REGULATION OF CEREBRAL CORTEX DEVELOPMENT AND EXPANSION BY*MIR3607*

Doctoral Thesis presented by

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PhD Program in Neuroscience

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INSTITUTO DE NEUROCIENCIAS

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This Doctoral Thesis, entitled "*Regulation of cerebral cortex development and expansion by miR3607"* is presented under the conventional thesis form with the following quality indicators:

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Yours sincerely

Kaviya Chinnappa

DIRECTOR'S REPORT

Sant Joan d'Alacant, 08 Sep 2021

Dr. D. Victor Borrell Franco, Director of the doctoral thesis entitled "*Regulation of cerebral cortex development and expansion by miR3607"*

CERTIFIES:

That Ms. Kaviya Chinnappa has carried out under my supervision the work entitled "*Regulation of cerebral cortex development and expansion by miR3607***"** in accordance with the terms and conditions defined in her Research Plan and in accordance with the Code of Good Practice of the University Miguel Hernández of Elche, satisfactorily fulfilling the objectives foreseen for its public defence as a doctoral thesis.

I sign for appropriate purposes, at ..... , ... of ..... of 202…

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That Ms. Kaviya Chinnappa has carried out under the supervision of our PhD Programme the work entitled "*Regulation of cerebral cortex development and expansion by miR3607"* in accordance with the terms and conditions defined in its Research Plan and in accordance with the Code of Good Practice of the University Miguel Hernández de Elche, fulfilling the objectives satisfactorily for its public defence as a doctoral thesis.

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CONTENTS

ABBREVIATIONS

AGO	Argonaute
APC	Adenomatous Polyposis Coli
BMP	Bone Morphogenetic Protein
BrdU	Bromodeoxyuridine
bRGCs	basal Radial glial cells
CC	Corpus callosum
CP	Cortical plate
CTX	Cortex
Cux1	Cut Like Homeobox 1
DAPI	4´,6-diamidino-2-phenylindole
DEGs	Differentially expressed genes
E	Embryonic day
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast Growth Factor
GFP	Green Flourescent Protein
GW	Gestational weeks
INM	Interkinetic Nuclear Migration
IPCs	Intermediate progenitor cells
ISVZ	Inner Sub-ventricular Zone
IUE	*in utero* Electroporation
IZ	Intermediate Zone
KD	Knockdown
LS	Lateral Sulcus
miRNA	microRNA
MZ	Marginal Zone
NECs	Neuro-epithelial cells

OE	Overexpression
OSVZ	Outer Sub-ventricular Zone
P	Postnatal day
Par3	Partitioning defective 3
Pax6	Paired box 6
PH3	Phospho-histone H3
pre-miR	Precursor-microRNA
P-Vim	Phospho-vimentin
RA	Retinoic Acid
RGCs	Radial Glial cells
RISC	RNA Induced Silencing Complex
SCR	Scrambled
SG	Spenial gyrus
Shh	Sonic hedgehog
SNORD	C/D box small nucleolar RNA
SP	Subplate
STR	Striatum
SVZ	Sub-ventricular Zone
Tbr1	T-box brain transcription factor 1
Tbr2	T-box brain protein 2
TRBP	Transactivation response element RNA binding protein
TUD	Tough decoy
UTR	Untranslated region
VZ	Ventricular Zone
Wnt	Wingless/ Integrated
β-catenin	beta catenin

SUMMARY

MicroRNAs (miRNAs) are a class of non-coding RNA molecules increasingly recognized to play varied roles in development, physiology and disease. The majority of miRNAs are expressed in the brain, have fast turnover rates and the ability to regulate several genes in a spatio-temporal fashion. This makes them important regulators of gene expression during the extraordinarily complex process of brain development. The identification that novel miRNAs emerged during speciation in mammalian evolution helps to understand their importance and roles in the developmental processes leading to the formation of folded brains with increasing complexity in higher mammals. Our previous transcriptomic analyses of the developing ferret cortex identified candidate genes and miRNAs differentially expressed across germinal layers, with potential relevance in cerebral cortex expansion. We identified *MIR3607* as highly and differentially expressed between embryonic germinal layers of the large and folded human and ferret cortex, but not expressed in the lissencephalic small mouse cortex. Experimental expression of *MIR3607* in the developing cerebral cortex of mouse embryos at E14.5 affected neurogenesis, and transcriptomic profiling revealed increased Wnt/βCatenin signaling and decreased apical adhesion as the major underlying factors. Expression of *MIR3607* at E12.5, when progenitor cells expand, dramatically effected amplification and delamination of apical progenitors, as predicted, leading to rosette formation. This was rescued by co-expressing Adenomatous Polyposis Coli (APC), repressor of canonical Wnt signaling and a direct target of *MIR3607*. A similar phenotype was produced in human cerebral organoids, indicating the conservation of this function. Experiments of loss of *MIR3607* function in ferret severely impaired polarity of apical progenitor cells and induced their delamination and ectopic mitosis, defects phenocopied by overexpressing APC. Our findings demonstrate that *MIR3607* activates Wnt/βCatenin signaling in apical progenitor cells, promoting their amplification and the sustained expansion of the Ventricular Zone to form a large and complex cerebral cortex in higher mammals.

RESUMEN

Los microARNs (miARNs) son una clase de moléculas de ARN no codificantes cada vez más reconocidas por desempeñar funciones variadas en el desarrollo, la fisiología y la enfermedad. La mayoría de los miARNs se expresan en el cerebro, tienen tasas de renovación rápidas y la capacidad de regular espacial y temporalmente múltiples genes. Esto los convierte en importantes reguladores de la expresión génica durante el proceso extraordinariamente complejo del desarrollo del cerebro. La identificación de que nuevos miARNs surgieron durante procesos de especiación en la evolución de los mamíferos, ayuda a comprender su importancia y su papel en los mecanismos de desarrollo que conducen al plegamiento de cerebros con una complejidad creciente en mamíferos superiores. Análisis transcriptómicos previos de nuestro laboratorio de la corteza del hurón en desarrollo identificaron genes candidatos y miARNs expresados diferencialmente a lo largo de las capas germinativas, con potencial relevancia en la expansión de la corteza cerebral. Identificamos *MIR3607* como altamente expresado y de forma diferencial entre las capas germinativas embrionarias de la corteza humana y de hurón, caracterizadas por su gran tamaño y claro plegamiento, pero no presente en la pequeña corteza de ratón, animal lisencefálico. La expresión experimental de *MIR3607* en la corteza cerebral en desarrollo de embriones de ratón tras 14.5 días de gestación (E14.5) afectó el proceso de neurogénesis, y la caracterización transcriptómica reveló un aumento de la vía de señalización de Wnt / βCatenina y una disminución de la adhesión apical como principales factores subyacentes. La expresión de *MIR3607* en ratón a E12.5, cuando las células progenitoras aumentan en número de manera importante, afectó dramáticamente a los procesos de amplificación y delaminación de los progenitores apicales, tal y como predijimos, lo que condujo a la formación de rosetas. Este efecto fue revertido mediante la coexpresión de Adenomatous Polyposis Coli (APC), represor de la vía de señalización canónica de Wnt y diana directa de *MIR3607*. Un fenotipo similar se produjo en organoides cerebrales humanos, lo que indica la conservación de esta función. Experimentos de pérdida de función de *MIR3607* en hurones alteraron gravemente la polaridad de las células progenitoras apicales e indujeron su

delaminación y mitosis ectópica, defectos fenocopiados por la sobreexpresión de APC. Nuestros hallazgos demuestran que *MIR3607* activa la vía de señalización Wnt / βCatenina en células progenitoras apicales, promoviendo su amplificación y la expansión de la Zona Ventricular, lo que da lugar a la formación de una corteza cerebral grande y compleja en mamíferos superiores.

INTRODUCTION

The cerebral cortex is a highly organized structure in the brain that controls complex cognitive functions. Across evolution, the cerebral cortex has undergone dramatic changes from a small and simple cortex of reptiles and birds to increasingly larger and folded neocortex of mammals including primates, which is essential for higher order functions. The formation of this elaborately structured and functional neocortex with increasing complexity across evolution is subject to genetic regulation. This requires the interplay of several coding and non-coding genes, including genes emerged in recent evolutionary lineages. These gene regulatory mechanisms control a series of different steps during brain development, including the generation of several different types of progenitor cells and their proliferative activity to determine neuronal output *(de Juan Romero and Borrell.,2015; Borrell and Calegari.,2014; Sun and Hevner.,2014; Cardenas et al.,2018; Cardenas and Borrell.,2020).*

1. Cerebral cortex development and gyrification

1.1 Early specification of the neural tube

The early embryo forms as a single sheet of cells which, upon gastrulation, form the three embryonic germinal layers, namely the outer ectoderm, middle mesoderm and inner endoderm. Gastrulation defines the midline and different axes of the embryo (anterior-posterior, dorsal-ventral and medial-lateral), which are important for formation of different organs including the brain. The formation of the notochord at the midline of the embryo as the mesoderm invaginates is important for the formation of the nervous system and determines its position. The inductive signals from the notochord cause the overlying ectoderm to differentiate into neuroectoderm, which gives rise to the entire nervous system. The midline ectoderm that contains the neuroectodermal precursor cells thickens into a distinct columnar epithelium called the neural plate, in a process called neurulation. The lateral margins of the neural plate fold inward and form the neural tube, which gives rise to the brain, spinal cord and most of the peripheral nervous system *(Gilbert.,2010; Purves et al.,2012).*

The major brain regions are formed by bending, folding and constriction of the neural tube as a result of morphogenetic movements as shown in Figure 1. The anterior part of the neural tube forms the brain and the posterior part forms the spinal cord. The neural tube is majorly divided into three vesicles: prosencephalon, mesencephalon and rhombencephalon. The rostral prosencephalon gives rise to the telencephalon, which is divided in two bilaterally symmetric telencephalic vesicles. The dorsal telencephalon becomes the cerebral cortex and hippocampus, and the ventral telencephalon leads to the formation of basal ganglia, basal forebrain nuclei and olfactory bulb. The caudal part of the prosencephalon becomes the diencephalon, which forms the thalamus and hypothalamus. The mesencephalon gives rise to superior and inferior colliculi, and the collection of nuclei called the midbrain tegmentum. The rhombencephalon forms the cerebellum, pons and medulla *(Purves et al.,2012).*

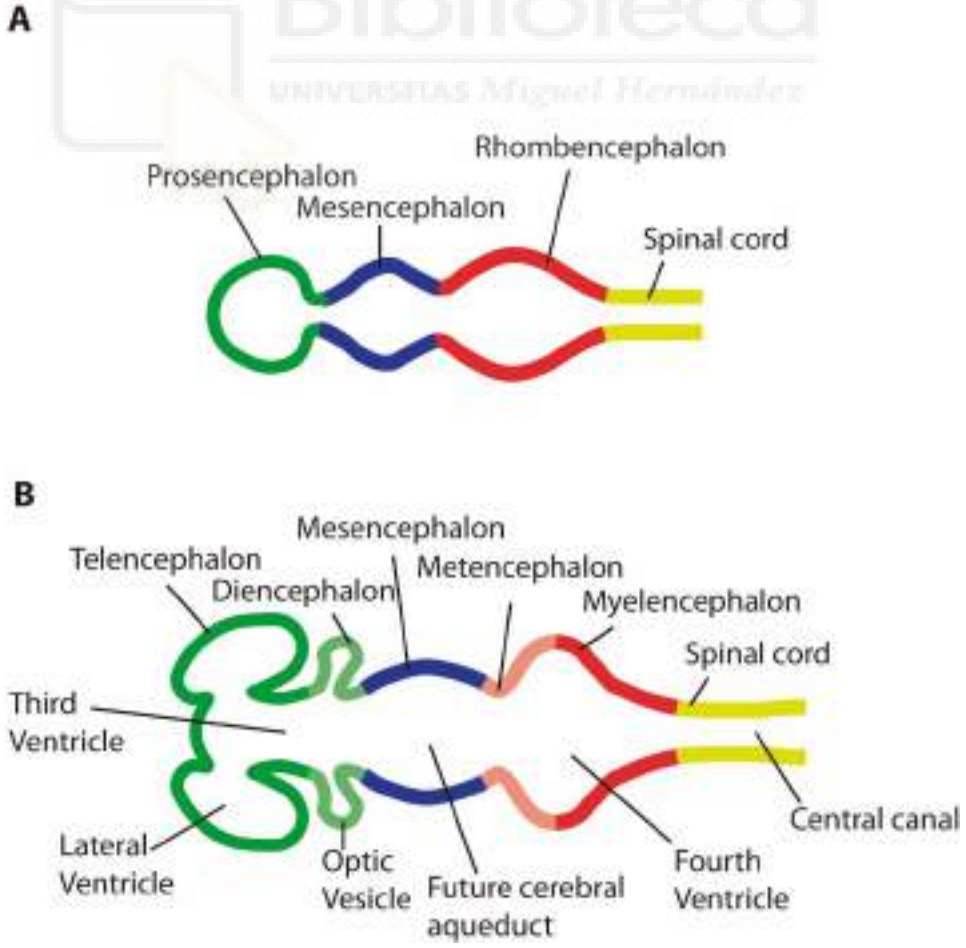


Fig. 1. Regional specification of the neural tube. (A) Early in development, the neural tube is subdivided into prosencephalon, mesencephalon and metencephalon. The posterior region forms the spinal cord. (B) Later in development, further divisions occur giving rise to telencephalon and diencephalon on the anterior side, and metencephalon and myelencephalon on the posterior side. These structures give rise to major functional structures later and the spaces eventually forming the ventricles *(Modified from Purves et al.,2012).*

1.2 Neurogenesis

Early in development, the neural plate and neural tube are formed by a single layer of neuroepithelial cells (NECs), which are the primary neural stem cells. NECs show epithelial features, apical-basal polarity, express specific transmembrane proteins such as prominin-1 (CD133), and are attached to each other by tight junctions and adherens junctions *(Weigmann et al.,1997).* The highly polarized neuroepithelial cells extending their apical process attached to the ventricle and basal process extending to the basal lamina undergo cell-cycle dependent intekinetic nuclear migration(INM). Their nuclei undergo apical to basal migration during G1, exit S-phase at the basal part, then undergo basal to apical migration during G2 before undergoing mitosis at the apical ventricular surface *(Arai and Taverna.,2017; Farkas and Huttner.,2008).* They initially undergo symmetric proliferative divisions, each producing two daughter stem cells of the same type. Over time, NECs become Radial Glia Cells (RGCs), progenitor cells more fate restricted that will undergo asymmetric neurogenic divisions, giving rise to renewed RGCs as well as basal progenitors and neurons *(Wodarz and Huttner,2003).* In the early mouse telencephalon, NECs undergo mitoses while retaining and splitting the basal process, whereas in human NECs retract their basal process during mitoses, which is regrown following division *(Kosodo et al.,2008; Kosodo and Huttner,2009; Subramanian et al.,2017).*

As NECs transform into RGCs and neurogenesis begins, the single-layered neuroepithelium transforms into a multilayered tissue. The layer next to the ventricle is called Ventricular Zone and houses the cell body of RCGs. The transformation of NECs into RCGs involves the downregulation of certain epithelial features, including tight junctions and certain plasma membrane proteins that are important for apical basal polarity *(Aaku-Saraste et al.,1996)*.

Other neuroepithelial features are maintained, including the expression of neuroepithelial markers such as the intermediate filament protein Nestin and proteins associated with adherens junctions, such as Prominin-1. Also, maintenance of features important for apical-basal polarity including the expression of apical complex proteins such as Par3/Par6/aPKC, and maintenance of contact with the basal lamina *(Wodarz and Huttner.,2003; Weigmann et al.,1997; Halfter et al.,2002)*. RCGs also display several astroglial properties, including glycogen granules and expression of several astrocytic markers such as the astrocyte specific glutamate transporter (GLAST), Ca^{2+} binding protein S100β, glial fibrillary acidic protein (GFAP), vimentin and brain lipid binding protein (BLBP) *(Kriegstein and Götz.,2003; Campbell and Götz.,2002; Götz.,2003)*. In mice, this transition occurs between embryonic stages E10 and E12, while in humans it is proposed to occur as early as 8-10 weeks of gestation *(Subramanian et al.,2017).* RCGs, also possessing apical and basal processes spanning the cortical surface, undergo INM like neuroepithelial cells, undergoing mitosis at the apical surface and migrating basally for S phase, but this movement is confined within the VZ. Most of the neurons in the brain are derived either directly or indirectly from RGCs, in addition to their role as scaffold for the radial migration of neurons and as precursors of astrocytes *(Götz et al.,2002; Malatesta et al.,2000)*.

A secondary type of cortical progenitors are Intermediate Progenitor Cells (IPCs), which are born from the mitoses of RGCs and starting at E13.5 form a secondary germinal layer basal to the VZ, called subventricular zone (SVZ). IPCs express characteristic transcription factors such as Tbr2, and undergo symmetric neurogenic divisions away from the apical surface to form two neuronal daughter cells *(Götz and Huttner,2005).* The importance of signaling factors and mechanisms governing the emergence of IPCs, thereby increasing the neuronal output and impacting the evolution of cortical size from reptiles to birds and mammals, has been recently demonstrated relating to the levels of Robo and Delta1 signaling, showing the importance of basal progenitors in determining the size and complexity *(Cardenas et al.,2018).*

A second important class of basal progenitors are called basal Radial Glial Cells. These are very abundant in the OSVZ of large and gyrified brains, and have been

found to be an important determinant factor of cortical folding *(Reillo et al.,2011; Fietz et al.,2010; Hansen et al.,2010; Kelava et al.,2012).* Initially identified by similarity with apical RGCs but with only a basal process, without apical proces, further studies in primates have demonstrated that these cells may present multiple morphologies, including apical and basal processes, as well as a number of short lateral processes that have been linked to the proliferative capacity of these progenitor cells *(Betizeau et al.,2013; Kalebic et al.,2019).* In primates, bRGCs undergo mitotic somal translocation (MST), where the nucleus moves basally along the basal process prior to mitosis, and they have the capacity to undergo multiple self-renewing divisions *(Hansen et al.,2010 Betizeau et al.,2013)*. At the molecular level, bRGCs are characterized by expression of Pax6 (like aRGCs) or Pax6 and Tbr2 (*Reillo and Borrell.,2012)*. Single cell sequencing studies identified several markers specific to bRGC, such as *HOPX, PTPRZ1, TNC, FAM107A* and *MOXD1 (Pollen et al.,2015).* Interesingly, aRGCs generating bRGCs destined to form the OSVZ occurs only during a specific restricted time window of cortical development, when aRGCs undergo self-consuming divisions, that is they form bRGCs at the expense of aRGCs *(Martinez-Martinez et al.,2016)*. Figure 2 summarizes the main types of progenitor cells found in the mouse and ferret/human cerebral cortex.

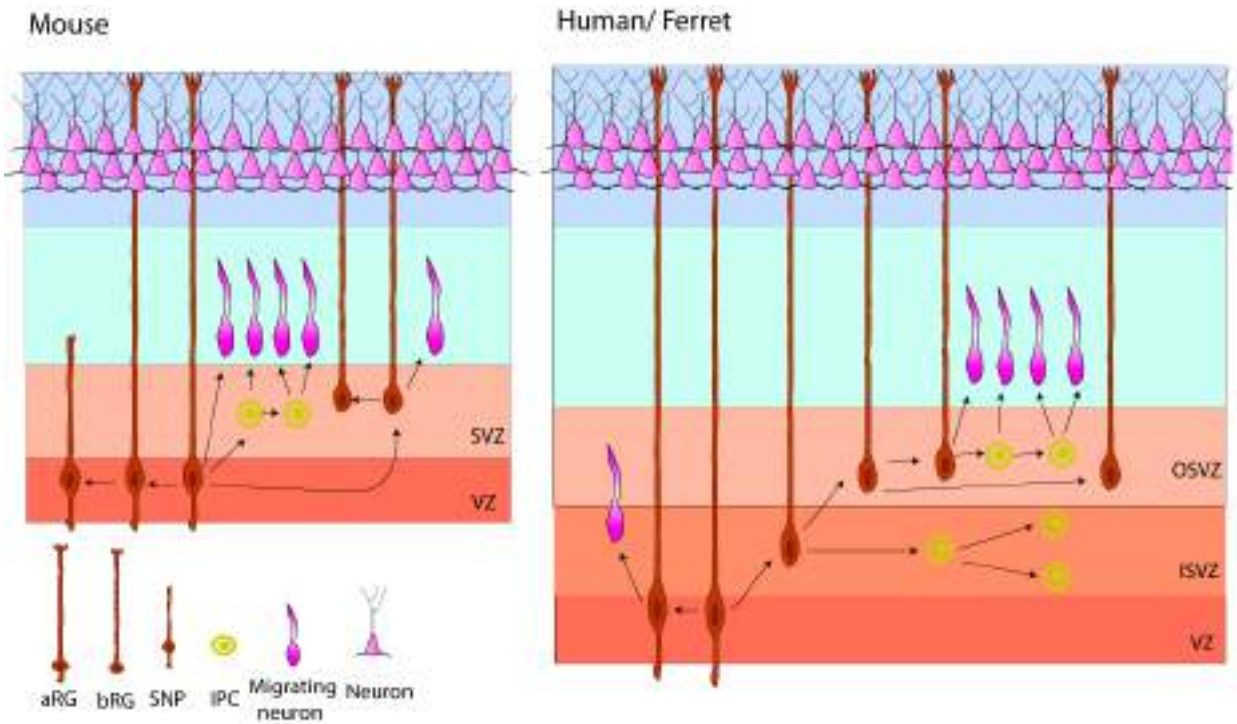


Fig. 2. Cerebral cortex neurogenesis in mouse and ferret/human. The progenitors in the cerebral cortex include apical radial glial cells (aRG),short neural precursors (SNP), intermediate progenitors (IPC), and basal radial glial cells (bRGC) that give rise to neurons which migrate radially to the cortical plate and positioned in an inside-out pattern. The basal radial glial cells which are very few in mouse cortex but abundant and form an extended OSVZ in higher order species including human, increases the neuronal output, also helping with tangential dispersion of neurons, forming gyri and sulci *(Modified from Borrell and Reillo.,2012)*

1.3 Neuronal migration and subtype specification

Projection neurons in the cerebral cortex are produced from progenitor cells in a temporal order, from E11.5 to E17.5 in mouse. The earliest born neurons form a transient layer called preplate, which soon afterwards splits into a superficial marginal zone (MZ) and a subplate (SP). Postmitotic neurons formed subsequently will migrate radially away from the germinal layers to place themselves in between MZ and SP, eventually forming the multilayered cortex with the later born neurons migrating past the early born neurons *(Angevine and Sidman.,1961; Caviness and Takahashi.,1995; Molyneaux et al.,2007)*. Neurons produced very early in development inherit the apical-basal polarity of their mother RGCs and migrate to the cortical plate by somal translocation. As the thickness of the cortical primordium increases, this mode of migration is replaced by a multi-phase mode. Neurons first exhibit a short bipolar migration from their birthplace to the SVZ. Then they undergo multipolar migration, extending and retracting multiple neurites and frequently changing direction, but overall moving towards the cortical plate. Finally, they re-acquire apical-basal polarity and move towards the pial surface by cell locomotion, alternating the elongation of a thick leading process with the translocation of the cell nucleus, and using the basal process of RGCs as their migration substrate and guide. As locomoting neurons approach the MZ, they undergo a final event of glia-independent somal translocation, when the leading process is attached to the pial surface and shortens upon upward movement of cell soma *(Nadarajah et al.,2001; Noctor et al.,2001; Kon et al.,2017).* In folded brains, the presence of basal processes from bRGCs provides additional radial fibers that transform the parallel array of radial fibers to a fanned conformation, thus contributing to the tangential dispersion of

radially-migrating neurons and gyrification *(Reillo et al.,2011)*. In agreement with the modification of the radial fiber scaffold, observations in the ferret cerebral cortex have revealed that in gyrencephalic species radially-migrating neurons display tortuous migrations to help with lateral dispersion just prior to the start of folding of the cerebral cortex *(Gertz and Kriegstein.,2015).* This seems to stem from increased branching of leading processes in gyrencephalic species, such as ferret, which may favor the tangential dispersion of neurons *(Martinez-martinez et al.,2018).* Migration speed and cell-cell adhesion between migrating neurons are also known to be key aspects during cortical folding *(del Toro et al.,2017).*

The different layers of the cerebral cortex contain different types of neurons, which are largely specified in progenitor cells. The existence of basal progenitors in mammals, and the extent of their proliferation, are important for the formation and expansion of supragranular layers (Layers II-III), although not solely linked to it *(Montiel et al.,2016; Cardenas et al.,2018; Cardenas and Borrell.,2019).* Moreover, the existence of the OSVZ in gyrencephalic mammals has been associated to, and found important for, the formation of complex supragranular layers *(Lukaszewickz et al.,2005; Smart et al.,2002)*. In primates, bRGCs self-amplify by proliferative divisions during mid-corticogenesis, enabling the formation of enlarged supragranular layers *(Betizeau et al.,2013).*

There are certain genes like Cux2, Cux1 and Svet1, being expressed in a subset of progenitors during the generation of upper layer neurons of the cerebral cortex suggesting that these markers might be markers of progenitors generating upper layer neurons *(Tarabykin et al.,2001; Nieto et al.,2004; Zimmer et al.,2004).* Further evidence that Cux2 progenitors are destined to produce upper layer neurons supported the fact that certain RGCs are intrinsically specified to become upper layer neurons independent of niche or birthdate *(Franco et al.,2012).* Expression of Foxg1 has been shown to suppress Tbr1 and in turn cause the progenitors to acquire deep layer Fezf2+/Ctip2+ identity, and the negative feedback signal from deep layer neurons represses Fezf2/Ctip2 and confer Satb2+ upper layer fate *(Toma et al.,2014).* Satb2 has been shown to be essential for determining the identity of upper layer neurons that contribute the corpus callosum, and the absence of which lead to ectopic expression of Ctip2 and contribution to corticospinal tract *(Britanova et al.,2008).* Sox5, Fezf2 and Ctip2

play an important role in the specification of subcortically projecting axons. Fezf2/Ctip2 and Satb2 appear to be mutually repressive, to ensure that the neurons adopt either a subcortical or callosal projection neuron identity *(Leone et al.,2008)*. However, studies have also shown that mutual regulation between Satb2 and Fezf2 enables Satb2 to promote subcerebral neuron identity in layer 5 neurons, but repress subcerebral characters in callosal projection neurons *(McKenna et al.,2015).*

1.4 Axon specification and guidance

Once neurons have been generated, and already as they undergo radial migration, they begin differentiating by growing out a main axon, which navigates to its final target following guidance cues via its growth cone *(Ye et al.,2019)*. There are three major classes of cortical excitatory projection neurons, depending on their axon projection targets: 1, Cortico-fugal projection neurons (CFuPN); 2, Callosal projection neurons (CPN); and 3, Ipsilateral projection neurons. CFuPNs are neurons born early that reside in deep layers and project away from the cortex. They are predominantly of two types: cortico-thalamic neurons that project to different nuclei of the thalamus, located mostly in layer VI but with a small proportion in layer V; sub-cerebral projection neurons located in layer V that project to the brain stem and targets in the spinal cord. CPNs reside majorly in cortical layers II/III and to a lesser extent in layers V and VI, project to the contralateral cortex through the corpus callosum and connect the two cerebral hemispheres. Ipsilateral or association projection neurons extend their axonal projections within the same cortical hemisphere of origin *(Molyneaux et al.,2007; Fame et al.,2011)*. The precise guidance of developing axons and the accurate connectivity of neuronal circuits is essential for proper functioning of the nervous system *(Dickson.,2002)*.

2. Extrinsic factors influencing cerebral cortex development

The spatial and temporal patterning of the cerebral cortex by expression of different set of genes is induced by several endogenous signaling factors. These signaling molecules are secreted by particular cell class or tissue, or embryonic structures like the notochord, roofplate, floorplate, neuroectoderm itself or the adjacent tissue structures. These signals can also form a gradient depending on the distance from the source and the graded signal shape the structure accordingly. These secreted signals are received by different classes of receptors in the cells, which then regulates the expression of intrinsic factors to control the process of neurogenesis and maturation of neurons *(Purves et al.,2012).*

The role of FGF signaling in area specification has been first demonstrated by Fukuchi-Shimogori and Grove. FGFs 3, 8, 17, 15 and 18 were found to be expressed at the rostral midline of the neocortex in the commissural plate and surrounding tissue from E9.5 until E12.5 *(Bachler and Neubüser.,2001)*. Increasing FGF8 in the rostral cortex lead to the expansion of rostral cortical areas at the expense of caudal areas while decreasing rostral FGF signal lead to a dramatic shrinkage of rostral neocortical areas and the ectopic expression of FGF8 in the caudal cortex lead to a duplication of more rostral somatosensory cortex caudally *(Fukushi-Shimogori and Grove.,2001)*. Moreover, FGF signaling is important for proper proliferation of progenitors and generation of neurons. Loss of FGF signaling is known to cause loss of RGCs and premature neurogenesis resulting in reduced cortical surface, whereas its activation causes cortical expansion with formation of fold in normally lissencephalic mouse cortex and extra fold in gyrencephalic ferret cortex *(Rash et al.,2011; Rash et al.,2013; Masuda et al.,2015).*

RA signaling is implicated in cellular differentiation, transitions between various classes of neural stem cells leading to their terminal division for neurogenesis. Excess RA signaling has been known known to cause severe birth defects, including defects in neural tube closure and early brain morphogenesis *(Linney and LaMantia.,1994; Purves et al.,2012)*. RA secreted by meningeal membranes promote neurogenesis and its blockade resulting in elongation of the neuroepithelium *(Siegenthaler et al.,2009).* BMP signaling controls the dorsal

midline specification, development of the choroid plexus and promote the dorsomedial patterning of the telencephalon in cooperation with Wnts *(Furuta et al.,1997; Hèbert et al.,2002; Panchision et al.,2001).* Apart from these, in the cerebral cortex, BMP signaling is reported to promote neuronal differentiation of RGCs, transition of multipolar to bipolar morphology of migrating neurons in the SVZ and IZ by regulating the expression of microtubule-associated protein CRMP2 *(Li et al.,1998; Sun et al.,2010; Agirman et al.,2017)*

An increase in Shh signaling in radial glial cells has been found to prolong their self-renewal at the expense of generation of basal progenitors, but the formed basal progenitors undergoing multiple round of divisions and, with formation of basal radial glial cells, leading to the folding of the mouse cortex. The reduction of Shh signaling in RGCs has been found to impair their proliferation along with the generation of basal progenitors and basal radial glial cells *(Wang et al.,2016; Komada et al.,2013).* Notch signaling effector, Hes genes are known to maintain the stemness characteric of radial glial cells by suppressing the expression of proneural genes *(Kageyama et al.,2008)*. Hes1 is expressed in radial glial cells in cell-cycle dependent fashion and its genetic loss lead to brain hypoplasia as a result of premature neurogenesis *(Ishibashi et al.,1995; Ohtsuka et al.,2001).* Hes1 gene is epigenetically silenced after divisions of radial glial cells to promote the differentiation of the daughter cells into neurons or intermediate progenitors *(Ochiai et al.,2009; Lopez at al.,2016).* Apart from these, other signaling mechanisms such as MAPK pathway, integrin signaling, PDGFD signaling, PI3K/PTEN/AKT pathway, Hif1α signaling have been shown to regulate cerebral cortex development and gyrification *(Penisson et al.,2019).*

One of the important pathways,the canonical Wnt/β-catenin signaling pathway, is known to play important roles during cerebral cortex development. Binding of Wnt ligands to a complex composed of Frizzled and the lipoprotein receptor-related protein (LRP) located on the plasma membrane of apical progenitors, lead to stabilization of β-catenin and translocation to the nucleus, where they associate with the TCF/LEF transcription factors and promote the transcription of effector genes. In the absence of Wnt ligands, β-catenin is phosphorylated, ubiquitinated and degraded by the complex of proteins composed of Axin, APC, the Ser/Thr kinases GSK-3 and CK1, protein phosphatase PP2A, and the E3-ubiqutin ligase

b-TrCP *(Harrison-Uy and Pleasure.,2012; Cadigan.,2012).* Wnt signaling activity is known to be distributed along a dorsally restricted medial to lateral gradient in the cerebral cortex and decreasing activity upon increasing developmental stages *(Machon et al.,2007; Mutch et al.,2009; Pöschl et al.,2013)*. Expression of stabilized beta-catenin in the neural precursor cells in the developing mouse cortex lead to their expansion with increased mitoses resulting in folds resembling gyri and sulci *(Chenn et al.,2002)*. Moreover, persistent expression of stabilized beta-catenin lead to the expansion of RC2and Pax6 expression resulting in the delay in maturation of radial glial cells into intermediate progenitors expressing Tbr2 thereby negatively regulating the formation of basal progenitors *(Wrobel.,2007; Mutch et al.,2010)*. Whereas inhibition of β-catenin signaling in neural precursor cells lead to premature cell cycle exit and differentiation of neurons *(Woodhead.,2006; Draganova.,2015).* Wnt/β-catenin pathway has been shown to promote differentiation of late neural precursor cells *(Hirabayashi.,2004)* and experimental activation of Wnt-signaling in late apical progenitors lead to respecification to an early fate *(Oberst et al.,2019).* Transient dowregulation of canonical Wnt signaling is also found to play an important role in polarizing the cells for radial migration *(Boitard et al.,2015).* Thus, the effect of Wnt signaling in cortical development is time and context dependent. Furthermore, the importance of Wnt signaling have been illustrated by experimental manipulation of several components of the pathway. CDK-5 dependent phosphorylation of Axin is known to control the pool size of IPs and their switch from proliferative to differentiation status via β-catenin activation *(Fang et al.,2013).* APC, a critical component of the pathway is known to regulate β-catenin in the early embryonic cortex and determines the size of proliferative pool, the polarity of radial glial cells and formation of primary cilium and thereby regulate orderly differentiation of cortical progenitors *(Ivaniutsin et al.,2009; Yokota et al.,2009; Nakagawa et al.,2017).*

3. Intrinsic factors influencing cerebral cortex development

A number of transcription factors are expressed in the dorsolateral wall of the developing telencephalon, including Lhx2, Foxg1, Emx2 and Pax6. These play important roles in establishing the polarity and identity of neocortical domains, by repressing dorsal midline and ventral fates *(Rallu et al.,2002)*. Loss of Lhx2 and Foxg1 lead to the expansion of structures limited to the dorsal midline such as hippocampus and cortical hem *(Bulchand et al.,2001; Muzio and Mallamaci.,2005)*. Loss of Emx2 and Pax6 lead to the expansion of ventral progenitor domains into the cortex *(Muzio et al.,2002).* Pax6 is expressed at high levels in progenitor cells of the cortical VZ, which is important for the proliferation of RGCs and the repression of the neurogenic transcription factor Tbr2. Accordingly, basal progenitors downregulate Pax6, which unleashes the expression of Tbr2 and neurogenesis *(Götz et al.,1998; Sessa et al.,2008; Molyneaux et al.,2007).*

Basic helix loop helix (bHLH) transcription factors, such as those belonging to the families Ascl, Neurogenin, NeuroD, Olig, Id and Hes, play important roles in the establishment of cell fates *(Ross et al.,2003).* Factors from the Hes and Id families maintain the proliferation of progenitor cells by repression of pro-neural genes *(Iso et al.,2003; Justice and Jan.,2002).* On the other hand, proneural genes commit progenitor cells to the neuronal lineage: in the dorsal telencephalon, neurogenins lead to the generation of glutamatergic neurons, and in the ventral telencephalon Ascl1 drives the generation of GABAergic interneurons. Proneual genes induce the expression of pro-neural genes like β-tubulin and Neurofilament-M, promoting neurogenesis at the expense of gliogenesis. The transcription factor NeuroD begins its expression in immature neurons, maintained thereon in the cortical plate *(Nieto et al.,2001; Sun et al.,2001).* Finally, Olig1, Olig2 and Ngn2 belong to bHLH transcription factors implicated in the formation of oligodendrocytes *(Takebayashi et al.,2002)*.

