description and possible causes of microcephaly, which is the topic of this thesis, will be treated in more detail in the following chapter.

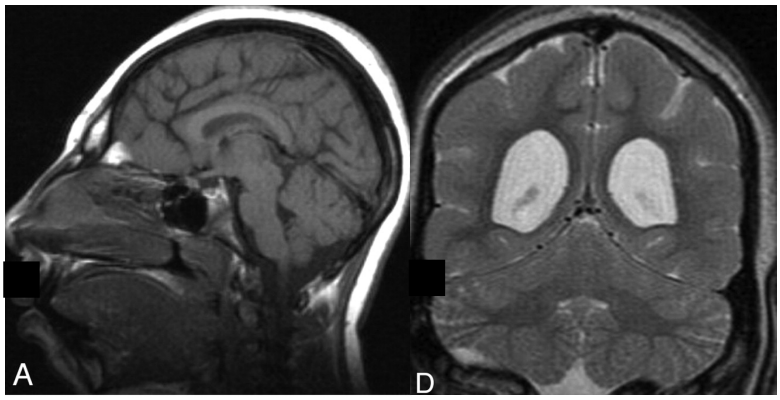


Figure 10 *MRI of a microcephalic brain showing a reduction in size and extreme simplification of gyrification (adapted from Barkovich et al. 2012)*

Megalencephaly: it is defined as an oversized and overweight brain that exceeds the mean by 3 SD (Mirzaa et al., 2014; Reijnders et al., 2017). Abnormalities in brain size can manifest during embryonic development or perinatally. The pathology sometimes evolves slowly during infancy (Fig 11). A variation of megalencephaly is hemimegalencephaly (Fig 12), a congenital malformation that is considered the result of abnormal cell proliferation leading to

complete or partial overgrowth of one single hemisphere. Clinically, megalencephaly can be classified in two main subtypes: anatomic and metabolic.

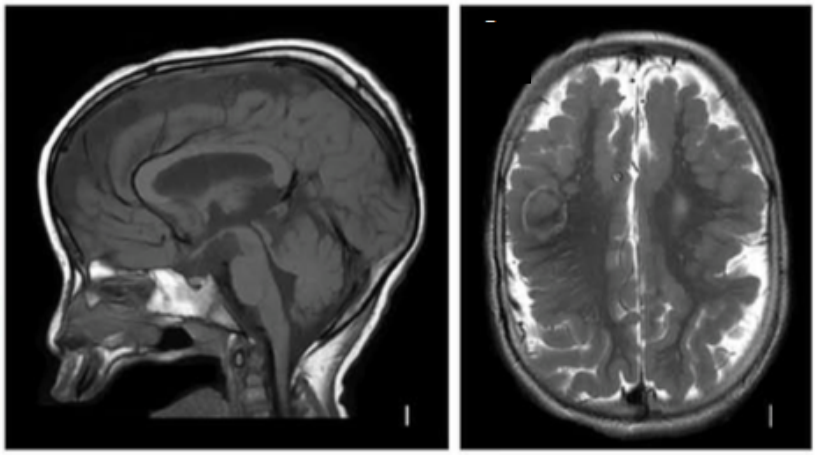


Figure 11 *MRI of a megalencephalic human brain showing an increase in size with consequent disorganization of the entire architecture of the brain (adapted from Barkovich et al. 2012).*

Anatomic cases consist in cellular defects displaying an increase in size or number of cells; metabolic subtypes are generally caused by the abnormal accumulation of metabolic substrates or metabolic residual in the cell. Disorders causing megalencephaly can be caused by genetic mutations in key nodes of several signaling pathways regulating cellular growth and proliferation, such as the PI3K-AKT-mTOR pathway. For example, AKT3 gain of function

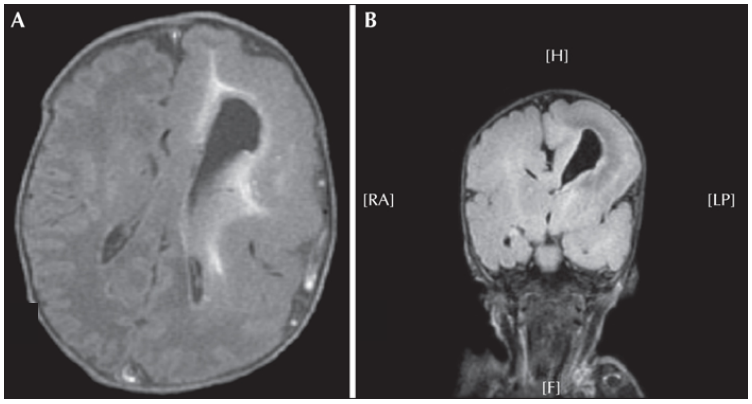


Figure 12 *MRI of an hemimegalencephalic human brain showing only half of the brain affected by the pathology, the left hemisphere is normal (adapted from Barkovich et al. 2012).*

mutations lead to the constitutive activation of AKT and as a result to the overproduction of Cyclin D2 and to overproliferation of neural progenitors (Mirzaa et al., 2014; Reijnders et al., 2017). Many mutations have recently been identified and most of them were found on genes involved in proliferation and cell growth, including: *PIK3R2, PTEN, AKT3, TSC1, TSC2, MTOR, CCND2*, (Mirzaa et al., 2014; Reijnders et al., 2017). Besides the overgrowth of the brain, these mutations also cause general cortical development impairments with severe mis-organizations of neuronal circuits and consequent intractable epilepsy.

2)Cell migration defects: Newborn neurons must migrate over long distances before reaching their final position. Defects in this process can lead to two main cortical malformations: lissencephaly and heterotopia.

Heterotopia. This condition is defined by the presence of focal accumulations of neurons in abnormal locations, as a consequence of altered neuronal migration (fig 13). The most common clinical presentation is seizures. Depending on the location of these foci, heterotopia can be classified as subependymal/periventricular nodular heterotopia (PNH) or subcortical heterotopia (SCH). PNH is the result of premature arrest of radial neuronal migration in the subventricular zone and intermediate zone of a developing neocortex (Carabalona et al., 2012; Sheen, 2001). Several mutations have been associated with PNH, the most common being in the X-linked *FLNA* gene, that encodes filamin A, a protein involved in cell-cell adhesion and polarity of radial glial cells(Carabalona et al., 2012). Mutations in the gene coding for this protein impairs b1 integrin mediated cell adhesion resulting neuroepithelium integrity defects due to radial glial progenitor fiber disruption and as well defects in neuronal migration (Carabalona et al., 2012; Sheen, 2001). SCH is characterized by the presence of two cortical

plates separated by a layer of white matter due to stalled migration of neurons in the IZ. The clinical presentation of this type of heterotopia largely depends on the extent of cortical involvement. In 2014 Kielar (Kielar et al., 2014) identified SCH related mutations in a gene called *Eml1*. The functions of the EML1 protein has not been studied in depth, however, even if the cellular mechanism that leads to such a pathology remains unclear, it has been proposed to be a regulator of Golgi trafficking and primary cilium formation (Collins et al., 2019).

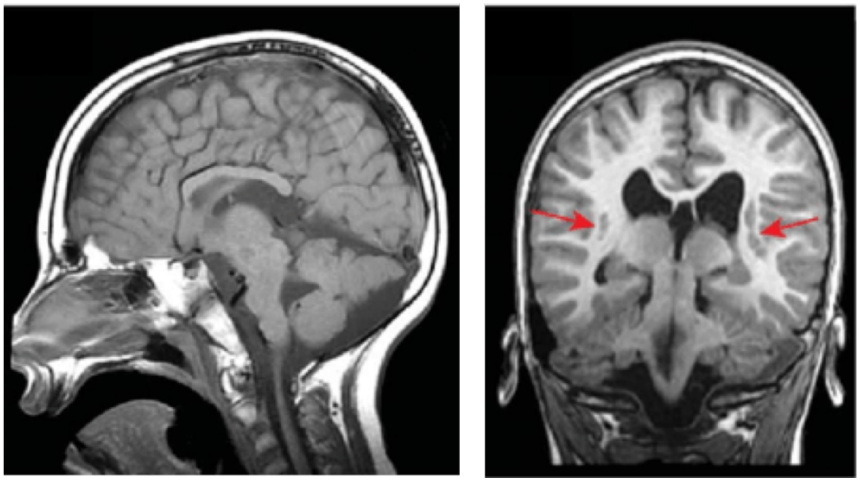


Figure 13 *MRI showing human adult brain affected by heterotopia; the red arrows show neural heterotopic foci (adapted from Barkovich et al. 2012).*

Lissencephaly: is described as a smooth brain. It is a severe form of abnormal neuronal migration characterized by the absence of gyri with a thickened cortex (agyria) or the presence of few broad fat gyri with a thickened cortex (pachygyria). Both of them leading to a relatively smooth brain (Fig 14). The pathology is characterized by an arrest of neuronal migration reducing the cortex in four pseudo layers (instead of six in healthy conditions). With an outer marginal layer of neurons, a superficial layer of pyramidal neurons, a variable cell sparse layer and a deep cellular layer composed by medium and small-sized neurons (Friocourt et al., 2011; Leventer, 2007).

Lissencephaly can be divided in two main groups:

Classical lissencephaly, type I is the most prevalent form of lissencephaly. Patients suffering from this syndrome present diffuse hypotonia, early developmental delay, spastic quadriplegia and severe mental retardation. They develop medical refractory epilepsy and complex seizures (Gleeson et al., 1999). The pathology shows smooth brain with a clear decrease in white matter throughout the whole brain (Barkovich et al., 2012). One of the most studied and most frequent lissencephaly is related to mutations in *LIS1*. This gene encodes for an important dynein partner; LIS1 directly interacts with cytoplasmic dynein modulating and regulating its activity (McKenney et al., 2010). When LIS1 is inhibited, it strongly impacts nuclear translocation leading to defects in INM and neuronal migration (Vallee & Tsai, 2006; Wynshaw-Boris, 2007). Another lissencephaly related gene is *DCX* and encodes for a microtubule associated protein essential for microtubule polymerization and stabilization (Moores et al., 2006). Interestingly when mutated *DCX* induces lissencephaly only in male while heterotopia in female. It has been shown that loss of function of DCX causes an impairment of radial neural migration in a non cell-autonomous way(Bai et al., 2003). Even though the exact cellular mechanism is not completely understood, it has been proposed that DCX may be required from the cell to organize an appropriate cytoskeletal response that direct neuronal migration like neuronal branching (Moores et al., 2006). Mutations in *LIS1* and *DCX* genes impairs microtubule organization and consequently defects in migration capability of neurons. The severity and pattern of the disease is slightly different depending on what gene is mutated. Mutations in *LIS1* result in extremely severe lissencephaly with a posterior-anterior gradient (Mei 2008) while mutations in *DCX* exhibit asymmetrically prominent lissencephaly that is more striking in the antero-posterior gradient(Mei et al., 2007).

Lissencephaly type II characterized by irregular brain surface caused by the presence of heterotopic tissue which results from over migration of neuroglial elements. In general, this pathology displays dilated ventricles, abnormal white matter, small brainstem, hypoplastic vermis and cerebellar polymicrogyria(Barkovich et al., 2012; Dobyns & Truwit, 1995).

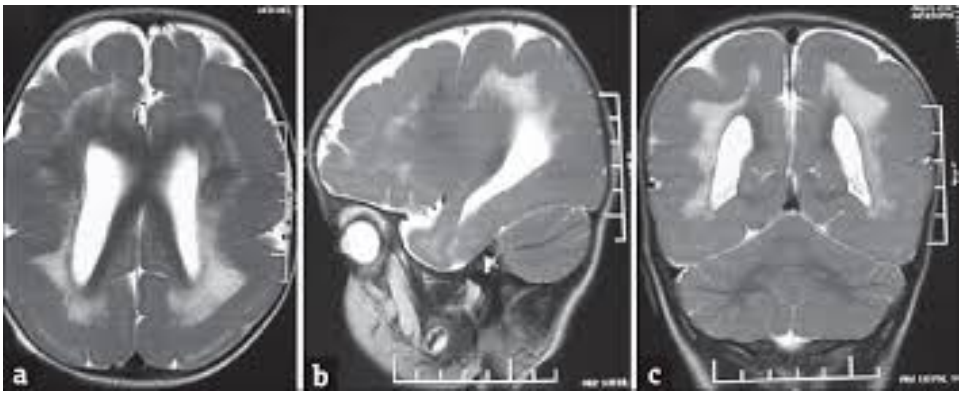


Figure 14 *MRI of a lissencephalic human brain showing an almost complete absence of gyrification (adapted from Barkovich et al. 2012).*

This pathology has been studied for the last two decades and the most frequently observed mutations were found in the classical dystroglycanopathies related genes *POMT* (involved in α-dystroglican modification), *POMGnT1, FKRP* and *LARGE*, all encoding for protein involved in glycosylation of transmembrane proteins. Mutations in any of these genes will impair the modifications of protein like α-dystroglican or glycoproteins causing loss of ECM adaptors required for essential interactions between the cell and its micro-environment, leading to migration defects (Barkovich et al., 2012; Juric-Sekhar et al., 2011).

3)Cell organization defects: organizational processes include neurite extension, synaptogenesis and neural maturation, which are essential for normal cortical morphology and function. Defects in these processes can lead to a group of pathologies classified as polymicrogyria and schizencephaly.

Polymicrogyria (PMG) can be caused by disturbances in the late stages of neuronal migration or early stages of cortical organization (and could therefore also be classified within the previous category). These defects lead to abnormal development of the deep layers, leading to the development of a cerebral cortex characterized by small gyri divided by small sulci, causing an irregular bumpy cortical surface (Barkovich et al., 2012) (Fig 15). The clinical features present microcephaly, hypotonicity and seizures with marked developmental delay (Squier & Jansen, 2014). Histologically, it is characterized by a reduction in thickness of the deep layers and an increase in separation within layers by an intermediate linear region of scattered cells and myelinated fibers. This results in an overall reduced thickness of the cortical plate. PMG has been linked to mutations in the *OCLN* gene, coding for occludin, an integral component of the tight junctions, *COL4A1/COL4A2* coding for collagen, an essential component of the ECM, as well as tubulin coding genes (*TUBA, TUBB*).

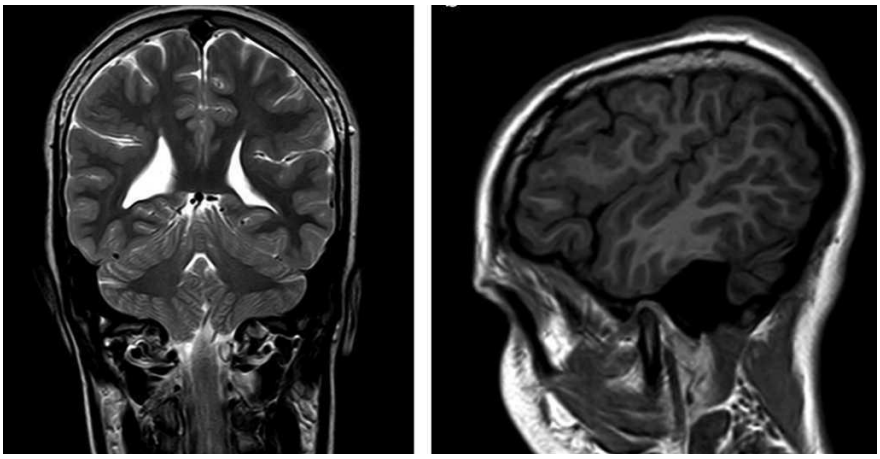


Figure 15 *MRI of a human brain affected by polimicrogiria showing a reduction of brain size together with aberrant gyrification (adapted from Barkovich et al. 2012).*

Schizencephaly: is a brain malformation characterized by a full-thickness cerebral cleft that will extend from the pial surface of the cortex to the ependymal lining of the lateral ventricle (Fig 16). This is usually related to abnormal delaminating grey matter. Common clinical

presentations are microcephaly, seizures, mental retardation and motor dysfunction. It has been reported to be related with mutations in the gene *EMX2* which is a regulator of transcription during neural development with many identified interactors(Groves et al., 2019).

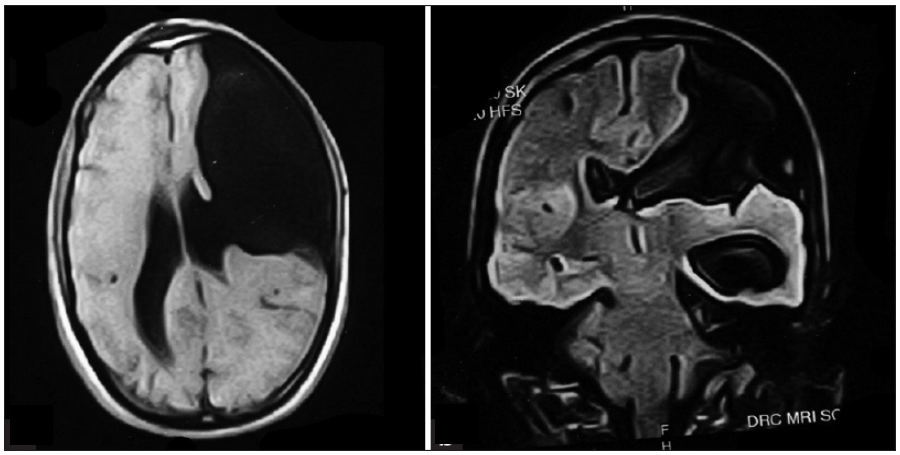


Figure 16 *MRI of a schizencephalic human brain showing a brain whose missing an entire emisphere (adapted from Barkovich et al. 2012).*

6. MICROCEPHALY

As described previously, microcephaly is characterized by a dramatic reduction in brain size. It can result from a plethora of etiologic factors such as: exposure to pathogens, toxins or teratogens, metabolic conditions and genetic syndromes. For the purpose of this thesis, only genetic causes for primary microcephaly will be reported. When microcephaly is present at birth it is called primary congenital microcephaly or MCPH (Microcephaly Primary Hereditary). The major putative causes of primary microcephaly have been proposed to account for the decreases neuronal output: premature differentiation, reduced proliferation or premature cell death. Cell death is by far the most common cause of microcephaly, while the two others have received much less experimental support. There are multiple families of genes that, when mutated, lead to microcephaly. The most frequent ones are listed below.

*Centrosomal protein*s. More than half of microcephaly cases are related to mutations in genes that encode for centrosomal associated proteins. These proteins control centriole biogenesis, duplication or function. Centrosomes consist of a mature mother centriole and a daughter centriole that will duplicate in G1/S phase and have a role in microtubule nucleation during mitosis (Nicholas et al., 2010). Common MCPH-associated centrosomal factors are *ASPM, WDR62, CDK5RAP2, SAS-4, PCNT* and others.

Centrosomes are indeed essential organelles for Radial glial cells, with key functions during mitosis, but also for polarity maintenance or INM. Mutations in genes that regulate their biogenesis often cause death of the progenitors and/or neurons, leading to microcephaly. One such example is SAS-4, a factor involved in centriolar biogenesis. Insolera and colleagues demonstrated that SAS-4 loss of function causes microcephaly, due to the loss

of radial glial cells that die prematurely (Insolera et al., 2014). The lack of SAS-4 impairs centriole biogenesis, affecting spindle pole assembly, causing mitotic delay with subsequent p53 upregulation and apoptosis of the radial glia cell. Conversely, a higher number of centrioles can also lead to microcephaly. This is the case following *Plk4* overexpression, a kinase involved in centriole duplication. Its overexpression induces an accumulation of extra centrosomes and aneuploidy due to chromosome mis segregation. In radial glial cells this causes massive cell death, decreasing the pool of progenitors and resulting in microcephaly (Marthiens et al., 2013).

Another case of microcephaly caused by mutations in centrosomal proteins is related to Cdk5Rap2, a factor involved in centriole maturation. Its loss causes primary microcephaly due to the loss of radial glial cells. Cdk5Rap2 recruits γ-TurC, which controls microtubules nucleation on the mature centrosome. Thus, when Cdk5Rap2 is missing the microtubule aster will not form giving rise to alterations of the mitotic morphology and orientation, affecting mitotic orientation and duration. Consequently, strong apoptotic cell death is observed in the Cdk5Rap2$^{-/-}$ mice(Lizarraga et al., 2010). Pcnt$^{-/-}$ mice also exhibit strong microcephaly with reduction of the thickness of all neuronal layers. *Pcnt* encodes for pericentrin, the core of a protein complex that assemble on spindle pole and control MT organization at this site. Without Pcnt the complex will not form, decreasing the efficacy of astral MT assembly and impairing proper spindle pole orientation and therefore the angle of division of neural progenitors. This angle is essential because it defines if a division is proliferative or neurogenic differentiative (Lancaster & Knoblich, 2012). Here the divisions will become differentiative giving rise to more neurons at the expenses of self-renewing divisions, decreasing the total neuronal output and leading to microcephaly (Chen et al., 2014).

DNA damage response. Factors associated with DNA replication, DNA repair, and maintenance of genome stability have been linked to microcephaly. Microcephalin 1 (*MCPH1*) was the first identified MCPH gene. SiRNA experiments and analysis of truncating mutations in primary fibroblasts from microcephalic patients showed that MCPH1 is involved in DNA damage response and regulates cell cycle progression upon DNA damage, affecting Cyclin A stability (Alderton et al., 2006; A. P. Jackson et al., 2002). The loss of MCPH1 causes the downregulation of the downstream effect of the ATR signaling pathway in which MCPH1 plays a role. MCPH1 activates Cdc25A, that activates Cdk-CyclinB1 for the entry in mitosis. Therefore, the lack of MCPH1 leads a delay in mitosis entry, slowing down the proliferation index of the neural progenitors decreasing the total neuronal final output.

Although beyond the scope of this thesis, it is worth noting that microcephalic primordial dwarfism (MPD) can be caused by key components of DNA replication machinery. This include *ORC1, ORC4, ORC6, CDT1* (causing, if mutated Meyer Syndrome), encoding proteins that are parts of the chromatin bound complexes and GMNN involved in the formation of the replication fork and initiation of DNA replication process(Bicknell et al., 2011; Burrage et al., 2015; Fenwick et al., 2016). Clinical studies and genomic analysis directly on patients showed that the loss of function of any of these proteins cause a delay in cell proliferation leading as well to a small increase in apoptosis, caused by a late initiation of DNA replication or a reduced capacity for the cell to overpass the *phases checkpoints* after a DNA damage. A slower cell cycle in neural progenitors will lead to a much lower output of neurons establishing, once again, the bases for microcephaly.

Proliferative factors: numerous growth/proliferative factors are released in the cerebrospinal fluid (CSF) from a specific area of the brain called the choroid plexus (Lehtinen et al., 2011).

Some of these factors bind to their receptor on the apical side of apical progenitors, stimulating their proliferation (Lehtinen et al., 2011). For example, Insulin-like growth factor 1 and 2 (IGF1 and IGF2) bind to the IGF receptor located at the apical surface of the RG cells, in direct contact with the CSF. Mutations in the Insulin receptor 1 (*IGF1R*) leads to reduced brain size in humans (Juanes et al., 2015). Studies in the developing mouse brain showed that, when IGFR is mutated, the activation of the Pi3K/Akt signaling cascade is affected, leading to a decrease in progenitor proliferation, and to microcephaly (Juanes et al., 2015; Lehtinen et al., 2011).

Docking proteins: Other microcephaly genes encode proteins involved in intracellular transport. These include genes like *COH1, TRAPPC9, CHMP1A* (Kolehmainen et al., 2003; Ganeshwaran H. Mochida et al., 2009, 2012). Mutations in these genes affect intracellular trafficking, giving rise to autosomal recessive primary microcephaly, together with several other clinical features that affect other organs (Kolehmainen et al., 2003; G. H. Mochida et al., 2004). The exact mechanisms by which defects in intracellular transport can affect cortex development is not clear, but it has been postulated that aberrant sensing of neurotrophic signals such as TGFa could be involved, leading to decreased proliferation rates of the aRGs (Ganeshwaran H. Mochida et al., 2009).

Scaffolding protein: examples of genes involved in MCPH causing genes is ALFY. This gene encodes for autophagy scaffold proteins controlled by the downstream signaling pathway of Wnt and it is as well responsible for a negative feedback loop on the Wnt signaling itself (Kadir et al., 2016). Experiments *in vivo* in drosophila showed that mutations in ALFY lead to massive apoptosis due to the misregulation of the Wnt pathway (Kadir et al., 2016). In this aberrant

system, it will happen that ALFY will stimulate and favorize the autophagic pathway increasing massive autolysis of the cells bringing them to auto-disintegration leading to premature cell death.

Protein/amino acids syntases: the last identified category of microcephaly causing genes are the ones involved in amino acid and/or protein synthesis. Some examples are: *QARS* (Zhang et al., 2014) a glutaminyl-tRNA synthetase, *AARS* essential for alanyl t-RNA aminoacylation reaction (Nakayama et al., 2017), *PYCR2* involved in proline biosynthesis (Nakayama et al., 2015). These genes, if mutated will all cause MCPH but accompanied with a variety of clinical features: cerebellar and cortical atrophy (QARS), hypomyelination and untreatable seizures (AARS) hypomyelination and general cerebral increased apoptosis (PYCR2). Loss of function mutations in these genes induce dramatic accumulation of misfolded proteins ultimately leading to neural cell death.

