PHF6 transcriptional regulation of neural stem cells and its misregulation in the rare developmental disorder of intellectual disability, Börjeson-Forssman-Lehmann syndrome

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English abstract

Intellectual disability (ID) encompass a spectrum of neurodevelopmental conditions characterized by impairments in cognitive functioning and adaptive behaviour, with an onset in early childhood. The focus of this thesis has been investigating the role of the plant homeodomain zinc-finger protein-6 (PHF6), whose mutations have been implicated in Börjeson-Forssman-Lehmann Syndrome (BFLS), a rare X-linked intellectual disability (XLID) that is characterized by moderate to severe intellectual impairments as well as physical phenotypic characteristics, and seizure susceptibility.

Neurogenesis is a crucial process in the development of the central nervous system (CNS) that includes proliferation, differentiation, and migration of neural stem cells (NSCs). Guided by a tightly regulated network of transcription factors, signalling pathways, and cellular interactions, disruptions in any of these regulatory mechanisms can lead to profound and non-profound neurodevelopmental disorders with varying severity. Understanding NSC behaviour during embryonic stages sheds light on fundamental biological principles and provides insight into the pathogenesis of IDs.

Studies presented in this thesis has led to key discoveries that advance our current knowledge of BFLS pathogenesis. First, analysis of genome-wide binding of PHF6 in the developing brain led to identification of PHF6 binding sites in regions close to genes involved in CNS development and neurogenesis. Via employing BFLS mouse models of patient-related mutations, R342X (PHF6 truncation) and C99F-m (PHF6 point mutation), we observe impairments in neurogenesis including an increase in embryonic NSC (eNSC) self-renewal, along with a decrease in the number of neural progenitors. Our follow up studies led to the discovery of Ephrin receptors (EphRs) as direct transcriptional targets of PHF6. We revealed that

PHF6-mediated regulation of EphRs is disrupted both in BFLS mice and in conditional tamoxifen-induced *Phf6* knockout (KO) models. Furthermore, knockdown (KD) of *EphR-A* phenocopies the PHF6 loss-of-function effects, altering eNSC self-renewal, whereas its forced expression ameliorates these defects in eNSCs from BFLS mice. These results suggest that a novel PHF6/Eph-A pathway modulates eNSC dynamics in the developing brain, a process that is compromised in BFLS. This research not only advances our knowledge of NSC transcriptional regulation, but also sets a foundation for potential therapeutic strategies targeting EphR in BFLS.

The findings presented in this dissertation advance the field of neurodevelopmental research by detailing the role of the transcriptional regulator, PHF6, and its downstream targets, by which PHF6 alters cell population dynamics. These insights contribute significantly to our understanding of the genetic underpinnings of IDs and lay the groundwork for future research aimed at developing therapies for BFLS and related disorders. Building on these findings and given the role of EphR in the modulation of NSC dynamics, future studies should focus on investigating the therapeutic efficacy of modulating EphR activity to ameliorate the effects of PHF6 mutations and continue the investigation into the molecular mechanisms of NSC regulation and their disruption in BFLS.

RÉSUMÉ (French abstract)

Les troubles intellectuels (TIs) englobent un spectre de conditions neurodéveloppementales caractérisées par des déficiences du fonctionnement cognitif et du comportement adaptatif, avec un début dans la petite enfance. L'objectif de cette thèse a été d'étudier le rôle de la protéine à doigt de zinc de domaine homéodomaine de plante-6 (PHF6), dont les mutations sont impliquées dans le syndrome de Börjeson-Forssman-Lehmann (BFLS), un trouble intellectuel rare lié à l'X (XLID) caractérisé par des déficiences intellectuelles modérées à sévères ainsi que des caractéristiques phénotypiques physiques et une susceptibilité aux crises d'épilepsie.

La neurogenèse est un processus crucial dans le développement du système nerveux central (SNC) qui inclut la prolifération, la différenciation et la migration des cellules souches neurales (CSN). Guidée par un réseau de transcription régulé de manière stricte, des voies de signalisation et des interactions cellulaires, les perturbations de l'un de ces mécanismes régulateurs peuvent entraîner des troubles neurodéveloppementaux profonds et moins profonds de gravité variable. Comprendre le comportement des CSN pendant les stades embryonnaires éclaire les principes biologiques fondamentaux et fournit des informations sur la pathogenèse des TIs.