Several proteins regulating the cytoskeleton control different aspect of neuronal migration, and their malfunction leading to dramatic brain malformations. These include doublecortin (DCX), filamin A alpha (FLNA), platelet-activating factor acetylhydrolase 1b, regulatory subunit 1 (PAFAH1B1, also LIS1), and genes

encoding tubulin subunits (TUBA1A, TUBB2B and TUBB3). Mutation in one or more of these genes leads to developmental cortical malformations in humans such as lissencephaly (absence of folds) or neuronal heterotopia, either periventricular or in cortical band *(Fernandez et al.,2016; Francis and Cappello.,2021; Guarnieri et al.,2018)*.

The laminar fate of cortical neurons is specified by distinct transcription factors, such as *Cux1, Cux2* and *Lhx2* being expressed in layers II/III to IV, *Brn2* in layer II/III and V, *Rorβ* in layer IV, *Opn3* in layer V and *Foxp2* in layer VI *(Molyneaux et al.,2007).*

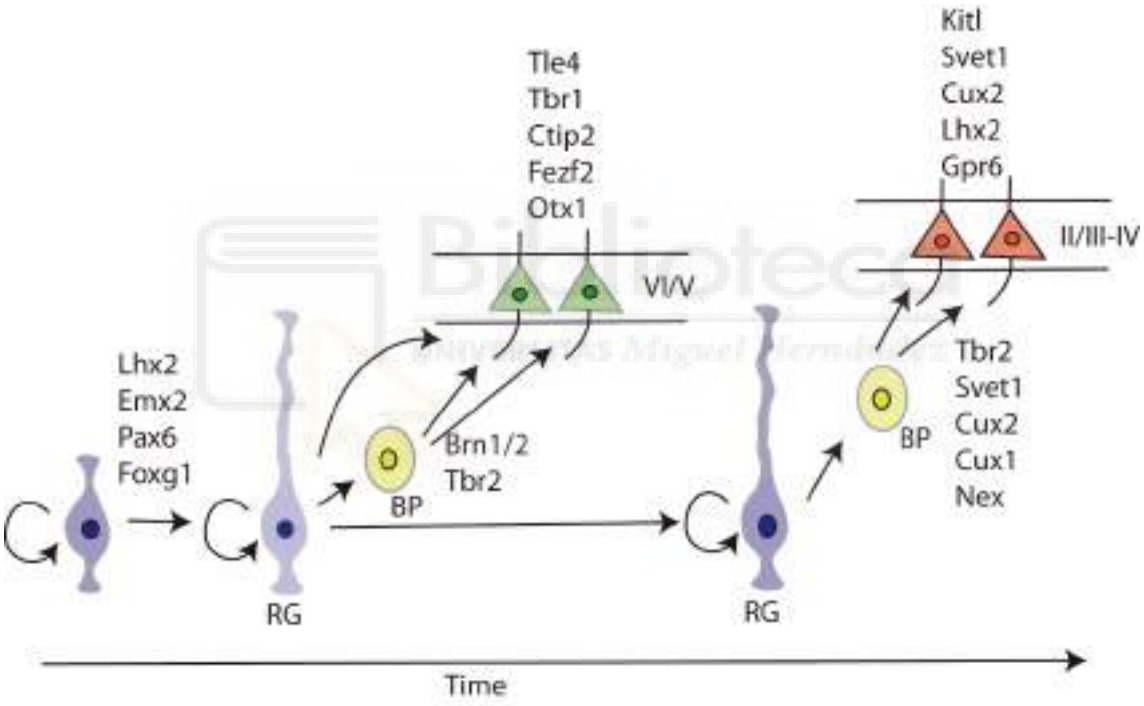


Fig. 3. Transcription factors important for neurogenesis. Different transcription factors that play important role in the self-renewal of radial glial cells, formation of intermediate progenitors, and thereby specification of different neuronal subtypes *(Modified from Molyneaux et al.,2007).*

Regarding the navigation and guidance of growing axons, this is signaled by a variety of attractive and repulsive molecular guidance factors, including netrins, ephrins, semaphorins, slit, neurotrophic factors, morphogens and neural cell adhesion molecules (L1, L2/HNK-1). These have been found to act on the growth

cone, regulating axon guidance through multiple signaling pathways *(Ye et al.,2019)*.

Single cell transcriptomic analyses of mouse cortex at different stages along neuronal differentiation identified the sequential dynamic expression of a core set of genes involved in proliferation, neurogenesis and neuronal differentiation, where newborn neurons show stage specific expression of different transcription factors during maturation (Figure 4; *Telley et al.,2016).* These analyses also identified differences in apical progenitors between early and late developmental stages. At early stages of development, processes related to cell cycle and nucleus/chromatin are predominant in apical progenitors, which determines the increased self-renewal capacity of progenitors. At late stages, there is an increased expression of membrane receptors, cell-cell signaling related proteins, and excitability related proteins in apical progenitors, which make them more responsive to environmental signals *(Telley et al.,2019).*

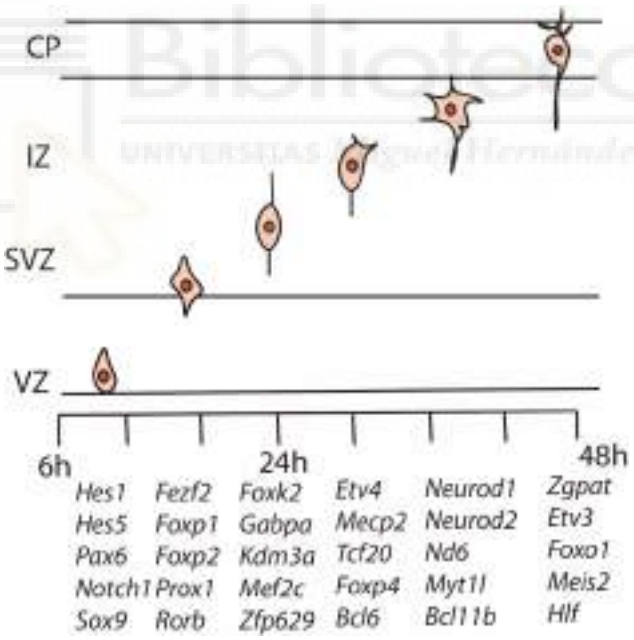


Fig 4. Sequential expression of different neuronal transcription factors post mitosis. Sequential expression of different transcription factors with increase in developmental time across stages of development *(Modified from Telley et al.,2016)*.

Several transcriptomic analyses have identified genes potentially relevant for the expansion and folding of the mammalian cortex, during phylogeny and ontogeny. These include genes specific to the human lineage, genes differentially

expressed between lissencephalic and gyrencephalic species, and genes differentially expressed between the germinal layers of prospective gyrus and sulcus regions. Some examples are shown in Figure 5 *(Fiddes et al., 2018; Fietz et al., 2012; Florio et al., 2015; Florio et al., 2017; Florio et al., 2018; Suzuki et al., 2018; de Juan Romero et al.,2015; Pollen et al.,2015)*. These genes include *Trnp1*, *Cdh1*, *Akna*, *ARHGAP11B*, *TMEM14B*, *Flrt1/3*, *NOTCH2NL*, *Fgf2* and *Hopx*, and have been found to be important for the process of gyrification in higher mammals (*Del Toro et al., 2017; Rash et al., 2013; Martinez-Martinez et al.,2016; Stahl et al., 2013; Camargo-Ortego et al.,2019; Florio et al.,2015; Liu et al.,2017; Vaid et al., 2018; Fiddes et al.,2018; Florio et al.,2018; Suzuki et al.,2018).*

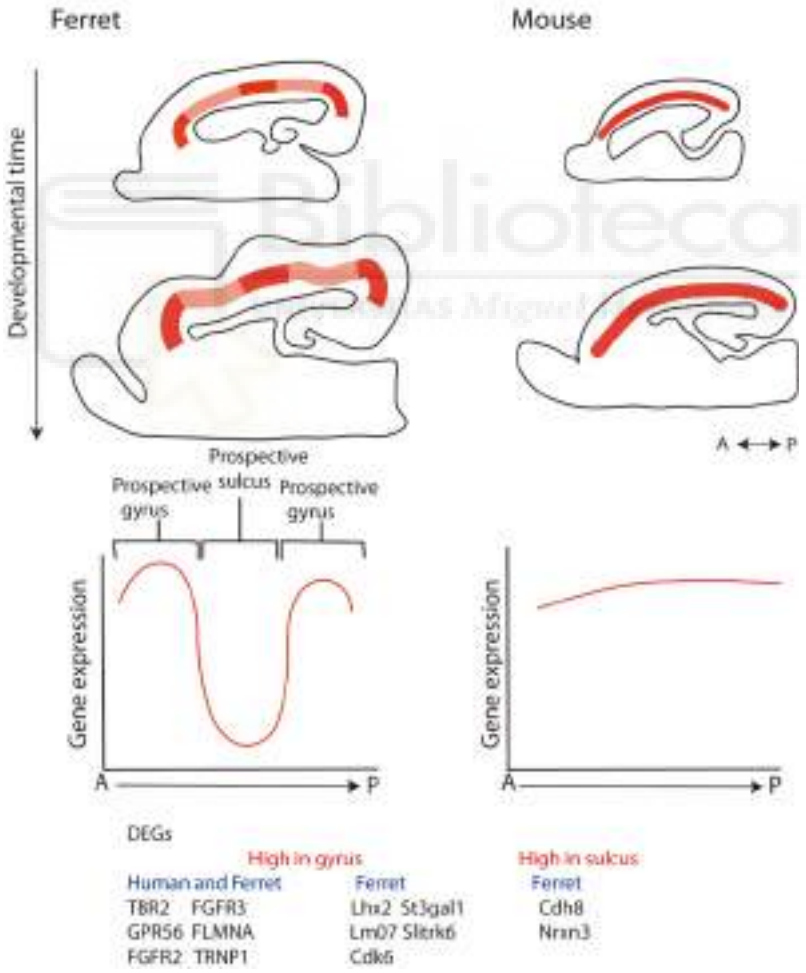


Fig.5 Genetic Patterning of cerebral cortex gyrification. Certain genes that show homogenous pattern of expression in lissencephalic mouse cortex, are found to exhibit modular expression pattern along Anterior-posterior axis in germinal zones of prospective folds and fissures. Examples of few genes that

show a varied pattern of expression in ferret and human are displayed here. *(Modified from Llinares-Benadero and Borrell.,2019)*

In addition to protein-coding genes, an increasing number of non-coding RNAs, including microRNAs, have been found to be expressed in neural cells and to be important for modulating the expression of several neural development genes, thereby regulating the development of cerebral cortex *(Qureshi and Mehler.,2012)*, as discussed below in detail.

5. MicroRNAs

Most protein coding DNA sequences remain highly conserved between species, even among those exhibiting a significant divergence of developmental complexity, whereas the amount of non-coding sequences increase consistently with complexity in development *(Taft et al.,2007)*. Among these non-coding sequences, several classes of regulatory factors have been discovered, including microRNAs (miRNAs). MiRNAs are endogenous short single stranded non-coding RNA molecules (20-23 nucleotides) that regulate gene expression through post transcriptional gene silencing, with important roles in a variety of physiological and developmental processes *(Bartel.,2004; Winter et al.,2009)*. The rate of acquisition of miRNAs is far greater along vertebrate evolution than any other genetic factor explaining chordate evolution, and this coincides with the dramatic increase in organism complexity of chordates *(Heimberg et al.,2008)*. Therefore, the increase in developmental complexity across evolution has been proposed to have resulted, at least in part, from the increased numbers and diversity of non-protein coding RNA molecules.

5.1 MicroRNA biogenesis

5.1.1 Canonical pathway

MiRNA genes are transcribed by either RNA pol II or pol III into primary miRNA transcripts (Pri-miRNA) *(Lee et al.,2004; Borchert et al.,2006)*. Pri-miRNAs

consist of a double stranded hairpin stem, a terminal loop on one side and two single stranded flanking regions on the other side of the hairpin *(Han et al.,2006; Zeng and Cullen.,2003).* Pri-miRNAs are cleaved in the nucleus by a microprocessor complex formed by the RNase III enzyme Drosha and DGCR8, which contains two double stranded RNA binding domains *(Denli et al.,2004; Gregory et al.,2004; Han et al.,2004)*. DGCR8 interacts with the pri-miRNA and functions as a molecular ruler to determine the precise cleavage site. The two RNase domains of Drosha cleave the 5´ and 3´ arms of the pri-miRNA hairpin, by cleaving 11 bp away from the junction between singe stranded RNA and double stranded RNA at the base of the hairpin, resulting in the formation of pre-miRNA *(Han et al.,2004)*.

Pre-miRNAs are then exported to the cytoplasm by Exportin-5 (XPO5), in complex with Ran-GTP *(Yi et al.,2003)*, and are subject to further maturation by the RISC loading complex (RLC). RLC is a multiprotein complex that consists of the RNase Dicer, the double stranded RNA binding domain proteins TRBP (Tar RNA binding protein) and PACT (Protein activator of PKR), and the core component Argonaute-2 (Ago2) *(Gregory et al.,2005; Macrae et al.,2008)*. The RNase III Dicer cleaves off the loop of the pre-miRNA and produces a microRNA duplex of the size of roughly 22 nucleotides with two nucleotide overhangs at each 3´ end *(Ketting et al.,2001)*. Dicer and its interactors TRBP/PACT dissociate from the miRNA duplex after Dicer-mediated cleavage. The double stranded duplex then unwinds and the functional guide strand is loaded into RISC. This process is facilitated by multiple helicases *(Salzman et al.,2007; Robb and Rana.,2007).* The complementary passenger strand mostly get degraded, although sometimes it can act as functional strand as well. Therefore, the functional RISC complex contains a single stranded mature miRNA that guides the complex to the target mRNAs.

The relative expression of each of the two miRNA strands may vary between tissues *(Biasiolo et al.,2011).* The functional strand is normally chosen based on the thermodynamic stability of the base pairs at the two ends of the duplex. The strand with the less stable base pair at its 5´ end is loaded into the RISC complex *(Khvorova et al.,2003).* The mature miRNA then guides the RISC to the target mRNA and cause degradation/ translational inhibition and or mRNA decay

(Winter et al.,2009). Most studies have shown miRNAs binding to 3´UTR of mRNAs, but in some cases miRNAs binding to 5´UTR or coding sequence have also been reported. Exceptionally, miRNA-mediated upregulation of gene expression has also been reported *(O´Brien et al.,2018).*

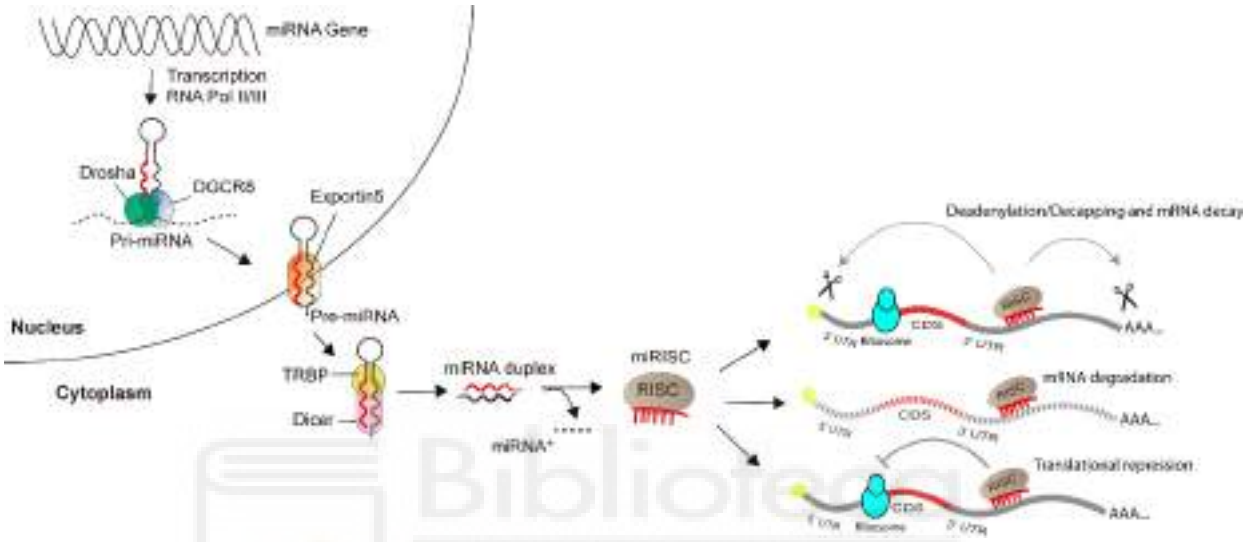


Fig.6. Canonical miRNA biogenesis pathway. miRNA gene is transcribed and cleaved in the nucleus to form pre-miRNA, which is exported into cytoplasm, where it is further cleaved to form mature miRNA which targets mRNA and cause target cleavage, translational repression or mRNA deadenylation, decapping and degradation *(Modified from Winter et al.,2009).*

5.1.2 Non-canonical pathways

Apart from miRNAs that go through the canonical biogenesis pathway described above, some miRNAs have also been identified to be processed through non-canonical pathways independent from Drosha or Dicer *(Miyoshi et al.,2010)*. Intron-derived miRNAs, called mirtrons, are released from the host transcripts as a result of splicing, bypass Drosha cleavage and are processed further by Dicer in the cytoplasm. These comprise a fraction of miRNAs in mammals and *Drosophila (Kim and Kim.,2007; Okamura et al.,2007).* MiRNAS have also been found to be produced from other classes of small RNAs such as snoRNAs, endo-shRNAs, tRNAs and endo-siRNAs, without the involvement of Drosha, in several

organisms including mammals and *Drosophila (Miyoshi et al.,2010)*. MiRNAs encoded by murine γ-herpesvirus 68 have been found to be processed by the tRNA processing enzyme tRNAse Z, rather than by Drosha *(Bogerd.,2010)*. MiRNAs like miR-451 and those derived from tRNAs have been known to be produced through a Dicer independent pathway *(Cifuentes et al.,2010; Haussecker et al.,2010).* Different non-canonical pathways of miRNA biogenesis that occur in mammals are illustrated in Figure 7.

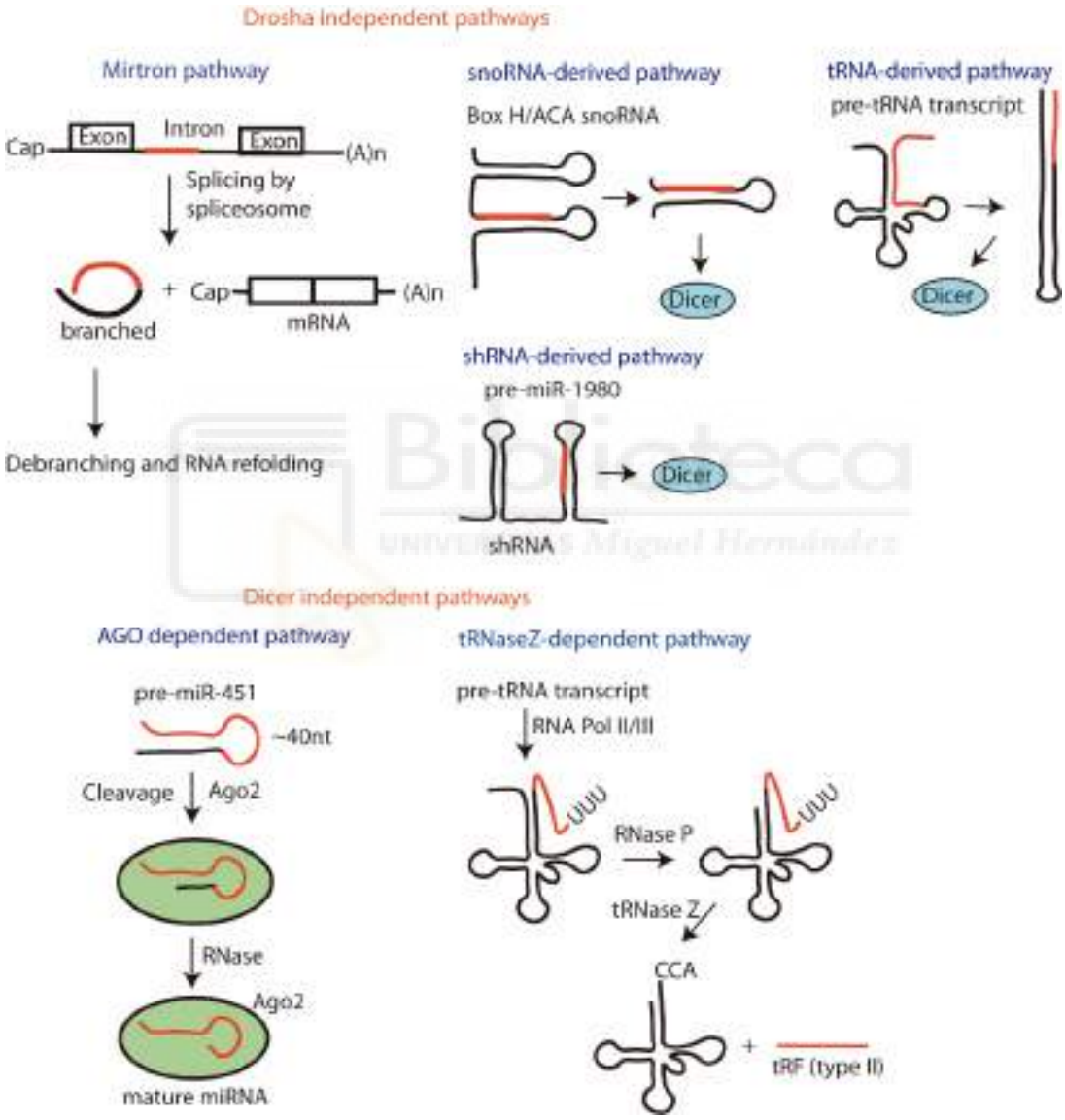


Fig. 7. Non-canonical miRNA biogenesis pathways. Several non-canonical miRNA biogenesis pathways that occur in mammals, which are either independent of Drosha or Dicer processing are depicted here *(Modified from Miyoshi et al.,2010)*.

5.2 MiRNA function

MiRNAs can either completely shut down gene expression or act as fine-tuners. Most miRNAs are pleiotropic, where one miRNA targets several genes causing mild to moderate effects on each. However, miRNAs usually act cooperatively, where multiple miRNAs target the same gene, therefore causing strong repression. In the context of brain development, the highly conserved and brain-enriched miRNAs miR9 and miR124 represent good examples of miRNAs regulating multiple genes in a context-dependent manner, thereby controlling multiple stages of neural development *(Gao.,2010; Radhakrishnan and Alwin Prem Anand.,2016)*. MiRNAs produced from a given genomic cluster, or enriched during a particular spatial-temporal window, may act together to regulate key developmental genes *(Tsang et al.,2010; Cherone et al.,2019)*, or they may target different components of a common signaling pathway and cause alterations to that pathway (*Gebert and MacRae et al.,2019).* Examples of miRNA pleiotropy of regulation in the context of neurogenesis are shown in Figure 8.

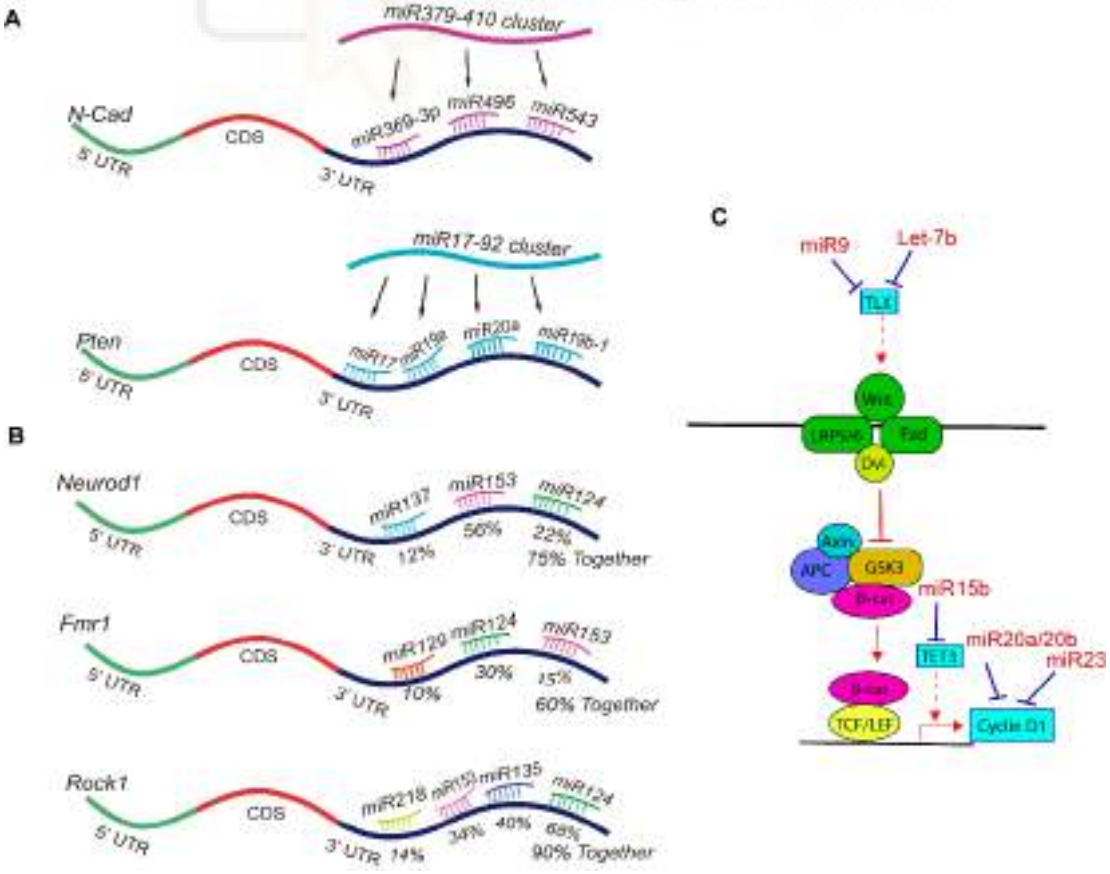


Fig. 8. MiRNA Targeting mechanisms. (A) MiRNAs produced from the same cluster targeting genes together causing strong repression. (B) Independent microRNAs cooperatively targeting different genes together, Suppression of particular gene caused by cooperative binding of different miRNAs together is greater than that caused individual miRNAs. Each miRNA is depicted in a different color. Each microRNA can target several genes and a particular gene can be repressed by several microRNAs together. (C) Different components of canonical Wnt signaling being targeted by different miRNAs.

MiRNAs form a functional unit with an AGO protein. The mammalian genome encodes four AGO proteins (Ago1-4), with Ago2 being most highly expressed. AGO is a single polypeptide chain composed of four domains: the amino-terminal domain (N), the Piwi-Argonaute-Zwille domain (PAZ), the middle domain (MID) and the P-element induced wimpy testes domain (PIWI). Two linker domains L1 and L2 connect the N and PAZ domains, and the PAZ and MID domains respectively. The MID and PIWI domains hold the 5´ end of the miRNA and the PAZ domain binds its 3´ nucleotide *(Schirle and MacRae.,2012; Sheu-Gruttadauria and MacRae.,2017; Diederichs and Haber.,2007).* The degree of complementarity between the miRNA and the target mRNA (miRNA response element) determines the Ago2-mediated processing of target mRNAs, where complete complementarity leads to AGO2 endonuclease activity and mRNA cleavage, whereas minimal pairing leads to translational inhibition and/or mRNA decay. Most miRNAs target the 3´UTR of mRNA targets, which depends on the complementarity to the seed sequence (position 2-8). Canonical target sites include those that are complementary to miRNA nucleotides 2-8 or 2-7, with or without A at its first position before complementary nucleotides to the seed sequence. ´A´ at the first position of the target site is not recognized by the miRNA, but by the binding pocket within AGO *(Agarwal et al.,2015; Wong et al.,2015).* Previous studies suggested that target recognition is achieved by a two-step mechanism, where the first 2-6 nucleotides of the seed are organized by the MID and PIWI domains, which allows rapid initial binding and complementarity to nucleotides 7-8 lead to a stable binding. In addition to the seed sequence complementarity, nucleotides 13-16 in the miRNA are called supplemental region and can also contribute to target binding. Apart from these, non-canonical binding sites with minimal seed pairing have also been reported *(Grimson et al.,2007; Helwak et al.,2013; Kim et al.,2016).*

Gene silencing caused by AGO-miRNA binding to the 3´UTR occurs through mRNA decay or translation repression. mRNA decay is responsible for 66-90% of silencing *(Guo et al.,2010)*, and it involves recruitment of a member of the glycine-tryptophan protein of 182 kDa (GW182) protein family by AGO. GW182 interacts with polyadenylate–binding protein (PABPC) and promotes mRNA deadenylation by recruiting the poly(A) nuclease 2 and 3, and carbon catabolite repressor protein 4-NOT complexes. Deadenylation is then followed by decapping by a complex composed by the mRNA-decapping enzyme subunits 1 and 2, which makes the mRNA susceptible to rapid degradation by 5´-3´ exoribonuclease 1 *(Rehwinkel et al.,2005; Chen et al.,2009; Behm-Ansmant.,2006)*. CCR4-NOT complex recruitment also leads to translation repression by recruiting RNA helicase DDX6 *(Mathys et al.,2014)*. Although the mechanism of translation inhibition seems unclear, some studies suggest that miRNA-RISC complex inhibits translational initiation by inducing the dissociation of eukaryotic initiation factors 4A-1 and 4A-2, thereby inhibiting ribosome scanning and assembly of the eIF4F translation initiation complex *(Meijer et al.,2013; Fukaya et al.,2014; Fukao et al.,2014).*

5.3 MiRNA regulation

MiRNA biogenesis and activity may be affected by differential regulation of cellular processes that alter the sequence of a miRNA or its precursor. Alternative processing and variation of the cleavage site by Drosha and Dicer have been shown to alter the sequence of some miRNAs, involving shift of the seed position and causing the formation of isomirs *(Kim et al.,2017; Neilsen et al.,2012).* MiRNA precursor sequence may undergo RNA editing, leading to the generation of isomirs and changes in biogenesis leading to sequence variations, deamination being the most commonly observed editing process. A-to-I editing by adenosine deaminase, and C-to-U editing by cytidine deaminase, acting on RNA have been shown to interfere with miRNA cleavage by Drosha or Dicer, and hence to edit the seed sequence *(Nishikura.,2016; Blow et al.,2006; Kawahara et al.,2008)*. Non-template addition of nucleotides to the miRNA, particularly adenylation or uridylation at 3´ ends by several enzymes, is known to modulate miRNA stability

(either increasing stability or promoting degradation) *(Katoh et al.,2009; Katoh et al.,2015; Jones et al.,2009; Gutierrez-Vazquez et al.,2017)*. The stability and turnover rates are miRNA specific and can be tissue specific. Some miRNAs are generally stable, while some are intrinsically unstable. Some miRNAs are stable until degraded in response to specific developmental cues, and miRNA stability also seems to depend on tissue context, with fast turnover rates observed particularly in neuronal miRNAs *(Guo et al.,2015; Krol et al.,2010).* A mechanism of miRNA destabilization called target RNA-directed miRNA degradation (TDMD) works via extensive pairing between the miRNA's 5´ and 3´ ends to their target RNAs, and it is often found associated with 3´ NTA, or tailing and with 3´-5´ trimming. TDMD inducers can be of viral origin or endogenous *(Ameres et al.,2010; la Mata et al.,2015; Haas et al.,2016)*. Apart from the above mechanisms, phosphorylation of miRNA has also been found to regulate its activity, such as for miR-34 *(Salzman et al.,2016).*

In addition to changes in miRNA sequence, post-translational modification of different residues of AGO proteins also affects the activity of miRNAs. Phosphorylation of Ser387 in the L2 region of Ago2 has been reported to stimulate the assembly of miRISC, whereas phosphorylation of Tyr393 in the L2 region and Tyr529 in the MID domain blocks miRNA loading. Phosphorylation of residues of S824-S834 cluster in the PIWI domain regulates the release of target mRNAs, that prevents overly long association with targets *(Zeng et al.,2008; Rüdel et al.,2011).* Other post-translational modifications including hydroxylation, PARylation, ubiquitination and SUMOylation have also been shown to regulate miRNA activity *(Gebert and MacRae.,2019).*

Endogenous RNAs including lncRNAs, pseudogenes, mRNAs and circRNAs have been known to sequester miRNAs, allowing the expression of their targets in development and cancer *(Thomson and Dinger.,2016).* In addition to changes in the miRNA sequence and the machinery components, RNA editing can alter the target sites, and formation of 3´ UTR isoforms that add or remove target sites alter the susceptibility of mRNAs to miRNA in a cell-type or tissue-type specific manner *(Zhang et al.,2016).*

6. MiRNAs in development

The first small RNA identified, *lin-4*, which was later identified as a miRNA, was discovered in *C.elegans* by Lee and colleagues as responsible for downregulation of Lin-14 protein levels through a post-transcriptional regulatory mechanism involving antisense pairing, which is essential for the developmental transition from first larval stage to the subsequent stages of development *(Lee et al.,1993).* Subsequently, a small RNA called *let-7* was identified as being important for controlling developmental timing in *C.elegans*, by negatively regulating the expression of heterochronic genes *(Slack et al.,2000, Reinhart et al.,2000)*, and its expression was detected across a wide range of animal species *(Pasquinelli et al.,2000).* Since then, many miRNAs have been identified to be conserved across several different species as master regulators for fine-tuning the expression of several genes involved in development.

Global elimination of miRNAs by full knock-out of *Dicer* in fruit flies, Zebrafish and mice, causes embryonic lethality, growth arrest and abnormal development of organs, indicating their critical importance in development *(Kloosterman and Plasterk.,2006; Bernstein et al.,2003).* Several miRNAs are known to regulate the cell cycle of embryonic stem cells, maintaining their pluripotency and differentiation. MiRNAs are also important for the proper development of different organs, including the nervous system, heart, lung, skeletal muscle and skin *(Bhaskaran and Mohan.,2014; Rago et al.,2019)*.

7. MiRNAs in cerebral cortex development

7.1 Conditional knock-out studies

Several studies involving conditional knock-out in mice of components of the miRNA biogenesis machinery demonstrate the importance of miRNAs for the appropriate development of the cerebral cortex. Studies where Dicer was conditionally deleted from cortical progenitors at different developmental time points shown a diversity of phenotypes with different degrees of severity.

Studies using Foxg1-cre deletion of Dicer, which eliminated Dicer in neural progenitor cells around E11.5, showed decreased expression of markers of RGCs such as Nestin, Sox9 and ErbB2, disorganization of IPCs and neurons, and increased apoptosis over time, although the relative proportion of different neuronal subtypes appeared normal *(Nowakowski et al.,2011)*.