6.1 Microcephaly and endocytosis

In addition to the causes listed above, few studies directly connect the onset of microcephaly with the loss of protein involved in endocytosis. One such example is the complex formed by Filamin A (FlnA) and Formin-2 (Frm2). Filamins are actin-binding proteins generally serving as a scaffold for membrane proteins (Gorlin et al. 1990). Formins are a group of protein involved in the polymerization of actin and associated with fast growing filaments (Dettenofer et al. 2008). FlnA and Frmn2 KO mice showed a severe form of microcephaly with no cell death but strongly reduced proliferation of neural progenitors and delayed differentiation (Lian et al. 2016). The two proteins, indeed, are essential for the stabilization on cellular and vesicular

membrane of the Wnt associated protein Lrp6, having an effect on the regulating the Wnt pathway. A dual loss of them will therefore cause a destabilization of Lrp6 with consequent loss of Lrp6 endocytosis and downregulation of the Wnt pathway inducing a decrease in radial glial progenitors' proliferation and differentiation.

Another example is ALG-2 interacting protein-X (Alix). It has been reported to be required during neurodevelopment where its loss results in microcephaly onset. In fact, Alix KO mice have smaller brains that display thinner deep and upper neuronal layers (Laporte et al. 2017). This is due to a reduction in neural progenitors that do not present any decrease in proliferation rate but show a massive cell death at the very early phases of neurodevelopment (E12.5) with consequent reduction of intermediate progenitors and newborn neurons. The molecular cause of this massive apoptosis seems to be related to the Alix involvement in Clatrhin Independent Endocytosis. Studies in mouse embryonic fibroblasts and neural progenitors derived from Alix KO mice indeed demonstrated how loss of Alix delays EGFR degradation (Mercier et al. 2016) and alters the levels of activation of the downstream MAPK pathway (ERK1/2) (Laporte et al. 2017). This impairment in growth signaling could affect cell survival in early phases of development leading to cell death. The authors however, do not exclude that the loss of Alix could affect as well other signaling pathways that could be involved in the massive cell death observed (Laporte et al. 2017).

Another form of microcephaly related to proteins involved in endocytosis is the Microcephaly-capillary malformation (MIC-CAP) syndrome. It takes its name from the fact that the affected patients exhibit, together with microcephaly, multiple small capillary malformations. It is an autosomic recessive form of microcephaly diagnosed in compound heterozygote subjects

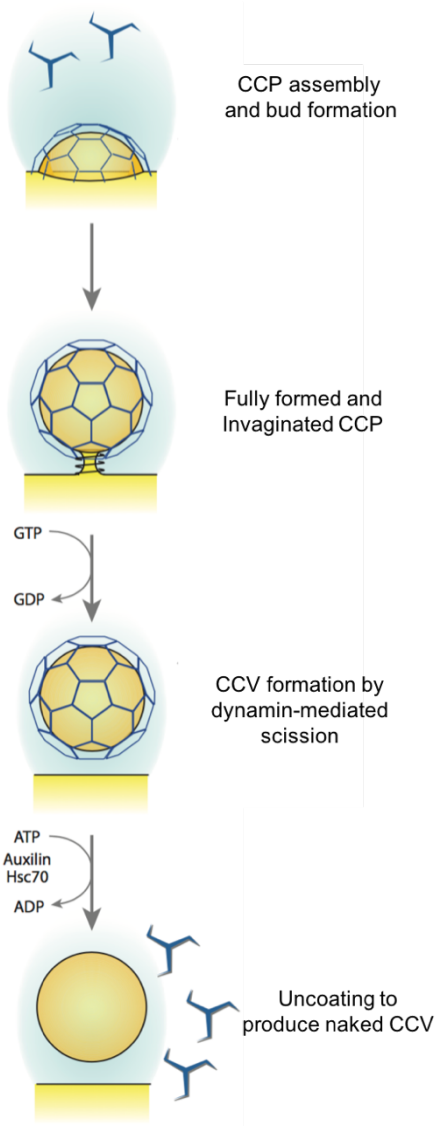
carrying mutations in the gene *STAMBP,* encoding for the deubiquitinating (DUB) isopeptidase STAMBP. This protein, as part of the ESCRT machinery, plays a key role in cell surface receptor-mediated endocytosis and sorting. Experiments on patients derived cell lines, showed an accumulation in ubiquitinated aggregated protein together with apoptosis and desensitization to the RAS-MAPK and PI3K-AKT-mTOR that were found overactivated. The authors report that this latter finding can be explained by the unbalancing effect that the loss of function of STAMBP can have on the endocytoses of growth factors (McDonell et al., 2013). In addition, previous studies demonstrated that the deletion of STAMBP in mouse would also bring to neurodegenerative lesions in the central nervous system caused by the accumulation of aggregated protein (Suzuki et al., 2011).

From the studies presented here, it is clear that endocytosis plays a critical role in neurodevelopment, and that its impairment causes disastrous effects. Endosomal trafficking is the process by which endocytosed vesicles are sorted to different routes including recycling and degradation. The protein of focus of this thesis, WDR81, is involved in this process. I will therefore first introduce endocytosis in general, followed by a deeper presentation of endosomal maturation.

7. ENDOSOMAL TRAFFICKING

7.1 Endocytosis

Endocytoses is the process by which plasma membrane lipids, surface proteins, and extracellular molecules are internalized by the cell via invagination of the plasma membrane, and detachment of the newly formed compartment for delivery into the cell. This process, together with exocytosis, is a major mode of interaction with the microenvironment in which the cell resides. Endocytoses regulates a multitude of cellular processes, such as antigen presentation, cell migration, intracellular signaling cascades, pathogens internalization and even mitosis (Schmid & McMahon, 2007).



There are different ways through which a cell can perform endocytosis. Because this thesis focuses on the WDR81 protein, and its effect on the intracellular processing of the EGF receptor, I will focus on the endocytic pathways used for receptor internalization.

Clathrin-Mediated Endocytosis (CME) is an endocytic pathway in which a Clathrin Coated Pit (CCP) undergoes progressive invagination before scission from the plasma membrane to form a Clathrin Coated Vesicle (CCV). The CCV will then lose its coat and release its content to a target cellular compartment (Schmid & McMahon, 2007).

A wide variety of transmembrane receptors and their ligands are internalized via CME. Adaptor and accessory proteins coordinate clathrin nucleation at sites of the plasma membrane which are destined to be internalized (Schmid & McMahon, 2007). The nucleation promotes the polymerization of clathrin in curved structures, stabilizing the deformation of the membrane. The polymerization of clathrin also helps in the formation and constriction of the newly formed vesicular neck. Here, an extremely important protein for the CME process known as Dynamin forms an helical polymer around the constricted neck and with its GTPase activity mediates the fission of the vesicle from the plasma membrane releasing the CCV into the cytosol (Praefcke & McMahon, 2004). The clathrin coat is then released from the vesicle by auxilin and hsc70. It is worth mentioning that clathrin alone cannot drive and maintain the deformation of the membrane. To do so, certain types of lipids like cholesterol need to be present, as well as accessory proteins such as members of the Epsin family or Adaptor protein-2 (AP2). CME appears to be the mechanism by which the cell endocytose most of its cargoes, including the transferrin receptor, the low-density lipoprotein receptor (LDLR) (Mettlen et al., 2018) or the EGF receptor (EGFR) (Sigismund et al., 2008).

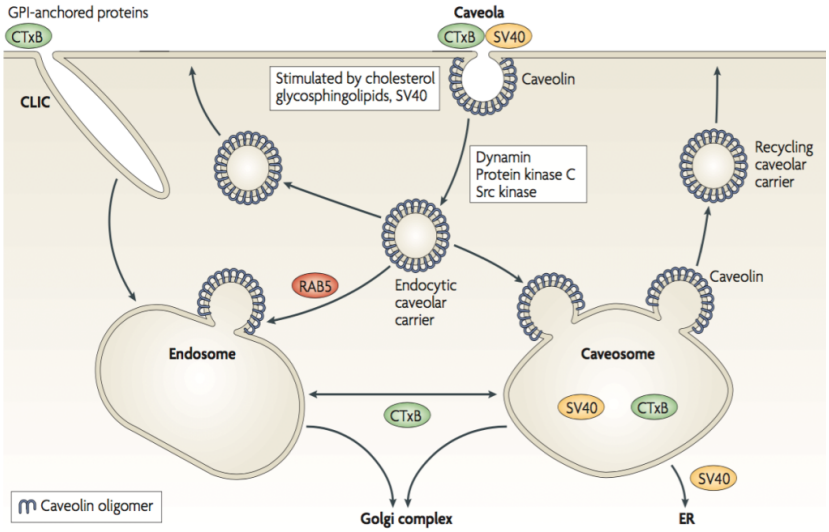


Figure 17 *Schematic representation of clathrin independent endocytosis (adapted from Parton et al. 2007)*

Clathrin independent endocytosis facilitates the internalization of large micrometer scale objects. It also allows the fast internalization of small cargoes and nutrients (Mayor et al., 2014). Clathrin independent endocytosis can be distinguished in three different mechanisms (Mayor et al., 2014):

Caveolin-associated endocytosis. The most commonly reported non-clathrin coated endocytic structures are known as caveolae. These structures are flask-shaped invaginations present at the level of the plasma membrane. They are 60 to 80 nm in diameter and can constitute approximately a third of the plasma membrane area of the cell in some tissues. Caveolins are enriched at the level of caveolae and control their biogenesis (Boscher, Cecile; Nabi, 2012), driving endocytic invagination and therefore permitting the formation of the caveolae themselves (Sohn et al., 2018). Aside from caveolins, other proteins are involved in caveolar genesis, including cavins and flottillins (Frick et al., 2007; Kovtun et al., 2015).

Clathrin and caveolin Independent endocytoses: this kind of endocytosis is cholesterol dependent. It is important for internalization of extracellular fluid, virions, receptors for immune response and hormones (Damm et al., 2005; Kirkham & Parton, 2005). This kind of endocytoses can bypass the conventional Rab-5 positive endocytic pathway, but the molecular mechanism is still unclear (Sandvig et al., 2018).

Macropinocytosis: On top of the mechanisms described above, which mostly control the internalization of receptors or proteins, cells can internalize larger volumes of nutrients by deploying larger volumes of membrane. The process involves the formation of a protrusion from the plasma membrane that subsequently fuses with itself, resulting in the uptake of extracellular components trapped between the membranes. PAK1 has been reported to be involved in that process and appears to be necessary and sufficient to induce

micropinocytosis, acting directly on the activation of PI3K in dependence of the small G protein Rac1 (Dharmawardhane et al., 2000; Sandvig et al., 2018).

In addition to the internalization mechanisms described above, it has become more and more clear that the plasma membrane itself is not merely a passive component but is also directly involved in the endocytic process. Indeed, its lipid content is very important and it has been shown that plasma membrane enriched phosphoinositide PtdIns(4,5)P2 is necessary for the invagination of CCPs and the bindings of many adaptor proteins involved in CME (Haucke, 2005). The other important lipid is cholesterol that – through its hydrophobic core - helps the formation of the invagination of the membrane during CIE (Parton & Simons, 2007). Sphingolipids also have an important role, and reduced levels on certain membrane domains will affect the endocytosis of certain receptors, such as IL2R (Spilsberg et al., 2007).

7.2 The endosomal machinery

The endocytic mechanism can be dissected into three major parts: a recycling circuit that reprocesses what has been previously endocytosed and metabolized by the cell; a degradative system for digestion of macromolecules; and a connecting, unidirectional pathway for transport of cargoes from the recycling circuit to the degradative system.

When a cargo is internalized, the majority is recycled back to the plasma membrane, or transferred to the trans Golgi network (TGN) for proteic modification before being recycled back to the membrane via early endosomes (EEs). Alternatively, cargos can be addressed to lysosomal degradation. In typical mammalian cells, the equivalent of 50–180% of the surface area of the plasma membrane is cycled in and out of the cell every hour (Doherty & McMahon, 2009; Hinze & Boucrot, 2018).

7.3 Early-to-late endosome conversion

Following endocytosis, cargoes accumulate in early endosomes (EEs), which provide the starting point for recycling or for lysosomal degradation, via maturation (or conversion) into late endosomes (LEs). EEs therefore act as the main sorting station in the endocytic pathway. (Maxfield & McGraw, 2004). EE are characterized by the presence of proteins that define many of its functional attributes. Rab5 is a key component together with its effector VPS34/p150, a phosphatidylinositol 3-kinase (PI(3)K) complex that generates the phosphoinositide (PI) PtdIns(3)P and thus helps to create the identity of the organelle (Behnia & Munro, 2005; Christoforidis et al., 1999; Zerial & McBride, 2001). During early to late endosome switching, a multitude of proteins are involved. At the very beginning of endosome formation, RAB-5 will be recruited and quickly activated by its Guanine nucleotide Exchange Factor (GEF) RABX-5, that will exchange GDP for GTP. This will allow for the recruitment of PI3K that will phosphorylate the Inositol present on the membrane of the endosome on its 3rd carbon giving rise to PtdIns3P (Chotard et al., 2010; Pfeffer, 2013). SAND-1 senses the PtdIns3P increasing levels on early endosomes and displaces RABX-5 from the endosome membrane, stopping its action on RAB5. In the meantime, SAND-1 and CCZ-1 form a complex that converts RAB-7 into a GTP-bound active form (Delahaye et al., 2014) permitting the maturation of the endosome into a late endosome. GTP-RAB7 recruits TBC-2, the GTPase Activating Protein (GAP) for RAB5, that will inactivate RAB-5 (Chotard et al., 2010). This mechanism where two RAB-GTPases respectively activate/inactivate each other has been called a RAB cascade.

By the time they fuse with lysosomes, some 10–40 min later, endosomes have completed a remarkable transformation in terms of membrane composition. The maturation process involves exchange of membrane components, movement to the perinuclear area, a

shift in choice of fusion partners, a drop in luminal pH, acquisition of lysosomal components, and a change in morphology. All these processes are closely coordinated and regulated by factors recruited to the surface of the limiting membrane from the cytosol.

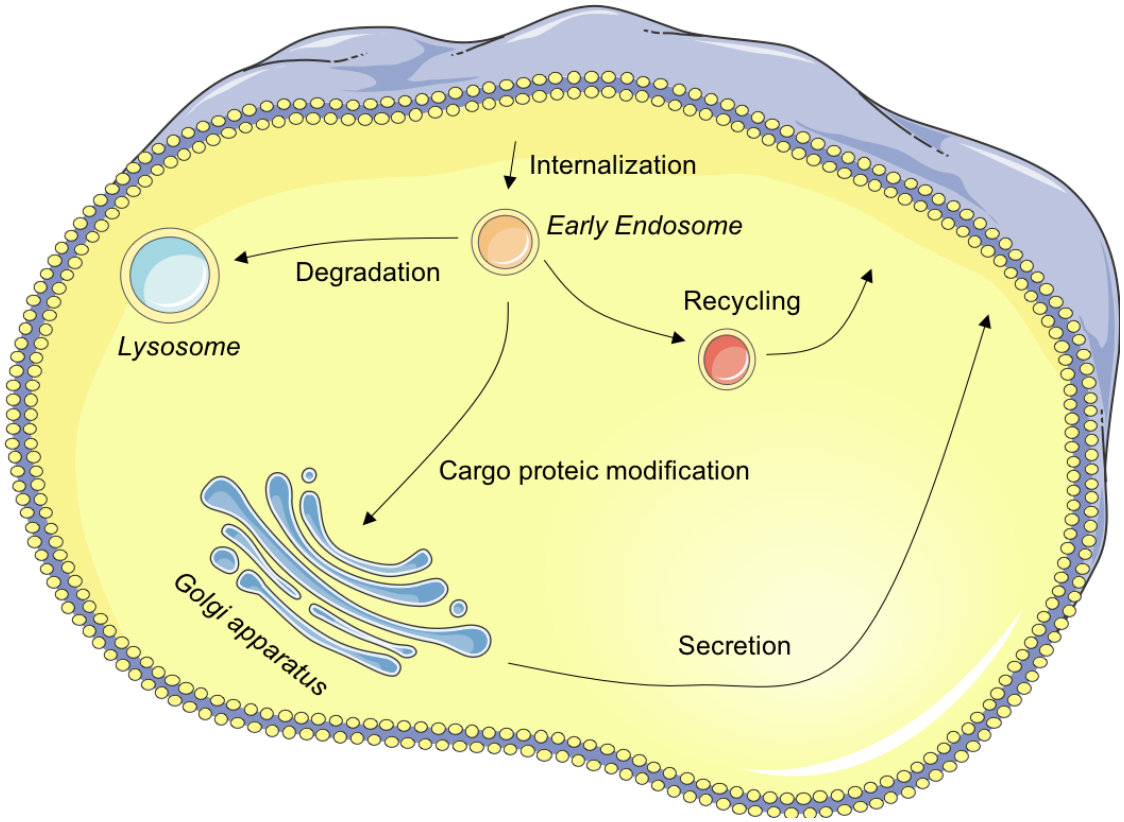


***Figure* 18** *schematic simplification of Early Endosomes possible pathways*

7.4 Regulation of endocytosis and Its physiological importance

Endocytosis regulates many processes such as nutrient uptake, cell adhesion, migration, pathogen entry, synaptic transmission, receptor downregulation, signaling, etc. In particular, endocytosis is crucial for regulating the activity of signaling receptors (e.g. recycling towards the PM versus degradation). Tyrosine kinases receptors (RTKs) signal via a plethora of pathways, fine tuning multiple cellular functions. Endocytoses has a role in

regulating RTKs signaling through their removal from the plasma membrane and subsequent internalization (von Zastrow & Sorkin, 2007), not only lowering the strength of the signal itself but even providing a spatial and temporal organization to the signaling event (Sigismund et al., 2008). One such example the EGF receptor (EGFR).

8. The EGF receptor (EGFR)

EGFR is a transmembrane receptor, first discovered of the TKRs part of the ErbB family, normally present under unstimulated conditions on the plasma membrane in an auto-inhibited, dimerization incompetent monomer. Ligand binding will promote receptor dimerization, which determines a series of structural rearrangements of the cytoplasmic domains allowing the formation of asymmetric dimers between the two adjacent catalytic regions of the receptor. These rearrangements consist in conformational changes that will lead to the trans-autophosphorylation of tyrosines, triggering the signaling cascade (Lemmon et al., 2014). In the meantime, EGFR will also be ubiquitinated on several lysine residues (Huang et al., 2006) by ubiquitination proteins like E3 ligase Cb1 in complex with Grb2 (Waterman et al., 2002). These intracellular modifications will lead to the recruitment of the endocytic machinery that mediates the receptor endocytosis (Barbieri et al., 2016). EGFR can heterodimerize with other ErbB family members, and each combination seems to have a different affinity for the various ligands of the family, and different signaling and trafficking properties (Lemmon et al., 2014; Lenferink et al., 1998).

The activation of EGFR will induce multiple signaling cascade pathways including Ras/MAPK and PI3K/AKT (Lemmon & Schlessinger, 2010). EGFR signaling is essential for several cellular functions, including survival, proliferation and differentiation depending on

the context (Lemmon & Schlessinger, 2010). EGFR signaling has to be regulated in order to avoid an overactivation of the pathways that could end up in overproliferative pathological/carcinogenic situations. The complete machinery that regulates EGFR activity is still under investigation (Sigismund et al., 2018), however, as mentioned in the previous paragraph, endocytosis plays an important role in compartmentalization and trafficking of the receptor, regulating its activity.

Both clathrin-mediated endocytosis (CME) and clathrin independent endocytosis (CIE) pathways are involved in EGFR internalization (Barbieri et al., 2016). Indeed, according to the EGF concentration, EGFR will be internalized by either one of these pathways. When EGF is present in low concentration, EGFR will be internalized by the CME and recycled back to the plasma membrane (Sigismund et al., 2008, 2018). When EGF is present at high doses, EGFR will be endocytosed via the clathrin independent machinery and routed to degradation (Barbieri et al., 2016; Sigismund et al., 2008). This latter case is important to induce negative feedback loops, avoiding a massive response to sustained signaling (Avraham & Yarden, 2011).

Once internalized, EGFR reaches the EEs and is sorted toward different fates: recycling or degradation in accordance with the way the receptors were endocytosed. Recycling is believed to be the default pathway, upon stimulation with low doses of EGF. Upon high doses of EGF, degradation of the receptor will be determined by the ubiquitination of EGFR, which will be actively recognized by the ESCRT complex that will sort the receptor into multivesicular bodies and lysosomes (Raiborg & Stenmark, 2009; Wollert et al., 2009).

Besides regulating the surface levels of EGFR, endocytosis plays a direct role in regulation of signaling of the receptor led to the concept that EGFR signaling is initiated at the plasma membrane but continues at the level of the endosomes(Oksvold et al., 2003; Villaseñor et al., 2015). Villaseñor (Villaseñor et al., 2015) demonstrated that the endosome

fusion and fission process could have a role in EGFR signaling keeping the number of EGFR clusters per endosome constant regardless of the concentration of EGF. This study reported that altering the number of EGFR clusters per endosome, chemically modifying cellular fission and fusion rate, critically impacts EGFR signaling output. For instance, they observed that increasing the clusters of EGFR would have as a consequence a sustained MAPK signaling over a long period of time, inducing the activation of differentiation pathway. Conversely reducing the clusters of EGFR would result in just a transient of MAPK activity resulting in sustained cellular proliferation (Cárdenas et al., 2018; Villaseñor et al., 2015).

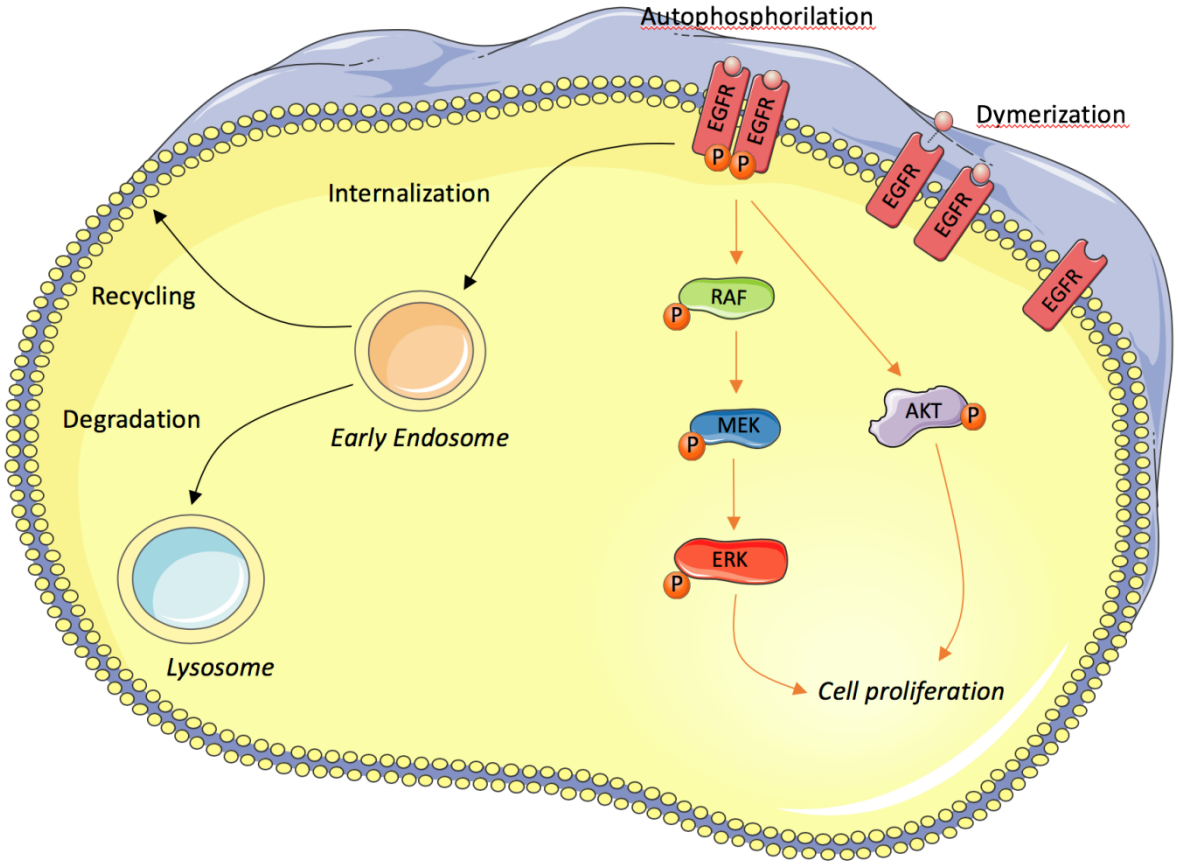


Figure 19 *schematic representation of EGFR endocytosis and main signaling pathways*

As EGFR, many other receptors are finely tuned via endocytosis, including TGFRs and PDGFR. Tumor Growth Factor Receptors (TGFRs) are also endocytosed by both clathrin dependent

and cholesterol/caveolin1-dependent pathways. CME of the receptors seems to happen in association with Smad protein anchor for receptor activation (SARA) and permits signaling, allowing interactions with downstream signaling components (Runyan et al., 2005). Another example is Platelet Derived Growth Factor Receptor (PDGFRs) that induces micropinocytosis at high concentrations of its ligand and CME at low concentrations. Low concentrations of PDGF will induce migration of the cell and higher doses will induce cell proliferation (De Donatis et al., 2008).