Les résultats de ces études ont conduit à des découvertes clés qui font progresser notre connaissance actuelle de la pathogenèse du BFLS. Tout d'abord, l'analyse de la liaison génomique de PHF6 dans le cerveau en développement a permis d'identifier des sites de liaison de PHF6 dans des régions proches de gènes impliqués dans le développement du SNC et la neurogenèse. En utilisant des modèles de souris BFLS avec des mutations similaires à celles des patients, R342X (troncation de PHF6) et C99F-m (mutation ponctuelle de PHF6), nous

observons des déficiences dans la neurogenèse, incluant une augmentation de l'auto-renouvellement des CSN embryonnaires (eCSN), ainsi qu'une diminution du nombre de progéniteurs neuraux. Nos études de suivi ont conduit à la découverte des récepteurs Ephrin (EphR) comme cibles transcriptionnelles directes de PHF6. Nous avons révélé que la régulation médiée par PHF6 des EphR est perturbée à la fois chez les souris BFLS et dans les modèles de knockout (KO) conditionnel induit par tamoxifène de *Phf6*. De plus, la réduction (KD) d'*EphR-A* reproduit les effets de la perte de fonction de PHF6, altérant l'auto-renouvellement des eCSN, tandis que son expression forcée améliore ces défauts chez les eCSN des souris BFLS. Ces résultats suggèrent qu'une nouvelle voie PHF6/Eph-A module la dynamique des eCSN dans le cerveau en développement, un processus compromis dans le BFLS. Cette recherche non seulement fait progresser notre connaissance de la régulation transcriptionnelle des CSN, mais pose également les bases de stratégies thérapeutiques potentielles ciblant EphR dans le BFLS.

Les résultats présentés dans cette dissertation font progresser le domaine de la recherche neurodéveloppementale en détaillant le rôle du régulateur transcriptionnel, PHF6, et de ses cibles en aval, par lesquelles PHF6 altère la dynamique des populations cellulaires. Ces informations contribuent de manière significative à notre compréhension des bases génétiques des HI et jettent les bases de futures recherches visant à développer des thérapies pour le BFLS et les troubles connexes. En s'appuyant sur ces résultats et compte tenu du rôle d'EphR dans la modulation de la dynamique des CSN, de futures études devraient se concentrer sur l'exploration de l'efficacité thérapeutique de la modulation de l'activité d'EphR pour atténuer les effets des mutations de PHF6 et continuer à enquêter sur les mécanismes moléculaires de la régulation des CSN et leur perturbation dans le BFLS.

Preface

The central body of this thesis comprises of one published research paper in EMBO Reports with me as first author.

Authors of Chapter 3: Dilan Rasool, Audrey Burban, Ahmad Sharanek, Ariel Madrigal, Jinghua Hu, Keqin Yan, Dianbo Qu, Anne K Voss, Ruth S Slack, Tim Thomas, Azad Bonni, David J Picketts, Vahab D Soleimani, Hamed S Najafabadi, Arezu Jahani-Asl

Contribution of authors to each chapter

Chapter 1: Dilan Rasool wrote the contents of this chapter, with edits from Arezu Jahani-Asl.

Chapter 2: Dilan Rasool wrote the contents of this chapter, with edits from Arezu Jahani-Asl, Hamed Najafabadi, and Ariel Madrigal.

Chapter 3: Dilan Rasool, Arezu Jahani-Asl, and Hamed Najafabadi wrote the contents of this chapter together. ChIP-Seq and RNA-seq (Figures 7-9) were conducted by Arezu Jahani-Asl, with data analysis by Vahab D Soleimani, Hamed S Najafabadi, and Ariel Madrigal. Figure 10A, 18B was conducted by Audrey Burban. Figures 11C, 11N-P, 11J, 16C-H were conducted together by Dilan Rasool, Audrey Burban and Ahmad Sharanek. Figure 12A-B, 12E-G was conducted by Jinghua Hu. Figure 12C, 12J was conducted by Keqin Yan. Figure 13-14 data analysis was conducted by Ariel Madrigal. Figure 15D was conducted together by Dilan Rasool and Audrey Burban. Figure 15E-G was conducted together by Dilan Rasool and Ahmad Sharanek. Figure 16I-J was conducted together by Dilan Rasool and Dianbo Qu. Dilan Rasool conducted all remaining experiments. The research program was conceived by Arezu Jahani-Asl. The conceptualization of the paper was done collaboratively by Arezu Jahani-Asl and Dilan Rasool, with Arezu Jahani-Asl supervising the project.

Chapter 4: Dilan Rasool wrote the contents of this chapter, with edits from Arezu Jahani-Asl

Contribution to knowledge and elements of original scholarship

Outlined below is the contribution to knowledge made to the field of neurogenesis and intellectual disability, specifically focusing on PHF6 transcriptional regulation of neural stem cells (NSCs) and its implications in Börjeson-Forssman-Lehmann Syndrome (BFLS). The findings represented in this dissertation were novel at the time of study.