Emx1-cre dependent deletion of Dicer, occurring in neuroepithelial cells from E10.5, caused the apopotosis of progenitor cells and defective differentiation of neurons at postnatal stages. Production of upper layer neurons was also decreased, the expression of the transcription factor Foxp2 was lost (a gene important for neuronal differentiation) and cortical connections were reduced and disorganized *(De Pietri Tonelli et al., 2008)*. Other studies using Emx1-cre dependent deletion of Dicer showed that this caused the reduction in thickness of germinal layers with increased apoptosis, increased production of deep layer neurons at the expense of the formation of upper layer neurons, and absence of callosal projections *(Saurat et al., 2013)*.

Deletion of Dicer slightly later during development using a Nestin-cre line led to a relatively milder phenotype, where cortical development was largely normal and defects occurring only in the production of late born neurons and neuronal migration *(Kawase-Koga et al.,2009)*. Other studies using Nestin-cre deletion of Dicer showed reductions in progenitor cell proliferation, defects in migration and differentiation of neurons, with precocious differentiation of astrocytes *(McLoughlin et al.,2012)*.

Dicer ablation using CaMKII-cre line led to increased apoptosis and microcephaly, enlargement of lateral ventricles, and defects in axonal path finding *(Davis et al.,2008)*.

More recently, a study from our laboratory using Rax-cre conditional Dicer knock-out, where the loss of Dicer occurred very early in the primordium of telencephalon at E7.5, revealed severe tissue disorganization. Highly proliferative rosettes formed in the rostral telencephalon as a result of downregulation of let-7 miRNAs, leading to the upregulation of IRS-2 and then to the overproliferation of progenitor cells. These results revealed the critical importance of miRNA timing at early stages to preserve the cellular homeostasis

and organization of the neuroepithelium at the rostral telencephalon *(Fernandez et al.,2020).*

Other studies using Drosha or DGCR8 conditional knock-outs reported a variety of cortical phenotypes that are distinct from the Dicer knock-out phenotypes. The depletion of miRNAs in these cases appears to be smaller compared to Dicer knock-outs, mostly implying miRNA independent functions of these microprocessor complex proteins *(Knuckles et al.,2012, Marinaro et al.,2017, Babiarz et al.,2011)*.

7.2 Specific miRNAs in cerebral cortex development

MiRNA profiling studies in the developing mouse and rat cerebral cortex identified several miRNAs being differentially expressed across stages of development, suggesting their involvement in specific aspects of cortical development *(Krichevsky et al.,2003, Nielsen et al.,2009, Yao et al.,2012).* Aside from their roles in progenitor cell proliferation, some miRNAs are specifically enriched in more mature cell types including neurons and glial cells, and involved in determination of cell fates during development *(Jovicic et al.,2013, He et al.,2012).* Figure 9 depicts examples of miRNAs and their target genes involved in cell fate determination of neural progenitors into neurons or glial, and their further subtype specification.

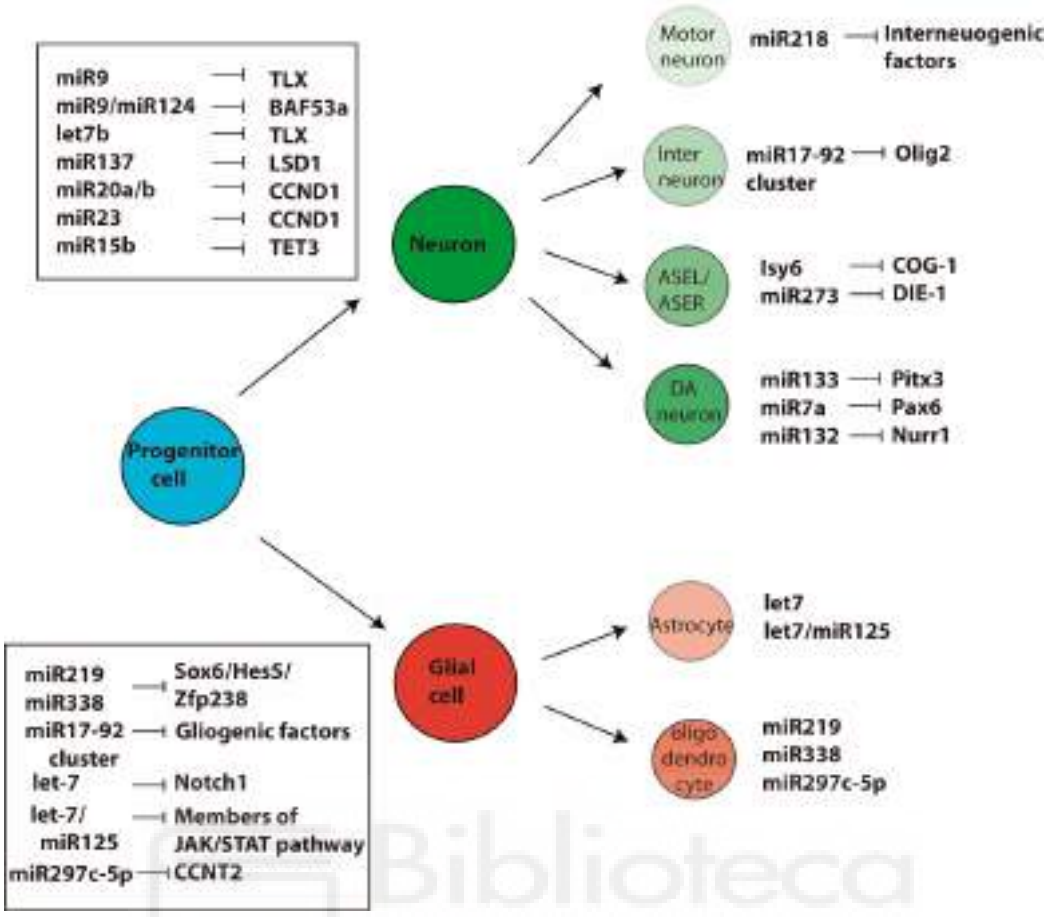


Fig. 9.microRNAs and their target genes involved in specification of cell fate. MiRNAs involved in the specification of progenitors into neurons and further into neuronal subtypes including motor neuron, interneuron, ASEL/ASER bilateral taste receptor neurons and dopaminergic (DA) neurons are indicated in blue. MiRNAs involved in the specification of progenitors into glial cell fate and further into different types of astrocytes and oligodendrocytes are indicated in red *(Rajman and Schratt.,2017).*

MiRNAs are important in several stages of neuronal development involving neurogenesis, polarization and migration of neurons, specification of different neuronal subtypes, axonal path finding, dendritogenesis and synapse development as follows.

7.2.1. Neurogenesis

miR-9, a miRNA highly conserved across mammals, is mostly expressed in neuronal precursors *(Delaloy et al.,2010).* Its overexpression causes neuronal

differentiation by directly inhibiting the nuclear TLX receptor, an upstream activator of the Wnt signaling pathway and important regulator of neural stem cell renewal. TLX in turn negatively regulates miR-9 expression through a negative feedback mechanism *(Zhao et al.,2009).* Also miR-137 has been shown to regulate TLX by directly inhibiting histone lysine-specific demethylase 1, a TLX transcriptional corepressor *(Sun et al.,2011)*. In addition, several miRNAs regulate Wnt signaling pathway, which is important for neurogenesis. miR-20a/20b and miR-23 have been shown to inhibit the expression of cyclin-D1 by directly binding to its 3´-UTR *(Ghosh et al.,2014).* miR-15b inhibits Tet methylcytosine dioxygenase 3 (TET3), which regulates the methylation status of the cyclin D1 promoter and in turn inhibits cyclin D1 expression *(Lv et al.,2014).* miR-9 has also been shown to promote progenitor cell apoptosis in the forebrain and to control the number of neurons in the cortex by repressing pro-gliogenic factors *(Bonev et al.,2011; Zhao et al.,2010)*. The miR17-92 cluster has been shown to be important to regulate the balance between RGCs and IPCs by targeting Pten and Tbr2 *(Bian et al.,2013).* miR-124, one of the miRNAs most highly expressed in the brain and highly conserved among mammals, is important for neuronal differentiation. miR-9 and miR-124 are known to drive neural progenitors towards the neuronal fate, by repressing their common target RE1-Silencing Transcription factor (REST) *(Visvanathan et al.,2007; Packer et al.,2008).* Additional miRNAs have been found to target the Notch pathway and thereby regulate neurogenesis. miR-34a and miR23b/24/27b repress Notch1, and miR-34a represses other regulators and effectors of the Notch pathway including Numb1, NeuroD1 and Mash1; finally, miR-9 represses Blbp, a downstream target of Notch1 *(Fukuda et al.,2005; Kuang et al.,2012).* MiRNAs including miR-34a and miR-29a are known to target p53 pathway and thereby control cell survival *(Raver-Shapira et al.,2007; Park et al.,2009).* Many of these miRNAs are also known to function differently by targeting different genes depending on the context.

7.2.2. Polarization and migration of neurons

Defects in neuron migration are known to cause severe neurodevelopmental disorders, and miRNAs have been found to be important in regulating factors key for neuronal migration. For example, miR-22 and miR-214 are important for the multipolar to bipolar transition of migrating neurons, facilitating their progression to the cortical plate, *(Volvert et al.,2014)*. miR-9 and miR-132 regulate the radial migration of cortical neurons by inhibiting the expression of Foxp2 *(Clovis et al.,2012).* Many miRNAs target doublecortin (Dcx), a microtubule associated protein well known for its effects on migration of neurons, and the mutation of which causes lissencephaly in humans. miR-34c, miR-204 and miR-134 target Dcx directly *(Veno et al.,2017; Gaughwin et al.,2011)*, whereas miR-22 and miR-124 target members of the CoREST/REST transcriptional repressor complex, thereby indirectly downregulating Dcx expression *(Volvert et al.,2014).* Members of the miR-379-410 cluster (miR-369-3p, miR-496 and miR-543) promote neuronal migration by targeting N-cadherin and reducing its expression *(Rago et al.,2014).*

7.2.3. Specification of neuronal subtypes

MiRNA profiling of glutamatergic and GABAergic neurons identified several miRNAs selectively enriched in these neuronal subtypes, suggesting their potential role in determining neuronal subtypes *(He et al.,2012)*. miR-9 represses Foxg1 and thereby promotes the generation of Cajal-Retzius cells and other early-born neurons *(Shibata et al.,2008)*. Recently, miR-128, miR-9 and let-7 have been shown to be expressed forming temporally opposing gradients, thus patterning the formation of cortical neuronal layers *(Shu et al.,2019)*. miR-125b and miR-181a promote the generation of dopaminergic neurons and miR-181a* inhibits this process *(Stappert et al.,2013).* miR-133b has been identified to regulate the differentiation of dopaminergic neurons by repressing the expression of the transcription factor Pitx3 *(Kim et al.,2007)*. Finally, miR-7a and miR-132

are important for differentiation of dopaminergic neurons by inhibiting Pax6 and Nurr1, respectively *(de Chevigny et al.,2012; Yang et al.,2012).*

7.2.4 Axonal growth and dendritogenesis

Once neurons acquire appropriate cell fate and position, they begin differentiation by growing axons and dendrites to establish functional connections. Two miRNAs localized in axons of rat cortical neurons, miR-338 and miR-181c, attenuate axonal growth by regulating the expression of factors involved in axon guidance *(Kos et al.,2016).* In mouse cortical neurons, miR-9 regulates the expression of microtubule associated protein 1B (MAP1B), which is important in axonal growth and branching *(Dajas-Bailador et al.,2012).* miR-134 overexpression in mouse brain reduces cortical pyramidal neuron dendritogenesis *(Christensen at al.,2010),* and miR-185 controls dendritic plasticity by suppressing Mirta22 *(Xu et al.,2013).*

Examples of miRNAs, their gene targets, and signaling pathways affected, that act in concert at several stages of formation and maturation of neurons to ensure the proper development of the cerebral cortex are shown in Figure 10.

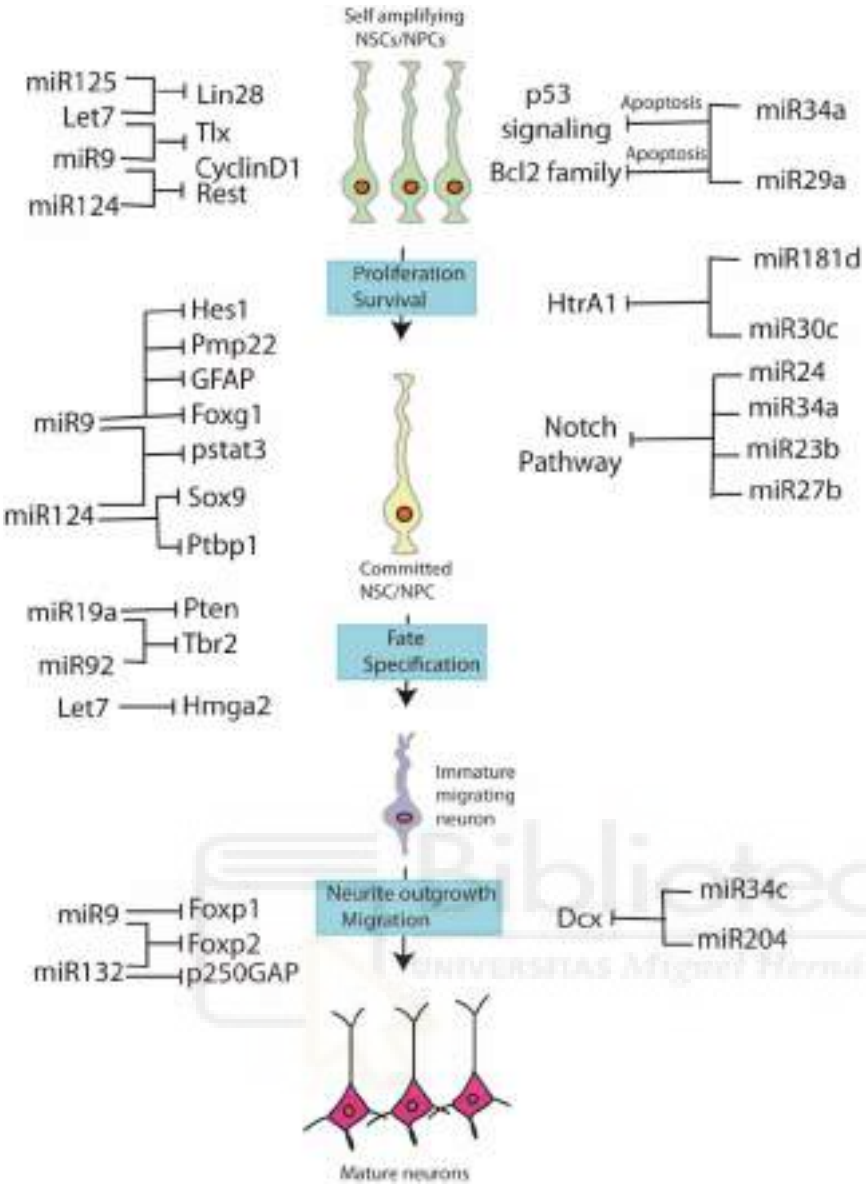


Fig. 10. MiRNAs and their targets in the developing cortex. MiRNAs and their target genes being regulated at different steps of neurogenesis *(Modified from Barca-Mayo et al.,2014).*

8. MiRNAs relevant for cerebral cortex evolution

During evolution, novel miRNAs were introduced at points of speciation divergence, suggesting an increase in the complexity of gene expression regulation and its potential contribution to the evolution of complex biological systems and organisms *(Heimberg et al.,2008)*. A single genomic cluster specific to eutherians (placental mammals) 12qF1, which encodes 15 miRNAs enriched

in corticospinal motor neurons (CSMN), coevolved with the motor cortex and corpus callosum. One of these miRNAs, miR-409-3p, is differentially expressed between developing CSMN and CPN (cortical projection neurons) and promotes CSMN development by repressing LMO4 *(Diaz et al.,2020)*. Similarly, numerous primate-specific miRNAs have been identified and shown to be expressed in the cortical germinal zones VZ and OSVZ of macaque embryos, while absent in the genomes of rodents. Some of these miRNAs appeared *de novo* in primates, while others resemble miRNAs in carnivores and may have been lost in rodents, and finally others resemble rodent miRNAs but with multiple mismatches, possibly impairing their function as miRNAs in rodents. The identification of primate miRNAs that target cell cycle regulation and proliferation/differentiation pathways suggests their importance in determining neuron production and cell diversity, thereby leading to the expansion and complexification of the cerebral cortex. For example, the primate-specific miRNA miR-1180-3p targets Kansl1, an evolutionarily conserved regulator of the chromatin modifier KAT8 that functions through histone H4 lysine 16 acetylation and dlx1, a homeodomain transcription factor that controls neurogenesis. Another primate-specific miRNA, miR-1301-3p, targets Mll1, an evolutionarily conserved histone H3 lysine 4 methyltransferase *(Arcila et al.,2014, Dehay et al.,2015)*. Other examples of primate-specific miRNAs that regulate cell cycle regulation are shown in Figure 11.

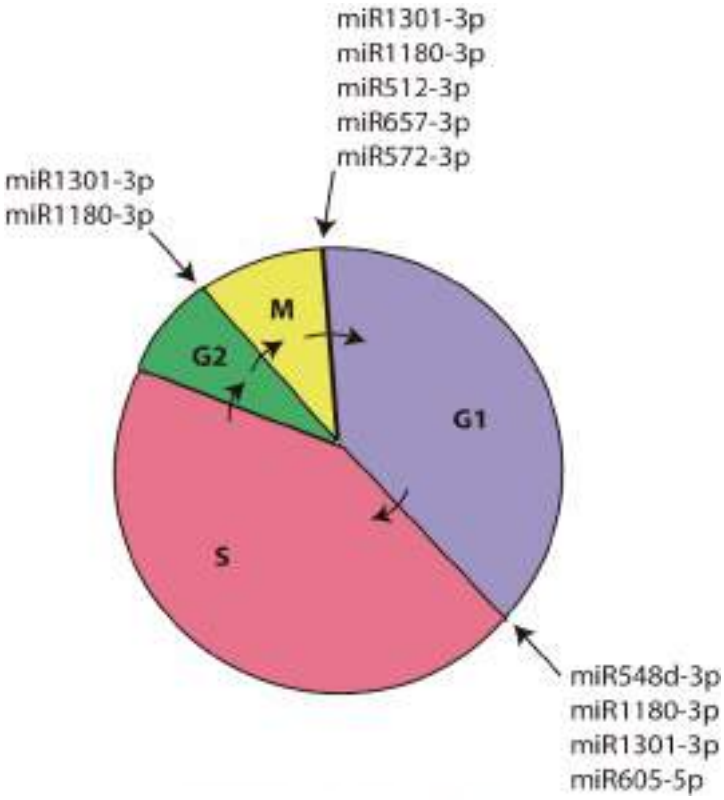


Fig. 11. Cell cycle regulation of primate-specific miRNAs. Arrows indicate different cell-cycle checkpoints where miRNAs act. G1-S-G2-M refer to the different stages of cell cycle *(Modified from Dehay et al.,2015).*

Studies carried out in human embryonic brain samples and a combination of analytical approaches, including high-throughput sequencing of RNA bound to AGO2 (AGO2-HITS-CLIP), single-cell RNA sequencing and computational analyses, have identified several miRNAs enriched in different cell types, and the target genes of which are expressed dynamically through different stages of development. In particular, two types of miRNA-mRNA interactions were identified: 1) where miRNAs expressed in a cell type regulate the expression of their target genes expressed in the same cell type; and 2) miRNAs expressed in a cell type to repress target genes that are not expressed in that same cell type. MiRNAs specific to great apes have also been identified, such as miR-2115, which is differentially expressed between embryonic stages with different proliferation capacity of VZ, suggesting the importance of this miRNA in regulating progenitor cell proliferation and, therefore, leading to the development of bigger brains. Indeed, miR-2115 targets ORC4, a known regulator of DNA replication whose mutations are linked to Meier-Gorlin syndrome, associated with

microcephaly *(Nowakowski et al.,2018).* Another primate specific miRNA, miR-934, has been recently identified and shown to be expressed at specific stages of early development and to control early neuronal differentiation *(Prodromidou et al.,2020).*

9. MIR3607

MIR3607-5p (annotated as small nucleolar RNA SNORD138) was initially identified in irradiated human cells *(Chaudhry et al.,2013)*. Expression of *MIR3607* was found to be significantly attenuated in prostate cancer specimens, and its low expression was correlated to disease progression. In this case, *MIR3607* was found to target and repress oncogenic SRC family kinases, LYN and SRC *(Saini et al., 2014)*. In contrast, it was also found highly expressed in human lung cancer cells and target APC, a negative regulator of the Wnt signaling pathway, thereby it promotes Wnt signaling leading to the upregulation of cyclinD1 and c-Myc, ultimately increasing cell proliferation *(Lin et al.,2017).* Recently, the dysregulated expression of *MIR3607* has been identified in colorectal cancer and hepatocellular carcinoma *(Lei et al.,2019; Dou et al.,2020).* The very different roles of this miRNA in different types of cancer suggest that its function is highly context-dependent, possibly by targeting different genes. *MIR-3607* was also identified as a novel miRNA in the human brain at fetal, young and adult stages, suggesting its likely importance in the development, organization and function of the human brain *(Moreau et al., 2013).*

OBJECTIVES

Several studies have previously related various types of cancer with deregulated expression of *MIR3607 (Saini et al., 2014; Lin et al.,2017; Lei et al.,2019; Dou et al.,2020)*. Expression of *MIR3607* has been detected in human brain and macaque cerebral cortex previously *(Moreau et al., 2013; Arcila et al.,2014)*, but not in mouse, and our previous transcriptomic analyses indicated its expression in the developing ferret, suggesting a conserved role in cortical expansion. Remarkably, the role of this promising microRNA in cerebral cortex development has never been studied. The main goal of this Project is to study the role of *MIR3607* in cerebral cortex development and expansion. This major goal was addressed with the following specific aims:

1. To determine the expression pattern of miR3607 in the developing mouse, ferret and human cerebral cortex.

2. To investigate the function of miR3607 in mouse cortical development.

3. To identify genes and signaling pathways targeted by miR3607 in the developing mouse cerebral cortex, and validate the best candidates by performing rescue experiments.

4. To investigate the evolutionary conservation of miR3607 function in cortical development, and its mechanism of action, by gain and loss of function experiments in gyrencephalic species, using ferret and human cerebral organoids as models.

MATERIALS AND METHODS

Experimental models

Mice

Wild-type mice used for experiments were maintained in an Institute for Cancer Research; Harlan Inc. (ICR) background. Mice were maintained on a 16h:8h light:dark cycle at the Instituto de Neurociencias de Alicante in accordance with Spanish (RD 53/2013) and EU regulations. Experimental protocols were approved by the Universidad Miguel Hernández Institutional Animal Care and Use Committee (IACUC). Healthy pregnant female mice used for *in utero* electroporation were at embryonic stages E12.5 and E14.5 according to the requirements of the project, and embryos were used without gender bias. The day of vaginal plug was considered as E0.5.

Ferret

Processed and cryopreserved brain sections of embryo (E35) and postnatal kit (P2) were used for *in situ* hybridization. Pregnant wildtype ferrets used *for in utero* electroporation were obtained from Euroferret (Copenhagen, Denmark), maintained on a 16h:8-h light:dark cycle at the Animal Facilities of the Universidad Miguel Hernández, treated according to Spanish and European regulations and experimental protocols were approved by the Universidad Miguel Hernández IACUC.

Human embryonic tissue

Processed brain sections embedded in paraffin used for *in situ* hybridization were obtained from spontaneous abortions of human embryos from the service of Pathology, Hospital Universitario “Principe de Asturias”, Alcalá de Henares, Spain. Tissue was extracted in accordance with the Spanish law on clinical

autopsies (Boletín Oficial del Estado [BOE] of 27 June 1980 and BOE of 11 September 1982).

Human cerebral organoids

Human cerebral organoids were generated following the Lancaster & Knoblich, 2014 protocol.

Plasmid constructs

The plasmid constructs mainly used for gain of function experiments were pCAG-GFP encoding GFP under the control of the CAG promoter, psil-pre-miRScr encoding scrambled control and psil-pre-miR3607 encoding miR3607-5p. Oligos encoding miR3607 (SIGMA) were designed with BamHI-HindIII sites and cloned into pSilencer2.1-U6 puro (Ambion). pSilencer puro negative control plasmid provided with the kit was used as a psil-pre-miRScr, encoding siRNA whose sequence is not found in the mouse, human, or rat genome databases.

pre-miR3607:

5´GATCCGACTGATTTCCTTCATGTCAAGCTTCAAGAGAGCATGTGATGAAG CAAATCAGTTTTTTT3´

The ligation products were transformed into competent *Escherichia coli*, plated on LB/Amp (Luria Broth/Ampicilin), amplified and sequenced to confirm the presence of the insert. Plasmid DNA was purified with an NucleoBond Xtra Midi kit (Cultech, 22740410.50), and resuspended in nuclease free water (SIGMA).

Other plasmids used were pCMV-GFP encoding GFP under CMV promoter, pCMV-Neo-Bam APC (Plasmid #16507, Addgene) encoding APC (Morin et al.,1997), pCAG-delta90GFP (Plasmid #26645, Addgene) encoding stabilized beta catenin (Wrobel et al.,2007).

The plasmid constructs used for loss of function experiments (TUDSCR control and TUD3607 to repress miR3607) were designed as per *Haraguchi et al.,2009*

and cloned into pSilencer2.1-U6 puro (Ambion) following the same protocol mentioned above.

TUDSCR

5´GATCCGACGGCGCTAGGATCATCaacTGGGCGTATAGACatctGTGTTCGT TCcaaGTATTCTGGTCACAGAATACaacTGGGCGTATAGACatctGTGTTCGTT CcaaGATGATCCTAGCGCCGTCTTTTTTGGAAA3´

TUD3607

5´ GATCCGACGGCGCTAGGATCATCaac ACTGATTTGCTTatctCATCACATG CcaaGTATTCTGGTCACAGAATACaacACTGATTTGCTTatctCATCACATGCca aGATGATCCTAGCGCCGTCTTTTTTGGAAA 3´

Validation of plasmid constructs using miRNA qRT-PCR

Human embryonic kidney293T (HEK293) cells were transfected with 2µg of psil-pre-miRScr or psil-pre-miR3607/ TUDSCR or TUD3607 with 2µg of pCAG-GFP using lipofectamine, harvested after 2 days, isolated RNA using *mir*Vana™ miRNA isolation kit (Cat #AM1560, Thermo Fischer Scientific), and carried out qRT-PCR to measure the expression of miR3607 using Taqman microRNA Assays (Thermo Fischer Scientific). Primers and probes for miR3607 (Assay #463448_mat) and U6 snoRNA control (Assay #001973) were ordered from Thermo Fischer Scientific (Cat #4427975).

in utero electroporation of mouse

The *in utero* electroporations were performed with ICR wildtype pregnant mice at the embryonic stages E12.5 and E14.5 in the neocortex. Surgical instruments were cleaned and sterilized. Pregnant mother was given intraperitoneal injections of 100µl of Buprenorphine (diluted to 1:10) and Ritrodine, deeply anesthetized, abdominal cavity was opened and the uterine horn exposed. Embryos were frequently bathed in pre-warmed 0.9% saline solution. DNA solution mixed with fastgreen (~ 2 µl) was injected using pulled glass micropipettes into the

telecephalic lateral ventricle of the embryos through the uterine wall, and square electric pulses (35V for E12.5, 45V for E14.5, 50ms on, – 950ms off, 5 pulses) were applied with an electric stimulator (Cuy21EDIT Bex C., LTD) using round electrodes (CUY650P5, Nepa Gene). Plasmid concentrations used for gain of function experiments: GFP(0.7µg/µl) combined with miRscr(1µg/µl) or miR3607(1µg/µl); for rescue experiments: miRscr(0.75µg/µl) or miR3607(0.75µg/µl) combined with APC(0.75µg/µl) and GFP(0.5µg/µl) or GFP(1.25µg/µl) alone. The embryos were then returned into abdominal cavity, sutured and the operated mice left to recover in clean warm cages until they regained their consciousness and maintained under standard conditions until the day of sacrifice.

in utero electroporation of ferret embryos

Ferret *in utero* electroporations were performed at the embryonic stage E35 in the neocortex. Pregnant females were deeply anesthetized with 2% isoflurane, abdominal cavity was opened and the uterine horn exposed. DNA solution mixed with fastgreen (~ 2 µl) was injected using pulled glass micropipettes into the telecephalic lateral ventricle of the embryos through the uterine wall, and square electric pulses (75V, 50ms on, – 950ms off, 5 pulses) were applied with an electric stimulator (Cuy21EDIT Bex C., LTD) using round electrodes (CUY650P7, Nepa Gene). Plasmid concentrations used: APC(1ug/ul)+GFP(0.75ug/ul); control GFP(1ug/ul); TUDScr(1ug/ul)+GFP(0.75ug/ul); TUDmiR3607(1ug/ul)+GFP 0.75(ug/ul). The embryos were then returned into abdominal cavity, sutured and the operated ferret left to recover in clean warm cages until they regained their consciousness and maintained under standard conditions until the day of sacrifice (2 days later).

Bromodeoxyuridine labeling experiments

Bromodeoxyuridine (BrdU, SIGMA) was diluted to 10mg/ml in 0.9% NaCl and administered at 50mg/Kg body weight. To identify progenitor cells in S-phase, a single dose of BrdU was injected at E13.5 and E15.5, embryos were fixed 30 min later. To calculate cell cycle re-entry, a single dose of BrdU was administrated 24 hr before fixation and the percentage of GFP/BrdU+/Ki67+ cells was calculated.

Tissue processing

Embryonic heads were dissected out in petri dishes filled with ice cold PBS and fixed immediately with 4% Paraformaldehyde (PFA) in Phosphate Buffer (PB) Ph 7.3 at 4ºC for 45 minutes to 3 hours depending on the embryonic stages. Postnatal pups were perfused transcardially and heads post fixed overnight at 4ºC. After fixation, brains were extracted out of the heads, washed with PBS, transferred to 30% sucrose and left overnight or until the brain sunk. For ISH, dissection was carried out under RNAse free conditions and brains left in 30% sucrose in 2% PFA. Brains were then embedded in an embedding chamber using cryo-medium NEG-50 and frozen under isopentane and liquid nitrogen. Cryostat was used for sectioning the brains into coronal sections of 20µm or 30µm.

Immunohistochemistry

The frozen brain sections were defrosted at room temperature, washed with PBS, permeabilized in PBS containing 0.25% Triton-X 100, blocked in 10% of Horse Serum and 2% Bovine Serum Albumin (BSA) for 2 hours, incubated with primary antibodies overnight in blocking solution, followed by appropriate fluorophore-conjugated secondary antibodies and counterstained with DAPI. Sections were then dehydrated by passing through ethanol in increasing concentrations and Xylol series and mounted the slides using cryomount solution. In case of co-staining with primary antibodies from the same host, sections were serially stained for two antibodies using conjugated Fab fragment secondary antibody for

the first antibody staining in order to block the available sites and prevent cross reaction. In case of BrdU staining, sections were pretreated with 4N HCL for 20 minutes at 37˚C, to disrupt the highly condensed chromatin structure. Primary antibodies used were: BrdU (1:500, Abcam ab6326), GFP (1:1000, Aves Lab GFP-1020), Ki67 (1:500, Abcam ab15580), phosphohistone H3 (1:1000, Upstate 06-570), Tbr1 (1:500, Abcam ab31940), Tbr2 (1:500, Millipore ab31940), Par3 (1:500, Millipore MABF28), Pax6 (1:500, Millipore AB2237), CUX1 (1:500, Santa Cruz), act-beta-catenin (1:500, Merk 05-665), PhVim (1:1000, Abcam),. Secondary antibodies used were from Vector Lab: biotinylated anti-Rat IgG (1:200, BA-9400), biotinylated anti-Rabbit IgG (1:200, BA-1000); from Jackson Inmunoresearch: Cy3 Fab fragment anti-Rabbit IgG (1:200, 711-167-003), Alexa488 anti-chicken IgY (1:200, 703-545-155), Cy5 Streptavidin (1:200, 016-170-084); from Invitrogen: Alexa555 anti-rabbit IgG (1:200, A-31572).

miRNA *in situ* hybridization

miRNA *in situ* hybridization (ISH) was performed as described in a book chapter by De pietri Tonelli et al., 2014. Cryostat sections or cryotome sections mounted on slides or deparafinized sections (parafinized sections rehydrated and deparafinized by passing through Xylol and alcohol series) were washed briefly with PBS, permeabilized with RIPA buffer (150Mm Nacl, TritonX-100 1%, 0.5% sodium deoxycholate,0.1% SDS, 1 mM EDTA, 50Mm Tris), pre hybridized at 54˚C for 1 hr with hybridization solution (50% Formamide (Ambion); 5x SSC (Sigma); 5x Denhardt´s (from a 50x stock; Sigma); 250µg/ml of Yeast RNA; 500µg/Ml salmon sperm DNA and hybridized overnight at 54˚C (30˚C below RNA Rm) with miRCURY™ LNA™ microRNA ISH Detection Probe (5´-3´-ACTGATTTGCTTCATCACATGC/3Dig_N/, product #612280-350 Exiqon) in hybridization solution. Washed slides 2x1 hour with pre-warmed post hybridization solution (50% Formamide; 2x SSC and 0.1% Tween), washed briefly with MABT buffer (1x maleic acid-Nacl pH 7.5, 0.1% Tween) and blocked with blocking solution (10% Bovine serum in MABT buffer) for 1 hr at room temperature and incubated with alkaline phosphatase coupled anti-digoxigenin Fab fragments (1:2000, Roche) in blocking solution overnight at 4˚C. After brief

washes with MABT and NTMT(100Mm Tris pH 9.5, 50Mm $MgCl_2$, 100Mm NaCl, 0.1% Tween) the sections were incubated in nitroblue tetrazolium (NBT)/ 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution [3.4 µg/ml from NBT stock and 3.5 µl/ml from BCIP stock in NTMT buffer (100 mg/ml NBT stock in 70% dimethylformamide; 50mg/ml BCIP stock in 100% dimethylformamide; Roche)]. Sections were then dehydrated by passing through ethanol and xylol series and mounted using cryomount solution.

hiPSC culture

Human iPSCs were cultured at 37°C, 5% CO_2 and ambient oxygen level on Geltrex coated plates in mTeSR1 medium (STEMCELL Technologies, 05850) with daily medium change. For passaging, iPSC colonies were incubated with StemPro Accutase Cell Dissociation Reagent diluted 1:4 in PBS for 4 minutes. Pieces of colonies were washed off with DMEM/F12, centrifuged for 5min at 300 xg and resuspended in mTeSR1 supplemented with 10µM Rock inhibitor Y-27632(2HCl) for the first day.