Endosomal maturation is a highly regulated process that impacts the fate of internalized receptors. My thesis was focused on a novel regulator of this process, the WDR81 protein.

9. WDR81: a new endosomal regulator

WD40 repeat protein 81 (WDR81) is, as previously mentioned, a poorly described protein. It is encoded by a gene located on chromosome 17: 1.716.523-1.738.599 forward strand in human and on chromosome 11: 75.440.944-75.454.717 reverse strand in mouse (Ensemble.org). The gene is composed of 10 exons and is extremely conserved from fish to mammals. Two isoforms have been identified in mouse and are generated through alternative splicing. No 3D-structure of the protein is available but in silico predictions (BLASTP, PASTA, CSpritz) indicate the presence of three major functional domains: a BEACH (Beige and Chediak-Higashi) domain involved in membrane trafficking (Khodosh et al., 2006; W. I. Wu et al., 2004) a transmembrane domain and multiple WD40 repeated domains mainly involved in scaffolding (Rutherford & Daggett, 2010).

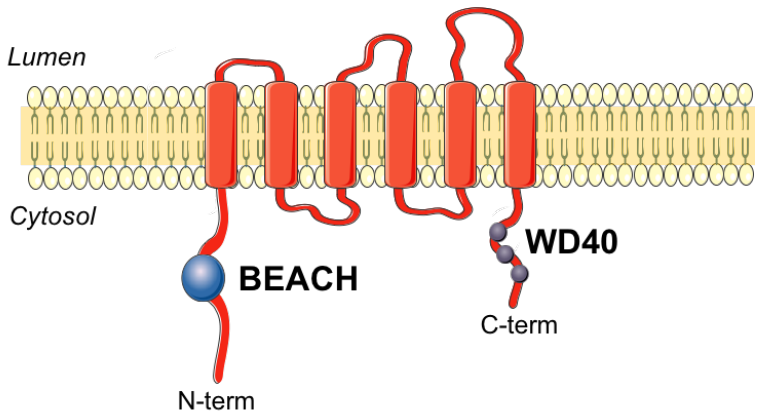


Figure 20 *schematic representation of WDR81 protein structure. It is composed by a transmembrane domain together with two functional domains: BEACH and WD40*

WDR81 has been very recently reported to be a regulator of endosomal maturation (K Liu et al. 2016, Rapiteanu et al. 2016). Kai Liu and colleagues in 2016 (K. Liu et al., 2016) demonstrated that WDR81 acts together with another protein of the same family called WD40 repeat protein 91 (WDR91) to negatively regulate the levels of Phosphatidylinositol 3-phosphate (PtdIns3P) at the membrane of early endosomes by inhibiting Phosphoinositol-3 kinase (PI3K) class III activity. PtdIns3P plays a key role in regulating the homeostasis of the endo-lysosomal system by promoting homo or heterotypic fusion of early endosomes through the actions of PtdIns3P-binding proteins like EEA-1 (Di Paolo & De Camilli, 2006; Schink et al., 2013) . This class of PI3K is mainly composed of two proteins: Vps34 and BECLIN-1 (BECN-1). BECN-1 is the regulatory subunit of PI3K class III and Vps34 is the kinase subunit responsible of the formation of PtdIns3P. The WDR81/WDR91 complex acts on BECN-1, negatively regulating the activity of PI3K class III. Kai Liu and colleagues reported that deletion of WDR81 inhibits the formation of the WDR81/WDR91 complex leading to an enrichment of BEC-1 subunit on PI3K complex activity, resulting in a prolonged existence of endosomal PtdIns3P and delay in early to late endosome conversion. PtdIns3P can drive fusion of early endosomes through its effectors, as already mentioned in chapter 7.3 (Subramanian et al., 2010). In the absence of WDR81 , early endosomes do not

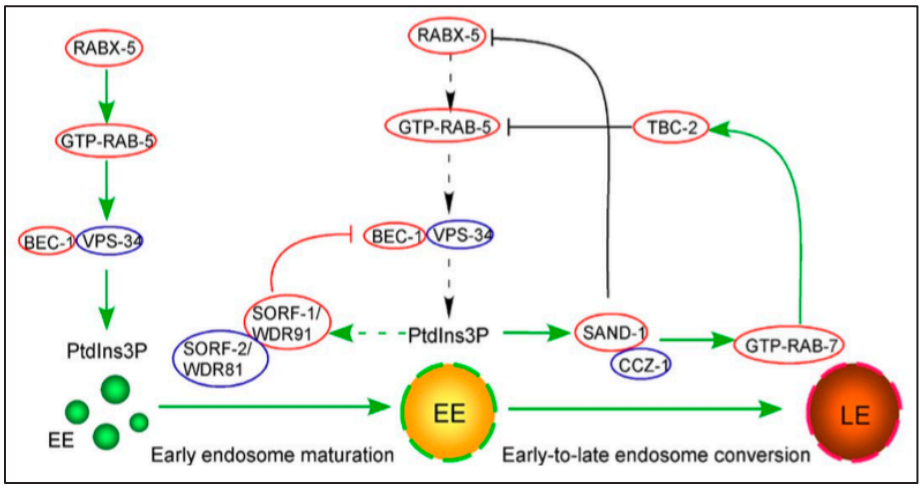


Figure 21 *schematic representation of WDR81 endosomal maturation regulation (adapted from Kai Liu et al 2016)*

mature into late endosomes and accumulate as swollen aberrant compartment (K. Liu et al., 2016; Rapiteanu et al., 2016).

In 2017 (K. Liu et al., 2017) Liu et al demonstrated that WDR91 is an active effector of Rab7 that couples the Rab cascade with the inhibition of endosomal PtdIns3P generation for early to late endosome conversion in the endosome-lysosome pathway. Studies in Hela cells demonstrated that loss of WDR91 leads to accumulation of PtdIns3P and the formation of greatly enlarged EEA-1/Rab7 intermediate endosomes preventing their fusion with LAMP-1 positive lysosomes suggesting that in physiological condition, WDR91 suppresses the activity of the endosomal PI3K complex. In addition, Liu et al investigated the role of WDR91 in the developing brain using conditional WDR91KO mice. Their results showed a reduction in cortical thickness due to a massive neuronal apoptosis perinatally and a reduction of neuronal arborization in the hippocampus and the cortex. In vitro analysis of hippocampal neurons corroborated this, showing a shorter axonal and dendritic length.

Further evidences of WDR81 involvement in hippocampal neurogenesis were reported by Wang et al., 2018. Indeed, the analysis of the adult hippocampus of tamoxifen inducible KO mice for WDR81 showed a reduced amount of adult neurogenic progenitors, due to a decrease of cells in S phase and an enrichment of G0/G1, with no elevation in cell death analyzed with caspase 3 immunostaining. The study excluded the involvement of apoptosis in WDR81 deficient mice and reinforced its involvement as an indirect cell cycle regulator via the endosomal conversion machinery control and therefore the regulation of proliferating factors. Wang et al indeed reported TGFβ Receptor to be regulated by WDR81 activity and showed how in WDR81 KO condition the receptor is delayed in early endosomes and ends up staying longer in the cell, signaling for a prolonged time. This results in an inhibition of adult hippocampal neurogenesis caused by a cell cycle G1 arrest or delay of adult progenitors.

In addition to its role as an endocytic regulator, WDR81 has been reported to have a function in controlling protein aggregate degradation, also known as aggrephagy (X. Liu et al., 2017). When misfolded proteins accumulate, they tend to aggregate and will be ubiquitinated to be sent to degradation thanks to the protein factor p62. Liu et al demonstrated that, in Hela cells, WDR81 interacts with p62 inducing a conformational change that enables p62 to be prone to (Ub)-aggregated proteins recruitment. This will permit the formation of a complex that will assemble with LC3 (the main regulator of autophagy) isoform C. Upon complex formation, LC3C will induce the formation of the autophagosome around the (Ub)-aggregated proteins, enabling their degradation. Interestingly, this latter function of WDR81 appears to be independent of WDR91, as opposed to what was observed for endosomal regulation. Hela cells KO for WDR81 show an abnormal accumulation of p62/Ub foci whereas WDR91 KO do not display such effect.

9.1 Wdr81 mutation causes microcephaly

In 2017, our collaborators (Cavallin et al., 2017) reported mutations in *WDR81* as a cause for an autosomal recessively inherited form of extreme microlissencephaly, a very severe neurodevelopmental disorder that combines microcephaly and lissencephaly. The study has been conducted on five French patients treated in Institut Imagine in Paris. All these patients display compound heterozygote mutation in *WDR81*. They were 3, 22, 13 and 17 years of age at the moment of their evaluation and all displayed a severe form of microcephaly. In all cases, microcephaly started with 1 to 3 standard deviations without overall body growth deficiency but reached 5 to 10 SD with aging. Neurological development was extremely impaired, and all patients displayed tetraplegia. Two of them developed untreatable epilepsy. MRI revealed extreme gyral simplification, with abnormal gyral pattern

comprising foci of extremely reduced sulcation and agyria, with increased subarachnoid spaces and deformed and enlarged ventricles with mid cerebellar atrophy. *WDR81* mutations were also found in fetal cases that were so severe that they led to medical termination of the pregnancy. Below is a detailed table of the cases and their clinical features:

Family	Family 1	Family 2	Family 3	Family 4			Family 5	
Patient	**Im-MCD_606**	**Im-MCD_752**	**CerID-22**	**Rdb-MIC_233**	**Rdb-MIC_234**	**Rdb-MIC_235**	**Nan-MCD-001**	**Published case**
Gender	Male	Foetus female (25 GW)	Female	Male	Foetus female (30 GW)	Foetus female (30 GW)	Female	
Gene (RefSeq) WDR81 (NM_001163809.1)								
Nucleotide variation 1	c.1882 C>T	c.2834_2837delTGTT	c.1582 C>T	c.1735G>A	c.1735G>A	c.1735G>A	c.3820_3835del	c.2567C>T
Nucleotide variation 2	c.3713 C>G	c.5464 C>T	c.4036_4041dup	c.1358 dup	c.1358 dup	c.1358 dup	c.5453G>T	
Protein variation 1	p.Gln628*	p.Phe946Serfs*17	p.His528Tyr	p.Gly579Arg	p.Gly579Arg	p.Gly579Arg	p.Pro1274Thrfs*56	p.Pro856Leu
Protein variation 2	p.Pro1238Arg	p.Arg1822*	p.Val1346_Thr1347dup	p.Tyr453*	p.Tyr453*	p.Tyr453*	p.Gly1818Val	
	Sporadic case	Sporadic case	Sporadic case	Familial case	Familial case	Familial case	Sporadic case	Familial cases (n = 5)
Ethnicity	European	European	European	European	European	European	European	Turkish
Transmission	AR	AR	AR	AR	AR	AR	AR	AR
References	This series	This series	This series	This series	This series	This series	This series	Gulsuner, 2011
Clinic								
Head circumference at birth, cm	30.5 (−2.7 SD)	NA	32 (−1.8 SD)	30 (−3 SD)	N/A	N/A	33 (−1 SD)	N/K
Birth weight, g	3620 (+0.5 SD)	NA	3180 (−0.4 SD)	3320 (−0.1 SD)	N/A	N/A	3700 (+1 SD)	N/K
Birth length, cm	48 (−1.4 SD)		50.5 (−0.3 SD)	49 (−1 SD)			51.5 (+1.5 SD)	N/K
Age at last evaluation	27 months	25 GW	22 years	13 years	33 GW	33 GW	17 years	28 years
Head circumference, cm	37.5 (−9 SD)	23 (−1 SD)	44 (−7 SD)	41 (<−8 SD)	25.7 (<−8 SD)	27.4 (−4 SD)	42 (−10 SD)	Disproportionate short stature (150 cm)
Growth parameter (weight)	9300 g (−1 SD)	970 g (+1 SD)	30 kg (−5 SD)	32 kg (−1.5 SD)	1561 g (−2 SD)	2056 g (+0.1 SD)	50 kg (−1 SD)	Normal
Growth parameter (height), cm	71	34.5 (+1 SD)	N/K	121 (−5 SD)	41.5 (−1.5 SD)	42.2 (−1 SD)	158 (−1 SD)	N/K
Level of neurological development	Virtually no development	N/A	Virtually no development	Virtually no development	N/A	N/A	Virtually no development	Intellectual disability
Neurological examination	Spastic tetraplegia	N/A	Spastic tetraplegia	Spastic tetraplegia	N/A	N/A	Spastic tetraplegia	No spasticity
Epilepsy	Absent	N/A	Absent	Infantile spasms (2.5 years)	N/A	N/A	Drug resistant > 1 seizures/day	Absent
Movement disorder	Generalized dyskinesia	N/A	Generalized dyskinesia	Dystonia	N/A	N/A	Absent	Cerebellar ataxia
Other signs	Nystagmus (neonatal period)	N/A	Nystagmus	Nystagmus (neonatal period)	N/A	N/A	Scoliosis, precocious puberty	Bilateral external ophthalmoplegia
MRI								
Age at MRI	22 months	25 GW (US)	14.5 years	4 years	30 GW (foetal MRI)	30 GW (foetal MRI)	6 years	
Cortex	LIS/extremely reduced gyration	Delayed primary gyration	Gyral simplification	LIS/extremely reduced gyration	Delayed primary gyration	Delayed primary gyration	Cortical atrophy	Normal/brain atrophy
Basal ganglia	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

***Table 1**: detailed description of WDR81 microcephalic patients (adapted from Cavallin M et al 2017)*

As reported before, the affected WDR81 patients are all compound heterozygote and all display different mutations varying from non-sense mutations, missence mutations, duplications and deletions.

Below a detailed table of the different mutations reported in Cavallin et al.:

Patient	1st mutation	2nd mutation	WDR81 domain affected
1	non sense 1182C>T Gln628*	missense 3713C>G Pro1238Arg	Downstream BEACH Transmembrane Loop
2	non sense deletion 2834_2837del Phe946*	non sense 5464C>T Arg1822*	Downstream BEACH WD40
3	missense 1582C>T His528Tyr	duplication 4036_4041dup Val1346_Thr1347dup	BEACH Transmembrane Loop
4	non sense 1358dup Tyr453*	missense 1735G>A Gly579Arg	BEACH
5	non sense 1358dup Tyr453*	missense 1735G>A Gly579Arg	BEACH
6	non sense deletion 3820_3835del Pro1274*	Missense 5453G>T Gly1818Val	Transmembrane Loop WD40

Table 2 *reporting all the patients analyzed by Cavallin et al. The mutations are all in compound heterozygote condition*

In this study, WDR81 was proposed to be required for proper mitotic progression. Indeed, the investigation of patient derived fibroblasts suggested increased mitotic index, together with an increase in prometaphase/metaphase cells at the expense of anaphase/telophase, suggesting mitotic progression delay.

A few other studies have linked *WDR81* mutations to pathological development. The first study was conducted in 2011, in consanguineous families displaying within 5 generations 177 cases of quadrupedal locomotion and a combination of clinical features, including

morphological abnormalities in the cerebellum (atrophy of the superior, middle and inferior peduncles of cerebellum), the corpus callosum, the precentral gyrus and the Brodmann areas BA6 BA44 and BA45 (Gulsuner et al., 2011). However, the authors do not mention any microlissencephaly but described the pathology as Cerebellar Ataxia, Mental Retardation and disequilibrium syndrome (CAMRQ2 [MIM 610185]). The mutation reported here is different from the one identified by Cavallin et al. It consists in a homozygote recessive missense mutation 2567 C>T that changes a Proline in position 856 with a Leucine in a connection domain downstream of the BEACH domain.

In 2013 Traka et al. generated a WDR81 ENU-induced Nur5 mouse carrying a homozygote recessive missense mutation 4046 T>C that would change a Leucine in position 1349 with a Proline. The mice displayed photoreceptor and Purkinje cells loss starting 24 days after birth of the mouse. TEM analysis of these cells revealed an aberrant accumulation of electron dense structures, later on identified as aberrant mitochondria showing mild to severe disorganization of the cristae. Subsequent immunostaining of WDR81 localized the protein at the level of the mitochondria of Purkinje cells.

In 2015 Doldur-Balli F et al reported how (at least in zebrafish) WDR81 seems to be highly expressed at the very early stages of embryonic development and then decreases in the final stages. In addition to that, via *in situ hybridization* they were able to assess that WDR81 is particularly highly expressed in the CNS, throughout development, suggesting its importance during neurodevelopment.

The last relevant study on WDR81 was from Shasheen et al. that that reported in 2017 2 more cases of WDR81 related malformations referring to two homozygote mutations: a missense mutation 845 G>A changing a Glycine with a Glutamine in position 282 in the BEACH domain; a non-sense mutation 1567 C>T inserting a STOP codon instead of an Arginine in position 523.

In this case, the authors reported severe congenital hydrocephaly and cerebellar hypoplasia diagnosed via perinatal ultrasound analysis.

Here below is a schematic representation of the different mutations described above:

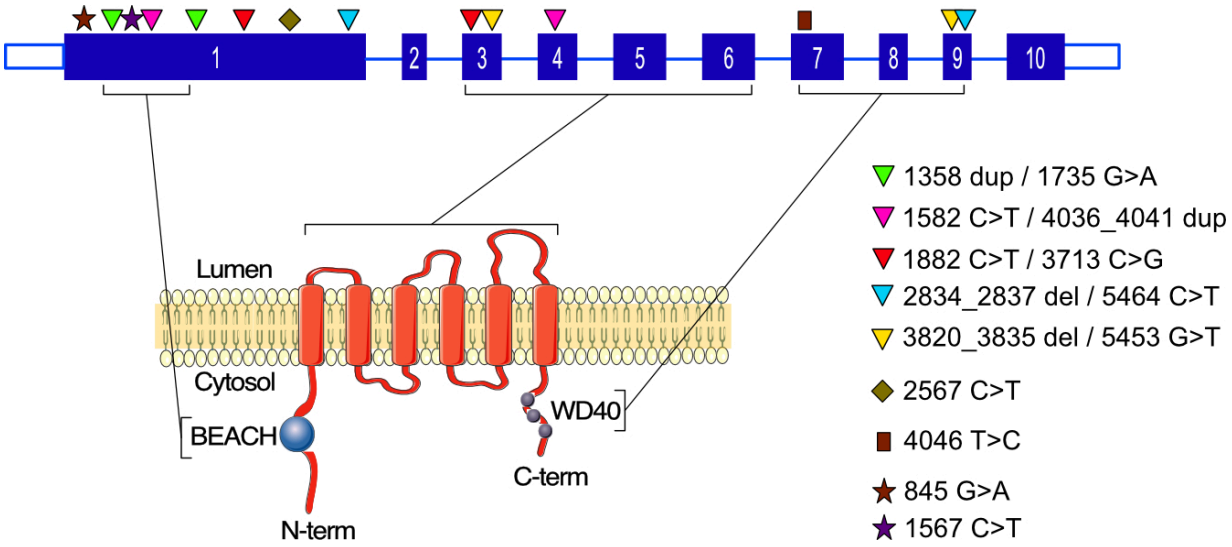


Figure 22 *Schematic representation of the published WDR81 genetic mutations. Triangles refers to Cavallin et al. 2017; Diamonds refers to to Gulsuner et al. 2011; Squares refers to Traka et al. 2013 ; Stars refers to Shaheen et al. 2017*

Taken together, all this data indicate that mutations in *WDR81* appear to severely affect development of the brain, and in particular of the neocortex. The aim of this thesis was to identify molecular and cellular mechanisms that cause altered brain growth upon WDR81 loss of function.

SCIENTIFIC QUESTIONS AND OBJECTIVES

Scientific questions and objective of the project

Neocortical development relies on the ability of neural progenitors to proliferate, self-renew and generate neurons. Neurogenesis is a finely regulated and time-limited process whereby all neurons must be generated in a given time window, with very little room to compensate for errors or delays. Therefore, any alteration in this process has the potential to lead to severe pathological malformations.

One such example is the WDR81-related microlissencephaly where patients show a small and smooth brain, with strongly affected cerebral function. These patients are amongst the most severely affected patients with brain malformations, not being able to communicate, eat or have an independent life.

The goal of this PhD project was to identify how mutation in *WDR81* may lead to microlissencephaly. For this project, I have decided to focus my analysis on the microcephaly part of the phenotype, to understand what leads to reduced brain size. My work can be separated in two main questions:

1. How can *WDR81* mutation cause a reduction of neurons that leads to microcephaly? In other words, which is the neurodevelopmental step that is affected, leading to reduced brain size?
2. What are the molecular causes that lead to this brain development defect?

Creating a CRISPR/Cas9 *WDR81*$^{-/-}$ mutant mouse that largely recapitulates the human phenotype, I have shown that WDR81-related microcephaly is due to a reduced proliferation of neural progenitors, but not to apoptotic cell death or premature progenitor differentiation. I demonstrated that this mutation leads to endosomal maturation defects affecting EGFR

trafficking and the MAPK downstream signaling pathway. As a consequence, the cells are unable to correctly respond to the EGF growth factor and display reduced rates of proliferation. This will decrease the overall neuronal output leading to microcephaly.

MATHERIALS AND METHODS

Materials and Methods

Animals and knock-out strategy

WDR81$^{-/+}$ transgenic mice were generated using CRISPR/Cas9 at Institut Curie animal facility. Mice were bread following the European and French National Regulation for the protection of vertebrate animals used for experimental and scientific purpose (2010/63/EU). All animals were kept at the Institut Curie Specific Pathogen Free (SPF) animal facility for breeding. For all pregnant females, the day of mating was referred to 0.5 day of gestation (E0.5). Pregnant females from E13.5 to E17.5 were used in order to dissect wild type and WDR81$^{-/-}$ embryos.

Genotyping *WDR81*$^{-/-}$ animals

Mice DNA was extracted from a piece of hear (adult) or tail (dissected embryos), put at 96 degrees in lysis tampon overnight. The DNA was then amplified via PCR using WDR81 specific primers: for KO1 Forward: GGCGGAAAGTGGTTCTTACA, Reverse : AGCCACCTCCTGCATGAACC; for KO2 Forward: TGGGGAAGAGATTCAGATGG , Reverse : CGTCACTTCATCATCCTCCTGGT. For KO1 the amplicon was purified using the nucleospin purification kit (Machenery and Nagel) and then exposed to the restriction enzyme AfeI (New England Bioscience). The restriction enzyme only cuts the mutant DNA giving rise to two DNA pieces of 200bp. For KO2, the amplicon was sanger-sequenced using the GATC-Eurofins platform.

Real-time reverse-transcription PCR

Wild type and WDR81$^{-/-}$ cortices were dissected at E14.5 in 1 ml of TRIZOL (Thermo Fisher 15596026). The mRNA was isolated as follows: TRIZOL + sample solution was exposed to chlorophorm for 7 minutes at room temperature and centrifuged at 15.000g for 30 min at 4 degrees. The translucent solution formed was then transferred in 1ml of isopropanol, incubated 7 minutes at room temperature and centrifuged at 4 degrees 10.000g. The pellet of nucleic acid formed was then washed in ethanol 70% and centrifuged 5 min at 10.000g at 4 degrees. The pellet was then resuspended in water. The nucleic acids solution was purified from DNA using TURBO DNA-free Kit (Thermofisher). The mRNA obtained was then retrotranscribed using the RT reverse transcription Kit (Thermofisher). Real time RT-PCR was performed using the qPCR Master Mix kit (Thermofisher) and the WDR81 Forward/Reverse primers; GAPDH gene was used for internal control and for normalization. Primers used for

WDR81 isoform 1 are forward: AGTGGATCCTTCAGACAGCC, Reverse : GAAGCCAGCCACAACACTC. Primers used for WDR81 isoform 2 are Forward: AGTGGATCCTTCAGACAGCC, Reverse : CTGACTTGTAGTGGTGCGTG.

***In utero* electroporation of mouse embryonic cortex**

Pregnant mice at embryonic day 13.5 or 14.5 were anesthetized with isoflurane gas, and injected subcutaneously first with buprenorphine (0.075mg/kg) and a local analgesic, bupivacaine (2 mg/kg), at the site of the incision. Lacrinorm gel was applied to the eyes to prevent dryness/irritation during the surgery. The abdomen was shaved and disinfected with ethanol and antibiotic swabs, then opened, and the uterine horns exposed. Plasmid DNA mixtures were used at a final concentration of 1 µg/µl per plasmid, dyed with Fast Green and injected into the left lateral ventricle of several embryos. The embryos were then electroporated through the uterine walls with a NEPA21 Electroporator (Nepagene) and a platinum plated electrode (5 pulses of 50 V for 50 ms at 1 second intervals). The uterus was replaced and the abdomen sutured. The mother was allowed to recover from surgery and supplied with painkillers in drinking water post-surgery. Electroporated brain were harvested at E16.5 and E17.5.