1) Genome-wide binding of PHF6: This research has led to the characterization of the embryonic developing cortex, and for the first time, has demonstrated the genome-wide binding of PHF6 in regions close to genes involved in CNS development and neurogenesis within the developing cortex. This finding advances our understanding of how PHF6 regulates critical genes during brain development.
2) Impact of PHF6 mutations: Utilizing BFLS mouse models with patient-related mutations (R342X and C99F-m), this study observed an increase in embryonic NSC (eNSC) self-renewal along with a decrease in the number of neural progenitors. These insights reveal the specific impact of PHF6 mutations on NSC dynamics and contribute to the broader understanding of neurodevelopmental disorders of cognition.
3) Ephrin receptors as downstream targets: The research identified Ephrin receptors (EphRs) as direct downstream targets of PHF6. Disruptions in PHF6-mediated regulation of EphRs were observed both in BFLS mice and in conditional tamoxifen-induced *Phf6* knockout (KO) models. This discovery highlights a novel PHF6/Eph-A pathway that modulates eNSC dynamics in the developing brain, highlighting EphA as potential therapeutic targets.
4) EphA receptors phenocopy PHF6-mediated eNSC alterations: Knockdown (KD) of *EphR-A* phenocopied the PHF6 loss-of-function effects, altering eNSC self-renewal,

whereas its forced expression ameliorated these defects in eNSCs from BFLS mice. This suggests potential therapeutic strategies targeting EphR in BFLS.

5) Therapeutic Implications: The insights gained from this research set a foundation for potential therapeutic strategies targeting EphR in BFLS and related neurodevelopmental disorders. The findings emphasize the importance of the transcriptional regulator, PHF6, in maintaining NSC dynamics and offers new directions for developing effective treatments for intellectual disabilities.

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This Ph.D. journey has been a profound and transformative experience, one that would not have been possible without the unwavering support and love of many incredible individuals. First and foremost, to my supervisor, Dr. Arezu Jahani-Asl, and my co-supervisor, Dr. Vahab D. Soleimani, your guidance, wisdom, and assistance have been instrumental in shaping my Ph.D.

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My deepest gratitude goes to my beautiful family. To Abdulla, Dulbar, Brusk, and Gilles, your love and encouragement have been my foundation and you have been the greatest inspiration in my life, I dedicate this degree to you. To Kurdistan and all my extended family, I am proud of my heritage and grateful for the values it has instilled in me.

To my lifelong friends, thank you for the countless moments of laughter, support, and friendship. Hajer, Meena, Ladan, Reem, Ahmad, Hauna, Rangin, Rozan, Hawar, Avin and the entire Squad Pyramid, you have been my confidants and my support system.

To the entire Alqarni family, especially my sisters and soulmates, Ayael and Afnan, your love and constant presence throughout my life have been my sanctuary, even with thousands of miles between us. Thank you for always standing by my side and lifting me up when I needed it the most.

Lastly, I dedicate this thesis to my late friend, Tania, this accomplishment is as much yours as it is mine.

List of abbreviations

Acute lymphoblastic leukemia (ALL)

Acute myeloid leukemia (AML)

Adenosine Triphosphate (ATP)

Agouti-related peptide (AgRP)

Alzheimer's Disease (AD)

Assay for Transposase-Accessible Chromatin Using Sequencing (ATAC-seq)

Autism spectrum disorder (ASD)

Autologous umbilical cord blood (AUCB)

Basal progenitor (BP)

Basic Fibroblast Growth Factor (bFGF)

Bovine Serum Albumin (BSA)

Börjeson-Forssman-Lehmann Syndrome (BFLS)

Brain tumour stem cell (BTSC)

Caudal ganglionic eminence (CGE)

Central nervous system (CNS)

Cerebrospinal fluid (CSF)

Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation Sequencing (ChIP-Seq)

Comparative Genomic Hybridization (CGH)

Cre Estrogen Receptor T2 (CreERT2)

Cysteine-Rich Domain (CRD)

Cytosine-Adenine (CA)

Dentate gyrus (DG)

4',6-Diamidino-2-Phenylindole (DAPI)

Discs Large Homolog 1 (Dlg1)

Double stranded break (DSB)

Double stranded DNA (dsDNA)

Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12)

Embryonic day (E)

Embryonic neural stem cells (eNSCs)

Embryonic stem cells (ESCs)

Ephrin receptors (EphRs)

Epidermal Growth Factor (EGF)

5-Ethynyl-2'-Deoxyuridine (EdU)

Extreme Limiting Dilution Assay (ELDA)

E2 factor (E2F)

False Discovery Rate (FDR)

FGF receptor 1 (FGFR1)

Fibroblast growth factor (FGF)