Generation of human cerebral organoids

Cerebral organoids were generated as previously described *(Lancaster and Knoblich.,2014)*. Briefly, mycomplasma-free iPSCs were dissociated into single cells using StemPro Accutase Cell Dissociation Reagent (A1110501, Life Technologies) and plated at the concentration of 9000 single iPSCs/well into low attachment 96-well tissue culture plates in hES medium (DMEM/F12GlutaMAX supplemented with 20% Knockout Serum Replacement, 3% ES grade FBS, 1% Non-essential amino acids, 0.1mM 2-mercaptoethanol, 4ng/ml bFGF and 50µM Rock inhibitor Y27632) for 6 days in order to form embryoid bodies (EBs). Rock inhibitor Y27632 and bFGF were removed on the 4th day. On day 6, EBs were transferred into low attachment 24-well plates in NIM medium (DMEM/F12GlutaMAX supplemented with 1:100 N2 supplement, 1% Non-essential amino acids and 5µg/ml Heparin) and cultured for additional 6 days. On

day 12, EBs were embedded in Matrigel drops and then they were transferred to 10cm tissue culture plates in NDM minus A medium (DMEM/F12GlutaMAX and Neurobasal in ratio 1:1 supplemented with 1:100 N2 supplement 1:100 B27 without Vitamin A, 0.5% Non-essential amino acids, insulin 2.5µg/ml, 1:100 Antibiotic-Antimycotic and 50µM 2-mercaptoethanol) in order to form organoids. 4 days after Matrigel embedding, cerebral organoids were transfered into an orbital shaker and cultured until electroporation in NDM plus A medium (DMEM/F12GlutaMAX and Neurobasal in ratio 1:1 supplemented with 1:100 N2 supplement 1:100 B27 with Vitamin A, 0.5% Non-essential amino acids, insulin 2.5µg/ml, 1:100 Antibiotic-Antimycotic and 50µM 2-mercaptoethanol). During the whole period of cerebral organoid generation, cells were kept at 37°C, 5% CO_2 and ambient oxygen level with medium changes every other day. After transferring the cerebral organoids onto the shaker, medium was changed twice per week.

Electroporation of human cerebral organoids

Cerebral organoids were kept in antibiotics-free conditions prior to electroporation. Electroporations were performed in cerebral organoids at stage 37 days after the initial plating of the cells and fixed 7 days post electroporation. During the electroporation, cerebral organoids were placed in an electroporation chamber (Harvard Apparatus, Holliston, MA, USA) under a stereoscope and using a glass microcapillary, 1-2µl of plasmid DNAs was injected together with Fast Green (0.1%, Sigma) into different ventricles of the organoids. Plasmid DNA concentrations were: GFP (0.7µg/µl), *MIRSCR* (1µg/µl), *MIR3607* (1µg/µl). Cerebral organoids were subsequently electroporated with 5 pulses applied at 80V for 50ms each at intervals of 500ms (ECM830, Harvard Apparatus). Following electroporation, cerebral organoids were kept for additional 24hr in antibiotics-free media, and then changed into the normal media until fixation. Cerebral organoids were fixed using 4% PFA for 1hr at 4ºC, cryopreserved with 30% sucrose and stored at -20ºC. For immunofluorescence staining, 16µm cryosections were prepared.

Western blotting

For western blot assays, Ferret Mpf cells (ATCC® CRL-1656™, passage 7) were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) with *MIR3607* expressing plasmid/ *MIRSCR* control plasmid and TUDSCR/TUD3607 plasmids. After 48 hours, cells were harvested, washed with cold PBS and lysate was prepared with pH 7,4 sterile cold lysis buffer (20mM HEPES, 150mM KCl, 1mM EGTA, 1mM EDTA, 0,1mM DTT, 40mM NaF, 1mM Na3VO4, 1% Triton-X and protease inhibitor). The amount of protein in each sample was then quantified as follows. The soluble fraction was collected from the lysate after centrifugation at 15000rpm for 15 minutes. The protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; Ref: #23227). Samples were heat treated at 95ºC for 5 minutes in Laemi Sample Buffer 1x (LSB). 25µg of total protein per well and per sample condition was run on a 8% SDS-PAGE gel for 2 hours at 120mV in Running Buffer 1x (Tris-Glicine 1X, SDS 0,1%). The wet transfer to a 0.45µm nitrocellulose membrane (GE Healthcare Life Science, Ref: 10600002) was carried out overnight at 4ºC, 30mV in cold transfer buffer (Tris-Glicine 1x, Methanol 1%, SDS 0,01%). After washing and fixing with Ponceau S, the membrane was blocked in 5% milk TBS-T buffer in shaker for one hour at room temperature. Antibodies were diluted on blocking solution at a concentration of 1:1000 anti-APC rabbit IgG (Cell Signaling, Ref: 2504S), 1:1000 anti-cMyc mouse IgG (Santa Cruz, Ref: sc40), 1:5000 Goat Peroxidase anti-rabbit IgG (Thermo Fisher Scientific, Ref: 31462) and 1:2000 Goat Peroxidase anti-mouse IgG (Thermo Fisher Scientific, Ref: 31444). Primary antibody incubation was carried out overnight at 4ºC in a shaker and incubated with secondary antibody for 2 hours at room temperature. To detect the labeling, Chemiluminescent HRP substrate (Millipore, Ref: WBKLS0100) was applied and the membrane exposed in a AI680 Bioimager. The quantification was normalized to the tubulin amount using anti-Tub mouse primary antibody 1:1000 (Sigma, Ref: T5168) and 1:2000 Goat Peroxidase anti-mouse IgG (Thermo Fisher Scientific, Ref: 31444). Mean intensity was measured by ImageJ Fiji and statistically analyzed by unpaired student t-test.

FACS sorting of electroporated brains

miRscr and miR3607 electroporated regions of the mouse cerebral cortex in the same medio-lateral and rostral-caudal level was dissected out using fluorescence dissection macroscope under ice cold conditions in HBSS medium (Thermo Fischer Scientific). Tissue was then dissociated with 40µl Trypsin, 10µl DNase in 1ml of HBSS + 10%FBS+ 1%P/S medium at 37˚C incubator for 8 minutes. 2 ml of medium was then added and tissue was further dissociated using the micropipette, centrifuged, resuspended in 500µl of the medium, filtered and took to the flow cytometry to isolate the cells with GFP. Cells with high intensity of GFP were then FACS sorted (FACS Aria II, BD) into 300µl of neurobasal medium (+10%FBS+1%P/S, and RNA was extracted using Arcturus PicoPure™ RNA isolation kit (Thermo Fischer Scientific, Cat # KIT0202) according to the manufacturer´s protocol. RNA integrity was then analysed using Bioanalyzer (Agilent 2100) and RIN values more than 9.5 were chosen for RNA sequencing. Cells from two embryos pooled together in each sample and three independent samples for each condition was used for sequencing.

RNA sequencing and differential expression analysis

Libraries were prepared with the SMART-seq v4 Library Prep Kit (ultra low input category that uses 10pg – 10ng of RNA) and sequenced on Illumina HISeq 2500 using 50bp single reads to detect changes in gene expressed. Sequencing reads were aligned using HISAT2 v2.1.0 *(Kim et al.,2015)* to GRCm.38/mm10 mouse genome. Integrative Genomics Viewer *(Robinson et al.,2011)* was used to visualize aligned reads and normalized coverage tracks (RPM). Reads overlapping annotated genes (Ensemble GRCm38.93) were counted using HTSeq v0.11.1 *(Anders et al.,2015).* Differentially expressed genes (DEGs) were detected using DESeq2 v1.18.1 package *(Love et al.,2014)* in R *(Ihaka and Gentleman.,1996).* Genes with Adj. $p < 0.01$ were considered significantly differentially expressed. For some comparisons, mRNA abundance of RNA-seq data was obtained using transcript per million normalization (TPM) *(Wagner et al.,2012)*. RNA-seq data reported in this study are accessible through the Gene

Expression Omnibus (GEO) database with the GEO Series accession number GSE135321.

Gene set enrichment and pathway analyses

Identification of enriched biological functions and processes in DEGs was performed using clusterProfiler *(Yu et al.,2012)* package in R. Over-representation test *(Boyle et al.,2004)* was performed using enrichGO function of clusterProfiler R package with the following parameters: gene=DEGs; OrgDb=org.Mm.eg.db; ont=BP; pAdjustMethod=BH; pvalueCutoff=0.05; qvalueCutoff=0.1). Functional Annotation Analysis of DEGs was performed using DAVID v6.8 *(Huang da et al.,2009)* with the following annotations: 3 Functional Categories (COG_ONTOLOGY, UP_KEYWORDS, UP_SEQ_FEATURE); 3 Gene Ontologies (GOTERM_BP_DIRECT, GOTERM_CC_DIRECT, GOTERM_MF_DIRECT); 2 Pathways (BIOCARTA, KEGG_PATHWAY); 3 Protein Domains (INTERPRO, PIR_SUPERFAMILY, SMART). Visualization of interrelations of terms and functional groups in biological netwoks was perfomed using ClueGO v2.5.0 *(Bindea et al.,2009)* plug-in of Cytoscape v3.6.*0 (Shannon et al.,2003)* with the following parameters: gene list=DEGs; ontologies= GO_MolecularFunction-EBI-QuickGO-GOA_22.03.2018_00h00, GO_BiologicalProcess-EBI-QuickGO-GOA_22.03.2018_00h00, WikiPathways_10.01.2019, KEGG_10.01.2019, REACTOME_Reactions_10.01.2019, REACTOME_Pathways_10.01.2019; Statistical Test Used = Enrichment/Depletion (Two-sided hypergeometric test); Correction Method Used = Bonferroni step down; Min GO Level = 3; Max GO Level = 8; Number of Genes = 3; Min Percentage = 4.0; Combine Clusters With 'Or' = true; Percentage for a Cluster to be Significant = 60.0; GO Fusion = true; GO Group = true; Kappa Score Threshold = 0.4; Over View Term = Smallest PValue; Group By Kappa Statistics = true; Initial Group Size = 1; Sharing Group Percentage = 50.0. Gene Set Enrichment Analysis (GSEA) *(Subramanian et al.,2005)* was performed using GSEAPreranked tool and GSEA function on clusterProfiler package in R, on 21822 unique features (genes) that were pre-ranked on the log2 of the fold-change from DESeq2 analysis. The following

parameters were used for GSEA: exponent = 0 (scoring_scheme=classic), nPerm=1000, minGSSize=15, maxGSSize=500. GSEA was performed using MSigDB gene sets Hallmark (h.all.v6.2.symbols.gmt) and GO (c5.all.v6.2.symbols.gmt).

miR3607 target prediction

miRNA targets were predicted using available online tools TargetScanHuman 7.2 (Agarwal et al.,2015), miRDB (Wong and Wang.,2015), TargetScanMouse Custom (Version 4 and 5.2) and miRDB custom prediction for identification of mouse target genes. Predicted targets were then compared to the list of downregulated genes to identify the possible direct gene directs.

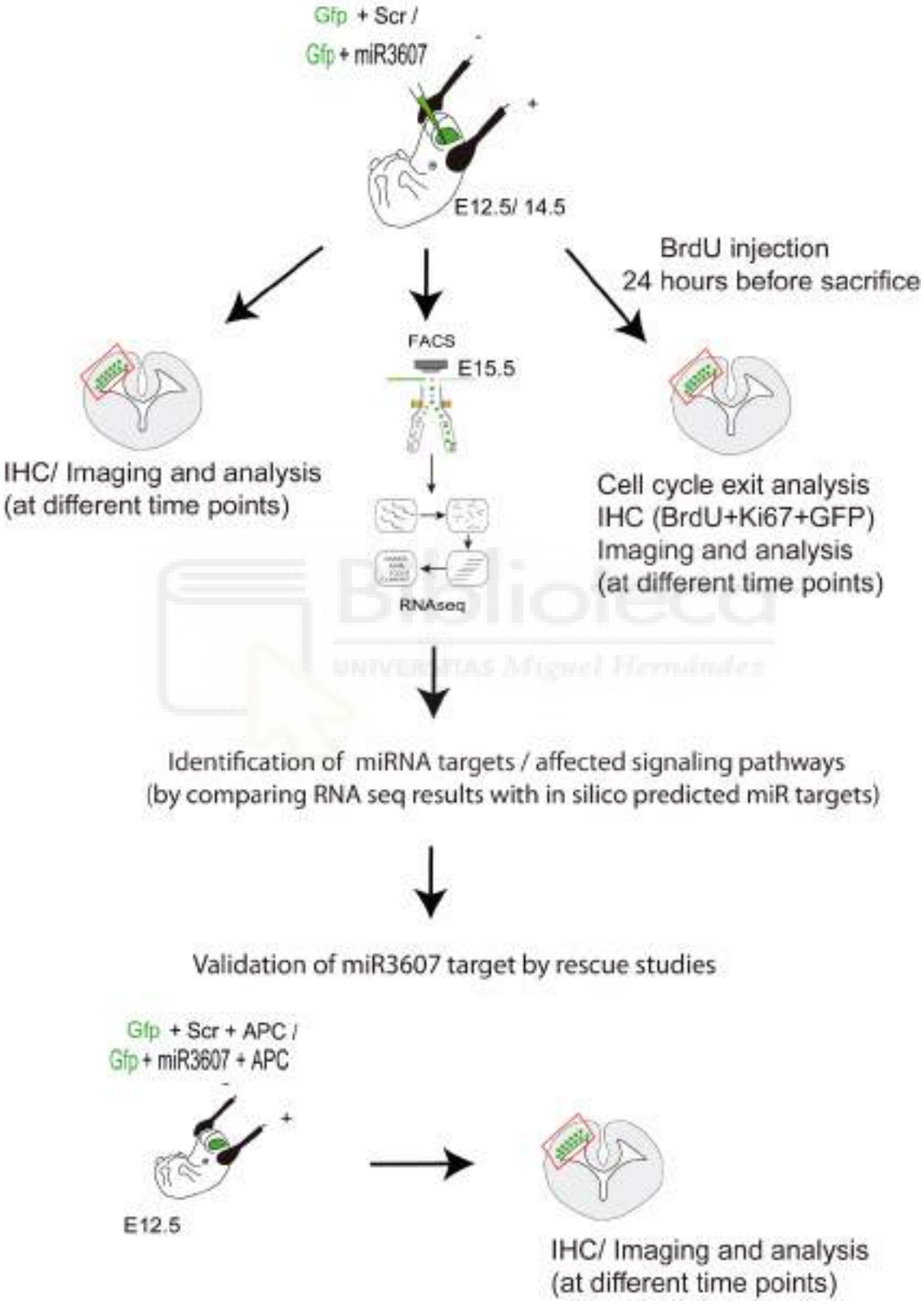
Image analysis, quantification and statistics

Images were acquired using a florescence microscope (Zeiss Axio Imager Z2) with Apotome.2 and coupled to two different digital cameras (AxioCam MRm and AxioCam ICc) or an inverted confocal microscope (Olympus FluoView FV1000). All images were analysed with ImageJ (Fiji). Co-localization studies were performed on single plane confocal images from 40X Z stacks. Cell distribution analyses were performed using Neurolucida and Neuroexplorer software (MBF Bioscience). All quantifications were performed at the same rostral-caudal and latero-medial levels on at least three different embryos from at least two independent litters. ISH images were acquired using Zeiss Axio Imager Z2. Brightness and contrast of the images shown in figures were homogeneously adjusted for clarity using Adobe Photoshop. Statistical analyses were carried out in Microsoft Excel or GraphPad Software using ANOVA with post-hoc Bonferroni correction (equal variances) or the Welch test with post-hoc Games-Howell (different variances), Kolmogorov-Smirnov test, χ^2-test or independent samples *t*-test, where appropriate and upon normality testing. Significance was set at p=0.05. In the analyses of Pax6 and Tbr2 co-expression, the influence of each protein's increased abundance over their increased co-expression in *MIR3607*-

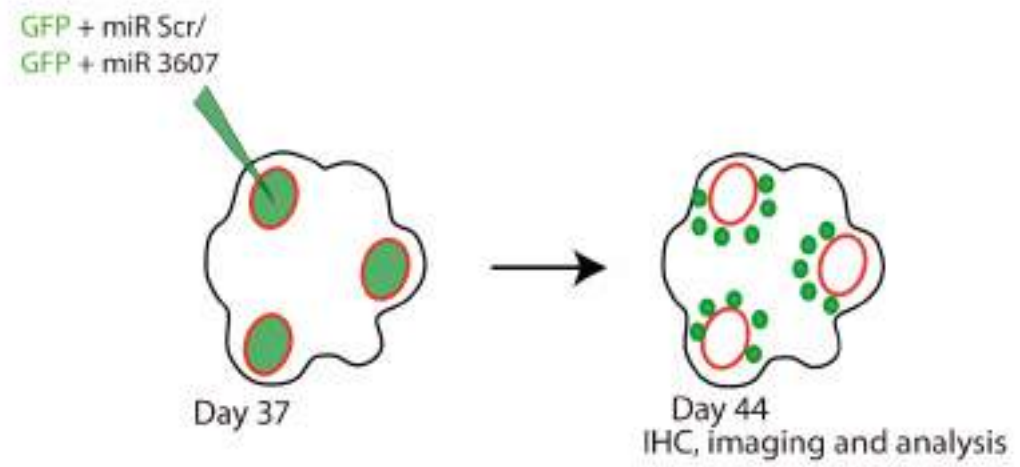
expressing embryos was tested by mathematical correction. This was performed by dividing the Pax6 intensity value of each cell by the average increase in Pax6 intensity between *MIR3607* and *Scr* embryos. The same method was used for Tbr2.

Methods Summary

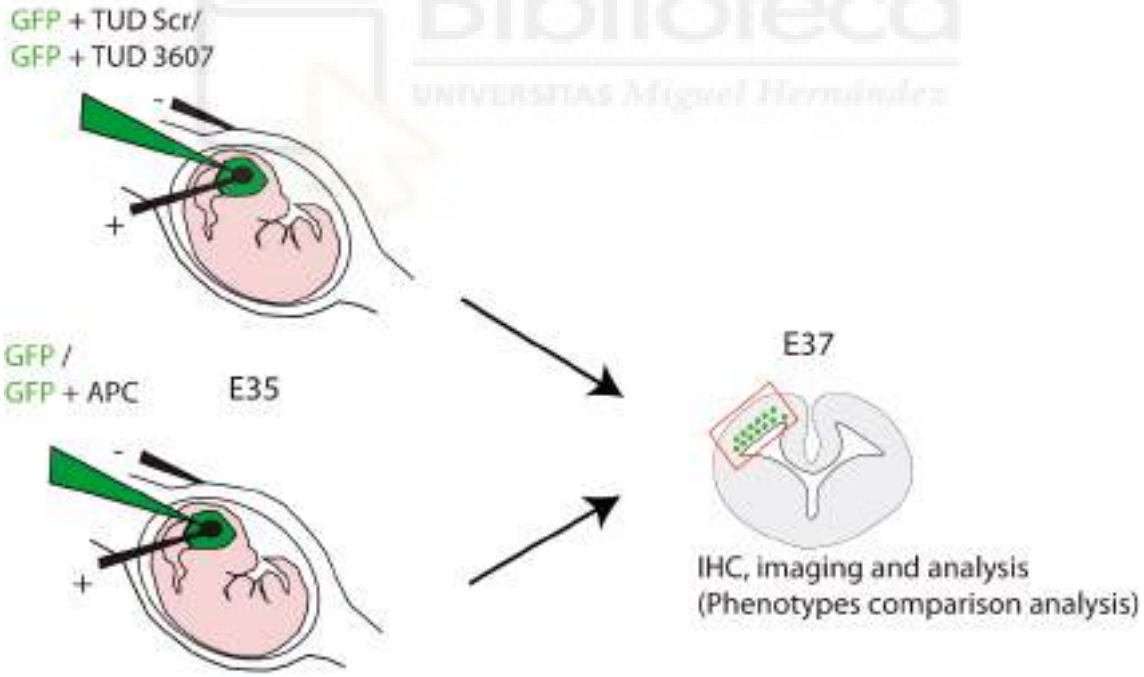
Gain of function studies *(Mus Musculus)*



Gain of function studies *(Human cerebral organoids)*



Loss of function studies *(Mustela putorius furo)*



Validation of APC as miR607 target in Ferret Mpf cells

MOTIVATION AND BACKGROUND

Previous transcriptomic analyses carried out in our lab identified several miRNAs expressed in all germinal layers of the ferret cerebral cortex at mid-to-late stages of cortical development, suggesting their importance in regulating the expression of several important developmental genes, their predicted targets. Among those miRNAs, pre-*MIR3607* was found to be expressed in all germinal layers of the ferret cerebral cortex, both during the embryonic critical period for formation of the OSVZ by massive production of bRGCs **(Fig. 1A)** *(Martinez-Martinez et al.,2016),* and at late postnatal stages preceding the expansion and folding of the cerebral cortex **(Fig. 1B)** *(de Juan Romero et al.,2015).* Previous reports confirm the expression of *MIR3607* in different human tissues, including the brain *(Saini et la.,2014, Lin et al.,2017; Moreau et al.,2013).* However, the expression of this miRNA in mouse had not been reported. All these evidences suggested the possibility that this miRNA might regulate the development and expansion of the cerebral cortex in higher mammals.

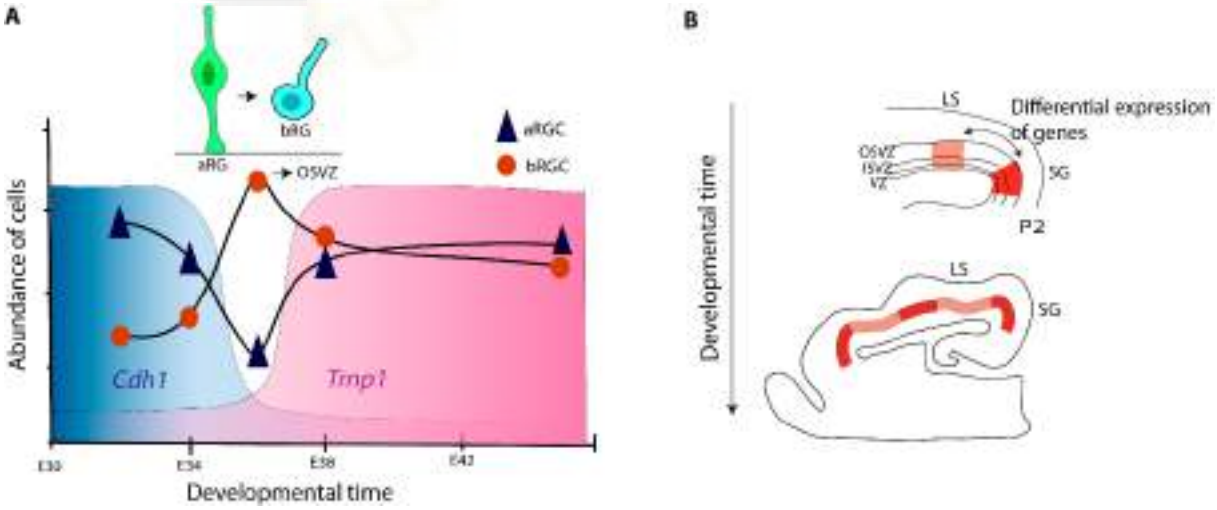


Figure 1. Differential expression of genes that are important for gyrification of ferret cerebral cortex. (A) Decrease in expression of *Cdh1* and Increase in expression of *Trnp1* in VZ determining the critical period in ferret cerebral cortex (E34-E38), when bRGCs of OSVZ are produced at the expense of aRGCs

(B) Differential expression of genes important for gyrification in the germinal zones of ferret cerebral cortex between prospective lateral sulcus and spenial gyrus. *(Modified from (Martinez-Martinez et al.,2016; de Juan Romero and Borrell.,2017; de Juan Romero et al.,2015; Llinares-Benadero and Borrell.,2019)*

When we analyzed the sequence conservation of human *MIR3607-5p*, as shown in Figure 2, we found it ranges between 100% in hominids and 92% in mouse.

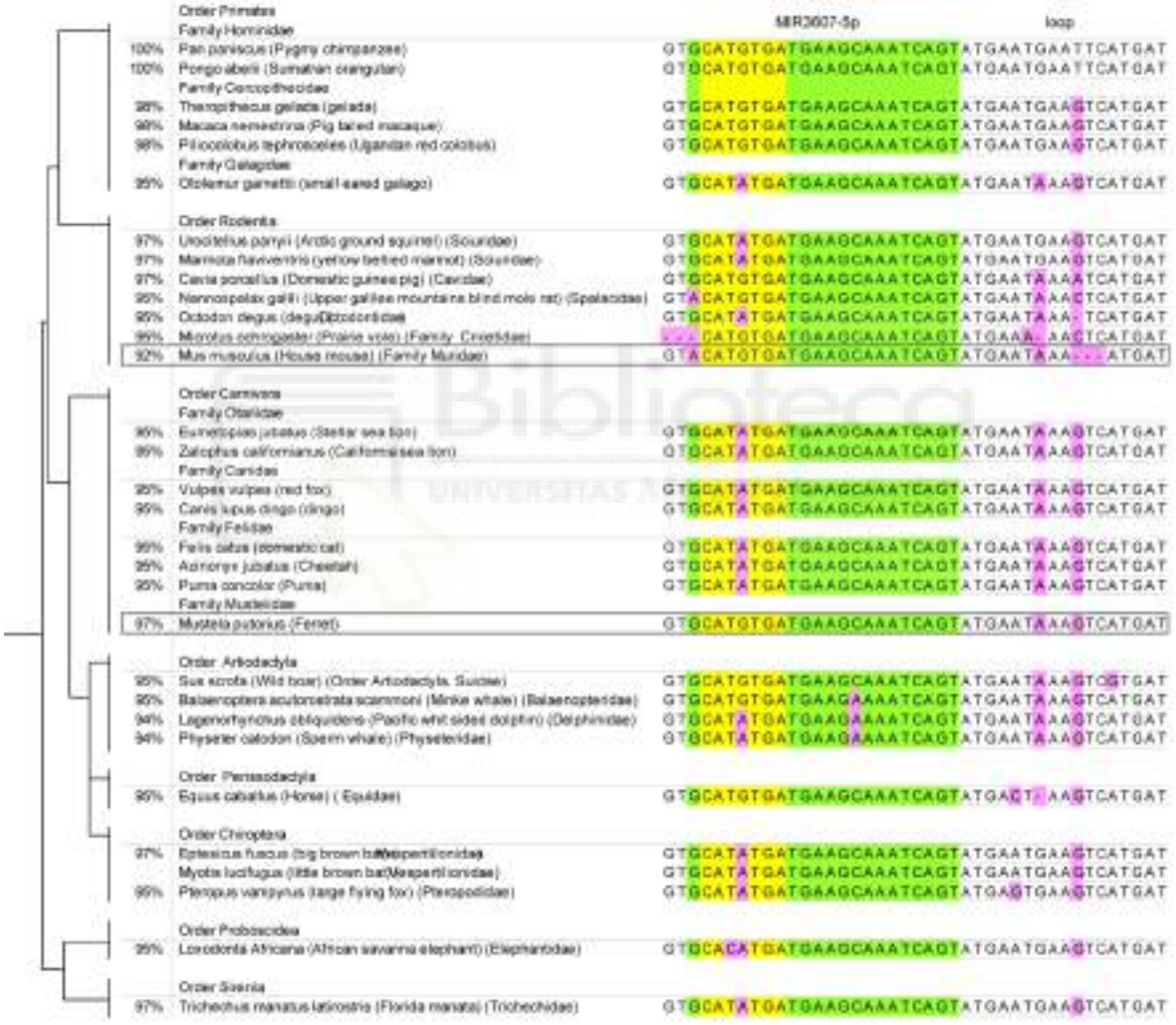


Figure 2. Comparison of *MIR3607* sequence across mammalian phylogeny. Comparison of nucleotide sequence of the *MIR3607* locus in individual species across representative orders and families of the mammalian lineage. The mature sequences for *MIR3607*-5p are highlighted in green, with the part of stem loop sequence. The seed sequence for *MIR3607*-5p is highlighted in yellow.

Nucleotide changes with respect to the human sequence are highlighted in purple. Numbers indicate percent of sequence conservation compared to human. Dendrogram on left indicates phylogenetic relationships among orders.

Nucleotide changes mostly occur in the 9th and 6th base of the loop sequence (in 29 and 19 species, respectively, out of 29 species with mismatches), and secondarily in the 4th base of the seed sequence (in 18 out of 29 species; always G to A changes). Outside primates, the mature *MIR3607*-5p sequence is 100% conserved in only 4 of the 25 species compared, which belong to three far-related orders: guinea pig (Rodentia), ferret (Carnivora), wild boar and horse (Artiodactyla). In mouse, the seed sequence is fully conserved, but the first base of the mature *MIR3607*-5p is changed (G to A), and four nucleotides are changed or missing in the loop sequence, as shown in the figure **(Fig. 2).** In addition to the exact similarity of the seed sequence (nt 2-8) of mature *MIR3607* in human, ferret and mouse, which is an important factor for binding to the target genes, *in silico* analyses revealed several predicted gene targets in common between human and mouse. All these were suggestive of a preserved functionality for this miRNA in the mouse brain and its targets, which prompted us to use mouse as a model organism to study the function of *MIR3607* in cerebral cortex development.

RESULTS

PART 1 - *MIR3607* AFFECTS NEUROGENESIS, NEURONAL MIGRATION AND MATURATION OF CORTICAL NEURONS

1.1. Mature *MIR3607* is expressed in the embryonic gyrencephalic cerebral cortex of human and ferret but not in the lissencephalic mouse

The above observations and evidences suggested the possibility that *MIR3607* might be relevant for the expansion and folding of the mammalian cerebral cortex. We began analyzing the expression level and pattern of the mature *MIR3607*-5p (*MIR3607* from hereon) in the developing brain of human, ferret and mouse using *in situ* hybridization (ISH), as the expression of pre-miRNA does not always parallel the expression of mature miRNA.

ISH stains of the embryonic human cortex at 16 gestational weeks (GW; at the peak of cortical neurogenesis) revealed the highest expression levels of *MIR3607* in the VZ and ISVZ, and in the cortical plate (**Fig. 3A**). Expression was also detected in the OSVZ, but at lower levels compared to the other germinal zones (**Fig. 3A'**).

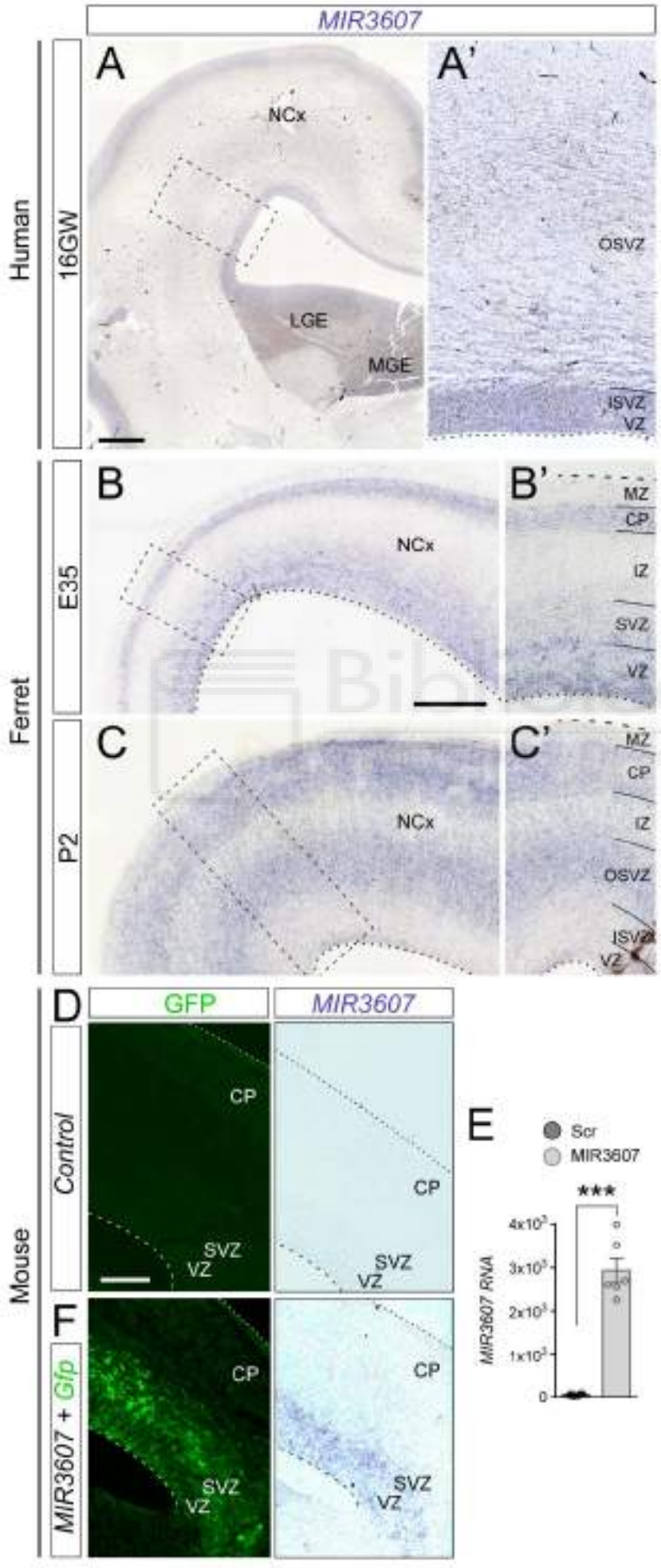

MIR3607
Human
16GW
A
NCx
LGE
MGE
A'
OSVZ
ISVZ
VZ
Ferret
E35
B
NCx
B'
MZ
CP
IZ
SVZ
VZ
P2
C
NCx
C'
MZ
CP
IZ
OSVZ
ISVZ
VZ
Mouse
D
GFP
MIR3607
Control
CP
SVZ
VZ
CP
SVZ
VZ
E
Scr
MIR3607
***
MIR3607 RNA
4x10-3
3x10-3
2x10-3
1x10-3
0
F
MIR3607 + Gfp
CP
SVZ
VZ
CP
SVZ
VZ

Figure 3. Mature *MIR3607* is expressed in cortical germinal layers of human and ferret but not mouse embryos. (**A,A'**) Coronal section of the human brain at 16 gestational weeks showing the expression pattern of *MIR3607*. Area boxed in (A) is shown in (A'). *MIR3607* expression is high in the three germinal zones: ventricular (VZ), inner and outer subventricular zones (ISVZ and OSVZ, respectively). (**B-C'**) Sagittal sections of the developing ferret cerebral cortex at the indicated ages showing the expression pattern of *MIR3607*. Areas boxed in (B,C) are shown in (B',C'). At E35, *MIR3607* is expressed at high levels in ventricular zone (VZ) and low levels in subventricular zone (SVZ). At P2, expression is undetectable in VZ and ISVZ, but high in OSVZ. Expression is high in cortical plate (CP) in both human and ferret. (**D,F**) Patterns of GFP and *MIR3607* expression in the cerebral cortex of an E15.5 mouse embryo in a control hemisphere (D), and in the contralateral hemisphere electroporated at E14.5 with *Gfp* plus *MIR3607*-encoding plasmids (F). Note the absence of endogenous expression in (D), and the high expression levels in VZ and SVZ in (F). (**E**) qPCR results of *MIR3607* expression levels (arbitrary units) in HEK293 cells transfected with psil-*Scr* and psil-*MIR3607*. Histograms indicate mean ± SEM; circles in plots indicate values for individual replicas. t-test, ***p=9.76x10^{-7}. Scale bars: 500µm (A), 200µm (B,C), 100µm (D,F).