Immunostaining of brain slices

Mouse embryonic brains were dissected out of the skull, fixed in 4% Pfa for 2 hours, and 80 µm-thick slices were prepared with a Leica VT1200S vibratome in PBS. Slices were boiled in citrate sodium buffer (10mM, pH6) for 20 minutes and cooled down at room temperature (antigen retrieval). Slices were then blocked in PBS-Triton X100 0.3%-Donkey serum 2% at room temperature for 2 hours, incubated with primary antibody overnight at 4°C in blocking solution, washed in PBS-Tween 0.05%, and incubated with secondary antibody overnight at 4°C in blocking solution before final wash and mounting in aquapolymount. Image analysis, modifications of brightness and contrast were carried out with Fiji. Statistical analysis was carried out with Prism. Figures were assembled in Affinity Designer.

Brdu/Edu labelling

For BrDU labelling experiments, BrDU (Invitrogen B23151) was injected at 50mg/kg intraperitoneally 30 min prior to harvesting embryos. For BrDU/EDU labelling experiments,

BrDU was injected at 50mg/kg intraperitoneally 4 hours prior to harvesting embryos, and EDU (Thermofisher Click-iT EDU Alexa Fluor 555) was injected at 50mg/kg 30 min prior to harvesting embryos. After fixation, brain slices were incubated in 2N HCL for 30 min at 37 degrees and then washed 3 times with PBS prior to immunostaining.

WDR81 patient cells and immunostaining

Control and WDR81 mutant primary fibroblasts were provided by Institut Imagine, Paris. The genotype of patient 1 cells was compound heterozygote 1882C>T/3713C>G and the genotype of patient 2 cells was compound heterozygote 1582C>T/4036_4041dup. Cells were grown in OPTIMEM + 10%FBS at 37 degrees in humid air containing 5% CO_2. Fibroblast were fixed in 4% paraformaldehyde for 20 min, treated with 50mM NH4Cl for 10min, washed three times with PBS and left in a blocking solution (PBS 1% donkey serum 0.1% Triton X) for 30 min. Cells were then incubated 1 hour at room temperature with primary antibodies, washed three times in PBS and incubate for 45 min at room temperature in blocking solution with Alexa Fluor coupled secondary antibodies. Cells were then washed and mounted.

Antibodies

Primary antibodies used: rabbit anti CUX-1 (Santa Cruz, discontinued), mouse anti Ctip-2 (Abcam ab18465), rabbit anti Pax6 (Biolegend 901301), Sheep anti TBR2/EOMES (R&D system AF6166), rabbit anti NEUN (Abcam ab177487), goat anti Phospho Histone3 (Santa Cruz SC-12927), rabbit anti BRDU (Abcam AB152095), rabbit cleaved caspase-3(CST 3661), rabbit anti Ki67 (abcam ab15580), rabbit anti EGFR (CST 4267),mouse anti p-ERK (CST 9106), rabbit anti GAPDH (Sigma AldrichG9545) and mouse anti EEA-1 (BD biosciences 610457). Secondary antibodies used: donkey Alexa Fluor 488 anti-mouse, anti-rabbit, anti-goat (Jackson laboratories 715-545-150, 711-165-152, 715-605-152), donkey Alexa Fluor 555 anti-mouse, anti-rabbit, anti-goat (Jackson laboratories 715-545-150, 711-165-152, 715-605-152), donkey Alexa Fluor 647 anti-mouse, anti-rabbit, anti-goat (Jackson laboratories 715-545-150, 711-165-152, 715-605-152).

Expression constructs and shRNAs

For WDR81 knockdown experiments, WDR81 shRNA was provided by Genecopoeia™. The small interfering RNA sequence was GGAGATAAGCAATTGGACTTC and was cloned in psi-

mU6.1 vector coexpressing mcherryFP. For WDR91 knockdown experiments shRNA was provided by Tebu-bio (217MSH024100-mU6). The small interfering RNA sequence was CCAGACAAACCGAAATGACAA and was cloned in psi-mU6.1 vector coexpressing mcherryFP. For p62 knockdown experiments shRNA was provided by Tebu-bio (217CS-MSH079315-mU6-01). The small interfering RNA sequence was GCATTGAGGTTGACATTGATG and was cloned in psi-mU6.1 vector coexpressing mcherryFP. Constructs were co-injected with GFP-pCagIG (Addgene 11159) at a concentration of 1ug/ul. Plasmids were introduced in the *in vivo* developing cortex by intraventricular injection and electroporation. For WDR81 rescue experiments, Cyclin D2 and Cyclin D2Thr280Ala were synthetized *in vitro* (Genescript). They were then cloned into GFP-pCagIG (Addgene 11159) after digestion by restriction enzymes EcoRI and EcoRV.

EGF pulse assay and EGF^{555} uptake assay

For EGF pulse assay, fibroblast cultures were EGF starved for two hours before the assay. EGF was added directly to the culture medium at 0,1mg/ml. Cells were then harvested at 0, 5, 15,30 ,60 ,120 minutes and processed for protein extraction. Proteins were then mixed with 4x Leammli (Biorad) and BME solution and used for Western Blot analysis. For EGF^{555} pulse assay, fibroblast cells were cultivated on glass coverslips; cells were starved for 24 hours and then exposed to at 0,1mg/ml EGF^{555} (Thermofisher E35350). Cells were fixed in paraformaldehyde 4% at 15, 30, 60, 120, 360 min and used for immunostaining.

RESULTS

Endosomal trafficking defects alter neural progenitor proliferation and cause microcephaly

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Abstract

Primary microcephaly and megalencephaly are severe brain malformations defined by reduced and increased brain size, respectively. Whether these two pathologies arise from related alterations at the molecular level is unclear. Microcephaly has been largely associated with centrosomal defects, leading to cell death. Here, we investigated the consequences of *WDR81* loss of function, which cause severe microcephaly in patients. We show that WDR81 regulates endosomal trafficking of EGFR, and that loss of function leads to reduced MAP kinase pathway activation. Mouse radial glial progenitor cells knocked-out for *WDR81* display reduced proliferation rates, leading to reduced brain size. These proliferation defects are rescued *in vivo* by the expression of megalencephaly-causing mutated Cyclin D2. Our results identify the endosomal machinery as an important regulator of RG cell proliferation rates and brain growth. They demonstrate that microcephaly and megalencephaly can be due to opposite effects on the proliferation rate of radial glial progenitors.

Introduction

Development of the neocortex relies on neural stem cells called radial glial (RG) cells, that generate the majority of cortical neurons (Uzquiano et al., 2018). Neuronal production is restricted to a short period during which all excitatory neurons are produced (Gao et al., 2014). This leaves little room for compensatory mechanisms to occur, and alterations during this critical period lead to brain malformations (Homem et al., 2015). Indeed, the developing neocortex is highly sensitive to perturbations, and a large number of mutations have been described to specifically alter its growth, but not that of other organs (Pirozzi et al., 2018).

Primary microcephaly is a severe neurodevelopmental disorder characterized by a head circumference that is more than 3 standard deviations (SD) below the mean (Jayaraman et al., 2018). The major molecular cause of microcephaly lies in defects in centrosomal number (Insolera et al., 2014; Marthiens et al., 2013), maturation (Lizarraga et al., 2010) and mitotic spindle regulation (Chen et al., 2014; Hu et al., 2014; Johnson et al., 2018), leading to apoptotic cell death. In fact, apoptosis appears to be the leading cause of microcephaly in animal models, irrespective of the upstream affected molecular pathway (Gruber et al., 2011; Kim et al., 2010; Mao et al., 2016). Reduced proliferation rates of progenitors, while proposed to be a putative cause of microcephaly (Reynolds et al., 2017), has received much less experimental support. One notable example is the gene encoding IGFR1, which is mutated in syndromic forms of microcephaly, and when deleted in mouse leads to reduced proliferation and small brain size (Juanes et al., 2014; Lehtinen et al., 2011).

On the opposite end of the spectrum, megalencephaly (MEG) is a neuronal disorder characterized by brain overgrowth (3 SD over the mean) (Dobyns and Mirzaa, 2019). The causes of megalencephaly are diverse, but activating mutations in the Pi3K-AKT-mTOR and the Ras-MAPK pathways have been identified as important underlying events (Kang and Lee, 2019; Mirzaa and Poduri, 2014; Rauen, 2013). Mouse and cerebral organoid models for these activated pathways demonstrated increased proliferation of radial glial cells leading to tissue overgrowth (Groszer et al., 2001; Hegedus et al., 2007; Li et al., 2017). Stabilizing mutations in the downstream target Cyclin D2 were also reported, and its ectopic expression in mouse brain stimulated progenitor proliferation (Mirzaa et al., 2014).

The EGF receptor (EGFR) and its ligands are major regulators of tissue growth (Sigismund et al., 2018). Accordingly, knock-out of EGFR leads to a dramatic atrophy of the cerebral cortex (Threadgill et al., 1995). Progenitor cells appear to become responsive to EGF

at mid-neurogenesis, while at earlier stages they rather exhibit FGR2 dependence (Tropepe et al., 1999). Endosomal trafficking of EGFR plays a major role in the regulation of its activity: while most EGFR signaling is believed to occur at the plasma membrane, internalization of EGFR is critical for signal termination (Tomas et al., 2014). Following endocytosis, internalized cargos follow different trafficking routes including recycling towards the plasma membrane or delivery to lysosomes for degradation (Cullen and Steinberg, 2018). Phosphatidylinositols (PtdIns) are major regulators of this process, defining endosomal compartment identity. Early endosomes are characterized by the presence of the small GTPase RAB5 and PtdIns3P, and late endosomes by RAB7 and PtdIns(3,5)P_2 (Stenmark, 2009). Recently, WDR81 and its partner WDR91 were shown to act as negative regulators of class III phosphatidylinositol 3-kinase (PI3K)-dependent PtdIns3P generation, therefore promoting early to late endosomal conversion (K. Liu et al., 2016). In WDR81 knock-out (KO) HeLa cells, endosomal maturation defects led to delayed EGFR degradation (K. Liu et al., 2016).

We recently reported compound heterozygous mutations in the human *WDR81* gene, that result in extreme microcephaly associated with reduced gyrification of the neocortex (Cavallin et al., 2017). Here, we generated a mouse knock-out model that largely recapitulates the human phenotype. Mutant brains are not only smaller but also display altered neuronal layering. We demonstrate that microcephaly is the result of reduced proliferation rates of radial glial progenitors, but not of cell death. Mechanistically, we show that WDR81 mutation delays EGFR endosomal trafficking and leads to reduced activation of the MAPK signaling pathway. These proliferation defects can be rescued by expressing a megalencephaly-causing mutated cyclin D2, indicating that microcephaly and megalencephaly can be due to opposite effects of the proliferation rates of radial glial cells.

Results

WDR81 KO mice display reduced brain size and altered neuronal positioning

In mice, two WDR81 isoforms have been identified. A long isoform (210 kDa) encompassing an N-terminal BEACH domain, a central transmembrane region, and a C-terminal WD40 repeat domain; and a shorter isoform (81 kDa) lacking the BEACH domain (**Figure 1A**). Measurements of mRNA isolated from embryonic E14.5 cortex extracts indicated that the long WDR81 isoform was highly dominant, with only trace levels of the short isoform (**Figure 1B**). We generated two WDR81 KO mice using gRNAs targeting the beginning of exon 1 (KO-1, affecting isoform 1), and the end of exon 1 (KO-2, affecting both isoforms)

(**Figure 1A)**. Both lines displayed frameshifts leading to the appearance of a premature STOP codon (**Figure S1A**). QPCR measurements in KO1 did not reveal any upregulation of isoform 2, indicating an absence of compensation (**Figure 1B**). Moreover, a strong reduction of isoform 1 mRNA levels was observed, likely due to non-sense mRNA decay (**Figure 1B**). WDR81 homozygote mutant embryos and pups were detected at sub-mendelian rates, and did not live for more than 21 days (**Figure S1B**).

We then analyzed brain size and organization in P7 WDR81$^{-/-}$ pups. Both KO lines were severely microcephalic, with a reduced hemisphere area (**Figures 1C and 1E**). Cortical thickness was also greatly reduced (by ~54%), suggesting defects both in tangential and radial expansion of the brain (**Figures 1D and 1F**). We next analyzed neuronal positioning in WDR81$^{-/-}$ P7 cortices. The localization of upper layer late-born neurons was severely affected, with a large number of CUX-1-positive neurons dispersed throughout the cortex (**Figure 1G**). Deeper neurons, which are born earlier during cortical development were however correctly positioned, as indicated by the localization of CTIP-2-positive neurons (**Figure 1H**). Overall, mice knocked out for WDR81 have reduced brain size and altered neuronal positioning, largely recapitulating the microcephaly and lissencephaly phenotypes reported in humans. These phenotypes were observed for both WDR81 KO lines and we therefore next focused our analysis on KO-1 (referred to as WDR81$^{-/-}$ from here on).

WDR81 KO alters radial glial progenitor proliferation

To identify the causes of reduced brain size in WDR81$^{-/-}$ pups, we tested for alterations of neocortex development at embryonic stages. To test for proliferation defects, we first measured the mitotic index of WDR81$^{-/-}$ radial glial progenitors, as defined by the percentage of phospho-Histone H3 (PH3)-positive cells out of total PAX6-positive cells. While at E12.5, proliferation appeared normal, mitotic index of WDR81$^{-/-}$ radial glial progenitors was severely reduced at E14.5 and E16.5 (**Figure 2A**). Strikingly, TBR2-positive intermediate progenitors appeared to cycle normally throughout development (**Figure 2B**). Therefore, WDR81 mutation specifically alters proliferation of radial glial progenitors at mid and late neurogenic stages. We next analyzed further these proliferation defects and measured the percentage of cells in S phase. A 30-minute BrdU pulse revealed an increased amount of WDR81$^{-/-}$ radial glial progenitors in S-phase (**Figures 2C and 2D**). To test whether this was due to a longer duration of S-phase, we performed a double BrdU-EdU pulse, in order to measure the rate of S-phase exit. Mice were first injected with BrdU, followed by a second injection with EdU 4 hours later. This assay revealed a decreased proportion of cells that exited S phase (BrdU+/EdU-) in

WDR81$^{-/-}$ brains, indicating a longer S phase in mutant radial glial progenitors (**Figures 2E and 2F**).

An alternative potential cause of reduced brain size is premature differentiation of progenitor cells. To test this, embryonic cortices were stained for PAX6 (radial glial progenitors), TBR2 (intermediate progenitors) and NEUN (neurons) at different developmental stages and the proportion of each cell population was measured. We did not observe any decrease in the proportion of progenitors out of the total cell population throughout development indicating they did not prematurely differentiate (**Figure 2G**). In fact, we even detected a reduction of the proportion of neurons at mid and late neurogenesis (**Figure 2G**). Finally, we analyzed apoptotic cell death in WDR81$^{-/-}$ cortices. Staining for cleaved caspase-3 (CC3) did not reveal any increased apoptosis, which remained almost undetectable both in WT and mutant embryos (**Figure 2H**). Therefore, reduced brain size in WDR81$^{-/-}$ mice is not the result of premature progenitor differentiation or increased apoptotic cell death, but appears to be a consequence of reduced radial glial progenitor proliferation rates.

Reduced proliferation and EFGR signaling in WDR81 patient cells

We next tested whether similar proliferation defects could be observed in patient cells mutated for WDR81. Two mutant primary fibroblast lines, derived from skin biopsies, were analyzed and compared to two control fibroblast lines. The mitotic index of both patient cells was strongly decreased, mimicking the mouse radial glial progenitor phenotype (**Figures 3A and 3B**). As an alternative measurement of proliferation, cells were stained for Ki67, which also revealed a substantial decrease for both patient cell lines (**Figures 3C and 3D**).

In radial glial progenitors, we detected proliferation defects from mid-neurogenesis (**Figure 2A)**, which fits with the time when these cells start responding to EGF (Tropepe et al., 1999). This observation suggested a potential alteration in the EGFR signaling pathway. In order to test this, we monitored the activity of this signalling pathway in control and patient fibroblasts. Strikingly, we observed that the protein levels of EGFR itself were drastically reduced in both patient cell lines (**Figures 3E and 3F**). We next measured the activation of the mitogen-activated protein kinase (MAPK) signaling pathway in response to EGF stimulation. Consistent with the decreased levels of EGFR, the phosphorylation of ERK was reduced in both patient fibroblasts following an EGF pulse (**Figures 3G-I and S2**). Therefore, WDR81 patient cells display reduced EGFR levels, leading to a reduced activation of the MAPK signalling pathway upon EGF stimulation.

The levels of EGFR are known to be tightly regulated, through complex feedback loops and the balance between recycling and degradation of the internalized receptor (Avraham and Yarden, 2011). We therefore asked whether reduced EGFR levels were a consequence of defects within the EGFR pathway itself. To test this, we EGF-starved cells and measured the levels of EGFR 24 hours later. In patient cells, starvation rescued EGFR levels to the ones of control cells (**Figures 3J and 3K**). These results indicate that reduced EGFR levels are only seen when the pathway is activated. Given that EGFR internalization upon EGF binding is a major regulator of the pathway, this data points towards intracellular processing defects of EGFR.

WDR81 is required for endosomal trafficking of EGFR

WDR81 is known to regulate endosomal maturation as well as autophagic clearance of aggregated proteins (aggrephagy) (K. Liu et al., 2016; X. Liu et al., 2017). Importantly, these two functions are independent and act via the WDR81 specific binding partners WDR91 and p62, respectively. We therefore tested whether one of these factors affected neocortex development similarly to WDR81. To perform this, we *in utero* electroporated shRNA-expressing constructs for WDR81, WDR91 and p62 in E13.5 developing brains and analyzed cell distribution at E17.5. Consistent with the KO data, WDR81 knock-down (KD) strongly affected neurodevelopment (**Figures 4A and 4B**). In particular, a large fraction of KD cells accumulated in the intermediate zone (IZ), at the expense of the germinal zones and cortical plate. This phenotype was phenocopied by WDR91 KD, but not by p62 KD which did not appear to affect cell distribution (**Figures 4A and 4B**). These results support the endosomal function of WDR81 as a critical player for proper neocortex development.

We next tested whether endosomal defects could be observed in WDR81 KO radial glial progenitors. Staining for various endolysosomal compartments revealed a specific alteration of EEA1+ early endosomes, which appeared strongly enlarged (**Figure 4C**). Quantification of their size confirmed this observation, revealing a 63% average increase (**Figure 4E**). To test whether this is a conserved feature of WDR81 patient cells, we measured early endosome size in mutant fibroblasts. Again, EEA1+ endosomes were found to be swollen, with an increased proportion of large endosomes (>0,5 µm) (**Figures 4D and 4F**). These results are consistent with previous observation made in KO HeLa cells and demonstrating a role for WDR81 in negative regulation of Class III Pi3K (K. Liu et al., 2016).

Because these endosomal defects are a potential cause of altered EGFR signaling, we next tested whether EGFR endosomal trafficking was affected in WDR81 mutant cells. Cells were first starved for 24 hours to restore EGFR to the levels of control cells, and subsequently pulsed with fluorescent EGF^{555}, to monitor internalization and clearance of EGF-bound EGFR. In both patient cells, EGF^{555} was shown to accumulate longer within EEA1+ early endosomes (**Figure 4G**). Quantification of the colocalization between EGF^{555} and EEA1 revealed that this delay was particularly important 120 minutes after EGF internalization (**Figure 4H**). Therefore, WDR81 is critical for endosomal homeostasis and trafficking of internalized EGFR following EGF binding.

Megalencephaly-causing mutation rescues progenitor proliferation in WDR81 mutant brains

Our results indicate that trafficking defects of EGFR can arise from mutations in WDR81, and lead to reduced activation of the MAPK signaling pathway. They further show that reduced radial glial progenitor proliferation is a cause of primary microcephaly. Megalencephaly is characterized by brain overgrowth and can be due to increased cell proliferation during development (Dobyns and Mirzaa, 2019). Major causes include gain-of-function mutations in AKT3 and its downstream target Cyclin D2 (Mirzaa et al., 2014; Rivière et al., 2012). Together, these data suggest that microcephaly and megalencephaly can be the consequence of opposite effects on the proliferation rates of radial glial progenitors. To further test this, we analyzed the effect of a megalencephaly-causing Cyclin $D2^{Thr280Ala}$ mutant on the proliferation of WDR81 KO radial glial progenitor. Degradation-resistant Cyclin $D2^{Thr280Ala}$, WT Cyclin D2 or a control vector were expressed using *in utero* electroporation in WT and WDR81-mutant mice brains at E 14.5, and the mitotic index of PAX6 cells was measured at E16.5. Following expression of the control empty vector, we confirmed the reduced mitotic index in $WDR81^{-/-}$ brains (**Figures 5A and 5B**). Moreover, expression of Cyclin $D2^{Thr280Ala}$ in WT brain increased mitotic index, indicating that this megalencephaly-causing mutation indeed stimulates radial glial progenitor proliferation rates (**Figures 5A and 5B**). Strikingly, expression of degradation-resistant Cyclin $D2^{Thr280Ala}$ in $WDR81^{-/-}$ brains rescued the mitotic index reduction (**Figures 5A and 5B**). WT cyclin D2 was also able to rescue proliferation, although to a lesser extent, indicating that large amounts of this protein, either due to overexpression or impaired degradation is able to restore proliferation (**Figures 5A and 5B**). Together, these results indicate that a megalencephaly-causing mutation can overcome the effect of a microcephaly-causing mutation on the proliferation of radial glial progenitors. These

two pathologies can therefore arise from a highly related cause: an imbalance in cell cycle regulation leading either to reduced brain growth or to brain overgrowth (**Figure 5C**).

Discussion

In this study, we investigated the mechanisms by which mutation in the *WDR81* gene leads to severe microcephaly in patients. We show that KO mouse recapitulates many features of the phenotype previously observed in patients and that the endosomal maturation function of WDR81 is critical for neocortex development. WDR81 is required for endosomal clearance of internalized EGFR and normal activation of the mitogenic MAPK signaling pathway. In the absence of WDR81, the proliferation rate of radial glial cells is affected, leading to reduced brain size. Importantly, cell death does not appear to contribute to this phenotype. Proliferation defects can be rescued by the expression of a megalencephaly-causing mutated cyclin D2, highlighting a tight functional link between these two pathologies.

Membrane trafficking has been poorly investigated in radial glial cells, albeit its predicted implication in many important processes including cargo polarized transport, secretion of extracellular matrix components, or endocytic processing of surface receptors for lysosomal degradation or recycling. We show here that the endosomal maturation machinery plays a critical role in the processing of internalized EGFR in RG cells, and is required for their proliferation. Neurogenesis depends on EGFR activity, with radial glial cells becoming responsive to EGF from mid-neurogenesis (Threadgill et al., 1995; Tropepe et al., 1999). Accordingly, we find that WDR81$^{-/-}$ RG cells are specifically affected at E14,5 and E16,5 stages of development. Why the proliferation rate of IPs was not affected is unclear but EGF is secreted into the cerebrospinal fluid from the choroid plexus and apical contact may be critical for responsiveness (Lehtinen and Walsh, 2011; Sun et al., 2005). EGFR was previously reported to be asymmetrically inherited during radial glial cell division, generating a daughter cell with higher proliferative potential (Sun et al., 2005). Later in development, EGFR also acts as an important regulator of astrocyte differentiation (Beattie et al., 2017). Our data point to the intracellular processing of EGFR as an important level of control for the regulation of proliferation in radial glial cells. WDR81 is likely to affect the trafficking of other cargos, which may also impact cell radial glial cell proliferation. Moreover, the trafficking of neuronal cargoes, such as adhesion molecules, is likely to lead to the altered neuronal positioning observed in KO mice, and to the lissencephaly phenotype in human.

In principle, microcephaly can be the consequence of premature progenitor differentiation, reduced proliferation rates, or cell death. While centrosomal defects leading to

apoptosis have been described, reduced proliferations rates have received little experimental support (Lehtinen et al., 2011). In mouse, RG cells produce eight to nine neurons during a short neurogenic period, before differentiating (Gao et al., 2014). We show here that *WDR81* mutation does not affect the modes of division of RG cells nor cell survival, but act solely through a reduction of their proliferation rate, leading to reduced brain size. This highlights the absence of compensatory mechanisms in the developing neocortex, where all neurons must be produced in a defined temporal window. During corticogenesis, G1 lengthening is associated with increased neurogenic divisions at the expense of symmetric amplifying divisions (Lange et al., 2009; Pilaz et al., 2009). We did not detect such cell fate changes in WDR81$^{-/-}$ brains. This is likely due to the fact that the proliferation rate of mutant RG cells is only affected at a stage where the vast majority of cells already perform neurogenic divisions (Uzquiano et al., 2018). At the macroscopic level, microcephaly and megalencephaly can appear as opposite phenotypes. However, whether they can originate from related underlying causes at the molecular level is unclear. We show here that microcephaly and megalencephaly can be due to opposite on the proliferation rates of RG cells, and can therefore be viewed as two sides of the same coin.