Fibronectin (FN)

Fluorescence-Activated Cell Sorting (FACS)

Fluorescent in-situ hybridization (FISH)

Fragile Mental Retardation Protein (FMRP)

Fragile X Syndrome (FXS)

Gene Expression Omnibus (GEO)

Gene Ontology (GO)

Gestational day (gd)

Glycogen synthetase kinase-3 beta (GSK-3β)

Glycosylphosphatidylinositol (GPI)

Green fluorescent protein (GFP)

Growth hormone (GH)

Growth hormone receptor (GHR)

Growth hormone-releasing hormone (GHRH)

GTPase-activating proteins (GAPS)

Guanine nucleotide exchange factors (GEFs)

Hank's Balanced Salt Solution (HBSS)

Hematopoietic stem and progenitor cells (HSPCs)

Hematopoietic stem cell (HSC)

Hemizygous (HEMI)

Heterozygous (HET)

Histon-3 (H3)

Homeobox (HOX)

Homozygous (HOMO)

Human umbilical cord tissue mesenchymal stromal cell (hCT-MSC)

Immediate-early gene (IEG)

Immunoprecipitation (IP)

Induced pluripotent stem cell (iPSC)

Inner SVZ (iSVZ)

Intellectual disability (ID)

Intelligence quotient (IQ)

Intermediate zone (IZ)

Janus kinase 2 (JAK2)

Juxta Membrane (JM)

Knockdown (KD)

Knockout (KO)

Lateral ventricles (LV)

Ligand-Binding Domain (LBD)

Limiting Dilution Assay (LDA)

Medial ganglionic eminence (MGE)

Mesenchymal stem cell (MSC)

MicroRNAs (miRNA)

Multiplex ligation dependent probe amplification (MLPA)

Neural progenitor cell (NPC)

Neural stem and progenitor cell (NSPC)

Neuroblastoma cell line (N2A)

Neurodevelopmental disorders (NDDs)

Neurogenic notch homolog protein 1 (Notch1)

Neurogenin (Ngn)

Neuroglycan C/Chondroitin sulfate proteoglycan 5 (NGC/CSPG5)

Non-homologous end joining (NHEJ)

Notch intracellular domain (NICD)

Nuclear localization sequences (NLS)

Nucleolar localization sequence (NoLS)

Nucleosome remodeling and deacetylase chromatin-remodeling complex (NURD)

Outer SVZ (oSVZ)

Patched (Ptc)

PDZ Binding Motif (PDM)

Phosphorylated Akt (p-Akt)

Phosphorylated ERK (p-ERK)

Postsynaptic Density-95, Dlg1, ZO-1 (PDZ)

Plant homeodomain (PHD)

Plant homeodomain zinc-finger protein (PHF6)

Platelet-derived growth factor receptor beta (PDGFRß)

Platelet-derived growth factors BB (PDGF-BB)

Polymerase associated factor 1 (PAF1)

Polymerase Chain Reaction (PCR)

Postsynaptic Density Protein 95 (PSD-95)

Prolactin (Prl)

Propidium Iodide (PI)

Quantitative Real-Time PCR (qPCR)

Reads per Kilobase Million (RPKM)

Receptor Binding Domain (RBD)

Receptor tyrosine kinase (RTK)

Rest (RE1-silencing transcription factor)

Rett Syndrome (RTT)

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Ribosomal RNA (rRNA)

RNA Polymerase II (Pol II)

RNA Polymerase II-associated factor 1 (PAF1)

Room temperature (RT)

Rostral migratory stream (RMS)

Receptor Tyrosine Kinase (RTK)

Rett syndrome (RTT)

Short tandem repeat (STR)

Signal transducer and activator of transcription 5B (STAT5B)

Small Interfering RNA (siRNA)

Stem Cell Frequency (SCF)

Sterile Alpha Motif (SAM)

Smoothened (Smo)

Sonic Hedgehog (Shh)

SRC family of kinases (SFKs)

Subgranular zone (SGZ)

Subventricular zone (SVZ)

Subventricular zone/rostral migratory stream/olfactory bulb (SVZ/RMS/OB)

Tandem repeat (TR)

T-cell Acute Lymphoblastic Leukemia (T-ALL)

Temporal lobe epilepsy (TLE)

Thyroid hormone (TH)

Transcription factors (TFs)

Transcription start site (TSS)

Transforming growth factor-beta (TGFb)

Transposable element (TE)

Tumour necrosis factor-alpha (TNFa)

Uniform Manifold Approximation and Projection (UMAP)

Untranslated region (UTR)

Upstream binding factor (UBF1)

Vascular endothelial growth factor receptor 2 (VEGFR2)