When we analyzed *MIR3607* expression in the developing ferret cortex, at embryonic day (E) 35, expression was high in VZ, low in SVZ and undetectable in IZ (**Fig. 3B,B'**). At this stage in ferret, there is no distinction between ISVZ and OSVZ, so this pattern was reminiscent of the difference between VZ and OSVZ in human embryos at 16GW. However, when we analyzed the expression pattern at postnatal day (P) 2, when ISVZ and OSVZ are clearly distinct in ferret, expression was reduced to background levels in VZ and ISVZ, while being high in OSVZ, and intermediate in IZ (**Fig. 3C,C'**). In addition, and similar to human, *MIR3607* expression was high at both developmental stages in CP, where cortical neurons finish radial migration and begin differentiating their dendritic and axonal arbors. It was evident from the expression pattern of *MIR3607* in germinal zones of both ferret and human during early development stages, that *MIR3607* may play important roles in neurogenesis. In addition, *MIR3607* expression in cortical neurons added the possibility that *MIR3607* may have a role in migration, specification and/or maturation of cortical neurons.

In contrast to human and ferret, our ISH stains revealed a complete absence of *MIR3607* expression in the embryonic mouse cortex. We were only able to detect the expression of *MIR3607* in the embryonic mouse cerebral cortex following overexpression of our artificial DNA vector construct encoding *MIR3607* (**Fig. 3D, D´**). Confirmation that *MIR3607* is not expressed in the developing mouse cortex prompted us to take advantage of this circumstance in order to investigate the role of this miRNA in cerebral cortex development, and its potential relevance in the limited growth of the mouse cerebral cortex as compared to ferret and human. Therefore, we reasoned that expression of *MIR3607* in the developing cortex of mouse embryos may shed light on its functional role in cortical development and the identification of potential gene targets important for these roles. For this purpose, We designed a DNA vector for expression of *MIR3607* and confirmed its expression in the cerebral cortex of electroporated mouse embryos by ISH, and in a human cell line by RT-qPCR, (**Fig. 3E,F**).

1.2. Effects on neurogenesis upon *MIR3607* expression in mouse cerebral cortex

To begin with, we performed *in utero* electroporation of mouse embryos to target the apical progenitors of cerebral cortex at E14.5, in order to study the effects of overexpression of *MIR3607* in neurogenesis. We expressed the mature form of *MIR3607* alongside a GFP reporter plasmid and compared to the scrambled-control electroporated embryos. One day after electroporation (E15.5), the majority of GFP+ cells in control *Scrambled*-electroporated embryos populated the VZ, with only half as many found in SVZ and very few in IZ (**Fig. 4A,B**). This distribution of GFP+ cells was similar in embryos expressing *MIR3607*, except for a clear tendency to having fewer cells in IZ and SVZ, while more in VZ (**Fig. 4A,B**). One day later (E16.5), we observed that more than half of all GFP positive cells were in the IZ with the remaining cells being distributed between VZ (20.5%) and SVZ (27.5%) in *MIR3607*-expressing embryos, as compared to control embryos, where the GFP positive cells wear nearly distributed equally in all three layers (**Fig. 4C,D**). The increase in percentage distribution of cells in IZ in case of miR3607, suggested the relative abundance of recently born migrating

neurons compared to control. Yet we reasoned if this increase in IZ cells is due to increased neurogenesis rather than faster multipolar-bipolar transition and migration of newborn neurons. Therefore, in order to confirm this, we performed cell-cycle exit analysis by BrdU and Ki67 labeling, which revealed a 29% increase in cell-cycle exit at E16.5 upon *MIR3607* expression at E14.5 compared to control (**Fig. 4E**), demonstrating more self-consuming, neurogenic divisions of progenitor cells at this stage.

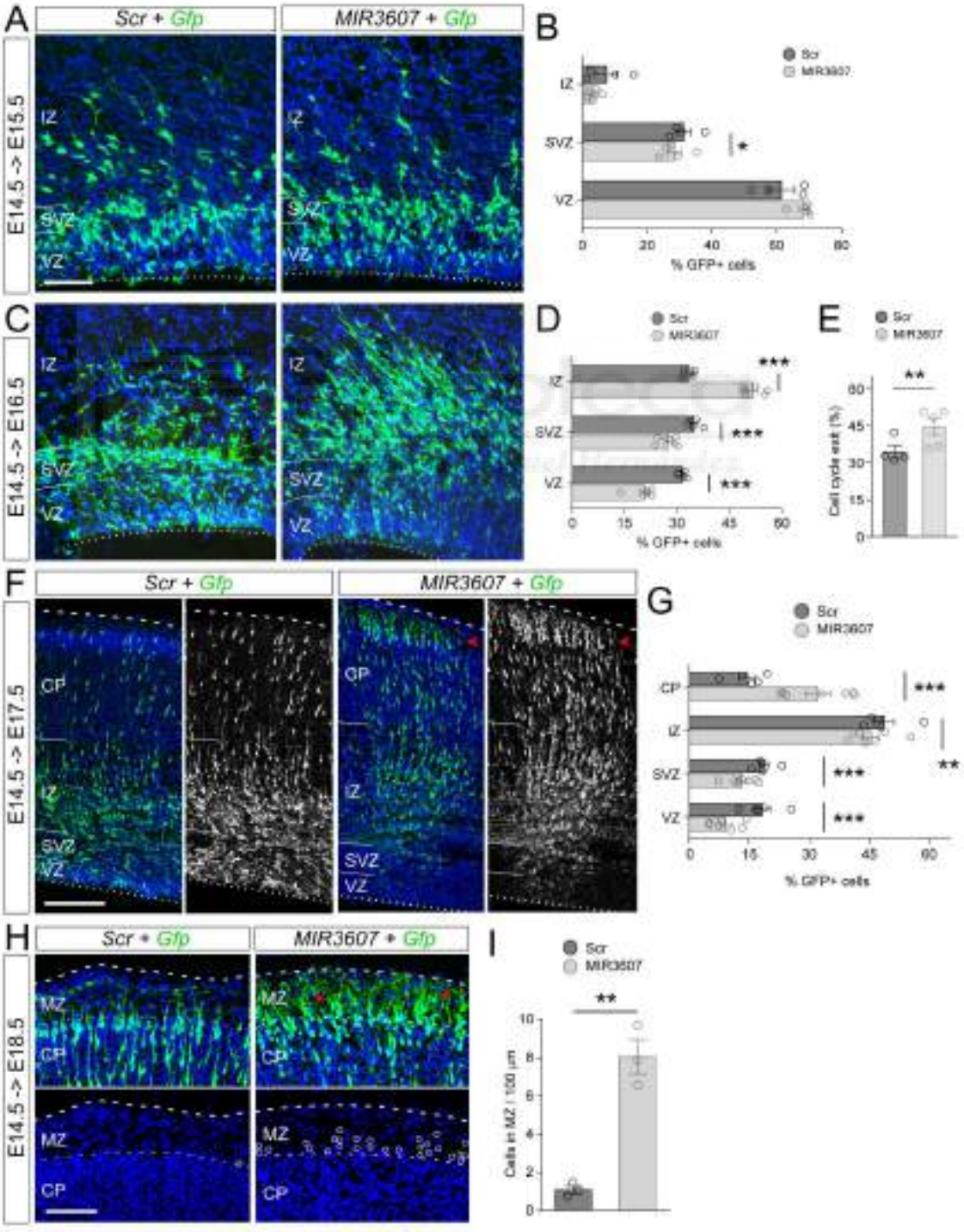


Figure 4. Defects in neurogenesis and neuron migration upon expression of *MIR3607* in mouse neocortex. (**A-D**) Sections through the parietal cortex of E15.5 (A) and E16.5 (C) mouse embryos electroporated at E14.5 with *Gfp* plus *Scrambled-* (*Scr*) or *MIR3607*-encoding plasmids, and quantifications of laminar distribution of GFP+ cells (B and D, respectively). Dotted lines indicate apical surface of the embryonic cortex. (**E**) Quantification of cell cycle exit rate at E16.5 for cells electroporated at E14.5 and receiving a single pulse of BrdU at E15.5. (**F,G**) Sections through the parietal cortex of E17.5 mouse embryos electroporated at E14.5 with the indicated plasmids (F), and quantifications of laminar distribution of GFP+ cells (G). Dotted lines indicate apical surface of the cortex, dashed lines indicate basal surface. (**H,I**) Detailed images of the marginal zone (MZ) and cortical plate (CP) of E18.5 mouse embryos electroporated at E14.5 with the indicated plasmids, showing high density of ectopic GFP+ cells within MZ in *MIR3607* embryos (H, open circles), and their quantification (I). Circles in plots indicate values for individual embryos. Histograms indicate mean ± SEM; n = 3-7 embryos per group; X^2 test (B,D,E,G), t-test (I); *p<0.05, **p<0.01, ***p<0.001. Scale bars: 100µm (A,C,H), 200µm (F).

Next, when we analysed at E17.5, there were lower proportions of GFP+ cells in VZ, SVZ and IZ compared to control, but a two-fold increase of GFP+ cells in CP. This was in agreement with the increase in cell cycle exit caused by miR3607 at E16, which lead to the depletion of progenitors and concomitant increase in the production of neurons, and their subsequent arrival in the CP ahead of control-electroporated cells (**Fig. 4F,G**). These results seemed to indicate that the primary effect of *MIR3607* expression was to promote neurogenesis, which secondarily translated into the premature radial migration and settling of neurons in the CP. However, analyses four days post electroporation at E18.5, revealed that many GFP+ cells in *MIR3607* embryos migrated past their boundary at the CP-MZ border, invading ectopically the MZ (**Fig. 4H,I**), which indicated a defective termination of radial migration. This prompted us to study the effect of *MIR3607* expression on migration and maturation of neurons.

1.3. Effects on neuronal migration and positioning upon *MIR3607* expression in mouse cerebral cortex

Once the neurons are formed, and undergo multipolar to bipolar transition, they migrate radially to the cortical plate and organize in an inside-out pattern, with the early born neurons occupying the lower layers and late born neurons settling superficially *(Molyneaux et al.,2007).* Given the migration defect observed at E18.5, we investigated the effects of expression of *MIR3607* at P5, when cortical lamination is complete. When we analysed for cell fate and positioning of GFP+ neurons, *MIR3607* electroporated GFP+ neurons were found in the CP (prospective layer 2/3 at this stage) and expressed the layer 2/3 marker Cux1, exactly like in control embryos, consistent with them maintaining a normal laminar fate (**Fig. 5A-A''**). However, when we analysed the location of cells, GFP+ neurons of *MIR3607* embryos mostly occupied the top of CP, whereas control-electroporated GFP+ neurons mostly were in the central positions within the CP (**Fig. 5B,C**).

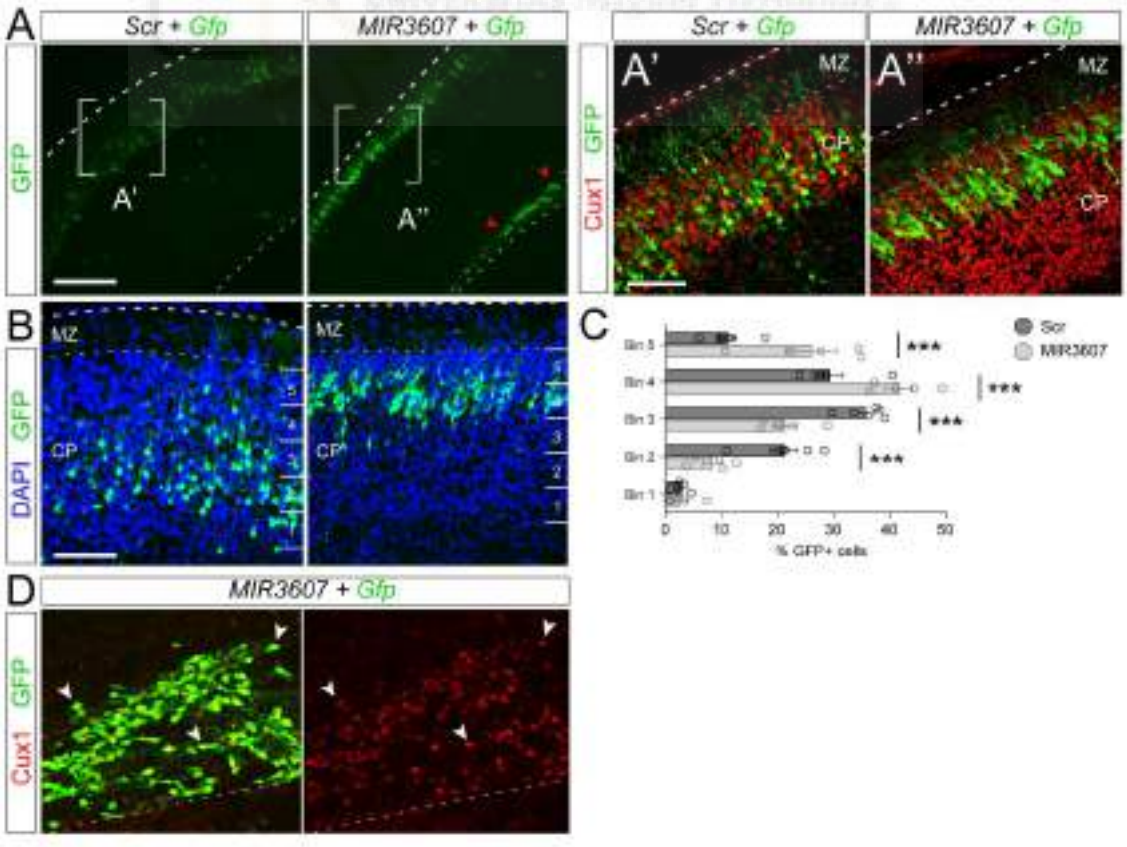


Figure 5. *MIR3607* expression causes long-term defects in neuron positioning. Sections through the parietal cortex of P5 mouse pups electroporated at E14.5 with *Gfp* plus *Scrambled*- (*Scr*) or *MIR3607*-encoding plasmids and stained as indicated, and quantification of the binned distribution of GFP+ cells within the CP (B, C). (A'-A'') are magnifications of the regions boxed in (A). (D) is a magnification of the white matter. All GFP+ cells in CP (normotopic) and white matter (ectopic) were positive for the upper layer marker Cux1 (red). Expression of *MIR3607* caused the overmigration and persistent presence of cortical neurons at the top of CP, and simultaneously the accumulation of ectopic neurons in the white matter (arrowheads in A and D), all of which maintained a correct upper layer fate. Circles in plots indicate values for individual embryos. Histogram indicates mean ± SEM; n = 5-6 embryos per group; X^2 test; ***p<0.001. Scale bars: 300μm (A), 100μm (A',A'',B,D).

Considering that expression of *MIR3607* induced premature neurogenesis, according to the inside-out gradient of cortex development this should have resulted in neurons accumulating in deep positions within the CP, not superficial. However, our result was consistent with the overmigration phenotype observed previously at E18.5, demonstrating that *MIR3607* expression causes the cells to migrate further than normal. In addition to defects in the CP, we also observed that many *MIR3607* neurons accumulated in the cortical white matter, forming subcortical ectopias (**Fig. 5A**). Marker analysis showed that the vast majority of these ectopic neurons expressed Cux1 (**Fig. 5D**), demonstrating retention of their normal fate for layer 2/3. Together, these observations confirmed that *MIR3607* expression altered radial migration of neurons in the developing mouse cerebral cortex.

1.4. Effects on neuronal maturation upon *MIR3607* expression in mouse cerebral cortex

Once the neurons finish their migration, they start their process of maturation by extending their axonal fibres to establish functional connectivity *(Ye et al.,2019)*. Given the effects of *MIR3607* on neurogenesis and neuron migration, we next enquired whether the maturation of neurons was also affected. We

electroporated E14.5 embryos to target progenitor cells producing layer 2/3 neurons, and then analyzed their growing axons across the Corpus Callosum (CC) at P5. Both in control and in *MIR3607* expressing embryos, GFP+ axons crossed the telencephalic midline at the level of the CC, and extended along the white matter of the contralateral hemisphere (**Fig. 6A**). However, the density of GFP+ callosal axons extending along the white matter was lower in *MIR3607* embryos than in controls. Importantly, the deficit in callosal axons increased as these approached the midline, while after midline crossing it remained largely unchanged (**Fig. 6B,C**). In the contralateral cortex, we further observed the invasion of axons from the white matter toward the CP in control embryos, which was virtually absent in *MIR3607* embryos (**Fig. 6D**).

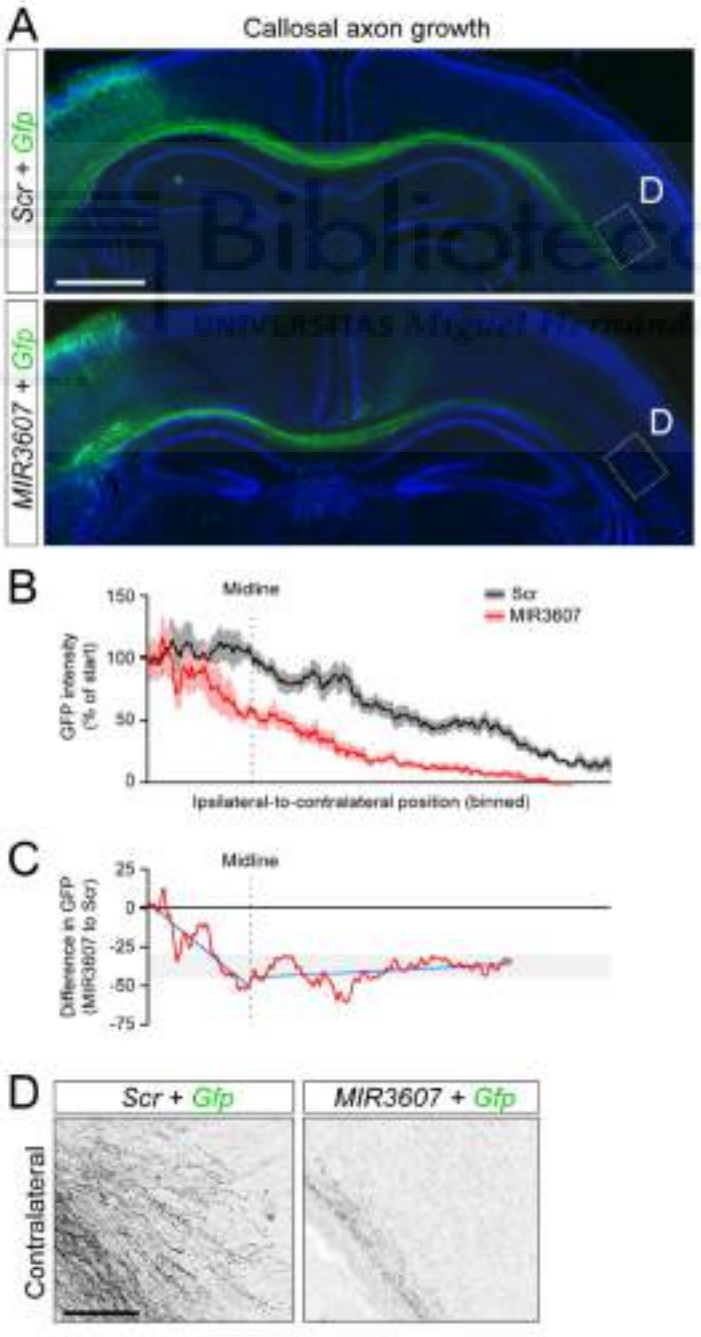
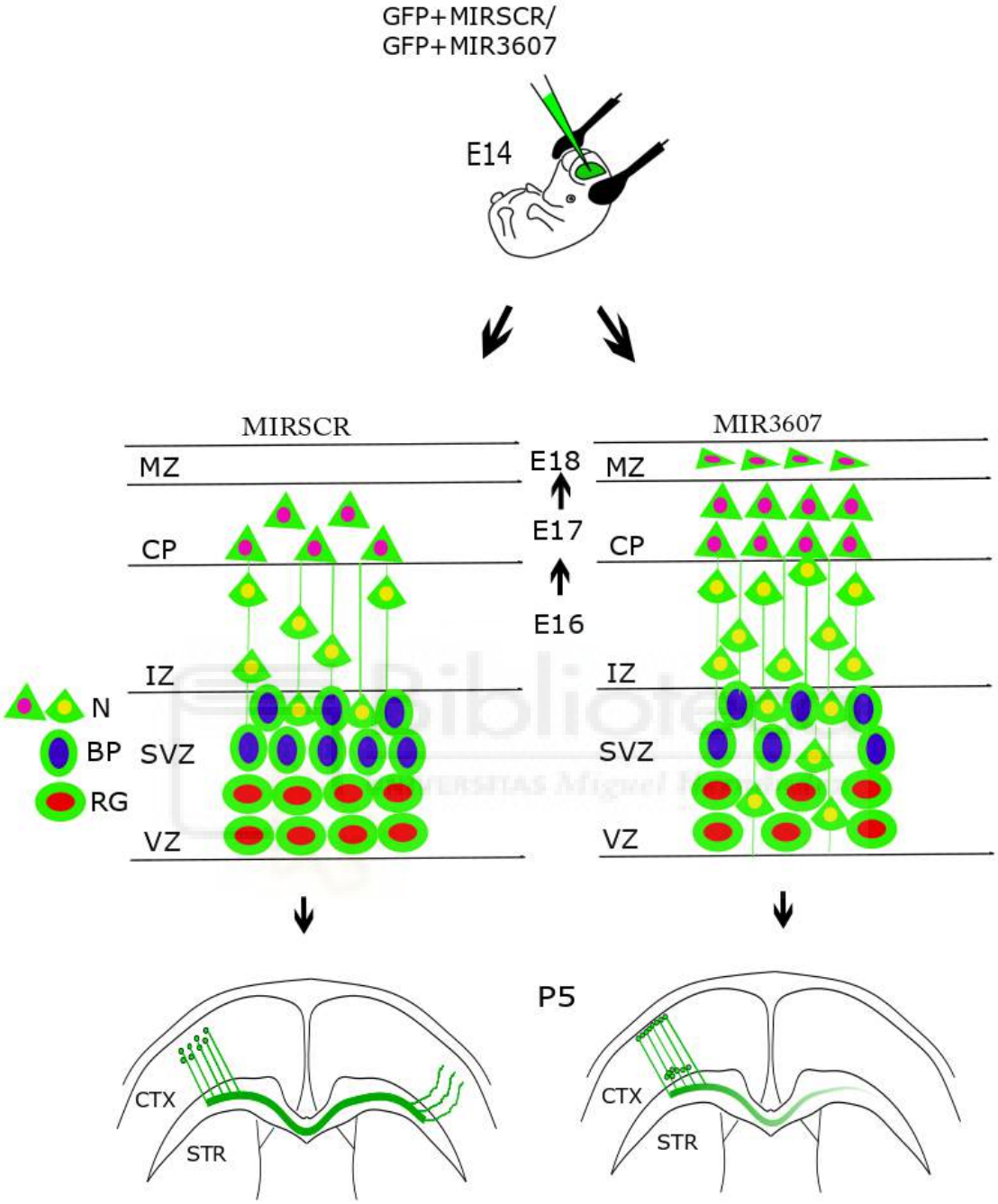


Figure 6. Expression of *MIR3607* in embryonic mouse cortex causes defects on callosal axon growth. (**A**) Coronal sections through the parietal cortex of P5 mouse pups electroporated at E14.5 with *Gfp* plus *Scrambled*- (*Scr*) or *MIR3607*-encoding plasmids, stained as indicated to reveal GFP labelling of callosal axons. Boxes indicate areas shown in (D). (**B,C**) Quantification of GFP intensity along the ipsi-to-contralateral track of callosal axons, relative to intensity at the start site (B; mean ± SEM), and difference between conditions (C). Dashed line indicates location of the midline. Blue is for trend lines before and after midline crossing. Data in (B) are mean ± SEM; *n* = 9-10 pups per group. The greatest defect was in axon growth towards the midline, while the difference to control animals remained stable after midline crossing. (**D**) Labelling of callosal axons in the contralateral layer 6, as indicated in (A), showing their scarce presence in *MIR3607* expressing pups. Scale bars: 1mm (A), 100µm (D).

Interim summary 1



PART 2 - *MIR3607* AFFECTS THE AMPLIFICATION OF PROGENITORS

2.1 *MIR3607* drives amplification of Pax6+ progenitor cells

In the above analyses, we found that expression of *MIR3607* at E14.5 in mouse cortical progenitor cells increased cell cycle exit and premature neurogenesis by E16.5, with a significant loss of cells in VZ and SVZ, and gain of IZ cells. However, it was intriguing that one day earlier at E15.5, we found more cells in VZ and fewer in IZ and SVZ, suggesting that the immediate early effect might be the opposite: reduced neurogenesis and increased aRGC amplification. In order to study this further, we performed BrdU labeling analyses, which revealed a 36% increase in BrdU-incorporating cycling progenitor cells in E15.5 embryos expressing *MIR3607* compared to control littermates (**Fig. 7A,B**), and cell cycle exit analyses revealed a 65% reduction in cell cycle exit between E14.5 and E15.5 (**Fig. 7B**). This confirmed that the earliest changes immediately upon *MIR3607* expression are reduced neurogenesis and dramatic amplification of progenitor cells. We next performed marker analysis of the cells present in the electroporated embryos at E15.5. In the mouse cerebral cortex, most progenitors present in VZ are aRGCs that express the transcription factor Pax6, and those in SVZ are IPCs that express Tbr2 *(Götz et al., 1998; Englund et al.,2005)*; Therefore, we first analyzed the expression of Pax6 in E15.5 embryos. In control embryos, a majority (76%) of GFP+ cells in VZ were positive for Pax6, as expected. Pax6 was also expressed by 42% of SVZ and 28% of IZ cells (**Fig.7C,D**). In embryos expressing *MIR3607*, the proportion of Pax6+ cells increased in all three layers, most dramatically in SVZ and IZ (1.7- and 2.6-fold, respectively) (**Fig.7C,D**). We next stained for Tbr2 and as expected, it was detected mostly in SVZ and IZ cells (74%), and to a lesser extent in VZ (44%), in case of control (**Fig. 7E,F**). Upon expression of *MIR3607*, Tbr2+ cell abundance also increased in VZ and SVZ, but much less than Pax6+ cells (**Fig. 7E,F**).

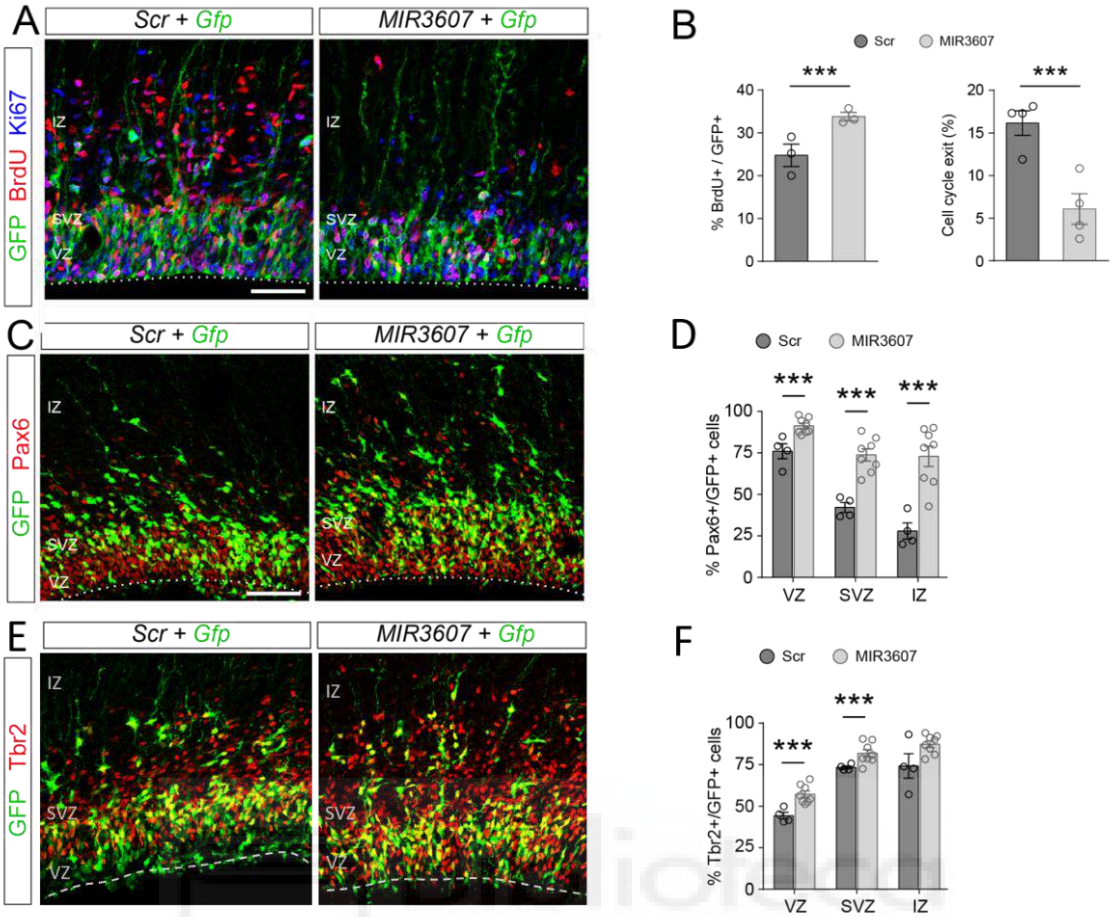


Figure 6. Amplification of Pax6+ progenitor cells immediately upon *MIR3607* expression. (**A,B**) Sections through the parietal cortex of E15.5 mouse embryos electroporated at E14.5 with *Gfp* plus *Scr*- or *MIR3607*-encoding plasmids stained as indicated, and quantifications of BrdU labelling index and cell cycle exit of GFP+ cells. Distribution and abundance of Pax6+ **(C,D)** and Tbr2+ cells **(E,F)** in the parietal cortex of electroporated embryos. GFP+ cells in *MIR3607*-expressing embryos had greater BrdU intake, lower cell-cycle exit, and displayed a dramatic increase in percentage of Pax6 expressing cells. Histograms indicate mean ± SEM; *n* = 3-8 embryos per group. X^2 test; *'*p*<0.02, ***p*<0.01, ****p*<0.001. Scale bars: 100µm

2.2 *MIR3607* increases co-expression of Pax6+Tbr2+ cells upon increased Pax6 expression

In the embryonic mouse cortex, Pax6 and Tbr2 mostly show mutually exclusive pattern of expression, with less proportion of cells co-expressing both the markers *(Englund et al.,2005; Arai et al.,2011).* Our marker analyses results showing high

proportion of cells expressing Pax6 without any reduction in Tbr2 expressing cells indicated the possibility of greater proportion of cells co-expressing both these markers upon *MIR3607* expression. Therefore, in order to study this in detail, we performed Pax6+Tbr2 co-staining and measured the intensities of both these markers in each cell. We made scatter plots of Pax6/Tbr2 expression level (log 10 Fold Change) over their mean intensity (arbitrary units) in individual GFP+ cells and frequency distribution plots for each of these parameters. Analyses in individual cells demonstrated an average 60% increase in Pax6 levels upon *MIR3607* expression, with only an 8% increase in Tbr2 levels (**Fig. 8A,B,C**). This selective change resulted in a significant increase in the abundance of cells co-expressing Pax6 and Tbr2 (**Fig. 8D).** Further, *in silico* analyses that corrected either for the average increase in mean intensity of Pax6, or of Tbr2, revealed that the increase in Pax6+Tbr2 co-expression upon MIR3607 expression was rescued by Pax6 correction and not Tbr2 correction **(Fig. 8E,F).** This demonstrated that the observed increase in Pax6 levels, but not Tbr2, was sufficient to explain the differences between control and *MIR3607* expressing embryos in Pax6+Tbr2 co-expression, and therefore the co-expression of Pax6 and Tbr2 is largely attributed to the increased Pax6 expression.

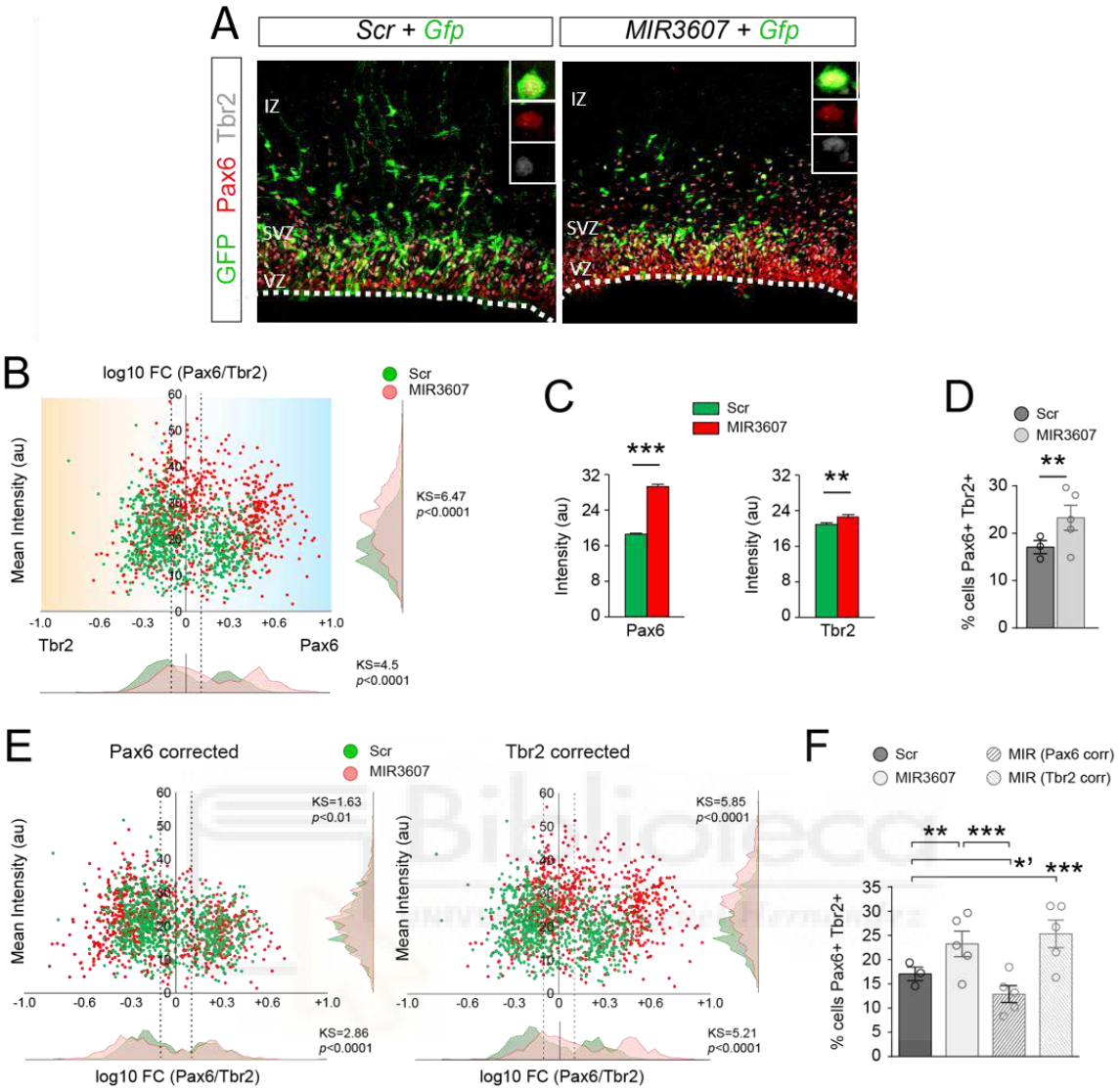


Figure 8. *MIR3607* expression cause increased Pax6 expression and Pax6/Tbr2 co-expression. **(A,B)** Scatter plots of ratio Pax6/Tbr2 expression level (log10 Fold Change) over their mean intensity (arbitrary units) in individual GFP+ cells and frequency distribution plots for each of the two parameters, from *Scr*- and *miR*-expressing embryos. Dashed vertical lines delimit Pax6/Tbr2 co-expression (-0.1<log10FC<+0.1). *N* = 738 cells, 3 embryos for *Scr*; 790 cells, 5 embryos for *MIR3607*. (**C**) Average expression intensity of Pax6 and Tbr2 among individual cells, and proportion of cells co-expressing both factors **(D),** as defined in (B). (**E**) Scatter plots and frequency distribution plots as in (B), but where Pax6 or Tbr2 values of each *MIR3607* cell were corrected to the average *Scr* value as in (C). (**F**) Proportion of cells co-expressing Pax6 and Tbr2, as defined in (E), for the indicated conditions. The increased Pax6/Tbr2 co-expression in *MIR3607* cells was rescued *in silico* with Pax6 correction, and hence it was due to

increased Pax6 intensity. Open circles in plots indicate values for individual embryos. Histograms indicate mean ± SEM; *n* = 3-5 embryos per group. X^2 test (D,F), t-test (C), Kolmogorov-Smirnoff test (B,E); *'*p*<0.02, **p<0.01, ***p<0.001.