Materials and Methods

Animals

All experiments involving mice were carried out according to the recommendations of the European Community (2010/63/UE). The animals were bred and cared for in the Specific Pathogen Free (SPF) Animal Facility of Institut Curie (agreement C 75-05-18). All animal procedures were approved by the ethics committee of the Institut Curie CEEA-IC #118 and by French Ministry of Research (2016-002.

Guide RNA selection and preparation

gRNA sequences targeting exon 1 of WDR81 have been identified and selected using the online software CRIPSOR (crispor.tefor.net). Forward and reverse oligonucleotides were annealed and cloned into px330 plasmid. gRNAs and Cas9mRNA have been produced as in Wang et al., 2013. Briefly, to generate Cas9 mRNA and gRNA, *in vitro* transcriptions were performed on the Cas9 pCR2.1-XL plasmid (Greenberg et al., 2017) and gRNA plasmids, using the mMESSAGE mMACHINE T7 ULTRA kit and the MEGAshortscript T7 kit (Life

Technologies), respectively. Cas9 mRNA and sgRNAs were then purified using the MEGAclear Kit (Thermo Fisher Scientific) and eluted in RNAse-free water. The gRNA and Cas9mRNA quality were evaluated on agarose gel.

Generation of WDR81 Knock-Out mice

Eight-week-old B6D2F1 (C57BL/6J × DBA2) females from Charles River France, were superovulated by intraperitoneal (i.p.) administration of 5 IU of Pregnant Mare Serum Gonadotropin followed by an additional i.p. injection of 5 IU Human Chorion Gonadotropin 48 h later. Females were mate to a stud male of the same genetic background. Cytoplasmic microinjection was performed into mouse fertilized oocytes using Cas9 mRNA and sgRNA at 100 ng/µl and 50 ng/µl, respectively in Brinster buffer (10 mM Tris-HCl pH 7.5; 0.25 mM EDTA). Microinjected zygotes were cultured in Cleave medium (Cook, K-RVCL-50) at 37°C under 5% CO2 and then implanted at one cell stage into infundibulum of E0.5 NMRI pseudo-pregnant females (25-30 injected zygotes per female). According to the genotyping strategy, 3 mice showed modified allele out of a total of 22 pups. The founders were then backcrossed to C57BL6/J.

Genotyping WDR81$^{-/-}$ animals

Mice DNA was extracted from a piece of hear (adult) or tail (dissected embryos), put at 96 degrees in lysis tampon overnight. The DNA was then amplified via PCR using WDR81 specific primers: for KO1 Forward: GGCGGAAAGTGGTTCTTACA, Reverse : AGCCACCTCCTGCATGAACC; for KO2 Forward: GGCTTGTAGTGGTTCTGTAC , Reverse : GATCCTTCTGCATTCCAA. For KO1 the amplicon was purified using the nucleospin purification kit (Machenery and Nagel) and then exposed to the restriction enzyme AfeI (New England Bioscience). The restriction enzyme only cuts the mutant DNA giving rise to two DNA pieces of 200bp. For KO2, the amplicon was sanger-sequenced using the GATC-Eurofins platform.

Real-time reverse-transcription PCR

Wild type and WDR81$^{-/-}$ cortices were dissected at E14.5 in 1 ml of TRIZOL (Thermo Fisher 15596026). The mRNA was isolated as follows: TRIZOL + sample solution was exposed to chlorophorm for 7 minutes at room temperature and centrifuged at 15.000g for 30 min at 4 degrees. The translucent solution formed was then transferred in 1ml of isopropanol, incubated 7 minutes at room temperature and centrifuged at 4 degrees 10.000g. The pellet of nucleic acid

formed was then washed in ethanol 70% and centrifuged 5 min at 10.000g at 4 degrees. The pellet was then resuspended in water. The nucleic acids solution was purified from DNA using TURBO DNA-free Kit (Thermofisher). The mRNA obtained was then retrotranscribed using the RT reverse transcription Kit (Thermofisher). Real time RT-PCR was performed using the qPCR Master Mix kit (Thermofisher) and the WDR81 Forward/Reverse primers; GAPDH gene was used for internal control and for normalization. Primers used for WDR81 isoform 1 are forward: AGTGGATCCTTCAGACAGCC, Reverse : GAAGCCAGCCACAACACTC. Primers used for WDR81 isoform 2 are Forward: AGTGGATCCTTCAGACAGCC, Reverse : CTGACTTGTAGTGGTGCGTG

***In utero* electroporation of mouse embryonic cortex**

Pregnant mice at embryonic day 13.5 or 14.5 were anesthetized with isoflurane gas, and injected subcutaneously first with buprenorphine (0.075mg/kg) and a local analgesic, bupivacaine (2 mg/kg), at the site of the incision. Lacrinorm gel was applied to the eyes to prevent dryness/irritation during the surgery. The abdomen was shaved and disinfected with ethanol and antibiotic swabs, then opened, and the uterine horns exposed. Plasmid DNA mixtures were used at a final concentration of 1 μg/μl per plasmid, dyed with Fast Green and injected into the left lateral ventricle of several embryos. The embryos were then electroporated through the uterine walls with a NEPA21 Electroporator (Nepagene) and a platinum plated electrode (5 pulses of 50 V for 50 ms at 1 second intervals). The uterus was replaced and the abdomen sutured. The mother was allowed to recover from surgery and supplied with painkillers in drinking water post-surgery. Electroporated brain were harvested at E16.5 and E17.5.

Immunostaining of brain slices

Mouse embryonic brains were dissected out of the skull, fixed in 4% Pfa for 2 hours, and 80 μm-thick slices were prepared with a Leica VT1200S vibratome in PBS. Slices were boiled in citrate sodium buffer (10mM, pH6) for 20 minutes and cooled down at room temperature (antigen retrieval). Slices were then blocked in PBS-Triton X100 0.3%-Donkey serum 2% at room temperature for 2 hours, incubated with primary antibody overnight at 4°C in blocking solution, washed in PBS-Tween 0.05%, and incubated with secondary antibody overnight at 4°C in blocking solution before final wash and mounting in aquapolymount. Image analysis, modifications of brightness and contrast were carried out with Fiji. Statistical analysis was carried out with Prism. Figures were assembled in Affinity Designer.

Brdu/Edu labelling

For BrDU labelling experiments, BrDU (Invitrogen B23151) was injected at 50mg/kg intraperitoneally 30 min prior to harvesting embryos. For BrDU/EDU labelling experiments, BrDU was injected at 50mg/kg intraperitoneally 4 hours prior to harvesting embryos, and EdU (Thermofisher Click-iT EdU Alexa Fluor 555) was injected at 50mg/kg 30 min prior to harvesting embryos. After fixation, brain slices were incubated in 2N HCL for 30 min at 37 degrees and then washed 3 times with PBS prior to immunostaining.

WDR81 patient cells and immunostaining

Control and WDR81 mutant primary fibroblasts were provided by Institut Imagine, Paris. The genotype of patient 1 cells was compound heterozygote 1882C>T/3713C>G and the genotype of patient 2 cells was compound heterozygote 1582C>T/4036_4041dup. Cells were grown in OPTIMEM + 10%FBS at 37 degrees in humid air containing 5% CO_2. Fibroblast were fixed in 4% paraformaldehyde for 20 min, treated with 50mM NH4Cl for 10min, washed three times with PBS and left in a blocking solution (PBS 1% donkey serum 0.1% Triton X) for 30 min. Cells were then incubated 1 hour at room temperature with primary antibodies, washed three times in PBS and incubate for 45 min at room temperature in blocking solution with Alexa Fluor coupled secondary antibodies. Cells were then washed and mounted.

Antibodies

Primary antibodies used: rabbit anti CUX-1 (Santa Cruz, discontinued), mouse anti Ctip-2 (Abcam ab18465), rabbit anti Pax6 (Biolegend 901301), Sheep anti TBR2/EOMES (R&D system AF6166), rabbit anti NEUN (Abcam ab177487), goat anti Phospho Histone3 (Santa Cruz SC-12927), rabbit anti BRDU (Abcam AB152095), rabbit cleaved caspase-3(CST 3661), rabbit anti Ki67 (abcam ab15580), rabbit anti EGFR (CST 4267),mouse anti p-ERK (CST 9106), rabbit anti GAPDH (Sigma AldrichG9545) and mouse anti EEA-1 (BD biosciences 610457). Secondary antibodies used: donkey Alexa Fluor 488 anti-mouse, anti-rabbit, anti-goat (Jackson laboratories 715-545-150, 711-165-152, 715-605-152), donkey Alexa Fluor 555 anti-mouse, anti-rabbit, anti-goat (Jackson laboratories 715-545-150, 711-165-152, 715-605-152), donkey Alexa Fluor 647 anti-mouse, anti-rabbit, anti-goat (Jackson laboratories 715-545-150, 711-165-152, 715-605-152).

Expression constructs and shRNAs

For WDR81 knockdown experiments, WDR81 shRNA was provided by Genecopoeia^{TM}. The small interfering RNA sequence was ggagataagcaattggacttc and was cloned in psi-mU6.1 vector coexpressing mcherryFP. For WDR91 knockdown experiments shRNA was provided by Tebu-bio (217MSH024100-mU6). For p62 knockdown experiments shRNA was provided by Tebu-bio (217CS-MSH079315-mU6-01). Constructs were co-injected with GFP-pCagIG (Addgene 11159) at a concentration of 1ug/ul. Plasmids were introduced in the *in vivo* developing cortex by intraventricular injection and electroporation. For WDR81 rescue experiments, Cyclin D2 and Cyclin D2Thr280Ala were synthetized *in vitro* (Genescript). They were then cloned into GFP-pCagIG (Addgene 11159) after digestion by restriction enzymes EcoRI and EcoRV.

EGF pulse assay and EGF^{555} uptake assay

For EGF pulse assay, fibroblast cultures were EGF starved for two hours before the assay. EGF was added directly to the culture medium at 0,1mg/ml. Cells were then harvested at 0, 5, 15,30 ,60 ,120 minutes and processed for protein extraction. Proteins were then mixed with 4x Leammli (Biorad) and BME solution and used for Western Blot analysis. For EGF^{555} pulse assay, fibroblast cells were cultivated on glass coverslips; cells were starved for 24 hours and then exposed to at 0,1mg/ml EGF^{555} (Thermofisher E35350). Cells were fixed in paraformaldehyde 4% at 15, 30, 60, 120, 360 min and used for immunostaining.

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Author contributions

J.A.C. and A.D.B. conceived the project. J.A.C. & A.D.B. analyzed the data. J.A.C., J.B.B. & A.D.B. wrote the manuscript. J.A.C. and A.D.C. did most of the experimental procedures. J.A.C., L.D.M. and F.E.M. created the mutant mouse lines. D.A. supervised the mouse mutant colonies and performed all the crossings. L.C. and J.A.C. set up *in utero* electroporation experiments. N.B.B. provided biological samples of the affected patients A.D.B. supervised the project.

Figure legends

Figure 1. WDR81 KO mice display reduced brain size and altered neuronal positioning

A. Schematic representation of mouse WDR81 isoforms and predicted structure. **B.** Quantification of WDR81 isoforms 1 and 2 mRNA levels in WT and WDR81$^{-/-}$ E14.5 cortices (n = 3 brains per genotype). Isoform 1 (ISO1) is the dominant isoform and its levels are strongly reduced in WDR81$^{-/-}$ cortices. **C.** WDR81$^{-/-}$ postnatal day 7 brains are microcephalic and display reduced cortical surface area as compared to WT brains. **D.** DAPI staining of P7 WT and WDR81$^{-/-}$ cross sections reveals reduced cortical thickness in mutants. **E.** Quantification of hemisphere areas in P7 WT and KO1 brains (n = 4 brains per genotype). **F.** Quantification of cortical thickness in P7 WT, KO1 and KO2 brains (n = 3 brains per genotype). **G.** CUX-1 staining in P7 WT and WDR81$^{-/-}$ cortices. Quantification of CUX1+ neuronal positioning reveals dispersion throughout the thickness of the neocortex (n = 3 brains per genotype). **H.** CTIP-2 staining in P7 WT and WDR81$^{-/-}$ cortices. Quantification does not neuronal positioning defects, with CTIP-2+ neurons still concentrated in the third bin (n = 3 brains per genotype). **(G, H)** WT and mutant cortices were divided into 5 bins of equal size to measure neuronal relative positioning, independently of cortical thickness. **p<0,01; ***p<0,001; ****p<0,0001 by unpaired t-tests.

Figure 2. WDR81 KO alters radial glial progenitor proliferation

A. PAX6 and PH3 double staining in E14.5 WT and WDR81$^{-/-}$ brains. Quantification of the mitotic index of PAX6+ cells reveals decreased proliferation of WDR81$^{-/-}$ radial glial progenitors at E14.5 and E16.5 (n = 5-8 brains per condition). **B.** TBR2 and PH3 double staining in E14.5 WT and WDR81$^{-/-}$ brains. Quantification of the mitotic index of TBR2+ cells indicates that proliferation of WDR81$^{-/-}$ intermediate progenitors in not affected (n = 3 brains per

condition). **C.** Schematic representation of the BrdU labeling experimental approach and Pax6 and BrdU staining in E14.5 WT and WDR81$^{-/-}$ brains. **D.** Quantification of the percentage of BrdU+ PAX6+ out of total PAX6 cells reveals increased number of cells in S phase in WDR81$^{-/-}$ radial glial progenitors at E14.5 (n = 3 brains per condition). **E.** Schematic representation of the BrdU / EdU double labeling experimental approach and PAX6, BrdU and EdU staining in E14.5 WT and WDR81$^{-/-}$ brains. **F.** Quantification of the percentage of of BrdU+ EdU- PAX6+ out of the total BrdU+ PAX6+ cells reveals a decreased proportion of cells that exited S phase following BrdU injection in WDR81$^{-/-}$ radial glial progenitors at E14.5 (n = 3 brains per condition). **G.** Staining for the cell fate markers PAX6 (radial glial progenitors), TBR2 (Intermediate progenitors) NEUN (Neurons) in E14.5 WT and WDR81$^{-/-}$ brains, and quantification of cell fate distribution at E12.5, E14.5 and E16.5 (n = 3-5 brains per condition). **H.** Staining for Cleaved Caspase-3 (CC3) and DAPI in E14.5 WT and WDR81$^{-/-}$ brains, showing an absence of apoptosis induction. *p<0,05; **p<0,01; ***p<0,001 by unpaired t-tests.

Figure 3. Reduced proliferation and EFGR signaling in WDR81 patient cells

A. PH3 and DAPI staining in control and WDR81 patient fibroblasts **B.** Quantification of the percentage of PH3+ cells reveals decreased mitotic index in patient cells (n = 3). **C.** Ki67 and DAPI staining in control and WDR81 patient fibroblasts. **D.** Quantification of the percentage of Ki67+ cells shows decreased proliferation in patient cells (n = 3). **E.** Western Blot for EGFR in control and WDR81 patient fibroblasts. **F.** Quantification reveals a strong reduction of EGFR levels in patient cells (n = 5). **G.** Time course of EGFR and P-ERK levels in control and WDR81 patient fibroblasts following an EGF pulse. **H.** Quantification of EGFR levels, normalized to control levels at T0 (n = 5). **I.** Quantification of P-ERK levels, normalized to control levels at T5 (n = 5). **J.** Western Blot for EGFR in control and WDR81 patient fibroblasts at steady state (+EGF) and cultivated for 24H in the absence of EGF. **K.** Quantification reveals a restoration of EGFR levels following starvation (n = 3).. *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001 by unpaired t-tests.

Figure 4. WDR81 is required for endosomal trafficking of EGFR

A. Expression of control shRNA and shRNA-mediated knockdown constructs for WDR81, WDR91 and p62. Plasmids were delivered by in utero electroporation at E13.5 and analysis was performed at E16.5. Ventricular Zone and Sub-Ventricular Zone (VZ +SVZ), Intermediate Zone (IZ) and Cortical Plate (CP). **B**. Quantification of electroporated cell distribution reveals major accumulation in the IZ following WDR81 and WDR91 knockdown (n = 4 brains per

condition). **C.** Ventricular zone of E14.5 WT and WDR81$^{-/-}$ mice cortices stained for EEA1 and Actin. **D.** Control and WDR81 patient fibroblasts stained for EEA1. **E.** Quantification of individual EEA1+ early endosomes in WT and WDR81$^{-/-}$ VZ reveals increased size in mutant brains (n = 3 brains per condition). **F.** Quantification of individual EEA1+ early endosomes in control and WDR81 patient fibroblasts reveals increased size in mutant cells (n = 20 cells per condition). **G.** EGF^{555} uptake assay in control and WDR81 patient fibroblasts, and stained for EEA1. **H**. Quantification of EGF^{555} and EEA1 colocalization during EGF^{555} uptake reveals prolonged colocalization between EGF and early endosomes in WDR81 patient cells (n = 28 cells per condition). *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001 by unpaired t-test (A & H) and Mann-Whitney tests (E & F).

Figure 5. Undegradable Cyclin D2^{Thr280Ala} rescues WDR81$^{-/-}$ proliferation index

A. Expression of Cyclin D2, Cyclin D2^{Thr280Ala} and empty vector in WT and WDR81$^{-/-}$ brains. Constructs were *in utero* electroporated at E14.5, and brains were fixed at 16.5 and stained for PAX6 and PH3. **B**. Quantification of the percentage of mitotic (PH3+) electroporated radial glial cells (PAX6+) out of total electroporated radial glial cells reveals rescue of mitotic index in WDR81$^{-/-}$ cells expressing Cyclin D2^{Thr280Ala} (n = 3 brains per condition). **C.** Model. WDR81 loss of function leads to reduced activation of the MAPK signaling pathway downstream of EGFR, to reduced radial glial progenitor proliferation, and to microcephaly. Gain of function in the Pi3K-AKT pathway or stabilizing mutations in Cyclin D2 lead to increased radial glial progenitor proliferation, and to megalencephaly. Cyclin D2 mutants can rescue proliferation defects in WDR81$^{-/-}$ brains, indicating that these two pathologies can arise from opposite effects on the proliferation rates of radial glial progenitor. *p<0,05; **p<0,01 by unpaired t-tests.

Supplemental figure 1. Genotypes and survival and WDR81$^{-/-}$ mice.

A. Sequencing of WDR81 knock-out animals reveals a 4 base pair deletion in KO1 and an 8 base pair deletion in KO2, both leading to frameshifts and premature STOP codons. **B.** Rate of WDR81$^{-/-}$ embryos and pups recovered throughout time. The expected rate is 25% (dashed line). By P21, no mutant was detected.

Supplemental figure 2. Quantification of EGFR levels and P-ERK levels for Control-2 and WDR81 patient-2 cell lines.

A. Quantification of EGFR levels, normalized to control levels at T0 for Control-2 and WDR81 patient-2 cell lines (n = 5). **B.** Quantification of P-ERK levels, normalized to control levels at

T5 for Control-2 and WDR81 patient-2 cell lines (n = 5). *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001 by unpaired t-tests.

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Figure 1

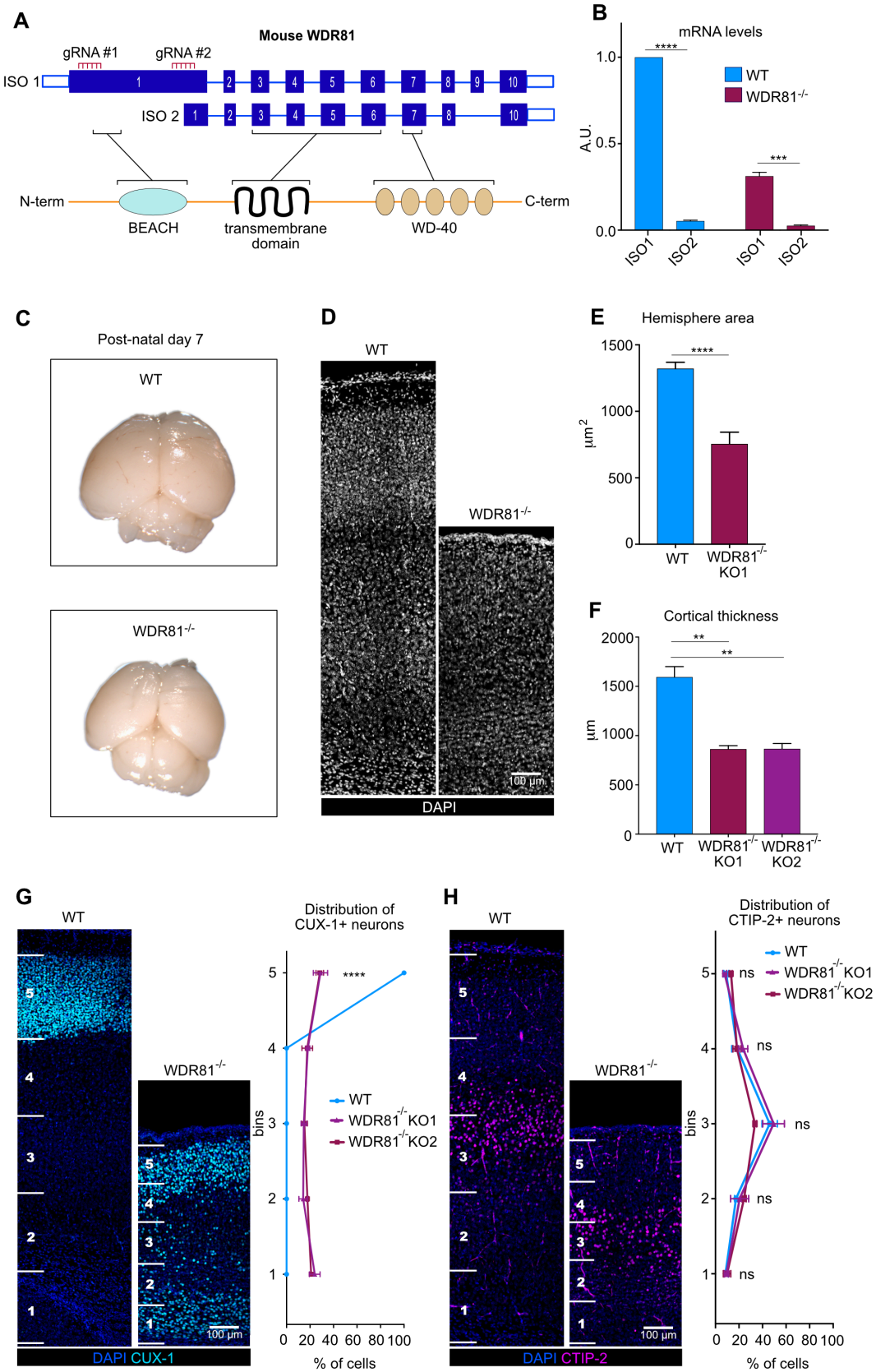

A
Mouse WDR81
gRNA #1
gRNA #2
ISO 1
ISO 2
N-term
C-term
BEACH
transmembrane domain
WD-40
B
mRNA levels
WT
WDR81-/-
A.U.
ISO1
ISO2
C
Post-natal day 7
WT
WDR81-/-
D
WT
WDR81-/-
100 µm
DAPI
E
Hemisphere area
µm2
WT
WDR81-/- KO1
F
Cortical thickness
µm
WT
WDR81-/- KO1
WDR81-/- KO2
G
WT
WDR81-/-
DAPI CUX-1
Distribution of CUX-1+ neurons
bins
% of cells
WT
WDR81-/- KO1
WDR81-/- KO2
H
WT
WDR81-/-
DAPI CTIP-2
Distribution of CTIP-2+ neurons
ns