Ventricular zone (VZ)

X-linked Intellectual Disability (XLID)

Zonula Occludens-1 (ZO-1)

CHAPTER 1

Introduction part 1 - Brain Development & Neurogenesis

1.1.1 The fundamentals of brain development

Embryonic brain development begins with the formation of the neural tube, from which the brain and spinal cord will develop. Research in the field of neurogenesis has employed rodents models, including mice, to gain a better understanding of mechanisms that regulate the formation of the brain. In humans, this process starts around gestational day (gd) 30, out of a total of up to 280 days, whereas in mice it starts at embryonic day (E) 9-9.5, out of a total of up to 21 days (**Fig 1**) (Semple et al., 2013; Zeiss, 2021). Central nervous system (CNS) developmental processes in both human and mice include neurogenesis, neuron death, myelination, synaptogenesis, and synaptic pruning (Zeiss, 2021). Mice reach many developmental milestones, including neurogenesis and the establishment of basic neural structures earlier as mice have a much shorter gestation period and lifespan. Furthermore, these process in mice occur in a 3–4-week timespan as opposed to human brain development, characterized by a long period of neurogenesis starting around gd42 and continues past birth (Stepien et al., 2021; Stiles & Jernigan, 2010), contributing to the complexity and depth of the human brain. The mouse neurogenic period starts from E9.5 to E18-E19.5 (Stepien et al., 2021; Zeiss, 2021). A commonality between humans and rodents is that the majority of neurogenesis is complete by birth (Zeiss, 2021). However, in structures like the hippocampus, there are notable differences between species. Human hippocampal development at birth is ~80% complete whereas in rodents it is only ~20% complete, representing challenges in cross-model comparisons of brain development (Zeiss, 2021). However, the fundamentals of brain development are conserved across humans and mice (Stiles & Jernigan, 2010).