When we next analyzed Pax6 and Tbr2 expression specifically in VZ, SVZ and IZ, we found an increase in Pax6 intensity in all three layers, with Tbr2 intensity mostly remaining unchanged, but showing significant slight increase in IZ. We also noted that in SVZ that there was nearly 50% increase in co-expression of Pax6 and Tbr2 upon *MIR3607* expression **(Fig. 9).**

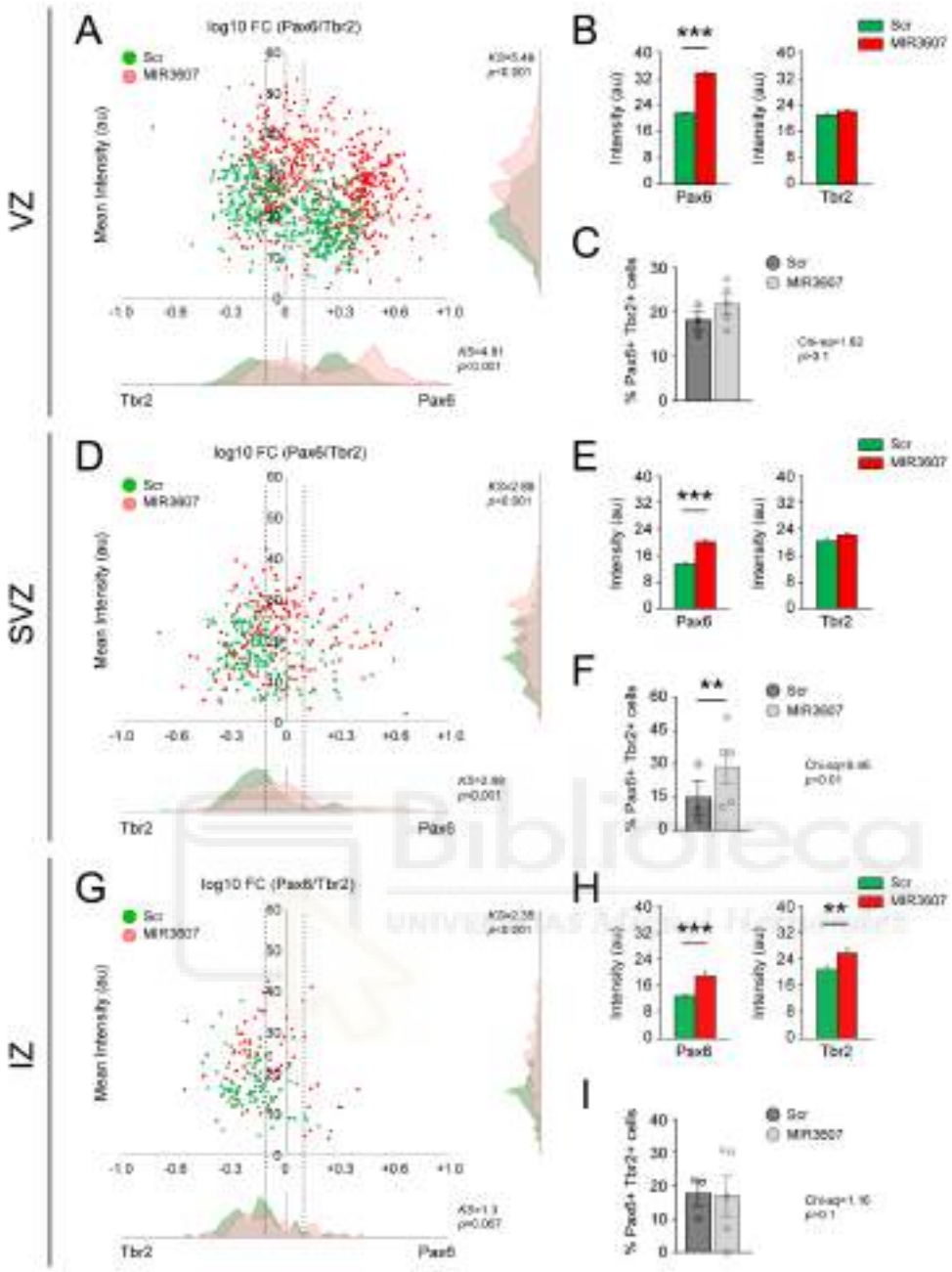
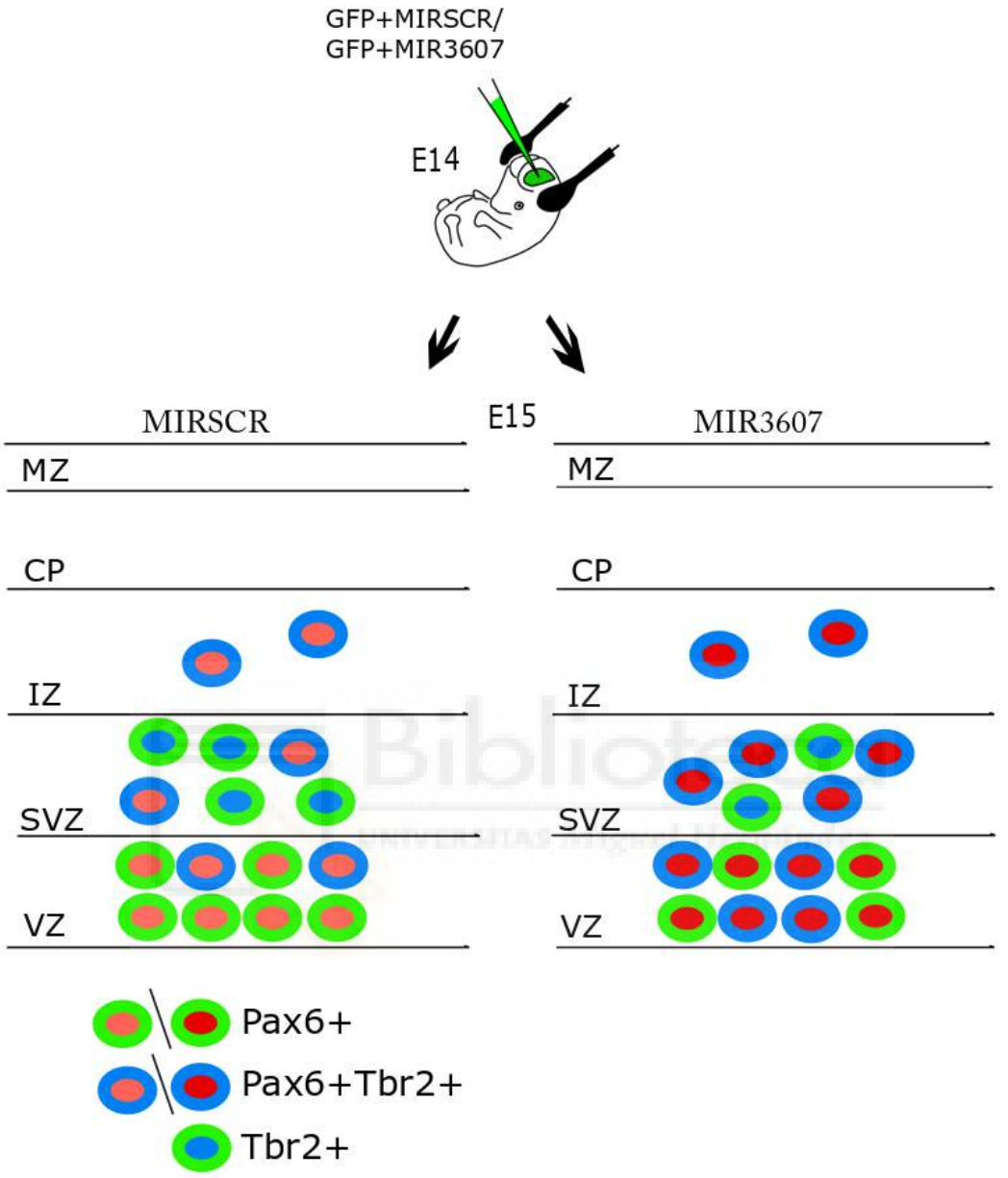


Figure 9. *MIR3607* drives overexpression of Pax6 in cortical cells. (**A,D,G**) Scatter plots of ratio Pax6/Tbr2 expression level (log10 Fold Change) relative to mean intensity of both markers (arbitrary units) in individual GFP+ cells, and frequency distribution plots for each of the individual parameters, at the indicated layers of the developing cortex. Dashed vertical lines delimit Pax6/Tbr2 co-expression (-0.1<log10FC<+0.1). (**B,E,H**) Average expression intensity of Pax6 and Tbr2 in individual cells at the indicated layers. (**C,F,I**) Proportion of cells co-expressing Pax6 and Tbr2 (-0.1<log10FC<+0.1). Differences in Pax6 expression intensity were largest in VZ, but differences in Pax6/Tbr2 co-expression were largest in SVZ. *N* = 471 cells VZ, 176 cells SVZ, 91 cells IZ, 3 embryos for *Scr*;

538 cells VZ, 176 cells SVZ, 73 cells IZ, 5 embryos for *MIR3607*. Kolmogorov-Smirnoff test (A,D,G), t-test (B,E,H), X^2 test (C,F,I); $^{**}p<0.01$, $^{***}p<0.001$.

Overall, our results showed a significant increase in co-expression of Pax6 and Tbr2, which is one of the defining features of OSVZ progenitors in ferret and primates *(Betizeau et al., 2013; Reillo and Borrell, 2012; Reillo et al.,2017).*

Interim summary 2



PART 3 – TRANSCRIPTOMIC ANALYSES TO IDENTIFY DEREGULATED SIGNALING PATHWAYS UPON *MIR3607* EXPRESSION

3.1 *MIR3607* activates signaling pathways driving proliferation and delamination of progenitors

In order to elucidate the regulatory pathways underlying the phenotypes observed above and mechanism of action of *MIR3607* leading to the changes in cortical progenitor cells reported above, we next investigated the impact of *MIR3607* expression at the transcriptomic level. We electroporated *in utero* the cerebral cortex of E14.5 embryos with plasmids encoding *MIR3607* or *Scrambled* plus GFP, and 24h later, we FACS-purified high GFP+ cells out of the electroporated area, isolated RNA and high quality RNA samples with RIN values above 9.5 were sent for RNA-sequencing to CRG, Barcelona (RNA-seq) (**Fig. 10A**). The results were analysed in collaboration with the research group of Dr. Jose Lopez Atalaya at the Instituto de Neurociencias-Alicante. We identified 173 genes differentially expressed (DEGs) in cortical progenitors upon *MIR3607* expression (FDR<0.01; **Fig. 10B**). A majority of DEGs were downregulated (58%), as expected from the action of a miRNA (**Fig. 10B**). Of the 76 genes in the mouse genome computationally predicted to be direct targets of *MIR3607* using available *in silico* target prediction tools, 63 were found to be expressed in our samples, and 9 of them were DEGs (**Fig. 10C**). The great majority of those DEGs were downregulated (8 out of 9), again as predicted from the action of a miRNA: Dnm3, Opcml, Pde4d, Tmem169, Cnr1, Bsn, Apc and Rnf38 (**Fig. 10C**). Several functional enrichment analyses were performed to capture biological information on DEGs. Gene Ontology (GO) analysis highlighted the Wnt signaling pathway and axon development as having the highest enrichment (**Fig. 10D**). Similarly, functional grouping of gene networks highlighted Wnt signaling pathway, neuroblast proliferation, regulation of neural precursor cell proliferation and L1CAM interactions (**Fig. 10E**). L1-CAM interactions are relevant for axon development, so these results were consistent with the observed deficient growth of callosal axons in P5 mice expressing *MIR3607* (**Fig. 10**). Functional annotation clustering analysis highlighted again, as top ranked, a cluster topped by the terms

Wnt signaling pathway, lateral plasma membrane and signaling pathways regulating pluripotency of stem cells (**Fig. 10F**).

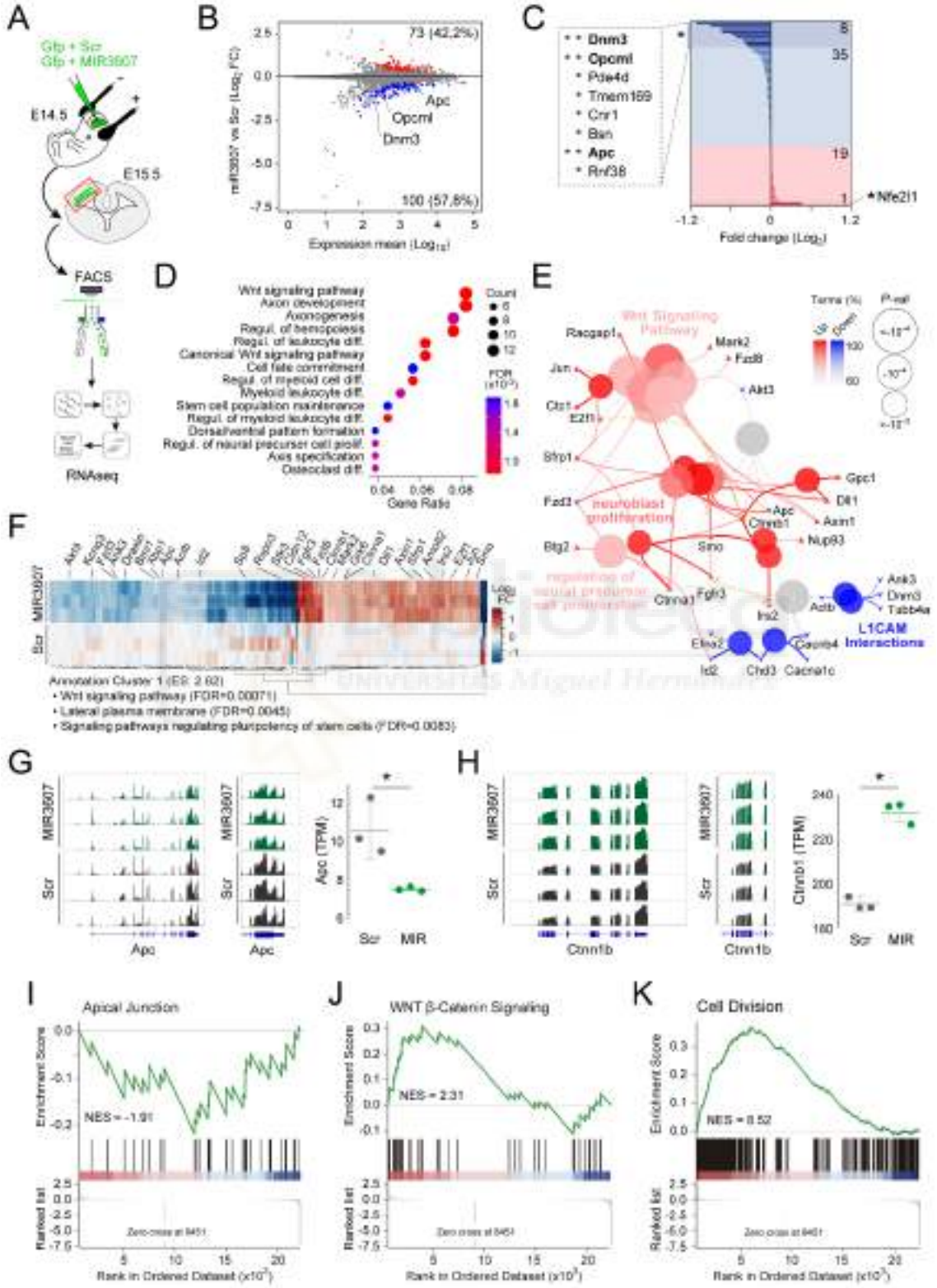


Figure 10. *MIR3607* promotes cell proliferation and activates Wnt signaling.

(**A**) Schema of experimental design: E14.5 mouse embryos were electroporated with *Gfp* plus *Scr*- or *MIR3607*-encoding plasmids, at E15.5 their brains were microdissected and dissociated, cells expressing high levels of GFP were purified by FACS sorting, and their pooled RNA expression profiles were analyzed by RNAseq. (**B**) Plot of Fold Change in gene expression (MAP estimate) in *MIR3607*- versus *Scr*-electroporated cells, over the gene's average expression level. Differentially Expressed Genes (DEGs; Adj. P < 0.01) are in red (upregulated, 73 genes) and blue (downregulated, 100 genes). Top three DEGs among *MIR3607*-predicted targets are named. (**C**) Fold change of 63 predicted *MIR3607* targets detected. DEGs are named (*Adj. *p*<0.1; **Adj. *p*<0.01); 8 genes were downregulated, and only one upregulated. (**D**) Functional enrichment analysis on DEGs, showing most significant enriched GO terms. Ontology: Biological Process. (**E**) Functionally grouped network based on functional enrichment analysis of DEGs. Size of nodes indicates statistical significance of the terms (Bonferrroni step down corrected P-values). Percentage of up- and down-regulated DEGs for each term is indicated by the color of the nodes on the network. (**F**) Heatmap of DEGs highlighting genes associated to the top ranked Annotation Cluster from Functional Annotation Clustering analysis (DAVID 6.8). The top three terms within this cluster and their statistical significance (FDR) are indicated. (**G,H**) Visualization of normalized coverage tracks from RNAseq data for whole transcript (left, middle), and expression levels (right) for *Apc* (G) and *Ctnn1b* (H) in *Scr*- and *MIR3607*-expressing cells. TPM, transcripts per million; *n* = 3 replicates per condition; Student two sample t-test; **p*<0.05. (**I-K**) Enrichment plots from GSEA for MSigDB Hallmark Apical Junction (NES = -1.91; *p*=0.01; Adj. *p*=0.021), WNT β-catenin Signaling (NES = 2.31; *p*=0.002; Adj. *p*=0.006) and GO term Cell Division GO:0051301 (NES = 8.52; *p*=0.002; Adj. *p*=0.008).

Altogether, these analyses revealed a prominent role of *MIR3607* in the regulation of Wnt/β-catenin signalling pathway, proliferative activity, axon development and lateral plasma membrane (**Fig. 10D-F**). All these biological functions closely matched the developmental processes that we found altered in our above phenotypic analyses of the developing cortex upon expression of *MIR3607* at E14.5. Indeed, *MIR3607* expression in cortical progenitor cells led to decreased expression of Adenomatous Polyposis Coli (APC), a key negative modulator of the canonical Wnt/β-catenin signalling pathway, and concomitantly to increased levels of Fgfr3, Fzd8, Ctnn1a and Ctnn1b transcription (**Fig.**

10B,C,E-H). Consistent with the GO analysis, gene set enrichment analyses (GSEA) *(Subramainan et al.,2005)* further confirmed a strong modulation by *MIR3607* of genes regulating Wnt/β-catenin signalling, the apical junctional complex and cell division in cortical progenitors (**Fig. 10I-K**). In summary, our transcriptomic analyses revealed that *MIR3607* expression causes dramatic changes in the expression levels of genes participating in biological functions and signaling pathways that are key for the amplification and delamination of cortical progenitor cells.

Part 4 - *MIR3607* CAUSES DELAMINATION AND AMPLIFICATION OF PROGENITORS IN THE EARLY EMBRYONIC CORTEX

4.1 Massive delamination and amplification of aRGCs in the early cortex by *MIR3607*

Progenitor cell amplification and self-renewal are singularly important at early stages of cortical development, prior to and at the onset of neurogenesis *(Telley et al.,2019).* Owing to the effects observed in the progenitors and significant pathways affecting proliferation being affected upon *MIR3607* expression, Wnt signaling in particular being highly active in the early embryonic cortex (*Mutch et al.,2009; Pöschl et al.,2013*; *Oberst et al.,2019)*, we next investigated the effects of expressing *MIR3607* in the early embryonic mouse cerebral cortex. To this end, we electroporated *MIR3607*-encoding plasmids into the cortical primordium of mouse embryos at E12.5 and investigated the immediate early effects in the cortex of electroporated embryos at E13.5. BrdU labeling experiments revealed that the cells in S-phase were perfectly aligned in the basal position of the VZ in control-electroporated and non-electroporated cortices (**Fig. 11A,B**). In contrast, expression of *MIR3607* caused the spreading of BrdU-incorporating cells over the entire thickness of the VZ, which revealed severe alterations in the organization of progenitor cells (**Fig. 11B**). Additionally, BrdU+ cells were frequently arranged in circles, resembling rosettes (**Fig. 11A**), which suggested delamination of progenitors, and increased rate of cell proliferation and amplification. Staining for Pax6 and Tbr2 revealed the disruption of both apical and basal progenitors, forming rosette like structures (**Fig. 11C, 11D**). Similarly, PHH3+ mitotic cells were displayed to abventricular positions across the VZ thickness in *MIR3607* embryos, compared to the normal arrangement of mitotic cells on the apical side of the VZ in control (**Fig. 11E)**. This disorganization was paralleled by a severe disruption of the apical adherens junction belt, as identified by Par3. In the area of cortex expressing *MIR3607*, Par3 was completely absent from the apical surface, instead forming small closed domains within the cortical parenchyma, in the lumen of rosettes (**Fig. 11F).**

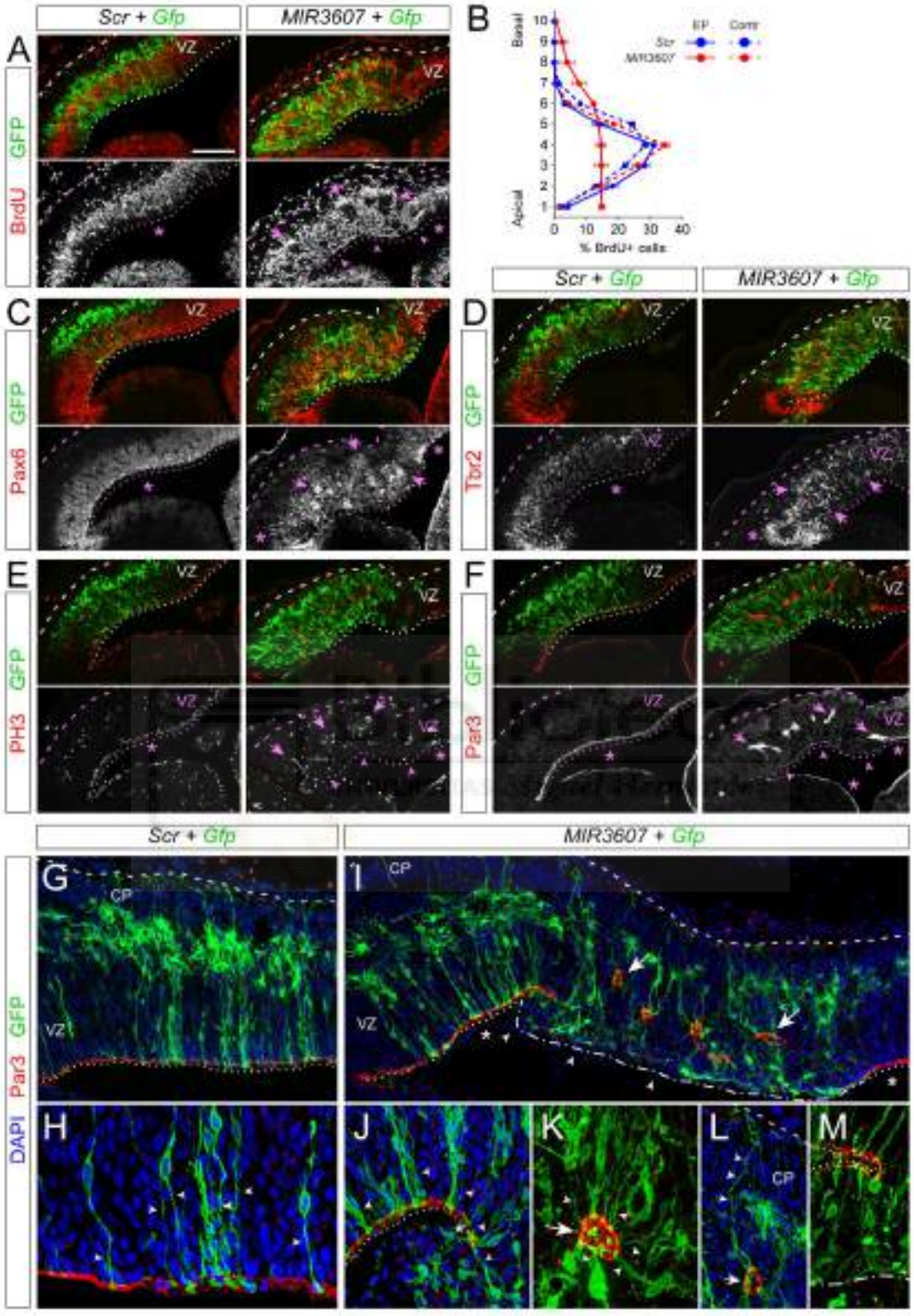


Figure 11. *MIR3607* drives massive delamination of apical progenitors and formation of rosettes. (**A,C-F**) Sections through the parietal cortex of mouse embryos electroporated at E12.5 with the indicated plasmids, analysed at E13.5 and stained as indicated. Expression of *MIR3607* caused a severe disruption of

the laminar organization of cycling progenitors (A), the Par3+ apical adherens junction belt (C) and the distribution of PH3 mitoses (D), Pax6+ RGCs (E) and Tbr2+ IPCs (F) (arrows). Arrowheads indicate areas with disrupted apical side of VZ. Arrows indicate rosettes of progenitor cells delaminated into the cortical parenchyma. Asterisks indicate areas with normal organization. (**B**) Quantification of binned distribution of BrdU+ cells across the cortical thickness. Data is from electroporated hemispheres (solid lines) and from non-electroporated, contralateral hemispheres (dashed lines). The typical accumulation of BrdU-incorporating cells in the basal side of the VZ (bins 3-5) was observed in *Scr*-electroporated (solid blue line) and contralateral hemispheres (dashed lines), but severely disturbed in *MIR3607* electroporated cortices (solid red line). Plots show mean ± SEM; *n* = 2,673 cells ipsi, 3,583 cells contra, 2 embryos, *Scr*; 5,150 cells ipsi, 5,485 cells contra, 4 embryos, *MIR3607*. Scale bar: 100µm (all panels).

When observed closely, we found the normal apical radial processes extending to the apical end of the VZ in controls. In contrast, upon *MIR3607* overexpression, in addition to the extension of normal radial processes by few cells, we found many cells resembling aRGCs extending their processes apically to the Par3 lumen within the cortical parenchyma and basally to the basal lamina at the cortical surface (**Fig. 11 G-M**). Altogether, these results revealed the clear formation of rosette structures upon *MIR3607* expression, with Par3 forming the core of rosettes, providing apical attachment surface to Pax6+ radial glial cells, undergoing mitosis locally forming Tbr2+ basal progenitors that migrate basally.

Next, we analyzed if the disorganization and rosette structures persisted at later stages (E15.5). To our surprise, the effects observed were even more dramatic. BrdU+ labeling revealed that progenitor cells failed to remain within the basal side of VZ, typical of control embryos, but were widespread through the SVZ and IZ, forming rosettes (**Fig. 12A**). Stains against Par3 demonstrated the persistent absence of apical adherens junction belt in VZ of *MIR3607* expressing embryos, and its presence as small circles at basal positions within the cortical parenchyma (**Fig. 12B**). Pax6 stains showed that in *MIR3607* expressing embryos, aRGCs no longer formed a compact VZ as in controls, but spread from the apical VZ surface to the IZ (**Fig. 12C**), forming cell clusters located basally, coincident with the location of rosettes. This massive disorganization of the VZ with delamination of

Pax6+ aRGCs also affected IPCs, identified with Tbr2 stains. Tbr2+ cells were also distributed ectopically in miR-expressing embryos, where they extended apically through the thickness of VZ to its apical surface, and basally through SVZ and IZ, where they formed distinct circular clusters (**Fig. 12D**).

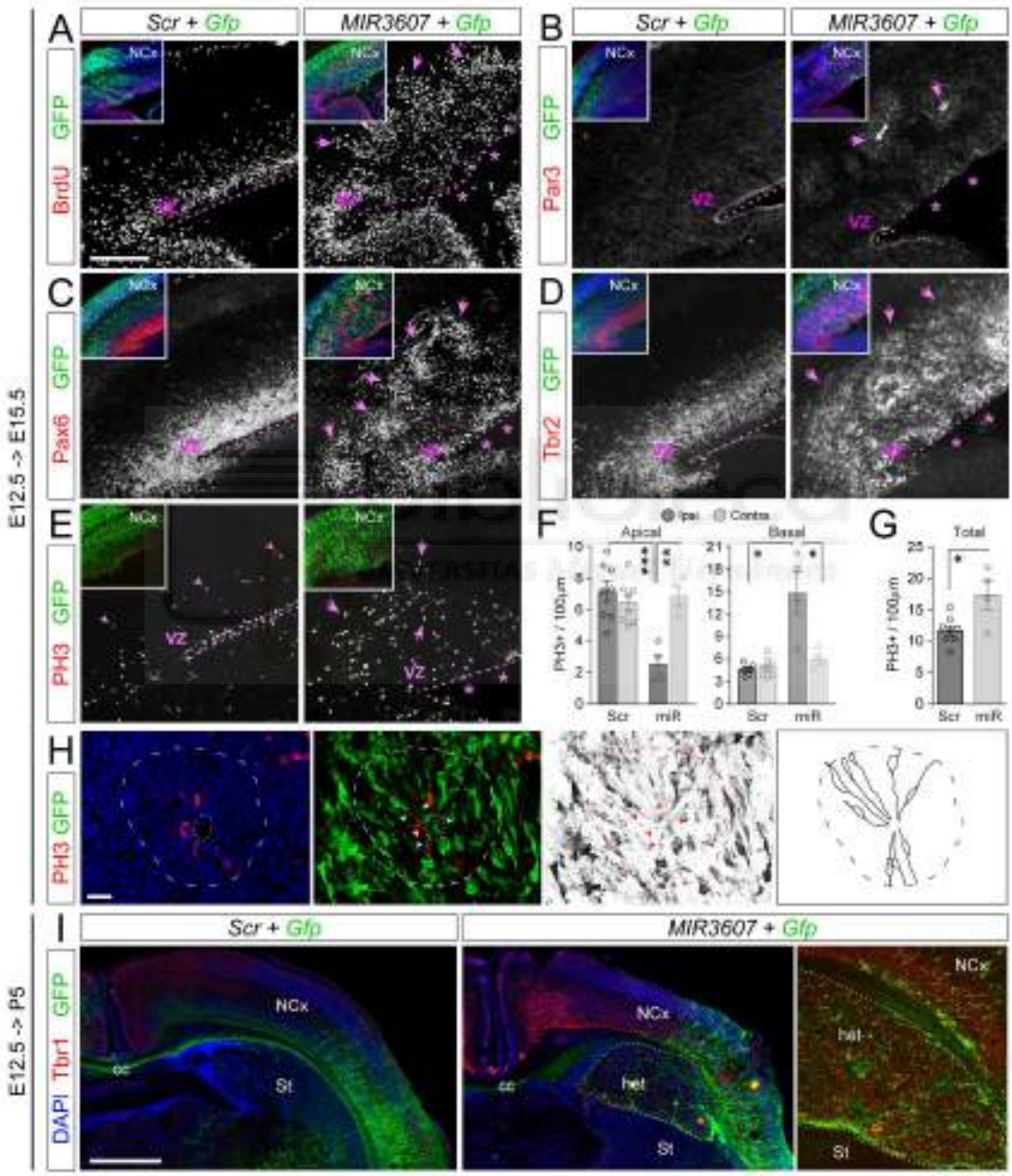


Figure 12. Severe disruption of apical junctions and formation of rosettes by *MIR3607* lead to subcortical heterotopia. (**A-D**) Sections through the parietal cortex of mouse embryos electroporated at E12.5 with the indicated plasmids, analysed at E15.5 and stained with the indicated markers. Expression

of *MIR3607* disrupted severely the laminar organization of VZ and the Par3+ apical adherens junction belt (asterisks). Arrows indicate rosettes of cycling (BrdU+), Pax6+ and Tbr2+ progenitor cells, containing a Par3+ lumen, delaminated into the cortical parenchyma. Insets in each image are low magnifications showing DAPI (blue) and GFP expression in the same area. (**E-G**) Distribution and quantification of mitotic cells in E15.5 embryos electroporated at E12.5 with the indicated plasmids. Histograms indicate mean ± SEM for the electroporated hemisphere (Ipsi) and the non-electroporated, contralateral hemisphere (Contra). Circles in plots indicate values for individual embryos. Expression of *MIR3607* severely decreased apical mitoses (asterisks) and increased basal mitoses (arrows), with an overall increase in mitotic density (G). *N* = 4-8 sections from 2-4 embryos per group; t-test; **p*<0.05, ***p*<0.01, ****p*<0.001. (**H**) Detail of a rosette (dashed line) in the cerebral cortex of an E15.5 embryo electroporated with *MIR3607* at E12.5, showing apical PH3 mitoses in the inner lumen (dotted line) surrounded by the apical processes of GFP+ aRGCs (arrowheads). Right panel shows line reconstructions of GFP+ cells within the rosette, demonstrating typical aRGC morphology with apical and basal processes radiating from the inner lumen. (**I**) Sections through the parietal cortex of mouse embryos electroporated at E12.5 with the indicated plasmids, analysed at postnatal day (P) 5, and stained for GFP and the deep-layer marker Tbr1. Note the mass of Tbr1+ neurons in the heterotopia (het) between the normal neocortex (NCx) and striatum (St) of the brain expressing *MIR3607*. cc, corpus callosum. Scale bars: 200µm (A-E), 10µm (H), 1 mm (I).

When we analyzed the rate of cell division by performing PH3 staining, expression of *MIR3607* led to a 65% loss of PH3+ apical mitoses alongside a 3-fold increase in basal mitoses, with an overall in mitoses by 48%, demonstrating increased progenitor cell proliferation. The mitotic cells were not aligned in a distinct VZ/SVZ, but spread basally through the IZ, occasionally forming small clusters (**Fig. 12E,F,G**). Overall, rosettes in the cortex of E15.5 *MIR3607* embryos displayed the same features as observed above in early embryos: a Par3+ lumen, an apical layer of Pax6+ cells, and a surrounding basal layer of Tbr2+ cells. Similar to the early embryos, GFP+ cells forming the core of rosettes confirmed their aRGC morphology, with distinct apical and basal processes radially aligned, and mitosis at the apical surface (**Fig. 12H**). Given the severe disruption of cells and formation of rosettes observed in the early embryonic cortex, we next examined the long-term consequences of this effect upon *MIR3607* expression.