Figure 2

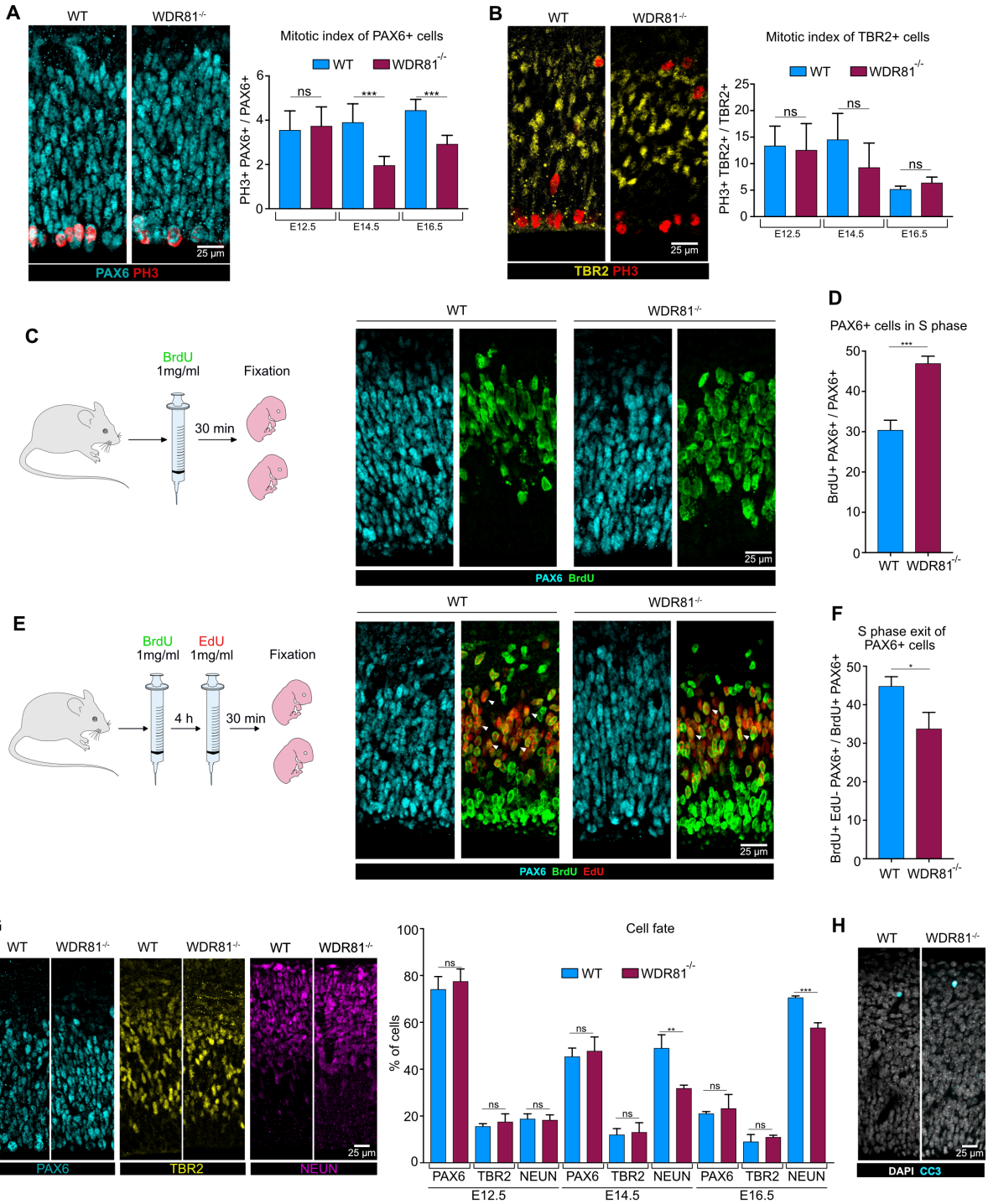

A
WT
WDR81-/-
Mitotic index of PAX6+ cells
WT
WDR81-/-
PH3+ PAX6+ / PAX6+
ns
***
***
E12.5
E14.5
E16.5
25 µm
PAX6 PH3
B
WT
WDR81-/-
Mitotic index of TBR2+ cells
PH3+ TBR2+ / TBR2+
ns
ns
ns
25 µm
TBR2 PH3
C
BrdU
1mg/ml
Fixation
30 min
WT
WDR81-/-
25 µm
PAX6 BrdU
D
PAX6+ cells in S phase
BrdU+ PAX6+ / PAX6+
***
WT
WDR81-/-
E
BrdU
1mg/ml
EdU
1mg/ml
Fixation
4 h
30 min
WT
WDR81-/-
25 µm
PAX6 BrdU EdU
F
S phase exit of
PAX6+ cells
BrdU+ EdU- PAX6+ / BrdU+ PAX6+
*
WT
WDR81-/-
G
WT
WDR81-/-
WT
WDR81-/-
WT
WDR81-/-
25 µm
PAX6
TBR2
NEUN
Cell fate
% of cells
WT
WDR81-/-
PAX6
TBR2
NEUN
E12.5
E14.5
E16.5
H
WT
WDR81-/-
25 µm
DAPI CC3

Figure 3

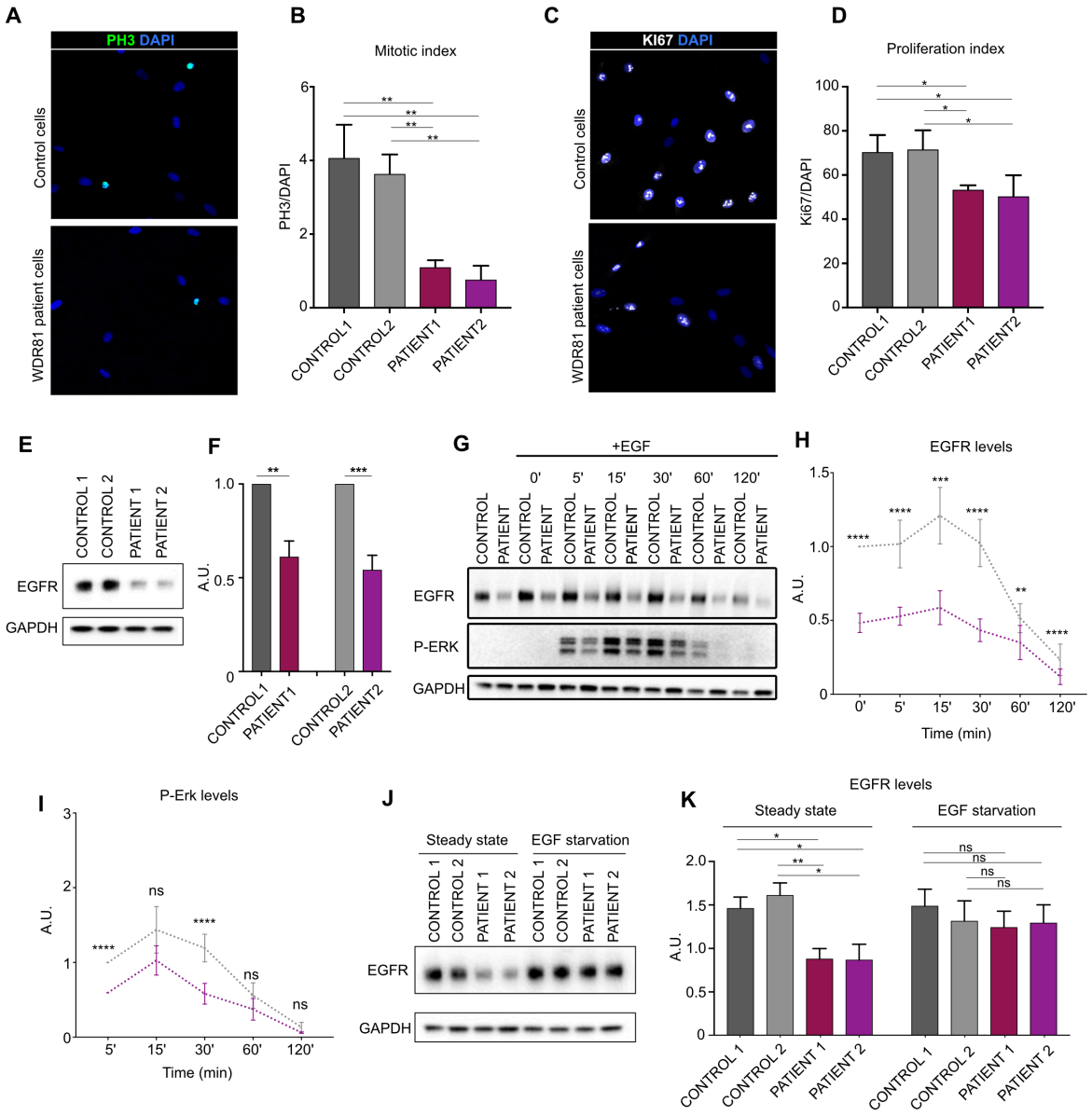

A
PH3 DAPI
Control cells
WDR81 patient cells
B
Mitotic index
PH3/DAPI
CONTROL1
CONTROL2
PATIENT1
PATIENT2
C
KI67 DAPI
Control cells
WDR81 patient cells
D
Proliferation index
Ki67/DAPI
CONTROL1
CONTROL2
PATIENT1
PATIENT2
E
CONTROL 1
CONTROL 2
PATIENT 1
PATIENT 2
EGFR
GAPDH
F
A.U.
CONTROL1
PATIENT1
CONTROL2
PATIENT2
G
+EGF
0'
5'
15'
30'
60'
120'
CONTROL
PATIENT
EGFR
P-ERK
GAPDH
H
EGFR levels
A.U.
Time (min)
I
P-Erk levels
A.U.
Time (min)
J
Steady state
EGF starvation
CONTROL 1
CONTROL 2
PATIENT 1
PATIENT 2
EGFR
GAPDH
K
EGFR levels
Steady state
EGF starvation
A.U.
CONTROL 1
CONTROL 2
PATIENT 1
PATIENT 2

Figure 4

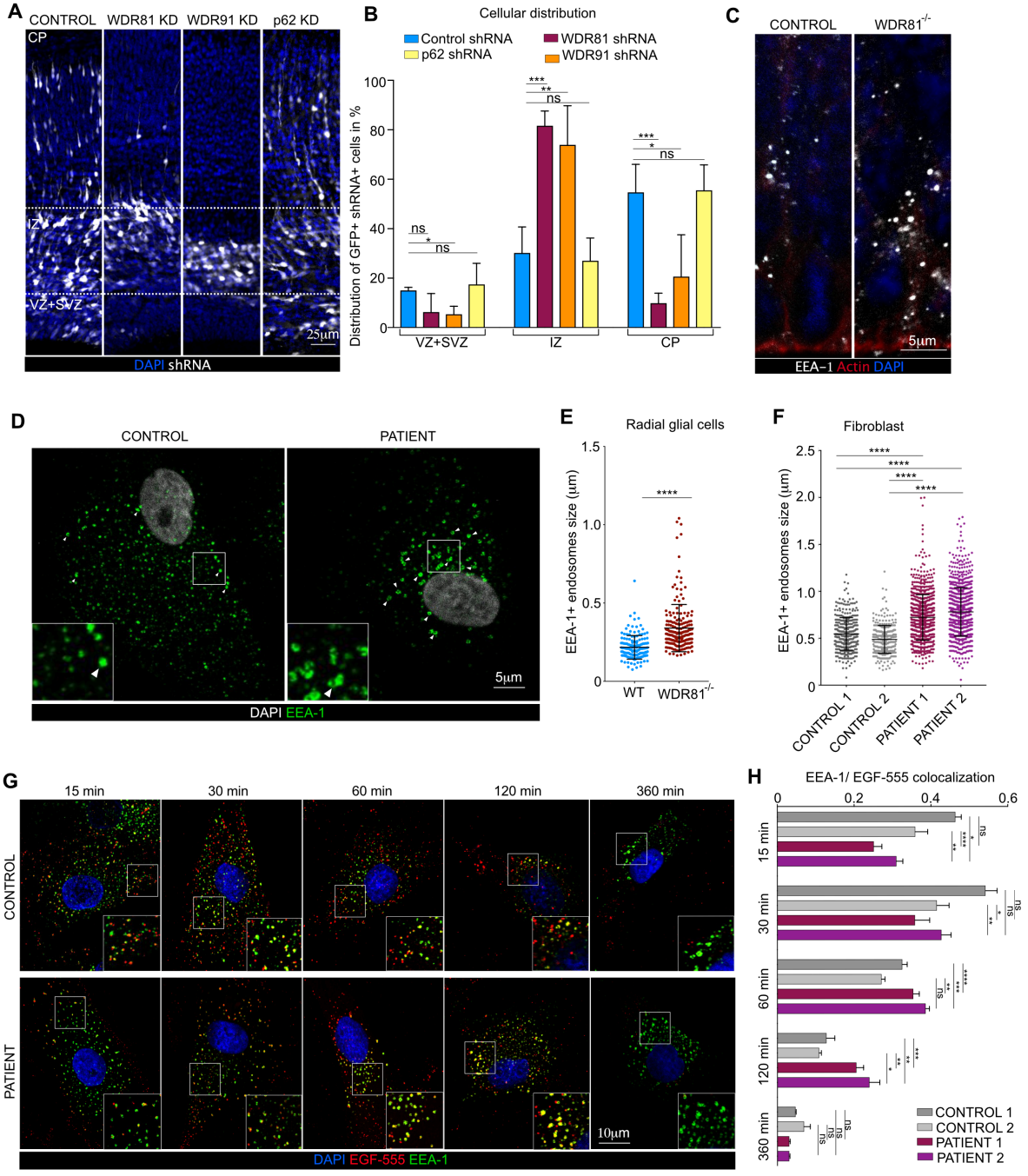
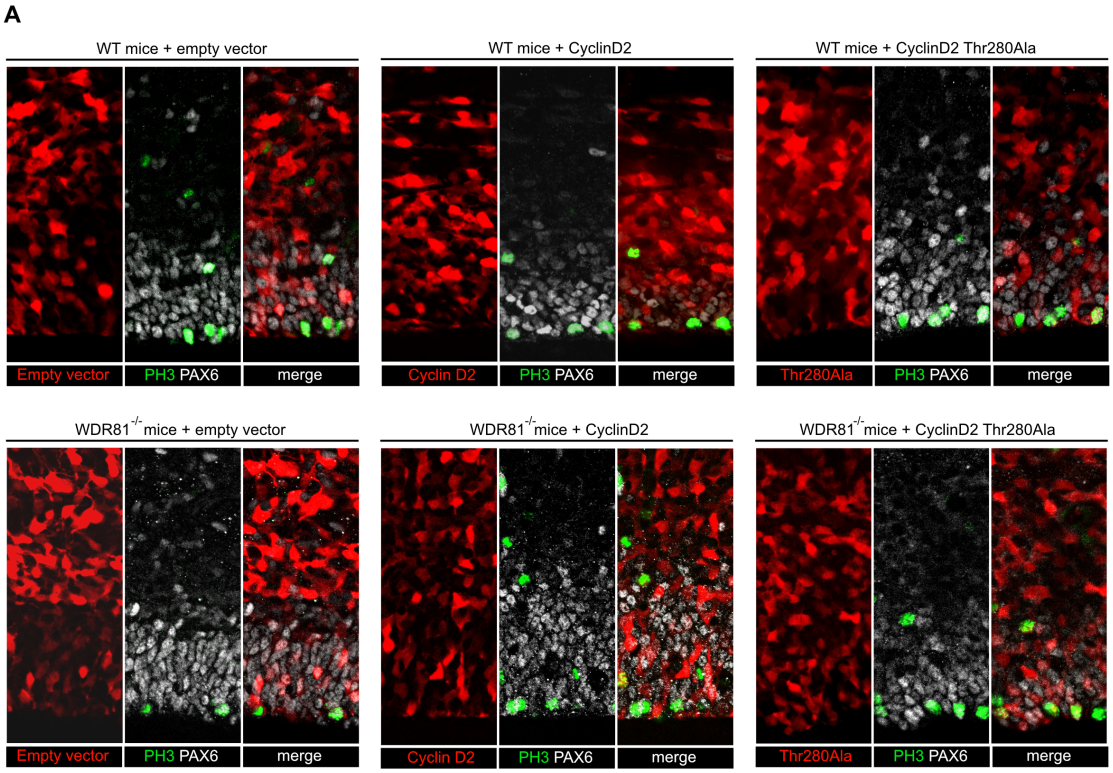
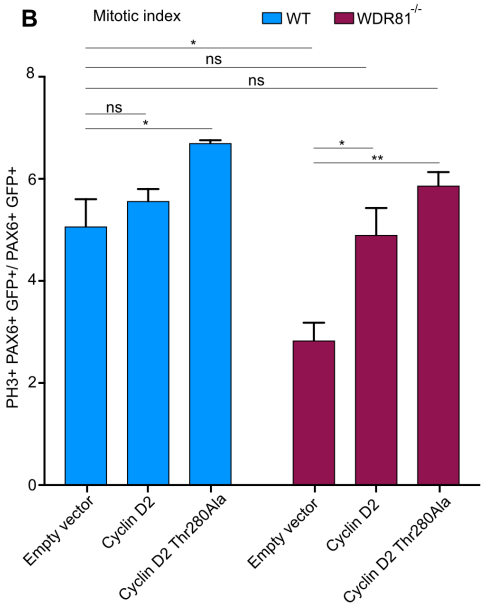
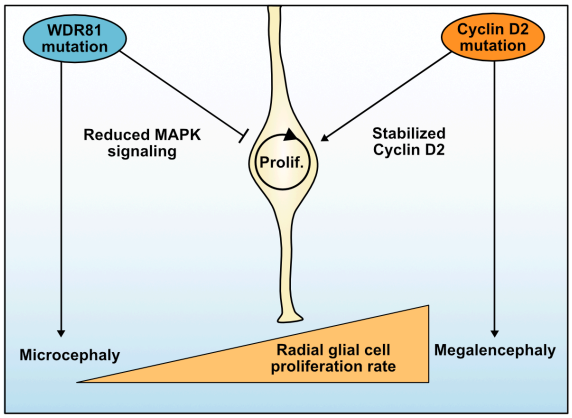

A
CONTROL
WDR81 KD
WDR91 KD
p62 KD
CP
IZ
VZ+SVZ
25µm
DAPI shRNA
B
Cellular distribution
Control shRNA
p62 shRNA
WDR81 shRNA
WDR91 shRNA
Distribution of GFP+ shRNA+ cells in %
VZ+SVZ
IZ
CP
C
CONTROL
WDR81-/-
5µm
EEA–1 Actin DAPI
D
CONTROL
PATIENT
5µm
DAPI EEA-1
E
Radial glial cells
EEA-1+ endosomes size (µm)
WT
WDR81-/-
F
Fibroblast
EEA-1+ endosomes size (µm)
CONTROL 1
CONTROL 2
PATIENT 1
PATIENT 2
G
15 min
30 min
60 min
120 min
360 min
CONTROL
PATIENT
10µm
DAPI EGF-555 EEA-1
H
EEA-1/ EGF-555 colocalization
15 min
30 min
60 min
120 min
360 min
CONTROL 1
CONTROL 2
PATIENT 1
PATIENT 2

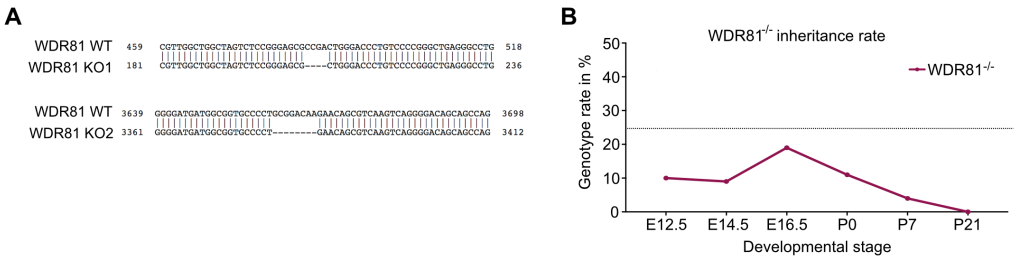
Figure 5


A
WT mice + empty vector
WT mice + CyclinD2
WT mice + CyclinD2 Thr280Ala
Empty vector
PH3 PAX6
merge
Cyclin D2
Thr280Ala
WDR81-/- mice + empty vector
WDR81-/- mice + CyclinD2
WDR81-/- mice + CyclinD2 Thr280Ala

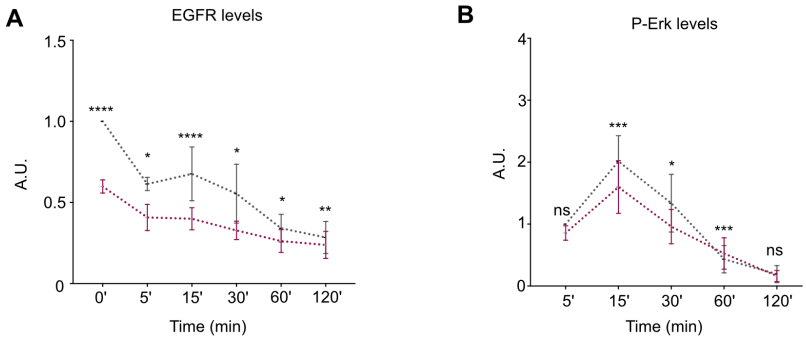

B
Mitotic index
WT
WDR81-/-
PH3+ PAX6+ GFP+/ PAX6+ GFP+
ns
*
**
Empty vector
Cyclin D2
Cyclin D2 Thr280Ala


C
WDR81 mutation
Cyclin D2 mutation
Reduced MAPK signaling
Stabilized Cyclin D2
Prolif.
Microcephaly
Radial glial cell proliferation rate
Megalencephaly

Supplemental figure 1



Supplemental figure 2



DISCUSSION AND CONCLUSIONS

DISCUSSION

The study presented in this thesis, reports a rare case where two pathologies (microcephaly and lissencephaly) are caused by mutations in a single gene called *WD Repeat domain protein 81 (WDR81).* It is an autosomal recessive disease that takes the name of Microlissencephaly. Here I focused more extensively on the microcephaly aspect of the pathology. Using the mouse developing neocortex as a model, as well as human fibroblasts derived from affected patients, I show that the loss of WDR81 causes defects in progenitor proliferation due to impairment of EGFR signaling, itself caused by defective endosomal maturation.

In this discussion I will first focus on the structure of the protein. I will then introduce and discuss the state of the art of the different findings relative to the description of the pathology, to then go deeply into the analysis of what are the possible cellular causes of WDR81 related microcephaly. After that, I will discuss the intracellular causes of such an effect on the development of the brain. I will then close by discussing how WDR81 could be involved in lissencephaly and in other cellular mechanisms.

Structure of WDR81

WDR81 is a poorly characterized protein, with no crystal structure available. It has been recently reported to be an endosomal maturation regulator (K. Liu et al., 2016) and as well an aggrephagy interactor (X. Liu et al., 2017).

Via online tools I predicted that WDR81 is a transmembrane protein with three functional domains: a BEACH domain, a transmembrane loop constituted of 6 α-helices and a repetition of 5 WD40 domains.

The transmembrane loop will be, by definition, the domain that will localize the protein at the level of the membrane of the endosome. Not so much is known about the cellular localization of WDR81. However, recent work from Liu et al and Rapiteanu et al reported WDR81 to be enriched on the surface of EEA1+ Early Endosomes in Hela cells (K. Liu et al., 2016; Rapiteanu et al., 2016) confirming its role as endosomal regulator.

Even though the exact function of this domain is not known, the BEACH domain has been reported to be of approximately 300 amino acids and to be found in proteins involved in vesicle trafficking, membrane dynamics and receptor signaling. This is most likely due to its ability to bind lipids, giving to the protein a membrane interaction domain (Wu et al., 2004). We could therefore speculate that the BEACH domain is specifically involved in binding to the early endosomes, acting as a sensor of the composition of the lipidic bilayer. However, to verify this hypothesis some experiments are needed. An *in vitro* expression of truncated WDR81 expressing only the BEACH domain could be used to perform a lipid binding assay, such as a lipid flotation assay.

The last structured domain that constitutes WDR81 is a repetition of WD40 domains that, as the BEACH domain, is well conserved throughout species. The W (tryptophan) D (aspartic acid) repeats give to this domain the structure of a β-propeller that acts as a platform for the stable or reversable association with binding partners (Gulsuner et al., 2011). One such partner is WDR91 that has been reported to bind to WDR81 via co-immunoprecipitation to form a complex essential for the inhibition of PI(3)K (K. Liu et al., 2016). Liu et al. also reported the crucial role of WDR81 in the formation of the complex. In Hela cells, the KO of WDR81 indeed caused the absence of both WDR81 and WDR91 at the endosomal level. Conversely, in WDR91KO Hela cells, WDR81 was reported to still be present at the level of the endosomes. This data together position WDR81 upstream in the initiation of the formation of the PI(3)K

inhibitory complex. To validate that the WD40 domain is responsible for the interaction between WDR81 and WDR91, it would be interesting to preform pull down assays with this domain and analyze its ability to bind WDR91.

Different WDR81 mutations cause different pathologies

The aim of this thesis was to understand how mutations in the poorly investigated *WDR81* gene can cause a recessive form of primary microlissencephaly. In 2017 Cavallin et al. reported the very first in depth clinical analysis focusing only on patients carrying WDR81 mutations. The most impressive finding of this paper is that even though all the patients are compound heterozygote for different mutations in WDR81 that span from non-sense mutation to duplication or depletion, they all display the same (with slightly different degrees of severity) microlissencephaly phenotype.

Primary fibroblast cell lines derived from skin biopsies of two of those patients have been included in the current study. They both display similar phenotype with virtually no neurological development, spastic tetraplegia, absent epilepsy, extremely reduced gyrification, thin corpus callosum (Cavallin et al., 2017). The first patient shows a point non-sense mutation on one allele introducing a STOP codon in the middle of exon 1 immediately after the BEACH domain; on the other allele a point mutation in exon 2 changes a Proline to an Arginine in the transmembrane domain loop. Therefore, only 50% of the full length WDR81 protein is expected to be present. In addition to that, the remaining 50% are likely affected due to the position of the mutation on the protein, and the fact that heterozygote individuals (parents) are not affected. The second patient displays a point mutation that changes a Histidine with a Tyrosine in exon 1, in the middle of the BEACH domain; and on the other allele, a 6 bases duplication in exon 4 that inserts a Valine and a Threonine into the

transmembrane domain loop. In this patient we could expect a milder phenotype since no STOP codon is present prematurely. However, the mutation in the BEACH domain (most likely to be the functional domain of WDR81) changes a hydrophilic positively charged amino acid (Histidine) with a hydrophobic amino acid (Tyrosine). This could induce a strong misfolding with a consequent loss of function of the BEACH domain and, by extension, of WDR81. Similarly, for the second allele, the two additional amino acids could unbalance the equilibrium of charges within the protein causing a conformational change affecting the orientation of the transmembrane domain and maybe the activity of WDR81 itself. For the reasons explained just above, it is therefore reasonable to think that in both patients, WDR81 is severely affected giving rise to the very same form of microlissencephaly.