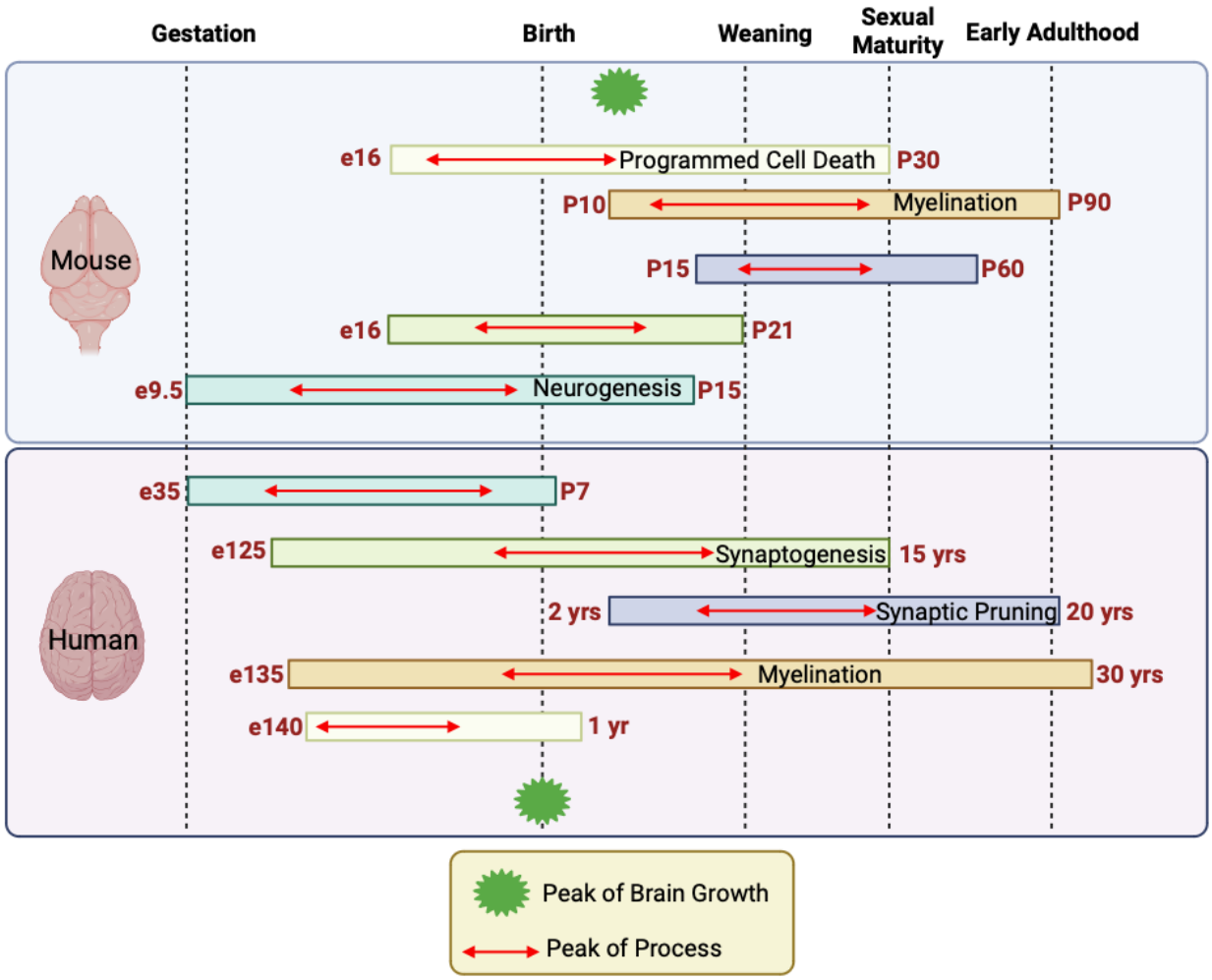

Figure 1: Illustration of the processes involved in brain development comparing human and rodent trajectories. Adapted from Zeiss, C.J, 2021. Made with BioRender.

1.1.2 Cortical development

There is an estimated 16-26 billion neurons, contributing to 20% of the total neuron number of the human brain, in the cerebral cortex alone (Stepien et al., 2021). Additionally, the development of the human neocortex is not constant throughout the whole structure and layers. Cortical regions such as the prefrontal and frontal areas that are located more rostrally are much larger in volume, surface area, and magnitude of white matter (Stepien et al., 2021). This enlargement in specific cortical regions is thought to be due to more connectivity between neurons rather than number of neurons (Stepien et al., 2021).

The formation of the cortical plate, otherwise known as corticogenesis, takes place during development and originates from a single layer of symmetrically dividing neuroepithelial cells (Rakic, 1995, 1995). Specifically, these neuroepithelial cells will become apical radial glial cells with cell bodies in the ventricular zone (VZ), and undergo further division to produce either basal progenitor cells or neurons (Noctor et al., 2007; Stepien et al., 2021). The migration of neurons from the VZ to the cortical plate is guided by the radial glial scaffold, which is a network of radial glial processes that extend from the VZ to the pial surface, passing through the intermediate zone (IZ), and directs the newly born neurons to their specific location in the developing cortex (Hatten, 1999; Rakic, 1972). Earlier-born neurons will form the deeper layers of the cortex, whereas later-born neurons will migrate further upwards to establish the upper cortical layers (Molyneaux et al., 2007). The subventricular zone (SVZ) acts in concert with the VZ wherein the intermediate progenitor cells housed here are prone to proliferate and increase the neuronal output necessary for cortical expansion, especially for neurons of the upper cortical layers (Haubensak et al., 2004).

The cerebral cortex is divided into six distinct layers and is classified by the specific cell types that populate it. The mature neocortex is made up of two broad categories of neurons, cortical projection neurons and interneurons (Stepien et al., 2021). The deepest layer, layer VI or multiform layer, is made up of a heterogenous mix of pyramidal neurons involved in sending feedback signals to the thalamus and thus modulating sensory input to the cortex. Pyramidal neurons are predominantly glutamatergic (excitatory) and function in intracortical communication (Douglas & Martin, 2004). In addition to these neuronal cell types, the cortex also houses non-neuronal cells including astrocytes, oligodendrocytes, and microglia; all of which work to support neuronal function and maintain homeostasis. Layer V, the internal pyramidal layer, houses large pyramidal neurons that are responsible for sending output signals to the various subcortical structures (Kasper et al., 1994). Next, Layer IV is the internal granular layer which is dense with stellate (glutamatergic) neurons, but also contains GABAergic neurons and serves as the main receiver of thalamocortical inputs (Molyneaux et al., 2007). Layer III is the external pyramidal layer which contains medium-sized pyramidal (glutamatergic) neurons that form corticocortical connections. Layer II is the external granular layer which holds small pyramidal neurons and stellate-shaped neurons. Finally, layer I, otherwise known as the molecular layer, is largely devoid of neurons and contains dendrites, axon terminals, and glial cells. This is the outermost layer and is important for signal transmission.

Glutamatergic neurons are the main excitatory neuron type of the cerebral cortex and are born in the VZ. This type of neuron originates from radial glial cells and migrates through the radial glial scaffold to populate the layers of the cortex (Greig et al., 2013; Molyneaux et al., 2007). GABAergic neurons are the main type of inhibitory neuron in the cortex, and they originate from the medial ganglionic eminence (MGE) and the caudal ganglionic eminence

(CGE). These neurons migrate perpendicularly to the radial glial scaffold to reach their precise location in the cortex. These inhibitory neurons are important for cortical excitability and sensory information processing (Anderson et al., 1997; Butt et al., 2005). The organization of glutamatergic versus GABAergic neurons within the cortical layers establishes the cortical excitatory-inhibitory balance important for motor control and cognitive functioning.