Examination of P5 cortices revealed the formation of subcortical heterotopia underneath the electroporation site. The size of this heterotopia varied between animals, but was never observed in *Scramble*-electroporated control mice. Heterotopias were largely composed by cells positive for Tbr1, a marker of deep layer neurons, confirming that the MIR3607 expression did not change the neuronal fate. But a majority of cells in the heterotopia formed of Tbr1+ neurons were negative for GFP, indicating that the mechanism of emergence of this phenotype had a significant non-cell autonomous component (**Fig. 12I**).

In summary, we have demonstrated that the *MIR3607* expression in the early developing cortex induced dramatic changes, as we expected according to our functional analyses, which revealed the impact of MIR3607 upon delamination and proliferation of progenitors. Overall, the effects observed reveal the function of *MIR3607* on neural progenitor cell proliferation, cell division, lateral plasma membrane and apical junction.

4.2 *MIR3607* promotes early cortical progenitor amplification and formation of rosettes by de-repression of β-Catenin signaling

It was clear from the above analyses that *MIR3607* promotes early cortical progenitor amplification and formation of rosettes. We next questioned the mechanism by which *MIR3607* acts to cause these phenotypes. *MIR3607-5p* has been shown to target the 3'UTR of *APC*, a key repressor of βCatenin signaling *(Lin et al.,2017)*, which was found to be significantly downregulated in our transcriptomic analyses, among other predicted target genes. Accordingly, our transcriptional profiling experiments revealed the strong and preferential activation of βCatenin signaling pathway at the transcriptional level in the developing mouse cerebral cortex upon *MIR3607* expression. Therefore, we suspected that the protein levels of activated beta catenin could have been upregulated upon downregulation of a beta-catenin destruction complex factor APC. As expected, our immunostains against activated βCatenin, revealed a two-fold increase in expression in the VZ of *MIR3607* expressing mouse embryos

24hr after electroporation (**Fig. 13A,B**). This further supported that the effects of *MIR3607* on cortical progenitor amplification and rosette formation might be caused by the overactivation of βCatenin signaling. Moreover, the effects of *MIR3607* expression in the embryonic mouse cerebral cortex causing a massive amplification of cortical progenitors and disturbance of VZ integrity, resembled the effect produced by overactivation of the Wnt/βCatenin signaling pathway *(Chenn and Walsh.,2002; Chenn and Walsh.,2003; Herrera et al.,2014; Poschl et al.2013; Woodhead et al.,2006; Wrobel et al.,2007).* To investigate this possibility, we electroporated E12.5 embryos with a constitutively active form of βCatenin (Δ90-βCat), to test if this would phenocopy the effects produced upon *MIR3607* expression.

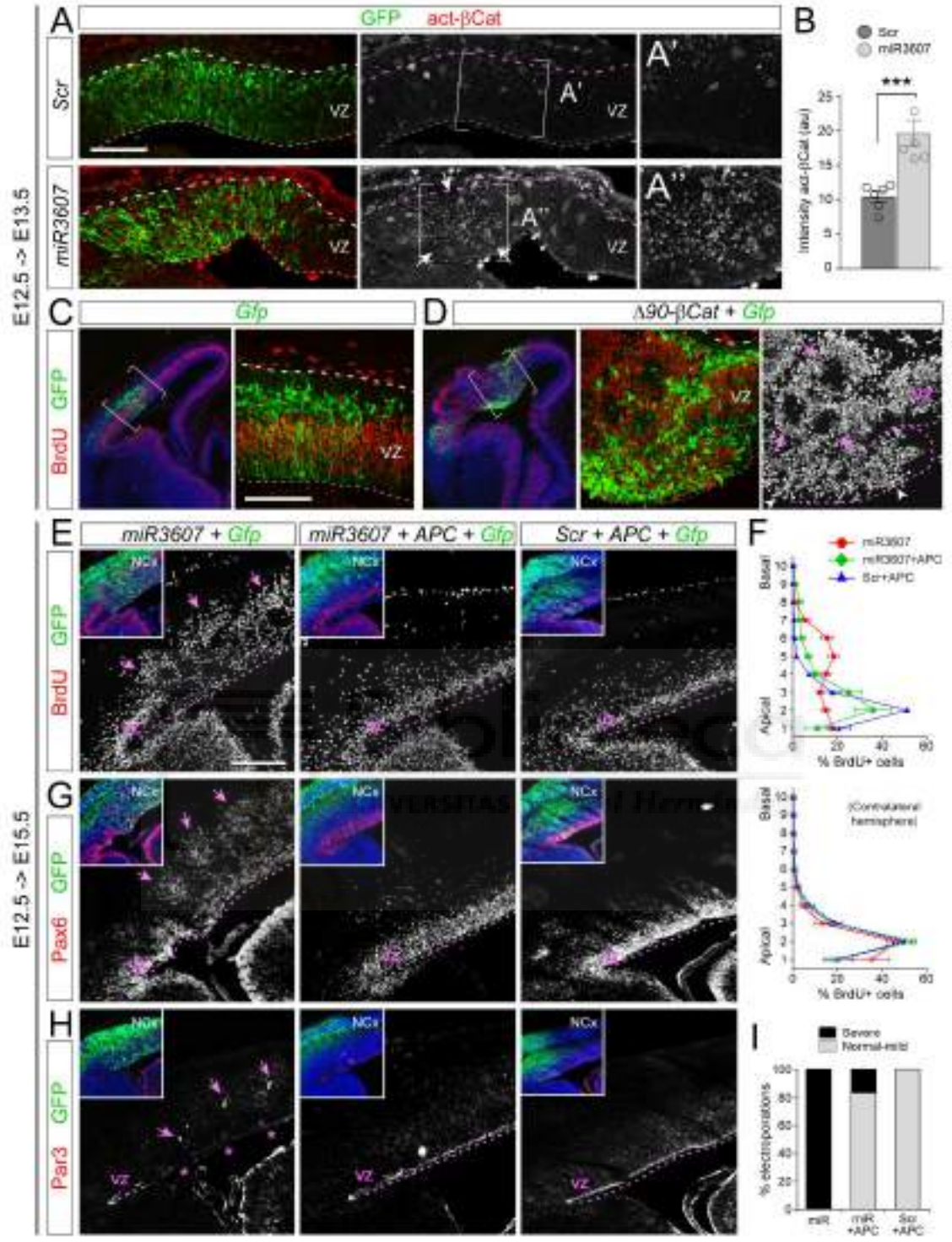


Figure 13. *MIR3607* promotes early progenitor amplification and formation of rosettes by de-repression of βCatenin signaling. (**A,B**) Sections through the parietal cortex of E13.5 mouse embryos electroporated at E12.5 with the indicated plasmids and stained against activated βCatenin, and quantification of signal intensity. (A',A'') are high magnifications of the corresponding areas boxed in (A). Histogram indicates mean ± SEM; circles indicate values for individual embryos; *n* = 5-6 embryos per group; t-test; ****p*<0.001. (**C,D**) Parietal cortex of

E13.5 mouse embryos electroporated at E12.5 with the indicated plasmids. Expression of constitutively active βCatenin very severely disrupted the integrity of the VZ (white arrowheads), with massive amplification and delamination of cycling (BrdU+) progenitor cells and formation of rosettes (pink arrows). (**E-H**) Sections through the parietal cortex of E15.5 mouse embryos electroporated at E12.5 with the indicated plasmid combinations and stained as indicated (E,G,H), and binned distribution of BrdU+ cells across the cortical thickness (F). In (F), top graph is data from electroporated, ipsilateral hemispheres; bottom is data from non-electroporated, contralateral hemispheres. Asterisks indicate the absence of the Par3+ apical adherens junction belt; arrows indicate rosettes. The severe disruptions caused by *MIR3607* expression were rescued in embryos co-expressing *MIR3607* and APC, and absent in embryos expressing *Scr*+APC. (**I**) Quantification of embryos with germinal layer disturbance. Scale bars: 100µm (A-D), 200µm (E,G,H).

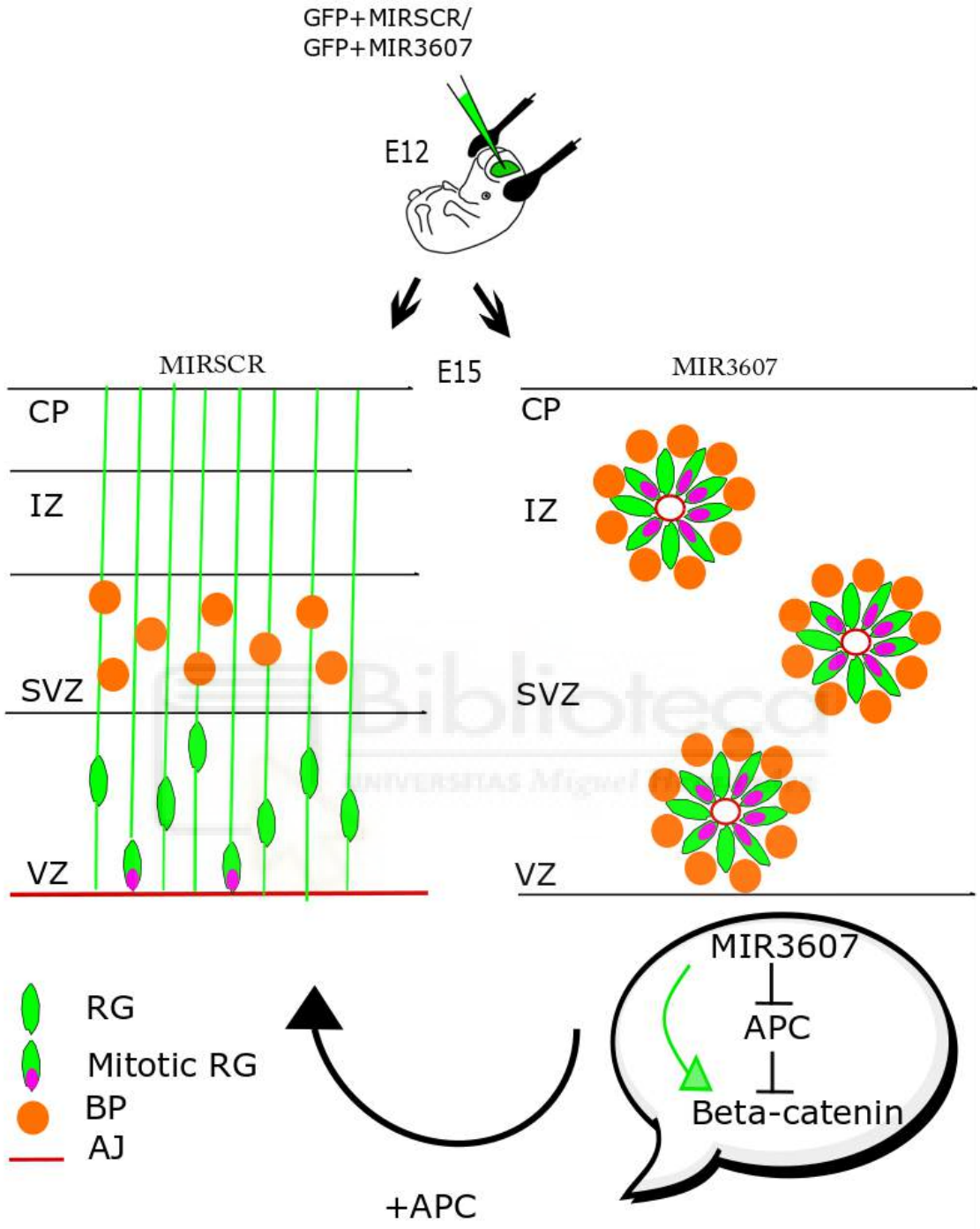
As expected, when analyzed one day after electroporation (E13.5) BrdU labeling revealed disorganization of cycling progenitor cells in the cerebral cortex, forming multiple proliferative rosettes (**Fig. 13C,D**), as observed upon *MIR3607* expression, but showing even greater level of disruption. This further supported the idea that the effects caused upon *MIR3607* expression was mediated by the overactivation of βCatenin signaling possible upon repression of *APC.*

Next, in order to investigate if the effects are mediated through repression of *APC*, we performed rescue experiments by additionally expressing APC alongside *MIR3607.* We performed electroporations in the E12.5 mouse cortex, and analyzed the effects at E15.5. As shown previously, expression of *MIR3607* alone caused a very severe disorganization of cycling progenitor cells forming rosettes, identified by BrdU labeling (**Fig. 13E,F**). Further, Par3 and Pax6 stains showed the formation of these proliferative rosettes (**Fig. 13G, H**). Remarkably, co-electroporation of *MIR3607* with APC completely rescued the formation of rosettes and lead to the formation of intact germinal layers in a majority of embryos, whereas expression of APC with a control scrambled miRNA sequence, had no effect on cortical progenitor cells nor on the normal organization of germinal layers (**Fig. 13E-I**).

Together, these results demonstrated that expression of *MIR3607* in the embryonic mouse cerebral cortex reduces the levels of expression of APC, which

is a part of βCatenin destruction complex. This leads to an abnormal accumulation of activated βCatenin and the overactivation of canonical Wnt pathway, causing the overproliferation of cortical progenitors and the instability of the VZ, forming delaminated proliferative rosettes.

Interim summary 3



PART 5 - CONSERVATION OF *MIR3607* FUNCTION IN GYRENCEPHALIC SPECIES

5.1 *MIR3607* expression in human cerebral organoids cause fragmentation of VZ into several proliferative closed ventricles

After elucidating the functional mechanism of *MIR3607* in cerebral cortex development using mouse as a model, we next investigated if the function of *MIR3607* is possibly conserved in human by causing overexpression of *MIR3607* in human cerebral organoids. Human cerebral organoids electroporated at 39 days in culture, when fixed and stained for Par3 seven days later, we identified fragmentation of ventricular zone leading to increased proliferative closed circles resembling rosettes, upon *MIR3607* overexpression **(Fig.14 B).** As a result, the density of ventricles increased upon *MIR3607* overexpression compared to control **(Fig.14 A,B,E)**. In addition to this, the perimeter of the ventricular surface was increased upon *MIR3607* overexpression compared to control **(Fig. 14 C,D,F).** Overall, the observed phenotypes resembled the effects observed in mice, forming rosette like structures, likely as a result of increased proliferation of apical progenitors along the ventricle. Also, the increase in perimeter of the ventricular surface resembled the effect caused by βCatenin overexpression in mouse neuroepithelium (*Chenn and Walsh, 2002*), which suggested the possibility that the mechanism of action of *MIR3607* is conserved, causing suppression of human APC, as its mRNA contains conserved target sites for *MIR3607* in the 3´ UTR.

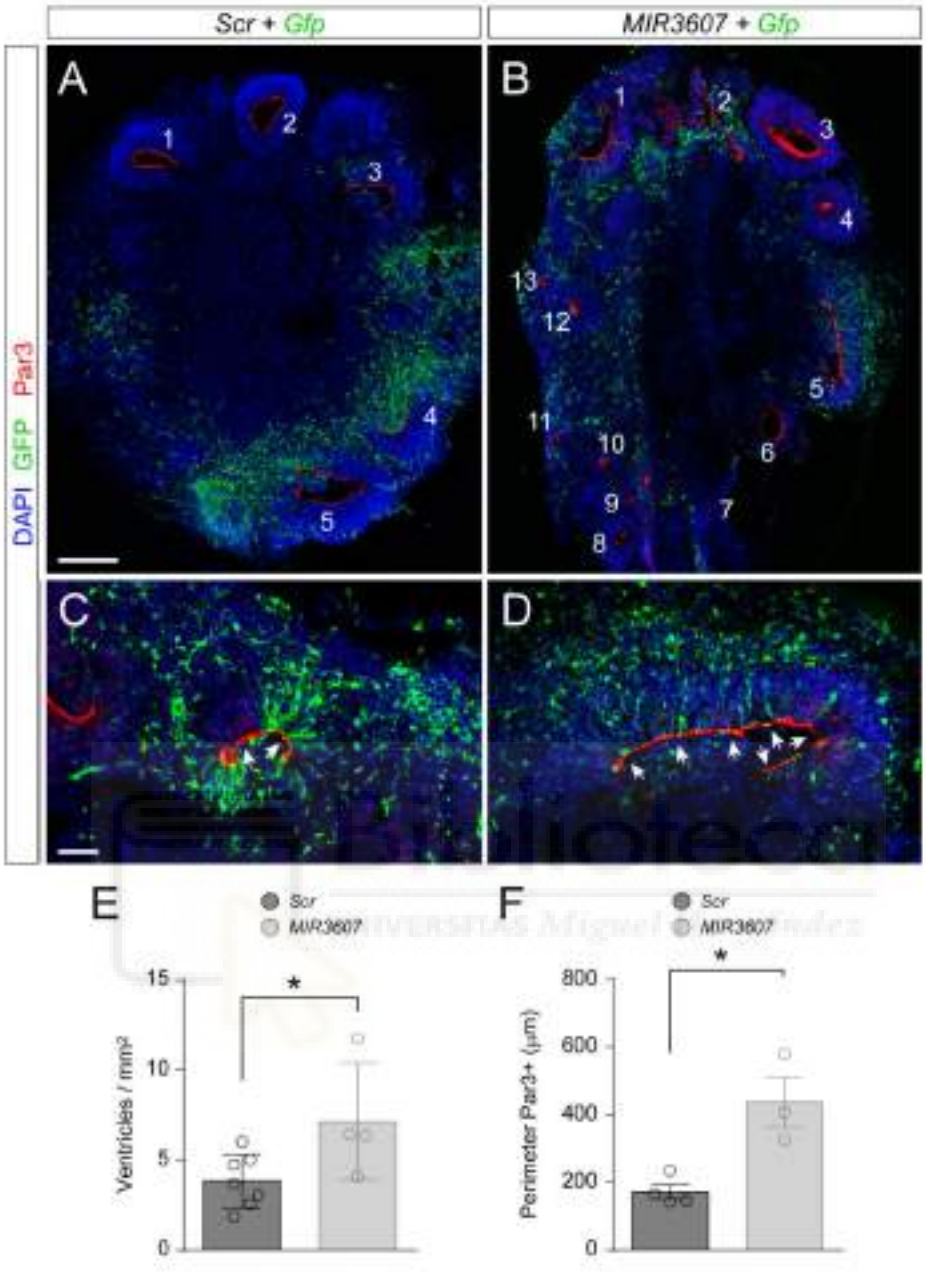


Figure 14. *MIR3607* promotes ventricular fragmentation and growth in human cerebral organoids. (**A-D**) Cross-sections through human cerebral organoids electroporated at 39 days in culture with *Gfp* plus *Scrambled-* (*Scr*) or *MIR3607*-encoding plasmids, fixed 7 days later and stained as indicated. Individual ventricles are numbered in panoramic views of representative organoids (A,B). Detailed views of representative electroporated ventricles are shown (C,D) with the apical surface identified by Par3 stain (red, arrows). (**E,F**) Quantifications of density of ventricles per surface area of organoid (E), and average length of Par3+ apical surface of individual electroporated ventricles (F). Histograms indicate mean ± SEM, and circles within indicate values for individual

organoids (in F, average value of ventricles per organoid); *n* = 3-7 organoids per group; t-test, **p*<0.05. Scale bars: 200µm (A,B), 50µm (C,D).

5.2 *MIR3607* targets APC in ferret and is required for aRGC amplification and maintenance of polarity

After studying the conserved function of *MIR3607* overexpression in human cerebral organoids, we next investigated if the molecular mechanism of action of *MIR3607* was also conserved, and what was the function of endogenous *MIR3607*. In order to do this, we first generated loss-of-function constructs against *MIR3607* (Tough-Decoy). Transfection of HEK cells with TUD-3607, or TUD-Scr as control, followed by qRT-PCR for *MIR3607*, showed a dramatic reduction of *MIR3607* levels **(Fig. 15A).** Then we transfected the ferret brain cell line Mpf with *MIR3607* encoding plasmids, Scrambled control plasmids, TUD-*MIR3607* or TUD-Scrambled controls, and measured the levels of APC protein by western blot. Ferret *APC* mRNA also contains target sites for *MIR3607* in its 3´ UTR. As expected, *MIR3607* overexpression caused a reduction in APC, and in turn expression of TUD-*MIR3607* lead to upregulation of APC, confirming APC as a conserved target of *MIR3607* in ferret, and that endogenous levels of *MIR3607* in ferret cells are sufficient to repress APC expression **(Fig. 15B).** Therefore, this further confirmed the notion that the mechanism of action of *MIR3607* is exerted through repression of APC, and that this function is conserved in ferret.

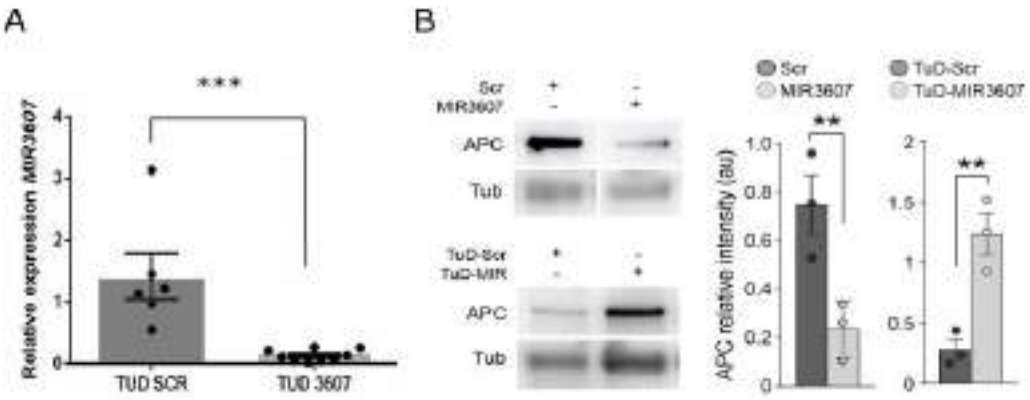


Figure 15. *MIR3607* regulates the expression of APC in ferret. (**A**) qPCR results of *MIR3607* expression levels (arbitrary units) in HEK293 cells transfected with psil-*Scr* and psil-*MIR3607.* circles in plots indicate values for individual replicas. (**B**) Western blots of APC in Mpf cells transfected with the indicated plasmids, and densitometry quantifications, showing reduction in expression of APC upon *MIR3607* expression and an increase upon downregulation of *MIR3607.* Histograms indicate mean ± SEM; (t-test, ***p*<0.01; ****p*=0.0008)

Finally, we investigated the importance of presence of *MIR3607* in ferret cerebral cortex by performing loss of function experiments using TUD-*MIR3607*. For this purpose, we performed *in utero* electroporations of pregnant ferret females at embryonic stage E35, when the expression of *MIR3607* was high in the VZ **(Fig. 3B, 16A).** Two days later at E37, preliminary analyses revealed defective apical detachment of the radial glial cells leading to increased number of cells accumulating in the VZ, and impaired interkinetic nuclear migration as revealed by decrease in apical mitoses and increase in sub-apical and basal mitoses, as compared to control **(Fig. 16B,C,D,E).** We next questioned if this effect is mediated through *APC* and therefore to answer that, we performed similar experiments with *APC* encoding plasmid/GFP control to check if we could phenocopy the results obtained upon TUD-3607 expression **(Fig. 15F).** As expected, expression of APC caused a similar phenotype but even more dramatic with higher percentage of cells accumulating in the Ventricular zone and concomitant increase in sub-apical mitoses upon reduced apical mitoses, compared to the control **(Fig. 16G,H,I,J).**

Overall, here we have demonstrated the essentiality of *MIR3607* in ferret cerebral cortex, whose absence would lead to impairment in detachment of apical radial glial cells, which would be essential at this stage to form OSVZ *(Martinez-Martinez et al.,2016).* Any impairment caused in the formation of OSVZ, would negatively impact the expansion and folding of the cerebral cortex.

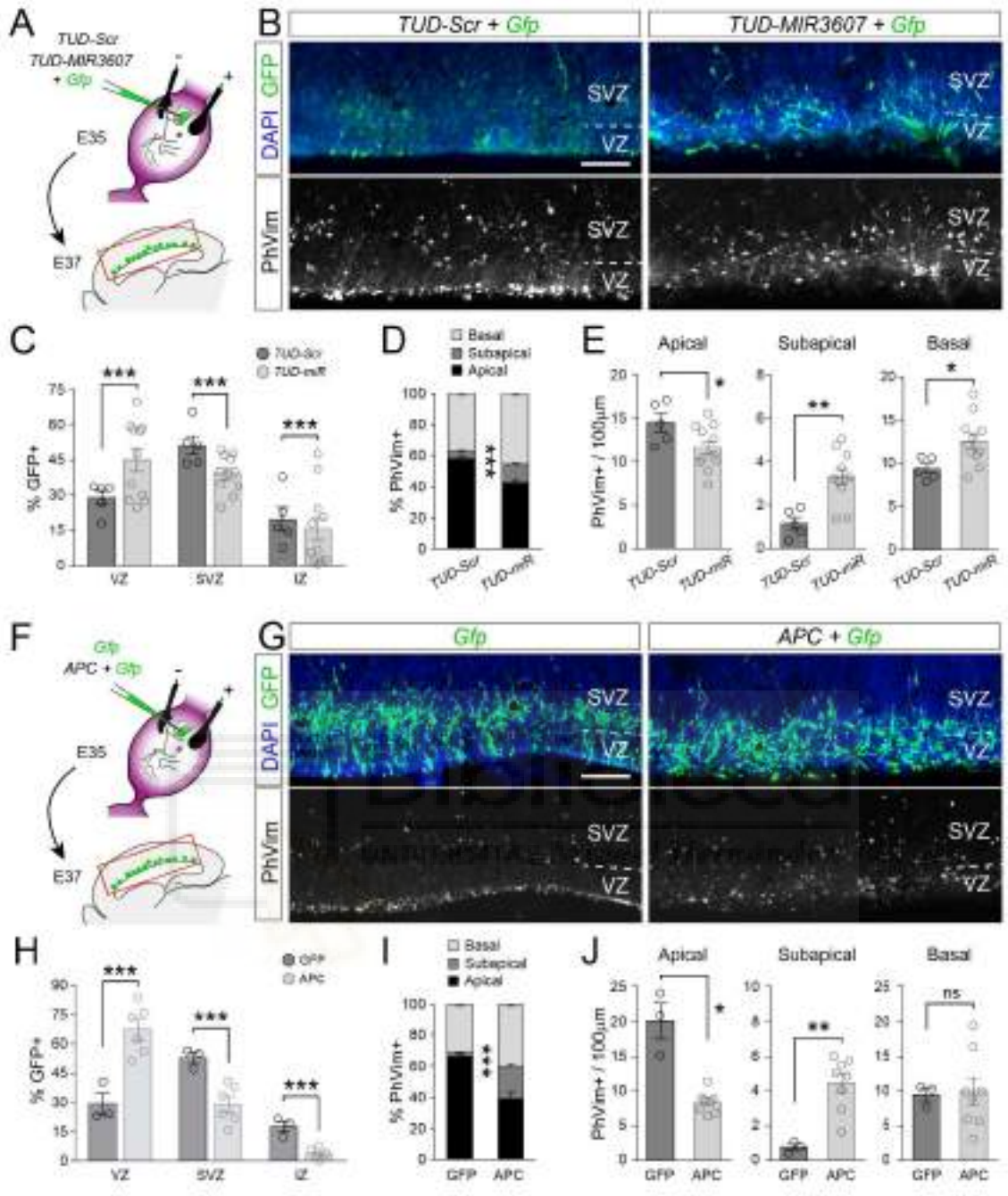
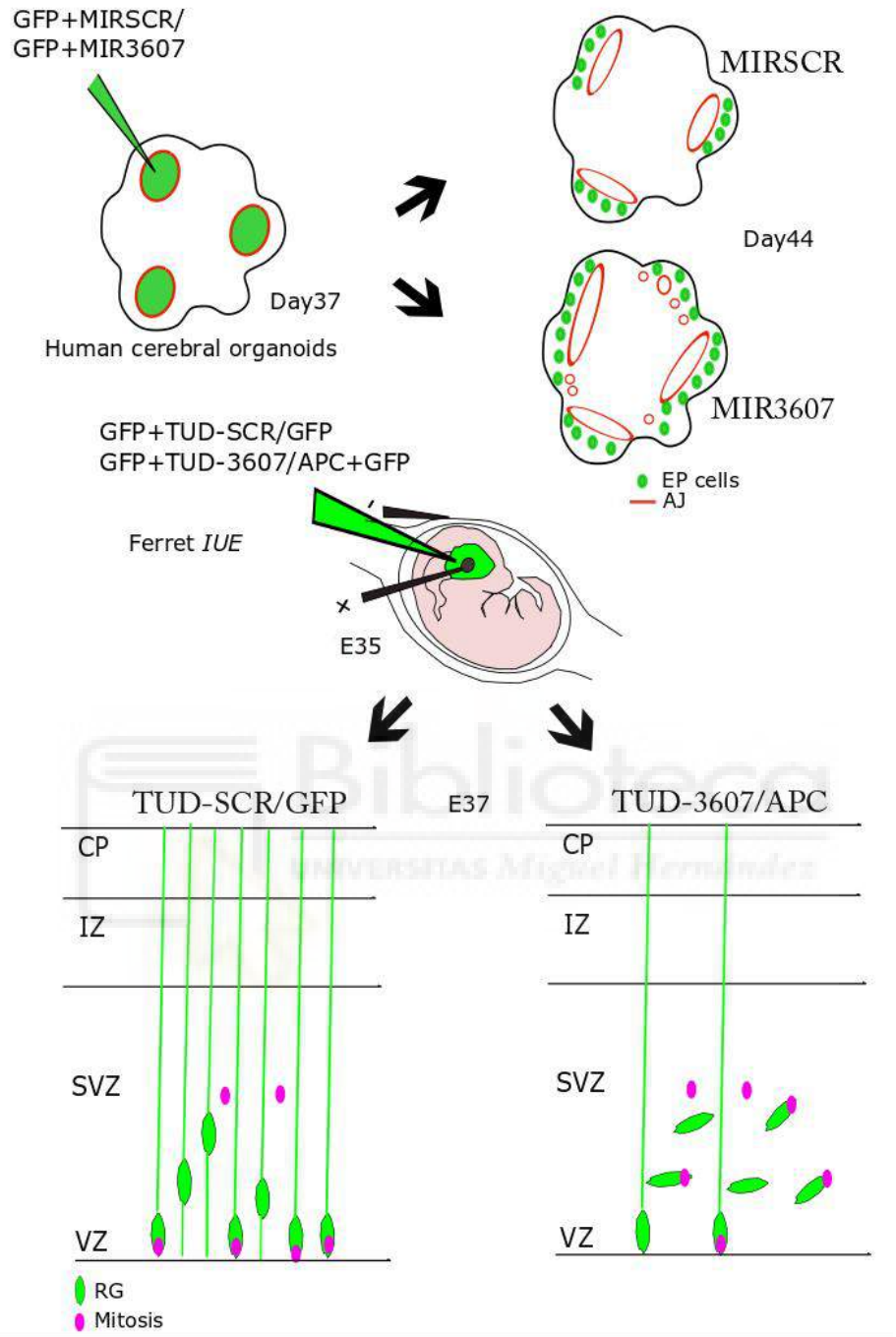


Figure 16. *MIR3607* is essential for proper delamination of progenitors. (**A,F**) Schema of Ferret in utero electroporation at E(35) and analyses at E(37) with the indicated plasmids. **(B,D,E**) Distribution of mitotic (PhVim+) cells in the ferret cerebral cortex at the apical, sub-apical and basal level between TUD-Scr and TUD-3607 electroporated embryos. (**C**) Distribution of GFP+ cells across different layers of the cortex (VZ, SVZ, IZ). **(G,I,J**) Distribution of mitotic (PhVim+) cells in the ferret cerebral cortex at the apical, sub-apical and basal level between GFP and APC electroporated embryos. (**H**) Distribution of GFP+ cells across different layers of the cortex (VZ, SVZ, IZ). Analyses indicate defective apical

delamination and radial migration of cells, resulting in increase in subapical mitoses upon expression of TUD-3607/APC.

Interim summary 4



DISCUSSION

Evolutionary significance of *miR3607* in cerebral cortex development

The diversity of neural progenitors and the extent of proliferation of these progenitors is an important determinant of the size of the cerebral cortex across evolution *(Cardenas et al.,2018; Cardenas and Borrell.,2020)*. The increased proliferation of aRGCs and basal progenitor cells has been linked to radial and tangential expansion respectively, thereby increasing the overall size of the cerebral cortex *(Chenn and Walsh.,2002; Nonaka-Kinoshita et al.,2013).* Furthermore, the presence of expanded and complex subventricular germinal layers (ISVZ and OSVZ), containing a high diversity of progenitors and a high proportion of bRGCs, is associated to species with large and folded cerebral cortex, including human and ferret *(Betizeau et al., 2013; Fietz et al., 2010; Hansen et al., 2010; Reillo & Borrell, 2012; Reillo et al., 2011; Smart et al., 2002)*. These bRGCs are characterized as self-amplifying proliferative progenitors that express either Pax6 or Pax6 plus Tbr2, with a basal process that induces a fanned array of additional radial fibers which cause the tangential dipersion of radially-migrating neurons *(Reillo & Borrell, 2012; Reillo et al., 2011)*.