However, the very first paper to report mutations in WDR81, described them in patients with cerebellar ataxia, mental retardation and disequilibrium syndrome (CAMRQ2) (Gulsuner et al., 2011). The author did not describe any features of the cortex, probably because of the limited access to the patients. Whether these patients display neocortical abnormalities remains an open question. However, based on the fact that a missense mutation in the BEACH domain impairs the function of the protein (Traka et al., 2013) causing microcephaly (Cavallin et al., 2017), we can speculate that the majority of the Gulsuner patients would display at least a mild form of microcephaly and or lissencephaly. In fact, as well Doldur Balli et al. in 2015 assessed how WDR81 is highly expressed in the brain and speculates how mutation in this gene could severely affect the brain. Another confirmation of this is the study of Shaheen et al. that reported how missense mutation and non-sense mutation of *WDR81* could both autonomously cause defects in the developing brain. This time, with the lack of cerebellum the study reported a severe hydrocephaly (Alkuraya et al., 2011). Together with it, looking at

the MRI, it is possible to identify a reduction of the thickness of the cortex as well that could be either the cause or the consequence of the reported hydrocephalus.

Studying the features of WDR81 in the developing neocortex

To have a better insight on how and if the brain is affected, we created a *WDR81* knock out mouse model using CRISPR/Cas9. The mouse has 2 predicted isoforms of *WDR81* that differ from one another by a different transcriptional starting point giving rise to a shorter exon 1 for isoform 2 and the absence of exon 9 (see figure 1 A of the result section). We created two WDR81 KO lines, both with a frameshift that creates a STOP codon downstream in the sequence.

I analyzed both lines to first investigate cortical size and neuronal positioning. The results showed that both WDR81KO mouse lines were strongly microcephalic showing a significative reduction of the thickness of the cortex. This data well recapitulates the microcephaly part of the pathology that the WDR81 human patients display. Together with this, I also identified a mis-positioning of the upper layer but not the deeper layer neurons. The first consideration in these datasets is that WDR81 mouse model is a good tool to study the pathology, recapitulating both the decreased in cortical size (a hallmark of microcephaly) and defects in neural positioning (a hallmark of lissencephaly).

However, a question arises from these later discoveries: What is the molecular cause of the reduced thickness and neuronal mispositioning? Because these are in fact two different questions, I decided to focus on the involvement of WDR81 in microcephaly.

What is the cause of WDR81-related microcephaly?

Microcephaly can be due to three major causes: cell death, premature progenitor differentiation, reduced proliferation.

Cell death, premature differentiation, or cell proliferation defects?

In the case of cell death, either the progenitors or the neurons die during neurodevelopment leading to an aberrant decrease of the cellular pool that constitutes the cortex. In Wang et al. 2018, it is reported how mutations in WDR81 do not cause aberrant apoptosis in neocortex. In line with this, I was not able to detect any aberrant cell death at any of the developmental stages analyzed (as shown in figure 2F). This result suggests that the decrease in thickness of the cortex is not due to aberrant apoptotic neural progenitors nor neurons. This finding is quite original as the vast majority mouse models for microcephaly display aberrant apoptosis (Jayaraman et al., 2016).

Another reported cause of microcephaly is premature differentiation, whereby cell fate decisions are affected. In this case, the pool of neural progenitors will decrease too fast during development due to the neural progenitors performing premature neurogenic divisions or terminal divisions. In WDR81 KO I have shown that at early mid and late developmental stages (E12.5, E14.5 and E16.5) the ratio of progenitors and intermediate progenitors remains the same between mutant and wild type brains. These data indicate that in WDR81 mutant mice neural progenitors do not exhibit any premature differentiation.

The last possible cause of WDR81 related microcephaly is reduced proliferation. Here neural progenitors should not proliferate enough within the reduced temporal window in which neurodevelopment occurs. As a result, the pool of neurons that should be produced in a developing brain will be strongly depleted, giving rise to a decrease in brain size. In the

present study, I provide evidences that WDR81$^{-/-}$ developing brain showed a decrease in the mitotic index of neural progenitors but only in the mid and late phases of development (E14,5 and E16,5). Using EDU/BrdU labeling I analyzed as well the S phase duration of radial glial progenitors. My data showed that WDR81 mutant brains progenitors spend more time in S phase if compared to the WT condition, which explain, at least in part, the reduced mitotic index. A longer S phase could indeed be the indicator of an overall lengthening of the time window that the progenitors would spend in interphase. An important ongoing experiment is therefore the measurement of the entire cell cycle length. Measuring G1 specifically, which is more challenging, would also be a critical experiment. It would be extremely informative, for example, to analyze the exact duration of each cellular phases using the *Fucci cell cycle sensor* coupled with live imaging.

Cell cycle and fate

Cell cycle duration and cell fate have been reported to be tightly connected, in particular in radial glial progenitors. For instance, work by Calegari and Huttner demonstrated that the lengthening of the neural progenitor's cell cycle by the downregulation of cdk activity is necessary and sufficient for neuronal differentiation, leading to early depletion of neural progenitors. Conversely the shortening of cell cycle (acting on the overall duration of G1 phase) with the acute overexpression of Cyclin D delays neurogenesis and enhances proliferative divisions (Lange et al., 2009). In light of these studies, one could have expected increased neuronal production and early progenitor depletion in WDR81KO mice. I however do not observe this and even registered, in mid and late developmental phases (E14.5 and E16.5), a reduction of the neuronal proportion. This result may be explained by a direct effect of WDR81 mutation on cell fate. Neurogenins are essential for neurogenic commitment and I

speculate that WDR81 may affect their expression. Indeed, FGFR has been reported to regulate Neurogenin 2 (*NEUROG2*) in the developing neocortex (Dennis et al., 2019; Li et al., 2014). As a TK receptor, FGFR needs to be endocytosed for its turnover and signaling. In a developing neocortex that misses an endosomal regulator like WDR81, FGFR signaling may be affected, leading to altered expression of *NEUROG2* and decrease neurogenic commitment of neural progenitors into neurons. To verify this hypothesis, it could be interesting to study the level of expression of neurogenin2 in the developing neocortex at the different developmental stages analyzed; and if there is any change to study the localization and the amount of FGFR in radial glial cells to assess to what degree the signaling coming from this receptor could regulate NEUROG2 in WDR81 KO brains.

WDR81 patient cells also display reduced proliferation

In addition to the *in vivo* characterization of radial glial cells of the WDR81 KO mouse model, I analyzed the features of cell cultures of fibroblasts primary derived from WDR81 related microcephaly affected patients.

In vitro, the two mutant cell lines showed a similar reduction of mitotic index (consistently with the *in vivo* WDR81KO mice data) and proliferation index when compared to wild type healthy cell cultures (Fig 3 B and C). This result is quite interesting because even though the *WDR81* mutation causing the putative defects on the protein is quite different in each patient, the phenotype expressed both *in vitro* and *in vivo* in human patients (causing the same pathology) is perfectly congruent (Cavallin et al., 2017 and the data reported in this thesis).

Surprisingly, analyzing the very same cell lines, Cavallin et al. reported no significative differences in mitotic index and a slightly increased proliferative index for both patients cell lines when compared to controls. These data are in evident discrepancy with our results. I

postulate that this difference is due to technical issues. Indeed, while the amount of foetal bovine serum (FBS) added to culture medium is usually 10% (including in my experiments), Cavallin et al. cultured the same cell lines with 1% of FBS which has been reported to induce 90% of cells in a culture to a G0/G1 stage (Mengual Gómez et al., 2010). In fact, this low level of FBS could have induced mitotic alterations *in vitro*, modifying the output of the experiment itself. Moreover, during the experiments with these cultures, I observed that the higher the passage of the cell culture the lower was the population doubling rate. This would probably be due to a process of aging of the *in vitro* culture that appears to gradually decrease the total level of EGFR (data not shown), reducing its capability in sensing proliferative factors present in the culture medium. It would be therefore interesting to know what are the passages at which Cavallin et al. analyzed the cell cultures for mitotic and proliferative index.

Cavallin et al reports an increase in prometaphase/metaphase and a decrease in anaphase/telophase in vivo in Drosophila neuroblasts. I did not check for mitotic alterations in our study, but this remains an intriguing possibility. How the endosomal defects generated by the lack of WDR81 could lead to defects in mitotic progression is however unclear. The current view is that endosomes play a role during mitosis: in prophase, Rab11+ endosomes accumulate around the microtubule organization center to prevent the unwanted transport of factors to the PM and help maintaining the correct protein reservoir for the two future daughter cells (Chen et al., 2014; Jongsma et al., 2015). In Anaphase some other Rab11+ recycling endosomes are brought in proximity of the cleavage furrow as a membrane reservoir providing the lipids required to complete cytokinesis (Hehnly & Doxsey, 2014; Simon & Prekeris, 2008). In light of this connection between endosomes identity and mitosis, proven that a cell lacking WDR81 will have delays in maturation of endosomes (K. Liu et al., 2016 and

our data), we could speculate an impairment of the correct composition of the membranes of these organelles, affecting mitotic progression.

What is the cause of cell proliferation defects?

WDR81 is known to be an endocytic regulator (X. Liu et al., 2017). In fact, in other presented studies the Knock Out of WDR81 showed to increase the size of Early Endosomes that swell aberrantly (K. Liu et al., 2016; Rapiteanu et al., 2016; Wang et al., 2018). Accordingly, I observed swellings of the early endosomes both *in vivo* in KO progenitors and *in vitro* in patient-derived fibroblasts primary cultures suggesting an impaired sorting and functioning of the endocytic machinery.

Numerous receptors need to be endocytosed by the cell for proper regulation of their activity. One of them is EGFR, a tyrosin-kynase receptor that has been reported to be essential in the mid and late phases of neurodevelopment when radial glial progenitor cells become responsive to EGF (Sibilia et al., 2003; Threadgill et al., 1995; Tropepe et al., 1999). In addition to that, EGFR has been reported to be asymmetrically inherited during radial glial cell division generating a daughter cell with higher proliferative potential (Sun et al., 2005). In this study it has been reported that in physiological condition a dividing progenitor will produce a cell with high levels of EGFR and a cell with low levels of EGFR. In mid phases of development, around E13.5 the cell with high levels of EGFR will migrate rapidly from the ventricular surface to the sub-ventricular surface to undergo in its final division and then differentiate. The cell with low levels of EGFR will remain in the ventricular zone and will continue to proliferate as a progenitor. The proliferative potential has been associated to the presence or absence on Numb that in early and late phases of development colocalizes with EGFR. Therefore, when EGFR is present at high doses, Numb will be highly present as well inactivating the Notch

pathway pushing the cell toward differentiation. When EGFR is present at low doses, Numb will not be inherited, with consequent activation of Notch giving to the cell a proliferative progenitor feature (Sun et al., 2005).

Our data show that overall levels of EGFR are strongly decreased in WDR81 affected primary derived fibroblasts. This lowering of EGFR is likely due to defects in maturation and sorting of the EGFR+ Early Endosomes and is rescued upon EGF starvation. Our data also show that internalized EGFR is sorted into Early Endosome but, in the absence of WDR81, is delayed in this compartment and as demonstrated by or data in Fig 4 G and H. It has been demonstrated that strong activation of EGFR leads to an increase of its degradation, while low doses favor its recycling (Sigismund et al., 2018). Once delayed in the cell, I hypothesize EGFR could hypothetically continue signaling, leading to an altered balance between recycling and degradation, in favor of excessive degradation of endocytosed EGFR. An alternative possibility is that the decrease of EGFR in the patients cells is consequence of a downregulation of EGFR expression, mediated through transcriptional or translational negative feedback loops (Avraham & Yarden, 2011a; Sênos Demarco et al., 2020; Villaseñor et al., 2015).

The data in Fig 4 G and H are in line with both hypotheses, as I demonstrated that the low EGFR levels in patient' fibroblasts can be rescued by a simple EGF starvation, indicating that altered EGFR levels are only detected when the pathway is active (and therefore EGFR endocytosed). To test between these theories, it would be extremely interesting to analyze the variations in time of EGFR mRNA levels after the activation of the receptor, as well as the variation of its surface and intracellular levels. The results coming from this data could be of great value not only for the study presented on this thesis but even for the field studying the cellular regulation of EGFR. Even though there are evidences that this kind of autoregulation

occurs, it is not entirely clear how this happens and which are the exact proteins involved in it (Avraham & Yarden, 2011b; Villaseñor et al., 2015).

Regulation of other signaling receptors by WDR81

Importantly, as a general endocytic regulator, WDR81 is likely to impair the intracellular trafficking affecting many other cargoes. One of them could be FGFR, another RTK that also goes through endocytic cycles. FGFR has been reported to be a major driver of early phases (from E10,5 to E12,5 in mouse) of cortical development (Tropepe et al., 1999). FGFR covers the function of major regulator during the process of arealization of the cortex (Arai & Pierani, 2014), so its processing impairment, together with others, could be one of the cause of the complete disorganization of the cortical areas reported in WDR81 patients (Cavallin et al., 2017).

WDR81 KO was shown to impair adult neurogenesis though the regulation of TGF-B signaling endosomes. Wang et al. (2018) reported that conditional WDR81 KO affects adult hippocampal neurogenesis, altering the mouse behavior and its learning capability. The phenotype was due to an impairment of the maturation/sorting of SARA endosomes, hubs for TGF-β receptor signaling. As a readout, the receptor stayed longer in the cell and the continuous signaling led to a decrease of neural proliferation and consequently reduced neural differentiation. Whether a similar mechanism is also at stake in radial glial cells is interesting possibility. This study also gives strength to the findings of Gulsuner, Traka and Cavallin, where WDR81 loss of function or ablation was reported to cause premature neurodegeneration in patients. Whether impairment of adult neurogenesis also participates in this phenotype remains to be investigated.

What is the cause of WDR81-related lissencephaly?

Cavallin et al reported that patients carrying *WDR81* mutations also display lissencephaly. Lissencephaly is described as a *smooth brain* characterized by the absence or simplification of gyrification. It is a severe form of abnormal neuronal migration where neurons will not organize themselves in the normal cortical layers, giving rise to a very disorganized cortex (Leventer, 2007). The most common and studied gene whose mutation leads to this pathology is *LIS1*, a dynein regulator. LIS1 loss interferes with the activity of dynein, impairing nuclear migration in migrating neurons, and thereby altering their final localization (Tsai et al. 2007).

WDR81-associated lissencephaly could be due to an impairment in the endosomal trafficking of adhesion molecules, such as Integrins or N-Cadherin. Both Integrins and N-cadherin are endocytosed from and recycled back to the PM, establishing adhesive junctions used by the cell for migration (Caswell et al., 2009; Y. Liu et al., 2018; Riento et al., 2003). They are also responsible for intracellular signaling cascades that participate in migration (Caswell et al., 2009). It has been shown that N-cadherin sustains the motility and polarity of newborn cortical neurons (Luccardini et al., 2013) and is also essential in radial glial cells for the establishment of a well-structured neuroepithelium (Gä Nzler-Odenthal & Redies, 1998; Luccardini et al., 2013; Teng et al., 2005).

Even if this hypothesis is appealing, I have not formally tested it in our system. Preliminary *in vivo* data in E14.5 brain show N-Cad aberrantly aggregating in enlarged foci in mutant mice, suggesting an accumulation of this adhesion molecule in one of the cellular compartments. It would be interesting to investigate in which compartment N-cad is sequestered, and whether early endosomal accumulation of the molecule may be an important cause of neuronal positioning defects.

In addition, another player that could have a role in the establishment of the lissencephaly phenotype could be EGFR once again. Indeed, after being asymmetrically inherited, EGFR has been reported to stimulate migration in the daughter cell that received the higher level of the receptor (Maretzky et al., 2011; Ohnishi et al., 2017; Sun et al., 2005). According to our results in fibroblasts, we could extrapolate that in WDR81KO cortices the flawed EGFR trafficking leads to an unbalance in the asymmetry of EGFR inheritance and impairment of the migration of newborn cells. This speculation further highlights the importance of the investigations of the levels of EGFR in the developing neocortex. In conclusion, the causes of lissencephaly in WDR81 mutant brains could be due to altered trafficking of different factors, including EGFR, N-Cad or integrins.

Microcephaly and Megalencephaly: two sides of the same coin?

So far, I showed that WDR81-related microcephaly is due to reduced proliferation of progenitor cells. Interestingly, Megalencephaly can be due to increased cell proliferation during development (Mirzaa et al., 2014; Parrini et al., 2016). An example is the Cyclin D2 related megalencephaly caused by a gain of function mutation that renders CyclinD2 resistant to degradation, stimulating the proliferation rates of neural progenitors and resulting in a general overgrowth of the brain (Mirzaa et al., 2014).

At a macroscopic level, microcephaly and megalencephaly can appear as opposite phenotypes. However, whether they can originate from related underlying causes at the molecular level is unclear. Both pathologies are complex and multicausal but can result from abnormal proliferation of radial glial cells. In this thesis I show that the WDR81 microcephaly is due to EGFR delayed sorting and clearance that affects its own signaling. I therefore thought to rescue our phenotype with the overexpression of the megalencephaly causing mutant

Cyclin D2 in WDR81KO mice. The data showed that indeed, the proliferation of radial glial cells is rescued (Fig 5A and B).

In fact, EGFR and Cyclin are interconnected in the same signaling EGFR pathway. Once EGFR is activated, the MAPK cascade will induce the expression of Cyclin D that will then lead to cell cycle progression enabling the cell to enter in S phase (Avraham & Yarden, 2011). We can imagine that in WDR81 related microcephaly, the downregulation of EGFR signaling will have a consequent decrease of Cyclin D expression affecting the cell cycle progression of the neural progenitors. In this regard, it could be interesting to test this hypothesis in WDR81KO brains checking the Cyclin D levels by western blot. Conversely, in the megalencephaly reported by Mirzaa et al., the undegradable Cyclin D induced an increase in cycling progenitor cells. Therefore, cyclin D could become a contact and interconnecting point for these two pathologies. In light of this, we could think that microcephaly and megalencephaly can be due to opposite effects in signaling pathways promoting cell proliferation and can therefore be viewed as two sides of the same coin. Thus, to complete the scientific investigation on the possible bridge existing in between these two pathologies, it could be interesting to knock down WDR81 in an undegradable cyclin D2 mouse model and check whether the progenitor's proliferation rate would decrease.

WDR81 involvement in other cellular functions

In 2017 Liu et al demonstrated that WDR81 is also involved in aggrephagy, a form of autophagy specialized in the degradation of aggregated proteins (X. Liu et al., 2017). For this cellular role, WDR81 acts together with another binding partner, p62. As done for WDR91, I tested whether p62 plays a role in neocortex development, similarly to WDR81, knocking it down using an shRNA approach. No effect on the developing neocortex after the depletion of

p62 was observed, confirming that it is the endosomal function of WDR81 that plays a critical role in neocortex, at least during development.

The role of WDR81 in aggrephagy seems to be similar to a chaperon protein that, recruiting p62, causes a conformational change, rendering the protein much more prone to binding ubiquitinated proteins. In addition to that, once bound to p62, WDR81 seems to have greater affinity for LC3-C, recruiting it in order to induce the formation of the autophagosome (X. Liu et al., 2017). Even if the knock down of p62 suggests that it does not play any role in neocortical development, when WDR81 is missing, p62 will accumulate in the brain in specific foci enriched in ubiquitinated aggregated protein and LC3 but only in neurons (X. Liu et al., 2017). In addition to that, the WDR81 $^{f/f}$ cKO used by this group did not show any obvious brain abnormalities and were mostly dying perinatally.

In light of the findings of Liu et al., I also performed immunostaining on WDR81KO mice and I registered perinatal accumulation of p62 linked to ubiquitinated aggregated protein foci in the brain (data not shown). Protein aggregation is a characteristic of many neurodegenerative disease like Alzheimer's disease, Parkinsons's disease, Huntington's disease and others. The aggregated oligomers appear to be toxic, causing injury or cell death. In general, the greater the degree of aggregation, the greater is the severity of the disease. Already in 2013, Traka et al. reported accumulation of electron dense structures foci-like in cerebellar Purkinje cells that would start to appear in mice at least 3 weeks after birth. Liu et al. in 2017 reported the accumulation of this foci perinatally but did not mention any p62 accumulation during development. With this and our evidences in mind we could speculate that the interaction WDR81/p62 has a role in preventing the accumulation of aggregated proteins in neurons as a protective function against neurodegenerative diseases, unraveling a

new exciting feature of WDR81 and opening the route for a new interesting study correlating aggrephagy and neural degeneration.

CONCLUSIONS

Neocortex development is a fascinating fine-tuned process that in its very initial phases relies on the proliferative potential of neural progenitors. However, these cells have a very limited time window to divide in order to give rise to the totality of cortical neurons of the brain. As a consequence, any cell autonomous or non-autonomous process disruption/modification that impairs the proliferation rate of neural progenitors will have disastrous consequences, leading to reduced brain growth, which cannot be compensate for later on.

Most studies report neural progenitor death of premature differentiation as the etiology of microcephaly, but reduced proliferation remains largely hypothetical. In the present work I have shown how WDR81 related microcephaly is due to reduced proliferation of neural progenitors in the mid and late phases of neurodevelopment. This is due to the reduced capability of progenitors in sensing the EGF proliferative signal. I indeed demonstrated that loss of function of WDR81 in radial glial cells alters endosomal maturation, due to its role in the regulation of the Class III PI3K. This gives rise to impaired intracellular trafficking of EGFR and reduced EGFR levels, leading to reduced MAPK activity.

FUTURE PERSPECTIVES

As stated in the discussion, a lot of questions still remain open. One of them concerns the EGFR defects in the brain of WDR81 KO mice. An important experiment would be to analyze EGFR clearance and signaling in neural stem cells cultures directly derived from WDR81KO mice at the different developmental stages analyzed (E12.5 E14.5 and E16.5). This would first give strength to the findings reported in human fibroblasts; and secondly unravel why at early developmental stages we do not observe any proliferation in mutant mice. Is it because in early development cells rely mostly on FGFR (Tropepe et al., 1999)? And if this is true, are there defects in FGFR clearance or signaling? In this regards it would be extremely informative to perform the same analysis done for EGFR even for FGFR in both systems: human cells and WDR81KO mice.

Then, to continue on the line of defective endosomal maturation, there could be strong potential in trying to treat WDR81KO mice with systemic administration of a PI(3)K class III inhibitor such as Wortmannin or LY294002. Indeed, since WDR81 loss of function was shown to lead to overactivation of PI(3)K, this way rescue the observed WDR81 phenotype. In particular, it would be interesting to test if the administration of PI(3)K inhibitors rescues the delay in maturation and sorting of the endosomes, the reduced proliferation rates of radial glial progenitors, and ultimately the microcephaly and lissencephaly phenotypes. If this is the case, this could represent a step forward not only in the comprehension of the phenotype, but even give a hint for the development of drugs able to treat WDR81-related pathologies.

We have seen how WDR81 loss can lead as well to lissencephaly. Some possible reasons have been already exposed in the discussion: like the possible involvement of

adhesion molecules such as N-Cadherin or Integrins, or the involvement of EGFR dosage in the dividing progenitors. These hypotheses are really exciting, and it would be of great value to perform experiments on developing brain to try to localize these molecules *in vivo* and then assess whether their cellular processing/sorting pathway is affected, in order to try to unravel the effects of WDR81 depletion on migrating neurons in the developing brain.

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DESCRIPTION DU PROJECT EN FRANCAIS

INTRODUCTION

Le néocortex est le centre des fonctions cérébrales supérieures, tel que la perception, la prise de décision et le langage. Son développement se produit par la prolifération de cellules souches neurales et la migration de neurones nouveau-nés sur des distances importantes. Chez la souris, les cellules gliales radiales apicales (aRG) sont les cellules souches du néocortex en développement et donnent naissance à tous les neurones néocorticaux, la plupart des cellules gliales et aux cellules souches adultes. Ces cellules neuroépithéliales sont fortement polarisées, et ont un processus basal s'étendant jusqu'à la surface piale du cerveau en développement et un processus apical qui reste en contact avec la surface ventriculaire.

On sait que les perturbations au cours des différents stades de développement conduisent à une multitude de malformations corticales. Parmi celles-ci, la microcéphalie primaire est caractérisée par un cerveau de taille réduite, mais par ailleurs structurellement normal. Elle résulte d'une réduction de la neurogenèse et / ou d'une augmentation de la mort neuronale. La mégalencéphalie, à l'inverse, se caractérise par une surcroissance cérébrale. Enfin, la lissencéphalie est caractérisée par un cerveau anormalement lisse, dépourvu des plis spécifiques du cerveau humain gyrencéphalique et résulte d'une altération de la migration neuronale. Des malformations corticales ont également été associées à la schizophrénie et à l'autisme.