Corticogenesis is completed when the progenitor cell population is depleted from continuous symmetric cell division or cell fate is switched as assembly of glial cells like astrocytes and oligodendrocytes has started (Stepien et al., 2021). The period of gliogenesis following neurogenesis shows varying levels of overlap across humans and mice.

1.1.3 Human versus mouse brain development

The rodent equivalence of a human baby at birth is ~P7-10 due to the peak of brain growth (as a percent of body weight) occurring postnatally in rodents whereas in humans it takes place at birth (Zeiss, 2021). By age 6, the human brain will have reached 90% of its adult size, however, structural changes in the grey and white matter will continue into adolescence (Semple et al., 2013; Stiles & Jernigan, 2010). In comparison, rodent brains reach 90% of their adult weight by weaning (Semple et al., 2013). The final adult brain weight for humans is reached at puberty, whereas in rodents it's in early adulthood (Zeiss, 2021).

In mice, neurogenesis and gliogenesis are mostly temporally separated, however in human brains, both processes continue in parallel for some time (Levitt et al., 1983). The prefrontal cortex is not fully formed until up to ~25 years of age in humans as synaptogenesis and myelination continues into early adulthood. This is the equivalence of ~P90 in rodents (Zeiss, 2021). Additionally, synaptic development of a newborn human is equivalent to synaptic

development at weaning age for rodents (Zeiss, 2021). During human infancy, there is gross synaptic proliferation that is 50% more than the adult average which is why synaptic pruning takes place soon after the peak of synaptogenesis (Zeiss, 2021). In rodents, this corresponds to the time of weaning. Interestingly, deficits in synaptic pruning are one cause of autism spectrum disorder (Tang et al., 2014). Finally, as a human is developing and learning, the brain is constantly changing due to myelination, as such, white matter is very prone to injury at this time which can lead to developmental abnormalities such as cerebral palsy (Volpe, 2003; Zeiss, 2021).

The neocortical grey matter is ~3 times thicker in humans compared to mice when factoring in brain size (Stepien et al., 2021). Additionally, humans show a unique pattern of cortical expansion and folding, known as gyrification, which increases the surface area of the cortex without increasing overall brain volume, and is crucial for advanced cognitive functioning (Sun & Hevner, 2014). This makes up ~80% of overall brain mass in humans with a folded surface area of up to 2000cm^2 for a grey matter thickness of 2.6mm (Stepien et al., 2021). Interestingly, the cortical columns of human brains has a much more conical shape (tip of the cone at the ventricle) than mouse cortical columns due to the high number of folds in the human cortices (Stepien et al., 2021). Gyrification in humans begins around 15 weeks gestation, and is mostly wrapped up by birth (Dubois et al., 2008). In contrast, the mouse cortex is smoother and has less folds than the human cortex, however the fundamentals of neuronal differentiation and migration are conserved across the species (Stiles & Jernigan, 2010), making mice viable models for studying brain development. In both species, NSCs generate neurons and glial cells, contributing to the formation and maintenance of neural circuits. The dynamic nature of stem cell activity, including their capacity for self-renewal and differentiation, is essential for adapting

to developmental needs. Thus, understanding the mechanisms governing stem cell behaviour can shed light on normal developmental processes while also providing insight into potential therapeutic strategies for neurodevelopmental disorders.

Introduction part 2 - Key mechanisms involved in embryonic and adult stem cell regulation

1.2.1 Mechanisms of stem cell division: balancing renewal and differentiation

Neurodevelopment involves the generation, differentiation, and integration of billions of neurons and glial cells. These cells will then form the neural networks important for cognitive function and behaviour. The corner stone of this process are stem cells which possess the ability to self-renew and differentiate for proper brain development. There are four types of stem cells; pluripotent can become any type of cell in the body, multipotent can become any type of cell in specific tissue (e.g. bone marrow), totipotent form the cells in the embryo and fetus (e.g. zygotes), and unipotent forms a single mature type of cell (e.g. sperm). Embryonic stem cells (ESCs), which arise from the inner cell mass of a blastocyst, are classified as pluripotent as they can differentiate to produce any cell type within the three germ layers; ectoderm, mesoderm, and endoderm, to produce all the tissues and organs of the body (Thomson et al., 1998). Neural development begins with the specification of ESCs into embryonic neural stem cells (eNSCs), which then give rise to the cell lineages that populate the brain, such as progenitors, neurons, and glial cells (Gaspard & Vanderhaeghen, 2010; Guillemot, 2007; Kamelska-Sadowska et al., 2019). A progenitor cell is considered committed since it has a destined fate lineage, while a NSC is uncommitted as it still has the propensity to self-renew or differentiate.