Transcriptome analyses across different species with smooth and gyrified cerebral cortex identified several protein-coding genes that are important regulators of cerebral cortex expansion and gyrification *(Fiddes et al.,2018; Fietz et al.,2012; Florio et al.,2015; Florio et al.,2017; Florio et al.,2018; Suzuki et al.,2018; de Juan Romero et al.,2015).* This includes genes that are differentially expressed in the germinal layers across different species, different types of progenitors and genes that are specific to primates or humans. Manipulation of some of these identified genes in small and smooth mouse cerebral cortex often led to the features specific to large and gyrified brains. Studies involving overexpression of *Fgf2*, human specific genes *ARHGAP11B/TMEM14B*, or downregulation of *Trnp1* or *Flrt1/3*, lead to cortical folding in mouse, with increase in expansion of basal progenitors including bRGCs, or due to changes in migration of neurons *(Del Toro et al.,2017; Rash et al.,2013; Stahl et al.,2013; Florio et al.,2015; Liu et al.,2017).* Similarly, overexpression of *Pax6*, *Hopx,* or

the human specific gene *NOTCH2NL*, in the mouse cerebral cortex leads to significant expansion of the cortical progenitor cells with features known to be relevant to large and folded brains *(Vaid et al.,2018; Wong et al.,2015; Fiddes et al.2018; Suzuki et al.,2018).*

Despite an increasing number of studies on primate or human specific protein coding genes, or genes differentially expressed across species, which are important for expansion and complexification of the cerebral cortex, little is known about the function of non-coding genes that emerged along different periods of evolution, or that are expressed only in higher order mammals. Given the potential of non-coding genes, including lncRNAs and miRNAs, in regulating the expression of important genes across different cell types and cerebral cortex development, their importance in the evolution of the cerebral cortex has not been studied enough. Important candidates in the context of evolution and expansion of the cerebral cortex include miRNAs that are abundantly expressed (with detectable expression levels in development compared to other non-coding RNAs) and novel miRNAs that emerged at lineage divergence points in evolution *(Heimberg at al.,2008; Fineberg et al.,2009; Dehay et al.,2015).* MiRNA profiling studies in primates identified several miRNAs differentially expressed across cell types and developmental stages, including primate-specific miRNAs regulating the expression of cell cycle and neurogenic regulatory proteins *(Nowakowski et al.,2018; Arcila et al.,2014; Moreau at al.,2013).*

In this context, our previous transcriptomic analysis of germinal layers of the developing ferret cortex uncovered pre-*MIR3607* as a non-protein coding gene with potential roles in evolution of the cerebral cortex, and thus an interesting candidate to study. Our *in situ* hybridization stains revealed the expression of the mature *MIR3607* in the germinal layers of the human embryonic cortex at 16 gestational weeks, around the period of peak neurogenesis. Its expression was also detected in the germinal layers of the developing ferret cerebral cortex. Its temporal pattern of expression is particularly interesting because it is highly expressed in VZ at E35, a stage corresponding to the critical period when OSVZ bRGCs are generated. Then, at P2, when bRGCs seeding the OSVZ are not generated from VZ anymore, we find expression of *MIR3607* low in VZ but high in OSVZ, when the already generated bRGCs undergo self-amplification within

the OSVZ *(Martinez-Martinez et al.,2016).* These expression patterns support a role for *MIR3607* in promoting the generation and amplification of bRGCs. Consistent with this interpretation, expression of *MIR3607* in the small and smooth mouse cortex at the peak of neurogenesis is undetectable, suggesting that it is limited to the development of large and folded brains. Although we find similar sequences of pre-*MIR3607* in the genome of mouse, with very few nucleotide changes, its absence of expression in the mouse suggests that other regulatory mechanisms repress (or simply do not drive) its expression. At the genomic sequence level, we find 100% conservation only among members of the primate family *Hominidae*, including humans, which may render additional advantages over other species beyond expression, potentially contributing to cortical complexification. Otherwise, the mature miRNA sequence is completely conserved among other members of the order *Primates* and few exceptions from other orders. The fact that dynamic nucleotide changes across phylogeny occur only in the predicted mature *MIR3607* sequence, with no changes observed in its complementary 3p counterpart (with less or no function) among the species analyzed, suggest the possibility of increased complexity of regulation and diverse functions exerted by *MIR3607* among higher order mammals.

Our results upon expression of *MIR3607* in the early embryonic mouse cerebral cortex suggest that *MIR3607* strongly promotes the proliferation of cortical progenitor cells and expansion of their pool, which is important at early stages of development, and drive their delamination from the VZ to basal positions, while retaining apical-basal polarity and Pax6 expression, typical of bRGCs. These are features greatly enhanced in the developing cerebral cortex of human, macaque and ferret, which may contribute to the abundant formation of bRGCs *(Fietz et al.,2010; Hansen et al.,2010; Reillo and Borrell.,2012; Reillo et al.,2011; Smart et al.,2002),* and to the enlargement of these cortices (*Fernandez et al.,2016; Nonaka-Kinoshita et al.,2013; Reillo et al.,2011)*. In agreement with this line of reasoning, overexpression of *MIR3607* in human cerebral organoids promoted the formation of closed proliferative ventricles, resembling delaminated rosettes, and increased apical surface area, supporting increased proliferation and aRGC expansion. On the other hand, downregulation of *MIR3607* in ferret cerebral cortex caused severe defects in proliferation, polarity and delamination of

individual aRGCs, impairing their self-amplification. In summary, our results suggest that the function of *MIR3607* is conserved in the developing cortex across species, and it is consistent with this miRNA favoring production of neurons for large brains across evolution.

MIR3607 controls different aspects of cerebral cortex development

A well-structured cortex requires a multi-step process that has to be perfectly coordinated for its effective functioning. This includes generating neurons in the right numbers and at the right time, migration of neurons to the right place, extension of dendrites and projection axons to different regions of the brain for establishing functional connectivity across different regions. Several miRNAs have been previously identified as potentially influencing several aspects of cortical development by targeting different genes and pathways *(Barca-Mayo and De Pietri Tonelli, 2014; Rajman and Schratt.,2017)*. The results of this Thesis suggest that *MIR3607* is capable of influencing all the above-mentioned aspects of development by regulating different genes and pathways. Our computationally predicted targets included a large set of genes important for neurogenesis and axon growth. In agreement with this, our gene ontology analysis of DEGs upon *MIR3607* overexpression highlighted axon development, cell fate commitment and regulation of neuronal precursor proliferation as highly enriched terms, among others. Moreover, the list of DEGs contained eight computationally predicted direct target genes that are downregulated, most of them having the conserved *MIR3607* target sites in their 3´ UTR, in human and other species, favoring the possibility that *MIR3607* is capable of targeting these genes across species.

Our results suggest that *MIR3607* overexpression in mouse causes delamination of progenitors and increased proliferation as immediate early effect, followed then by increased cell cycle exit and premature neurogenesis. Several miRNAs are known to be important for mammalian neurogenesis, including the brain enriched miRNAs let-7, miR-9, miR-124 and miR-137 *(Fernandez et al.,2020; Sun et al.,2015; Petri et al.,2014)*. Apart from these, miRNAs recently identified as

primate-specific, which are not expressed in mouse, mostly target cell cycle and neurogenesis regulating genes, thereby leading to increased neurogenesis, contributing to cortical expansion and folding (*Nowakowski et al.,2018; Arcila et al.,2014).* Similarly, in accordance with the phenotypes observed, *MIR3607* is found to alter the expression of genes associated with apical junctional complex and cell division in cortical progenitors, suggesting similar importance as primate specific miRNAs (**Fig. 16**).

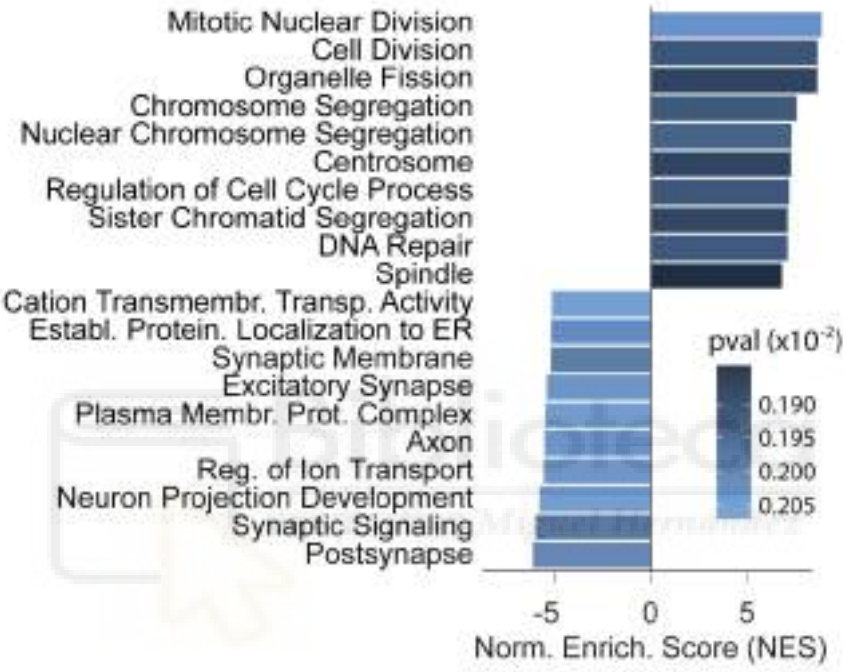


Figure 16. Gene Ontology analysis of DEGs from GSEA. Bar chart of top ranked GO gene sets according to normalized enrichment score (NES), from gene set enrichment analysis (GSEA). Bars are color-coded according to statistical significance (nominal *p* value) as indicated.

Our results also indicate that *MIR3607* affects the migration of neurons, causing overmigration with lamination defects, formation of ventricular heterotopia, and also affecting the extension of axonal projections. The effects that we observe are consistent with the results of our transcriptomic analyses, with significant changes in gene networks related to mTORC1 signaling (NES: 2.22; *p*-val: 0.0078; *q*-val: 0.0054), axon development and L1CAM interactions. Increased mTORC1 signaling is associated with several neurological and neurodevelopmental disorders with defects in organization of the cortex. Hyperactivation of mTORC1 signaling in mammalian models lead to disorganized

cortical lamination *(Orlova et al.,2010; Tsai et al.,2014; Moon et al.,2015),* and is known to be associated with the formation of heterotopia *(Broix et al.,2016).* Further, mTOR signaling is found to be important for axon specification and elongation, also controlling dendritic size and complexity *(Li et al.,2008; Kumar at al.,2005).* L1CAM plays a role in radial migration of neurons and its mutation associated with congenital hydrocephalus is found to dramatically impair axonal and dendritic arborization *(Tonosaki et al.,2014; Patzke et al.,2016)*. From an evolutionary perspective, the overmigration phenotype observed in mouse suggest that *MIR3607* may help neurons migrate long distances in large and gyrified cortices. This is important so these neurons have time to get settled before they initiate further maturation, extending their axonal and dendritic arbors, which is also consistent with our transcriptome analyses revealing processes related to late stages of maturation of neurons being downregulated **(Fig 16)**.

MIR3607 increases the proliferative capacity of progenitor cells by enhancing features of gyrified cortices

The proliferative capacity of cortical progenitors is critical, as it determines the final size of the brain. Several primate- and human-specific genes have been identified that enhance progenitor cell proliferation, including few miRNAs *(Fiddes at al.,2018; Florio et al.,2018; Suzuki et al.,2018; Nowakowski et al.,2018; Arcila et al.,2014; Prodromidou et al.,2020).* We have found that the expression of *MIR3607* enhances cell proliferation, and this effect is even more pronounced when it is expressed at early developmental stages, when self-amplifying progenitor divisions are comparatively high. This highlights the relevance of intrinsic genetic programs and their interaction with environmental signals among progenitor cells at different stages *(Telley et al.,2019).* Our results obtained with cerebral organoids indicate that this function of *MIR3607* is conserved in human. Another important factor linked to the increase in proliferation is the maintenance of Pax6 expression by cortical progenitors long after their delamination from the VZ. This is an important feature of proliferative bRGCs in species with large brains. Moreover, experimentally sustained expression of Pax6 in basal progenitors of the embryonic mouse cortex leads to an increase in cycling

progenitors in the SVZ along with increased bRGCs undergoing self-amplification, which is otherwise uncommon in mouse *(Wong at al.,2015).*

The transcription factors Pax6 and Tbr2 play key roles in cortical development, as for example they define the identity of aRGCs and IPCs, respectively *(Götz and Huttner.,2005).* Increasing levels of Pax6 in aRGCs favors basal progenitor cell genesis by positively regulating the levels of Neurog2 and Tbr2 *(Sansom et al.,2009).* But in mouse, the formation of basal progenitors and increased expression of Tbr2 rapidly downregulates Pax6 expression, suggesting a negative feedback loop between Tbr2 and Pax6 *(Sessa et al.,2017)*. Also, Pax6 seems to have auto-regulation by negative feedback *(Manuel et al.,2015).* As a result of the above, co-expression of Pax6 and Tbr2 at the protein level are near mutually-exclusive in mouse, with only a minority of cells expressing both proteins and at low levels *(Englund et al.,2005; Arai et al.,2011).* Intriguingly, many aRGCs within VZ express high levels of *Tbr2* mRNA but not protein, in mouse as well as in ferret, indicating the existence of post-transcriptional regulatory mechanisms (*de Juan Romero et al.,2015; Sessa et al.,2008).* Few miRNAs have been identified to be involved in this transition by directly blocking the expression of *Pax6* or *Tbr2 (Bian et al.,2013; Nowakowski et al.,2013; Needhamsan et al.,2014).* Interestingly, *miR-7* has been shown to inhibit Pax6 protein expression without altering its mRNA level, and the 3'UTR of Pax6 bears additional functional *miR-7* target site only in primates, supporting our idea that the increase in functional target sites during evolution is linked to brain expansion *(Needhamsan et al.,2014).* In contrast to mouse, Pax6 and Tbr2 protein are very frequently co-expressed within basal progenitors of the developing cortex of large-brained species such as macaque and ferret, essentially in bRGCs *(Betizeau et al.,2013; Reillo and Borrell.,2012)*. This suggests the absence, or override, of post-transcriptional regulation in these two particular genes, which may have contributed to the developmental features important for cortical expansion, including aRGC delamination and acquisition of bRGC fates.

We have shown that expression of *MIR3607* in the embryonic mouse cortex increases the frequency of cells double-positive for Tbr2 and Pax6 protein in VZ, and especially in SVZ. This is particularly dramatic for Pax6, which we also found is expressed at much higher levels relative to Tbr2 in *MIR3607* expressing

embryos. This suggests that existing mechanism of regulation between these two proteins may be modified or altered by *MIR3607*. Our results suggest that *MIR3607* indirectly promotes Pax6 translation, perhaps by blocking the post-transcriptional inhibition of Pax6 expression, as our transcriptional profiling shows no differential expression of Pax6, yet showing abundance of Pax6 mRNA levels compatible with post-transcriptional regulatory mechanisms. In this line of evidence, our GSEA analyses revealed mTORC1 among the most significantly altered pathways upon *MIR3607* expression. Importantly, mTORC1 regulates Pax6 protein levels in the chick retina, and this mTORC1 activation is downstream of Wnt/βCatenin signaling *(Zelinka et al.,2016).* Hence, this suggests the possibility that in the developing cerebral cortex Pax6 protein levels are indirectly increased by *MIR3607* via augmenting Wnt signaling and then mTORC. Moreover, βCatenin/TCF binding to Pax6 promoter is demonstrated to induce its expression in neural stem cells *(Gan et al.,2014).*

MIR3607 is a major regulator of the canonical Wnt/β-Catenin signaling pathway

Expression of *MIR3607* in the early embryonic mouse cortex drives the amplification and massive delamination of apical progenitor cells to basal positions, frequently forming conspicuous proliferative rosettes. Several signaling pathways have been known to be critical for neurogenesis including Shh, FGF, notch, BMP, Wnt signaling and so on *(Johansson et al.,2010, Kazanis et al.,2008)*. Our transcriptomic analyses revealed that several regulators and factors of Wnt/βCatenin signaling pathway were significantly altered, including upregulation of β*Catenin* itself, leading to hyperactivation of the pathway. Among several factors of this major signaling pathway that were altered, there was downregulation of mRNA levels of negative regulators of Wnt/βCatenin pathway such as APC, Draxin and Hecw1. APC was particularly interesting as it is a computationally predicted target of *MIR3607* in mouse and many other species including human **(Fig. 17A).** Also it is an experimentally verified target of *MIR3607*, and the resulting downregulation of APC is known to induce

hyperproliferation in human lung cancer cells by activating Wnt/βCatenin pathway *(Lin et al.,2017)*. Draxin and Hecw1 are also predicted targets of *MIR3607* in human, but not mouse, which might make them less likely to act as confident targets in our case, but suggests the possibility of stronger activation of Wnt/βCatenin signaling in human and other higher order species with conserved target sites of *MIR3607* for these genes. Several studies have demonstrated the critical role of Wnt/βCatenin signaling in cerebral cortex development, including its effect on promoting the proliferation of progenitors at early stages *(Chenn and Walsh.,2002; Chenn and Walsh.,2003; Woodhead et al.,2006; Wrobel et al.,2007; Mutch et al.,2009; Hirabayashi et al.,2004)*. In addition, APC, which is a component of the protein complex driving βCatenin for phosphorylation and proteasome degradation, is important for maintaining polarity of radial glial cells and control cerebral cortex neurogenesis, by tuning the levels of βCatenin via post-translational mechanisms *(Yokota et al.,2009; Nakagawa et al.,2017)*. Our results confirm the activation of the Wnt/βCatenin pathway upon expression of *MIR3607 in vivo* in the mouse cortex. Indeed, the effects of *MIR3607* overexpression in the early embryonic mouse cortex were phenocopied by overexpression of constitutively active βCatenin. The increased abundance of activated βCatenin upon *MIR3607* overexpression may result from the loss of negative regulation by APC. Consistent with these observations, the phenotype caused by *MIR3607* was largely rescued by co-expression of APC. With this, our study confirms the major role played by *MIR3607* in regulating APC and, in turn, critically regulating the Wnt/βCatenin pathway, a key pathway involved in cerebral cortex development **(Fig. 17B).**

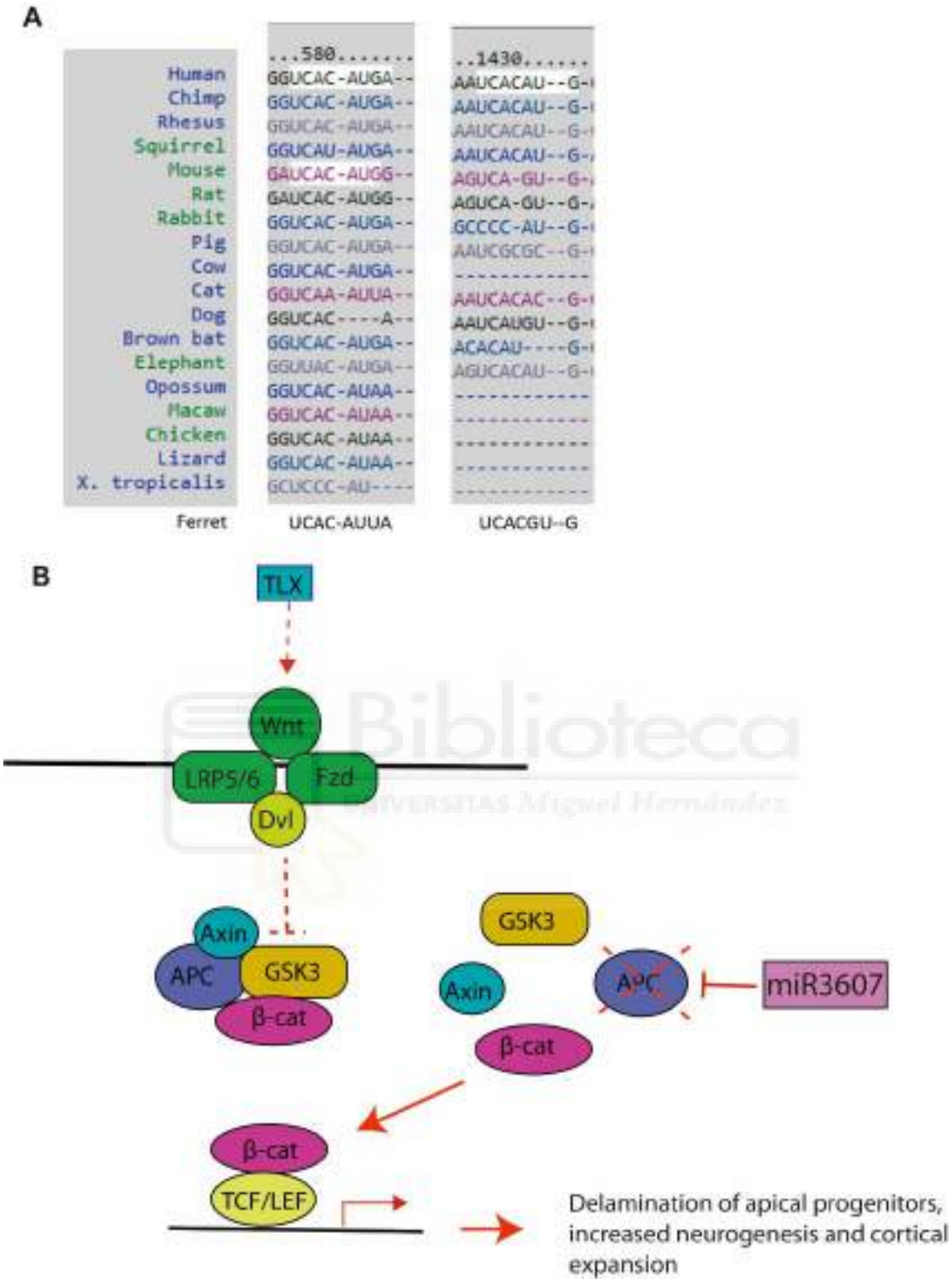


Fig.17 *MIR3607* targets APC and regulates Wnt/ β-Catenin signaling

(A) *MIR3607* target sites in two different locations of the 3'UTR of APC of different species. (B) By repressing expression of APC, *MIR3607* prevents the formation of the beta-catenin destruction complex, leading to the activation of this signaling pathway.

The effect of *MIR3607* on progenitor cells at different stages illustrate the existence of critical time windows during development

We have observed a significant difference in severity of phenotypes between early and mid developmental stages of mouse corticogenesis, where the Wnt/βCatenin signaling pathway is the main implicated. Our results suggest that the reduced severity of phenotypes as development progresses could be attributed to the decreased susceptibility of progenitors to Wnt/βcatenin signaling along development. This would be in agreement with previous reports showing that the transcriptional activity induced by Wnt/βcatenin signaling decreases over developmental time in the embryonic cortex *(Mutch et al.,2009; Pöschl et al.,2013).* A recent study showed that apical cortical progenitors respond differently to intrinsic versus extrinsic factors depending on developmental stage. In the early embryonic cortex (E12.5), aRGCs are more prone to respond to intrinsic signals, with cell-cycle and nucleus/chromatin-related processes being predominant. At later stages, with increased expression of membrane receptors and cell-cell signaling related proteins, cortical progenitor cells become more susceptible to environmental signals and become more neurogenic *(Telley et al.,2019).* Moreover, Wnt transcripts are expressed in a temporal early-high to late-low gradient in the developing mouse cortex, and late apical progenitors may be re-specified to an early fate by experimental activation of Wnt-signaling *(Oberst et al.,2019).*

Previous studies from our lab demonstrated the importance of temporally-dynamic changes in gene expression levels in the ferret cerebral cortex during a defined critical period, where aRGC self-consumption and delamination leads to the generation of massive amounts of bRGCs and the formation of the OSVZ. Again, mRNA levels of β-Catenin are upregulated in this critical period, further highlighting the importance of Wnt/βCatenin signaling in regulating aRGC-to-bRGC cell fate *(Martinez-Martinez et al.,2016).* The observed high expression of *MIR3607* at this particular stage further supports the critical importance of Wnt/βCatenin signaling activation by this miRNA at specific stages of development to regulate the extent of cell proliferation and the size of the cortex across evolution.

MIR3607 is important for the expansion of cerebral cortex in higher mammals

Delamination of apical progenitors from the VZ, while maintaining apical-basal polarity, is a key step for the formation of bRGCs and the OSVZ, critical for cerebral cortex gyrification *(Martinez-Martinez et al.,2016; Stahl et al.,2013).* Our loss-of-function experiments in ferret embryos indicate that *MIR3607* is important for proper RGC delamination and maintenance of polarity, at the stage when massive bRGC production and seeding of the OSVZ occurs. These results confirm the importance of *MIR3607* in assisting the formation of OSVZ and gyrification. Our western blot analyses confirmed the regulation of *APC* by *MIR3607* in ferret, which consequently regulates canonical Wnt signaling. Further confirmation of this mode of regulation comes from experiments overexpressing APC, which phenocopy the effects of loss of function of *MIR3607*, with the effect being even more dramatic as expected. The association of APC with microtubules has been shown to influence microtubule stability, and thus it may help to maintain the polarity of RGCs in mouse cerebral cortex *(Yokota et al.,2009).* APC is also known to regulate RGC morphology and the formation of primary cilia, via regulating βCatenin activity. In addition to its role as central nuclear effector of canonical Wnt signaling pathway, at the structural level βCatenin also regulates adherens junctions between the progenitors, thereby maintaining the integrity of VZ *(Nakagava et al.,2017).* Deregulated distribution of βCatenin upon dysregulation of its expression might disturb the integrity of VZ, as we observe here in mouse. Overall, our experiments in ferret confirm the conservation of function and mechanism of action of *MIR3607* by targeting APC and regulating canonical Wnt signaling.

Overexpression of *MIR3607* in organoids leads to the formation of many closed ventricles resembling the rosettes that we observed upon overexpression in the early embryonic mouse cortex, confirming the conservation of function of *MIR3607* in human cerebral organoids. *MIR3607* expression also increased apical surface area, possibly reflecting an increase in amplification (symmetric proliferation) of aRGCs. This is consistent with the experimental expression of

stabilized βCatenin in the early mouse cortex, which massively increases the surface area of the neuroepithelium *(Chenn and Walsh.,2002).* Altogether, past and present evidence strongly supports the notion that the molecular regulation of progenitor cell amplification and cortical expansion, via *MIR3607* expression, is a mechanism strongly conserved in evolution. Given the phylogenic relationships between human, mouse and ferret, our results also indicate that the absence of *MIR3607* expression in the embryonic mouse cerebral cortex is the result of a secondary loss during evolution, where this was expressed in the common mammalian ancestor.

CONCLUSIONS

1. *MIR3607* is expressed in the early embryonic cerebral cortex of the gyrencephalic ferret and human, but not the lissencephalic mouse.

2. Expression of *MIR3607* in embryonic mouse cortex cause premature neurogenesis, defects in migration, positioning and maturation of neurons.

3. *MIR3607* expression causes the amplification of cortical progenitors and increased co-expression of Pax6+Tbr2+ in SVZ cells in mouse, typical features of gyrencephalic brains.

4. Transcriptome analyses revealed changes in biological functions and signaling pathways that are key for the amplification and delamination of cortical progenitor cells, highlighting Wnt signaling as one of the major dysregulated pathways.

5. Expression of *MIR3607* in earlier embryonic mouse cerebral cortex causes the overactivation of βCatenin followed by the severe amplification of VZ progenitors and the destabilization of this germinal layer, forming proliferative rosettes.

6. The phenotypic effects of *MIR3607* overexpression in the early embryonic mouse cortex are rescued by co-expression of APC, functionally confirming that this miRNA targets APC and overactivates Wnt/βCatenin signaling.

7. Overexpression of *MIR3607* in human cerebral organoids also leads to the expansion of VZ progenitors and the formation of proliferative rosettes.

8. Endogenous *MIR3607* regulates the levels of APC in ferret brain cells, ensuring the amplification of aRGCs and maintenance of their polarity.

This function may guarantee the early expansion of the VZ prior to the peak of neurogenesis, leading to cortical expansion and possibly folding.

CONCLUSIONES

1. *MIR3607* se expresa de manera temprana en la corteza cerebral embrionaria de especies girencefálicas, como el hurón y el humano, pero está ausente en el ratón lisencefálico.

2. La expresión de *MIR3607* en la corteza embrionaria de ratón causa neurogénesis prematura, defectos en la migración, posicionamiento y maduración neuronal.

3. La expresión de *MIR3607* produce la amplificación de los progenitores corticales y el aumento de la coexpresión de Pax6+Tbr2+ en células de la SVZ en ratón, características típicas de cerebros girencefálicos.

4. Los análisis transcriptómicos revelaron cambios en funciones biológicas y vías de señalización claves para la amplificación y delaminación de las células progenitoras corticales, entre las cuales destaca la cascada de señalización de Wnt como una de las principales vías afectadas.

5. La expresión temprana de *MIR3607* en la corteza cerebral embrionaria de ratón produce la sobreactivación de β-catenina seguida de una amplificación sustancial de los progenitores de la VZ y la consecuente desestabilización de esta capa germinativa, dando lugar a rosetas proliferativas.

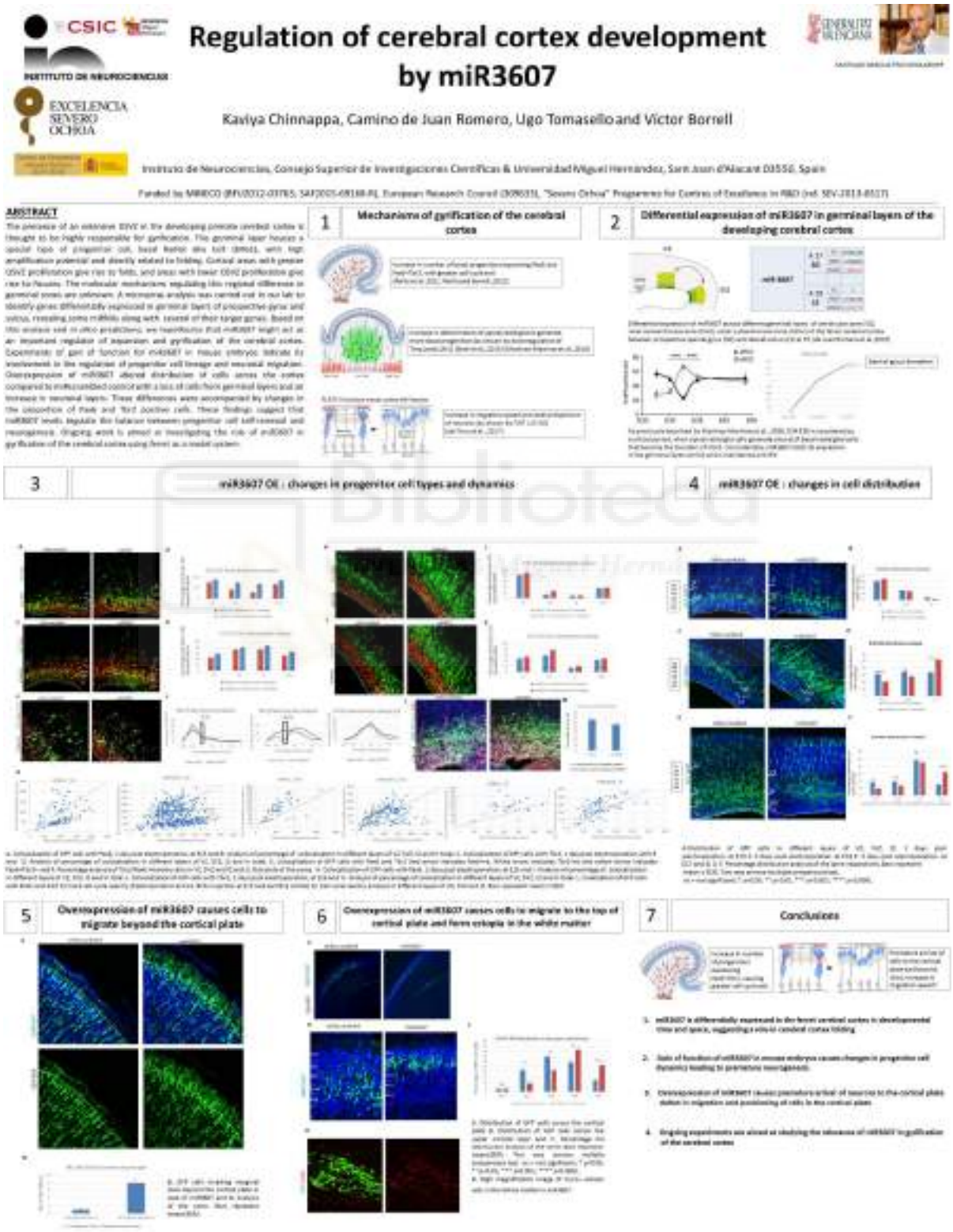
6. Los efectos fenotípicos producidos por la sobreexpresión de *MIR3607* en la corteza embrionaria temprana del ratón son rescatados mediante la coexpresión de APC, lo que confirma funcionalmente que este miARN tiene como diana APC y sobreactiva la vía de señalización de Wnt/β−catenina.

7. La sobreexpresión de *MIR3607* en organoides cerebrales humanos también conduce a la expansión de los progenitores de la VZ y la formación de rosetas proliferativas.

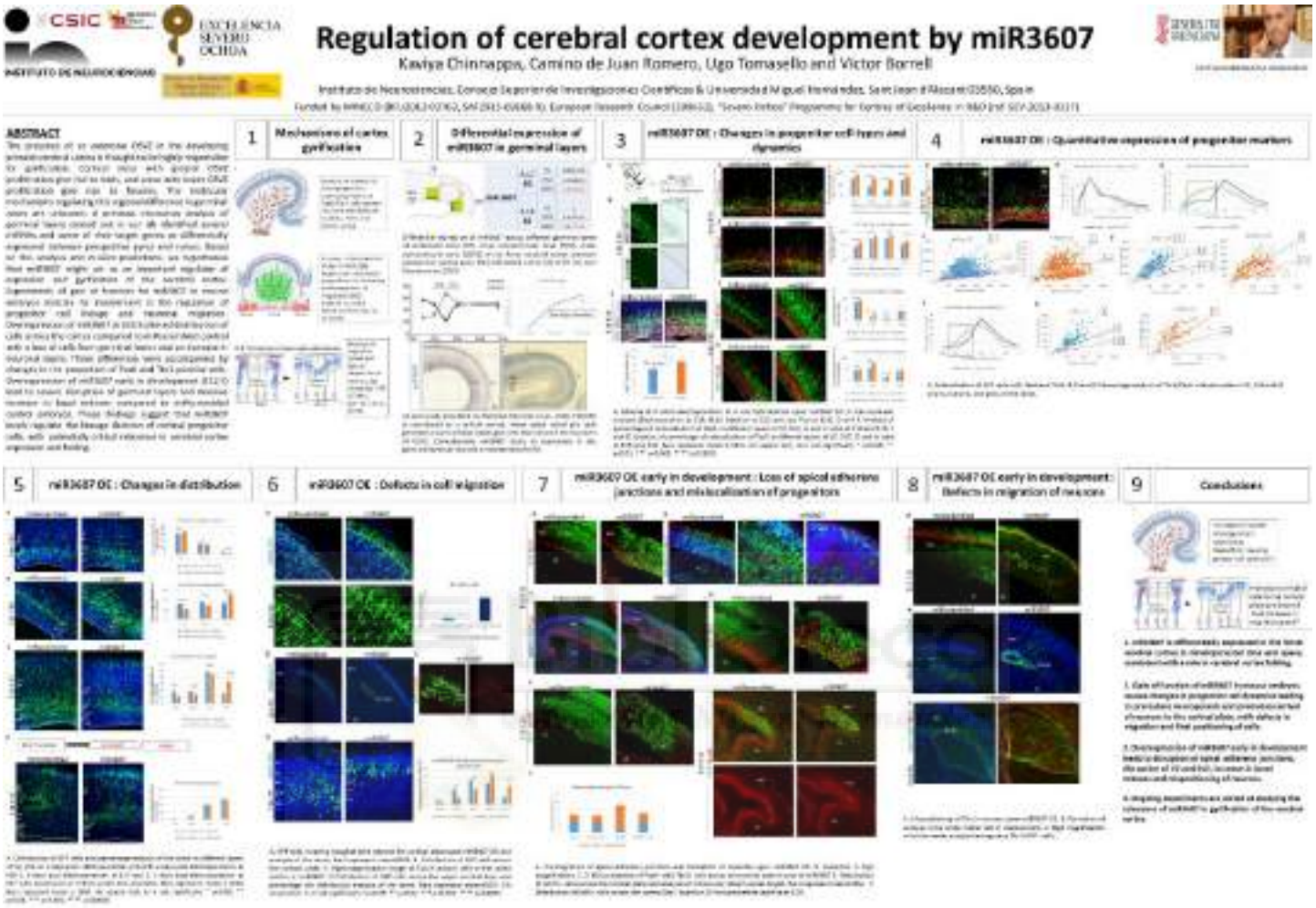
8. El *MIR3607* endógeno regula los niveles de APC en las células del cerebro de hurón, asegurando la amplificación de las aRGC y el mantenimiento de su polaridad. Esta función puede garantizar la expansión temprana de la VZ antes de que el proceso de neurogénesis alcance su máximo, lo que conduce a la expansión cortical y su posible plegamiento.

ANNEX I – AUTHOR´S CONTRIBUTIONS

Poster presented at EMBO AND SENC conferences 2017



Poster presented at FENS conference 2018



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