Avec l'avènement des technologies de séquençage de nouvelle génération, l'identification des mutations à l'origine de maladies s'est considérablement accélérée. Si cela offre la possibilité d'identifier de nouvelles voies biologiques importantes pour le développement du cerveau, l'étude de gènes, souvent mal caractérisée dans un contexte in vivo, reste un défi. En 2017,

Cavallin et al ont identifié 6 mutations de perte de fonction dans le gène WDR81, provoquant une microlissencéphalie extrême (microcéphalie + lissencéphalie). Ces patients sont parmi les patients les plus sévèrement touchés par des malformations cérébrales, aucun d'entre eux ne pouvant tenir leur tête, développer une utilisation volontaire des mains, avoir un contact visuel ou acquérir un langage.

WDR81 est une protéine transmembranaire mal caractérisée. Cependant, une étude récente a démontré qu'elle est un régulateur important de la maturation endosomale via son activité inhibitrice sur Pi3K la classe III.

Objectifs scientifiques

Le but de mon projet de doctorat est d'identifier comment une mutation dans WDR81 peut conduire à une microlissencéphalie. J'ai vérifié si cette mutation conduisait effectivement à des défauts endosomaux et dans quelles mesures. Par ailleurs, j'ai identifié la voie de signalisation moléculaire qui relie les altérations de la maturation endosomale à la malformation cérébrale. Pour ce projet, nous avons décidé de concentrer mon analyse sur la partie microcéphalie du phénotype, pour comprendre ce qui conduit à une réduction de la taille du cerveau.

RÉSULTATS

Les souris WDR81 KO présentent un cerveau de taille réduite et un positionnement neuronal altéré

On sait peu de choses sur le gène WDR81 mais, chez la souris, deux isoformes du WDR81 ont été identifiées : une isoforme longue (121 kDa) englobant un domaine BEACH N-terminal, une région transmembranaire centrale, un domaine de répétition WD40 C-terminal, et une isoforme plus courte (81 KDa) dépourvue du domaine BEACH (figure 1A). Les mesures de l'ARNm isolé à partir d'extraits embryonnaires de cortex E14.5 ont indiqué que l'isoforme WDR81 longue était fortement dominante, avec seulement des traces de l'isoforme courte (figure 1B). Nous avons donc généré une souris WDR81 KO en utilisant un ARNg ciblant le début de l'Exon 1 (KO-1). Etant donné qu'une traduction altérée de l'isoforme longue peut induire l'expression de l'isoforme courte, nous avons également généré un deuxième KO en utilisant un ARNg ciblant la fin de l'exon 1, après le site alternatif du début de traduction (KO-2) (figure 1A). Les deux lignées présentaient des décalages de trame conduisant à l'apparition d'un codon STOP prématuré. Nous avons ensuite validé l'absence d'expression du gène WDR81 dans les souris KO-1 en utilisant la technique de QPCR, qui a révélé une forte réduction des niveaux d'ARNm de l'isoforme 1, probablement due à une dégradation de l'ARNm non sens (Fig. 1B). Par ailleurs, aucune régulation positive de l'isoforme 2 n'a été observée, indiquant ainsi une absence de compensation. Des embryons mutants homozygotes pour le gène WDR81 ont été détectés à des taux sous-mendéliens, et au 21ème jour post-natal (P21), aucune souris mutante n'a été récupérée (Fig. 1C). Nous avons ensuite analysé la taille et l'organisation du cerveau chez les souriceaux WDR81 $^{-/-}$à P7. Les deux lignées KO étaient sévèrement microcéphaliques, avec une réduction de 39% de la zone hémisphérique (Fig. 1D).

L'épaisseur corticale a également été considérablement réduite, suggérant des défauts d'expansion tangentielle et radiale du cerveau (Fig. 1E). Nous avons ensuite analysé le positionnement neuronal dans les cortex WDR81 $^{-/-}$ à P7. La localisation des neurones tardifs de la couche supérieure a été gravement affectée, avec un grand nombre de neurones CUX-1 positifs dispersés dans tout le cortex (figure 1F). Les neurones plus profonds, qui naissent plus tôt au cours développement cortical, étaient cependant correctement positionnés, comme l'indique la localisation des neurones positifs pour CTIP-2 (Fig. 1G). En résumé, les souris mutantes pour WDR81 présentent une diminution de la taille du cerveau et un défaut du positionnement neuronal, ce qui rappelle fortement les phénotypes de microcéphalie et de lissencéphalie rapportés chez l'homme. Ces phénotypes ont été observés pour les deux lignées WDR81 KO et nous avons donc concentré notre analyse sur KO-1 (dorénavant appelé WDR81 $^{-/-}$).

WDR81 KO altère la prolifération des progéniteurs gliaux radiaux

Pour identifier les causes de la réduction de la taille du cerveau chez les souriceaux WDR81 $^{-/-}$, nous avons étudié les altérations du développement du néocortex aux stades embryonnaires. Les principales causes de microcéphalie primaire rapportées dans la littérature sont la prolifération réduite des progéniteurs, l'augmentation de la mort cellulaire ou la différenciation prématurée (Jayaraman 2018). Pour étudier les défauts de prolifération, nous avons d'abord mesuré l'indice mitotique des progéniteurs gliaux radiaux WDR81 $^{-/-}$, défini par le pourcentage de cellules positives à la phospho-histone 3 (PH3) sur le nombre total de cellules positives pour PAX6. Alors qu'à E12.5, la prolifération semblait normale, l'indice mitotique des progéniteurs gliaux WDR81 $^{-/-}$ radiaux était fortement réduit à E14.5 et E16.5 (Fig. 2A). Il est frappant de constater que les progéniteurs intermédiaires positifs pour Tbr2

semblaient avoir un cycle normal tout au long du développement (figure 2B). Par conséquent, la mutation WDR81 modifie spécifiquement la prolifération des progéniteurs gliaux radiaux aux stades neurogène moyen et tardif. Pour confirmer ces défauts de prolifération, nous avons ensuite mesuré le pourcentage de cellules en phase S. Une exposition de 30 minutes avec du BrdU a révélé une quantité accrue de progéniteurs gliaux radiaux WDR81 $^{-/-}$ en phase S (Fig. 2C). Pour vérifier si cela était dû à une prolongation de la durée de la phase S, nous avons effectué une double impulsion BrdU-EdU, afin de mesurer le taux de sortie des cellules de la phase S. Les souris ont d'abord reçu une injection de BrdU, suivi d'une seconde injection d'EdU 4 heures plus tard. Ce test a révélé une diminution de la proportion de cellules qui sortent de la phase S (BrdU + / EdU-) dans les cerveaux WDR81 $^{-/-}$, indiquant un ralentissement de la phase S dans les progéniteurs gliaux radiaux mutants (Fig. 2D).

Nous avons ensuite regardé si la différenciation prématurée des progéniteurs pouvait être une cause alternative de la réduction de la taille du cerveau. Des cortex embryonnaires ont été marqués pour PAX6 (progéniteurs gliaux radiaux), TBR2 (progéniteurs intermédiaires) et NeuN (neurones) à différents moments du développement et la proportion de chaque population cellulaire a été quantifiée. Nous n'avons observé aucune diminution des populations progénitrices au cours du développement, ce qui indique qu'elles ne se différencient pas prématurément (figure 2E). En fait, nous avons même détecté une réduction de la proportion de neurones au milieu et à la fin de la neurogenèse (Fig. 2E). Enfin, nous avons analysé la mort cellulaire apoptotique dans les cortex WDR81 $^{-/-}$. Le marquage de la caspase-3 clivée (CC3) n'a révélé aucune augmentation de l'apoptose, qui est restée presque indétectable à la fois dans les embryons WT et mutants (figure 2F). Par conséquent, la réduction de la taille du cerveau chez les souris WDR81 $^{-/-}$ n'est pas due à une différenciation précoce des progéniteurs ou à une augmentation de la mort cellulaire apoptotique, mais

semble être une conséquence de la réduction des taux de prolifération des progéniteurs gliaux radiaux.

Réduction de la prolifération et de la signalisation EFGR dans les cellules de patients WDR81

Nous avons ensuite vérifié si des défauts de prolifération similaires pouvaient être observés dans les cellules des patients ayant une mutation pour WDR81. Deux lignées de fibroblastes primaires mutantes, issues de biopsies cutanées, ont été analysées et comparées à deux lignées de fibroblastes témoins. L'index mitotique des deux lignées de patients était fortement diminué, imitant le phénotype observé chez les progéniteurs gliaux radiaux de la souris (figure 3A). Le marquage des cellules avec Ki67, autre technique utilisée pour étudier la prolifération, a également révélé une diminution substantielle pour les deux lignées cellulaires de patients (figure 3B).

Pour mieux comprendre les causes de la prolifération réduite chez les souris mutantes WDR81 et les fibroblastes de patients, nous avons effectué un profil d'expression génique comparant les cortex WT et mutants. En utilisant la technique de microarray permettant d'identifier les voies de signalisation potentiellement impliquées, nous avons observé une régulation négative de la voie de signalisation du récepteur du facteur de croissance épidermique (EGFR) (Sup Fig1). Afin de confirmer ces observations, nous avons suivi l'activité de cette voie de signalisation dans les fibroblastes témoins et de patients. Nous avons observé que le niveau d'EGFR lui-même, était considérablement réduit dans les deux lignées de patients (Fig. 3C). Nous avons ensuite mesuré l'activation de la voie de signalisation de la protéine kinase mitogène-activée (MAPK) en réponse à la stimulation à l'EGF. En accord avec la diminution du niveau d'EGFR, la phosphorylation de ERK est réduite dans les deux lignées de fibroblastes de patients après une exposition à l'EGF (Fig. 3D, E, F). Par conséquent, les cellules des patients

WDR81 présentent des niveaux d'EGFR réduits, entrainant une baisse de l'activation de la voie de signalisation MAPK lors de la stimulation avec l'EGF.

Les niveaux d'EGFR sont connus pour être fortement régulés par des boucles de rétroaction transcriptionnelles, et par un équilibre entre le recyclage et la dégradation du récepteur internalisé (Malartre 2016 Avraham 2011). Nous nous sommes demandé si la réduction des niveaux d'EGFR était une conséquence d'un défaut dans la voie EGFR elle-même. Pour vérifier cela, nous avons privé les cellules d'EGF et mesuré les niveaux d'EGFR 24 heures plus tard. Dans les cellules de patients, cette privation a permis de ramener le niveau d'expression d'EGFR à celui des cellules témoins (Fig 3G). Ces résultats indiquent que la baisse du niveau d'EGFR ne se produit que lorsque la voie est activée, et mettent en évidence des défauts de traitement intracellulaire du signal EGFR.

WDR81 est nécessaire pour le trafic endosomal d'EGFR

Le gène WDR81 est connu pour réguler la maturation endosomique et l'aggréphagie (Kai Liu 2016, Liu X 2017). Il est important de noter que ces deux fonctions sont indépendantes et agissent via les partenaires de liaison spécifiques de WDR81, respectivement WDR91 et p62. Nous avons donc vérifié si l'un de ces facteurs affectait le développement du néocortex de manière similaire à WDR81. Pour ce faire, nous avons électroporé in utero des constructions de shRNA exprimant WDR81, WDR91 et p62 dans des cerveaux en développement à E13.5 et analysé la distribution cellulaire à E17.5. En accord avec les données obtenues dans les souris KO, le knock-down (KD) WDR81 a fortement affecté le développement du néocortex (Fig. 4A). Une grande partie des cellules KD s'est accumulée dans la zone intermédiaire (IZ), au détriment des zones germinales et de la plaque corticale. Ce phénotype a été reproduit avec WDR91 KD, mais pas avec p62 KD qui ne semble pas affecter la distribution cellulaire (Fig. 4A).

Ces résultats confirment que la fonction endosomale de WDR81 est essentielle pour le développement correct du néocortex.

Nous avons ensuite vérifé si des défauts endosomiques pouvaient être observés dans les progéniteurs gliaux radiaux des cellules WDR81 KO. Le marquage des différents compartiments endo-lysosomaux a révélé une altération spécifique des endosomes précoces EEA1 +, qui semblaient gonflés. Pour évaluer quantitativement cela, et vérifier qu''il s'agit d'une caractéristique des cellules mutantes WDR81, nous avons mesuré la taille des endosomes précoces dans les fibroblastes de patients (figure 4C). Une fois de plus, les endosomes EEA1 + étaient gonflés, avec une proportion accrue de gros endosomes (figure 4B). Ces résultats sont cohérents avec le rôle de WDR81 dans la régulation négative de Pi3K classe III (Kai Liu 2016). Etant donné que ces défauts endosomaux sont une cause potentielle d'altération de la régulation de l'EGFR, nous avons ensuite regardé si le trafic endosomique de l'EGFR était affecté dans le mutant WDR81.Les cellules ont d'abord été affamées pendant 24 heures pour restaurer un niveau d'EGFR comparable à celui des cellules témoins, puis de l'EGF555 fluorescent a été ajouté, afin de suivre l'internalisation et le traitement de l'EGFR lié à l'EGF. Dans les deux lignées de patients, l'internalisation de l'EGF555 a été fortement retardée dans les endosomes précoces EEA1 + (Fig. 4D). La quantification de la colocalisation EGF555/EEA1 a révélé que ce retard était particulièrement important 2 heures après l'internalisation de l'EGF (figure 4E). Par conséquent, WDR81 est essentiel pour l'homéostasie endosomale et le trafic de l'EGFR internalisée après la liaison de l'EGF.

Une mutation causant la mégalencéphalie rétablit la prolifération des progéniteurs dans le cerveau des mutants WDR81

Nos résultats indiquent que les défauts de trafic de l'EGFR peuvent résulter de mutations dans le gène WDR81, et conduire à une baisse d'activation de la voie de signalisation MAPK. Ils montrent en outre qu'une réduction de la prolifération des progéniteurs gliaux radiaux est une cause de microcéphalie primaire. La mégalencéphalie est caractérisée par une surcroissance cérébrale et peut être due à une prolifération cellulaire accrue au cours du développement (Reijinders 2017). Les principales causes incluent les mutations de gain de fonction de l'AKT3 et sa cible en aval, la cycline D2 (Mirzaa et al 2014). Ces données combinées suggèrent que la microcéphalie et la mégalencéphalie peuvent être la conséquence d'effets opposés sur les taux de prolifération des progéniteurs gliaux radiaux. Pour comprendre davantage cela, nous avons exprimé la cycline D2 Thr280Ala non dégradable, la cycline D2 dégradable ou un vecteur témoin dans les cerveaux de souris WT et mutantes pour WDR81 à E 14,5, et mesuré l'indice mitotique des cellules électroporées PAX6 + à E16,5 (Fig.5A). L'expression du vecteur vide dans le cerveau WDR81 $^{-/-}$ a confirmé la réduction de l'indice mitotique. L'expression de la cycline D2 dégradable dans le cerveau WT a fortement fait augmenté l'indice mitotique, indiquant que la mutation causant la mégalencéphalie, augmente effectivement les taux de prolifération des progéniteurs gliaux radiaux. L'expression de la cycline D2 Thr280Ala non dégradable dans les cerveaux WDR81 $^{-/-}$ a compensé la diminution de l'indice mitotique due à la mutation WDR81. De façon frappante, l'indice mitotique était aussi élevé dans les cerveaux WT que dans dans les WDR81 $^{-/-}$, suggérant que la cycline D2 agit en aval de WDR81 (figure 5B). L'ensemble de ces résultats indiquent que la mutation provoquant une mégalencéphalie peut compenser l'effet d'une mutation induisant une microcéphalie, en agissant sur la prolifération des progéniteurs gliaux radiaux. Ces deux pathologies peuvent donc avoir une origine étroitement liée: un

déséquilibre dans la régulation du cycle cellulaire conduisant soit, à une réduction de la croissance cérébrale réduite, soit à une surcroissance cérébrale.

DISCUSSION

WDR81 est une protéine mal caractérisée qui, si elle est mutée, peut provoquer de graves défauts dans le développement du cerveau provoquant une microcéphalie. Il a récemment été signalé qu'elle était impliquée dans les endocytoses et l'autophagie, mais les mécanismes moléculaires sont peu connus. Cependant, pour pouvoir analyser au niveau cellulaire l'effet causé par la délétion de WDR81 dans un cerveau en développement, j'ai généré une souris WDR81 Knock Out en utilisant CRISPR / Cas9. Les souris générées ont un cerveau plus petit présentant des défauts évidents uniquement dans les couches supérieures mais étonnamment pas dans les couches profondes. Ces deux résultats reconstituent le phénotype observé dans les pathologies humaines de microcéphalie et de lissencéphalie. L'analyse des cerveaux de souris WDR81 $^{-/-}$ a montré une prolifération réduite des progéniteurs. Ce résultat suggère que la réduction de taille des cerveaux des souris WDR81 $^{-/-}$ est due à une diminution du pool de cellules produites à la fois pour les progéniteurs et les neurones. Cela affecte donc la production cellulaire globale conduisant ainsi au phénotype observé. J'ai ensuite vérifié si des défauts de prolifération similaires pouvaient être observés dans les deux lignées de fibroblastes primaires, portant la mutation pour WDR81. L'index mitotique et l'indice de prolifération des deux lignées étaient réduits, reproduisant ainsi l'effet observé dans les progéniteurs gliaux radiaux de la souris. Pour mieux comprendre les causes de la réduction de prolifération chez les souris mutantes WDR81 et les fibroblastes de patients, nous avons réalisé un profil d'expression génique comparant les cortex WT et mutants. À l'aide de la technique de microarray permettant d'identifier les voies de signalisation potentiellement

impliquées, nous avons observé une régulation négative de la voie du récepteur du facteur de croissance épidermique (EGFR). Afin de confirmer ces données, nous avons mesuré l'activation du récepteur EFGR en analysant les niveaux d'activation de MAPK dans les lignées cellulaires témoins et de patients, et en effet, nous avons pu observer une réduction de la p-ERK. Nous avons ensuite décidé de vérifier le niveau d'expression d'EGFR lui-même et nous avons également constaté une diminution dans les lignées des patients par rapport au contrôle. Ces données suggèrent que la délétion de WDR81 peut affecter la voie de signalisation EGFR et le renouvellement cellulaire du récepteur lui-même.

WDR81 est connu pour être un régulateur endosomique via Pi3K et on sait que les endocytoses font partie de la voie d'activation / signalisation d'EGFR. Nous avons donc regardé si l'implication de WDR81 dans les endocytoses pouvait également affecter le recyclage de l'EGFR. Pour cela, nous avons analysé la taille des endosomes et le trafic d'EGFR dans les lignées cellulaires de fibroblastes de patients. En comparant avec les lignées cellulaires contrôles, nous avons pu observer des endosomes gonflés ainsi que des défauts dans le trafic de l'EGFR. Ces deux résultats combinés suggèrent que WDR81 est effectivement impliqué dans la maturation des endosomes, ce qui entraîne un retard dans le processus de cargaison des endosomes, affectant et ralentissant la voie de signalisation.

La conséquence de ce retard dans l'amarrage de l'EGFR dans la cellule peut donc être à l'origine de la diminution de la prolifération des progéniteurs gliaux radiaux au cours du développement, provoquant ainsi une microcéphalie.

Nous avons ensuite tenté de comprendre si une croissance cérébrale diminuée (microcéphalie) et augmentée (mégalencéphalie) pouvaient être liées. En effet, de nombreuses mutations provoquant la mégalencéphalie se trouvent dans la voie AKT, conduisant sa régulation positive. Inversement, mes données indiquent une régulation à la

baisse, en aval des voies de signalisation de l'EGFR, provoquant une diminution de la prolifération chez les mutants WDR81.

En utilisant l'électroporation in utero pour sur-exprimer les mutations spécifiques de la mégalencéphalie (cycline D2) et causant une augmentation de la prolifération des progéniteurs, nous avons regardé si celles-ci permettaient de rétablir un taux de prolifération normal dans les mutants WDR81 KO, ayant des microcéphalies.

De toutes les constructions testées, seule l'expression de la Cycline D2 Thr280Ala non dégradable dans les cerveaux WDR81 $^{-/-}$ a compensé la diminution de l'indice mitotique due à la mutation WDR81, suggérant que la Cycline D2 agit en aval de WDR81. L'ensemble de ces résultats indiquent qu'une mutation engendrant une mégalencéphalie peut compenser l'effet d'une mutation induisant une microcéphalie, en agissant sur la prolifération des progéniteurs gliaux radiaux. Ces deux pathologies peuvent donc avoir une origine étroitement liée: un déséquilibre dans la régulation du cycle cellulaire conduisant soit, à une réduction de la croissance cérébrale réduite, soit à une surcroissance cérébrale.

RÉSUMÉ

Le développement du néocortex est un processus hautement réglé ou chaque type de cellule doit être correctement spécifié et positionné. Les altérations de ce processus peuvent entraîner diverses malformations corticales, y compris la microcéphalie (petit cerveau). Cette dernière pathologie a été récemment liée à des mutations dans le gène WDR81. WDR81 est une protéine transmembranaire mal caractérisée qui a été impliquée dans la maturation des endosomes ainsi que dans l'agrégation. On ne sait pas comment les mutations de WDR81 modifient la croissance du néocortex. Pour répondre à cette question, j'ai généré une souris knock-out CRISPR / Cas9 WDR81. Les animaux mutants pour WDR81 meurent juste après la naissance et récapitulent le phénotype de la microcéphalie humaine. Dans cette thèse j'ai démontre que la taille réduite du cerveau n'est pas due à une apoptose accrue ou à une altération du sort des cellules, mais à une réduction des taux de prolifération des cellules souches neurales. Les fibroblastes dérivés des patients affecte par cette type de microcéphalie présentent également une prolifération réduite, ce qui indique que cela peut être une caractéristique générale de la perte de fonction du WDR81. Dans ce système, j'ai identifié des altérations dans la voie de signalisation de la MAP kinase, qui montre une phosphorylation ERK réduite dans les cellules mutantes après stimulation par l'EGF. J'ai démontré en outre que ce défaut est dû à une forte réduction des niveaux d'expression du récepteur EGR (EGFR). Les niveaux d'EGFR peuvent être récupérés après une privation d'EGF, indiquant que cette régulation négative est due à une signalisation aval d'EGFR modifiée. En effet, j'observe des retards de clairance intracellulaire de l'EGFR suite à son internalisation après la liaison de l'EGF. J'ai démontré que l'EGFR s'accumule dans des endosomes précoces gonflés aberrants dans des cellules mutantes de patients WDR81, que nous observons également dans des cellules souches neurales de souris mutantes in vivo. Ensemble, ces travaux montrent que WDR81 régule le trafic endosomal d'EGFR, et que la perte de fonction conduit à une réduction de l'activation de la voie MAP kinase entraînant une prolifération réduite des cellules souches neurales et une microcéphalie liée à WDR81. Cependant, ces défauts de prolifération peuvent être sauvés in vivo par l'expression de la cycline D2 mutée provoquant la mégalencéphalie, démontrant que la microcéphalie et la mégalencéphalie peuvent être dues à des effets opposés sur le taux de prolifération des progéniteurs gliaux radiaux.

MOTS CLÉS

WDR81, Neurodéveloppement, Cellule souche neurale, Microcéphalie,

ABSTRACT

The development of the neocortex is a highly regulated process whereby each cell type must be correctly specified and positioned. Alterations in this process can lead to a variety of cortical malformations, including microcephaly (small brain). This latter pathology has been recently related to mutations in the WD repeat domain 81 (*WDR81*) gene. WDR81 is a poorly characterized transmembrane protein that has been involved in endosomal maturation as well as aggrephagy. It remains unknown how mutations in WDR81 alters the growth of the neocortex. To address this question, I have generated a CRISPR/Cas9 WDR81 knock-out mouse. Mutant animals for WDR81 die perinatally and recapitulate the human microcephaly phenotype. I demonstrate that the reduced brain size is not due to increased apoptosis or altered cell fate, but to reduced neural stem cell proliferation rates. Patient-derived fibroblasts also display reduced proliferation, indicating that this may be a general feature of WDR81 loss of function. In this system I identified alterations in the MAP kinase signaling pathway, which shows reduced ERK phosphorylation in mutant cells following EGF stimulation. I further demonstrate that this defect is due to a strong reduction of the EGR receptor (EGFR) expression levels. EGFR levels can be rescued following EGF starvation, indicating that this downregulation is due to altered EGFR downstream signaling. Indeed, we observe EGFR intracellular clearance delays following its internalization after EGF binding. I demonstrate that EGFR accumulates in aberrant swollen early endosomes in WDR81 patient mutant cells, which we also observe in mutant mouse neural stem cells in vivo. Together, this work shows that WDR81 regulates endosomal trafficking of EGFR, and that loss of function leads to reduced MAP kinase pathway activation causing reduced neural stem cell proliferation and WDR81-linked microcephaly. However, these proliferation defects are rescued *in vivo* by the expression of megalencephaly-causing mutated Cyclin D2 demonstrating that microcephaly and megalencephaly can be due to opposite effects on the proliferation rate of radial glial progenitors.

KEYWORDS

WDR81, Neurodevelopment, Neural stem cells, Microcephaly