Of the embryonic CNS, there are a population of progenitor cells called neuroepithelial cells which are a highly polarized cell type with apical ends that line the ventricular surface, interfacing with cerebrospinal fluid (CSF). Neuroepithelial cells, similar to stem cells, are able to self-renew and undergo symmetric division to expand their progenitor pool during early CNS development (Götz & Huttner, 2005). As embryonic CNS development continues, these neuroepithelial cells will differentiate into radial glial cells which will retain the polarity and

localization of the neuroepithelial cells; cell bodies located in the VZ with processes spreading to the pial surface. Radial glial cells function as scaffolding structures as well as neural progenitors, and their long radial fibers will act as guides for migrating neurons during cortico-development (Noctor et al., 2001). Radial glial cells will undergo asymmetric division, a characteristic not common in neuroepithelial cells, allowing them to self-renew while also giving rise to other cell types; initially neurons and then glial cells such as astrocytes and oligodendrocytes later on (Malatesta et al., 2000). The switch from neuroepithelial to radial glial cells allows for the diversification of the cell types that make up the brain.

The balance between stem cell self-renewal and differentiation is crucial for brain development and is a tightly regulated process of symmetric versus asymmetric cell division (Morrison & Kimble, 2006; Silva-Vargas et al., 2018). Symmetric division generates two identical daughter cells to broaden the pool of uncommitted stem cells or to enhance the committed progenitor pool, while asymmetric division results in one uncommitted daughter cell and a committed progenitor (**Fig 2**) (Inaba & Yamashita, 2012; Morrison & Kimble, 2006; Silva-Vargas et al., 2018).

Stem cell self-renewal and differentiation is influenced by cell fate determinants, most notably the Numb protein that is asymmetrically distributed during stem cell division, to ensure only one of the two daughter cells will inherit Numb expression. Numb expression inhibits Neurogenic notch homolog protein 1 (Notch1) signalling and promotes differentiation, whereas the daughter cell lacking Numb will maintain high Notch1 activity, thus sustaining it's stem cell identity (**Fig 2**) (Guo et al., 1996; Spana & Doe, 1996; Zhang et al., 2021). Notch signalling is an important factor in balancing differentiation and self-renewal during the development of the nervous system, where the Notch receptor binding to its respective ligands, Delta and Jagged,

will lead to transcriptional activation of target genes that regulate self-renewal, while inhibiting differentiation (Artavanis-Tsakonas et al., 1999; Shi et al., 2008).

Another important mechanism governing symmetric versus asymmetric self-renewal is cell polarity, where the orientation of cell division can dictate which form of division a stem cell will undergo (Chhabra & Booth, 2021; Yamashita et al., 2010). Cell polarity is established through a complex interplay of intrinsic factors, such as the partitioning of cell fate determinants, and extrinsic signals from the microenvironment, including cues from the extracellular matrix and neighbouring cells (Chhabra & Booth, 2021; Gönczy, 2008). During division, cell polarity is tightly regulated by the Par complex (comprising Par3, Par6, and aPKC) and other polarity proteins, which establish an axis of polarity (Chhabra & Booth, 2021). The positioning of the mitotic spindle must align parallel to the cell polarity axis to ensure cell fate determinates are distributed to only one daughter cell in asymmetric divisions, whereas perpendicular orientation allows for an equal distribution of cell fate determinants between the two daughter cells, promoting the expansion of the stem cell pool in symmetric divisions (**Fig 2**) (Chhabra & Booth, 2021). Additionally, proteins like LGN and NuMA are essential for the proper positioning of the spindle by interacting with the Par complex and guiding the orientation of the division axis (Zhu et al., 2011).

Defects in processes that regulate NSC fate specification, causing defective differentiation and self-renewal, can have major implications on proper brain functioning (Beachy et al., 2004; Niklison-Chirou et al., 2020; Rasool et al., 2024). For example, defective self-renewal, loss of neurons, impaired synaptic transmission, and degeneration of glial cells takes place in numerous neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's disease (Luo & O'Leary, 2005; Rasool et al., 2022; Winner & Winkler, 2015; Zhao

et al., 2008). Furthermore, patients suffering from these conditions will show dysfunctions in cognition or olfactory function which are linked to the main areas of adult neurogenesis- the hippocampus and olfactory bulbs (Hinnell et al., 2012; Simuni & Sethi, 2008).

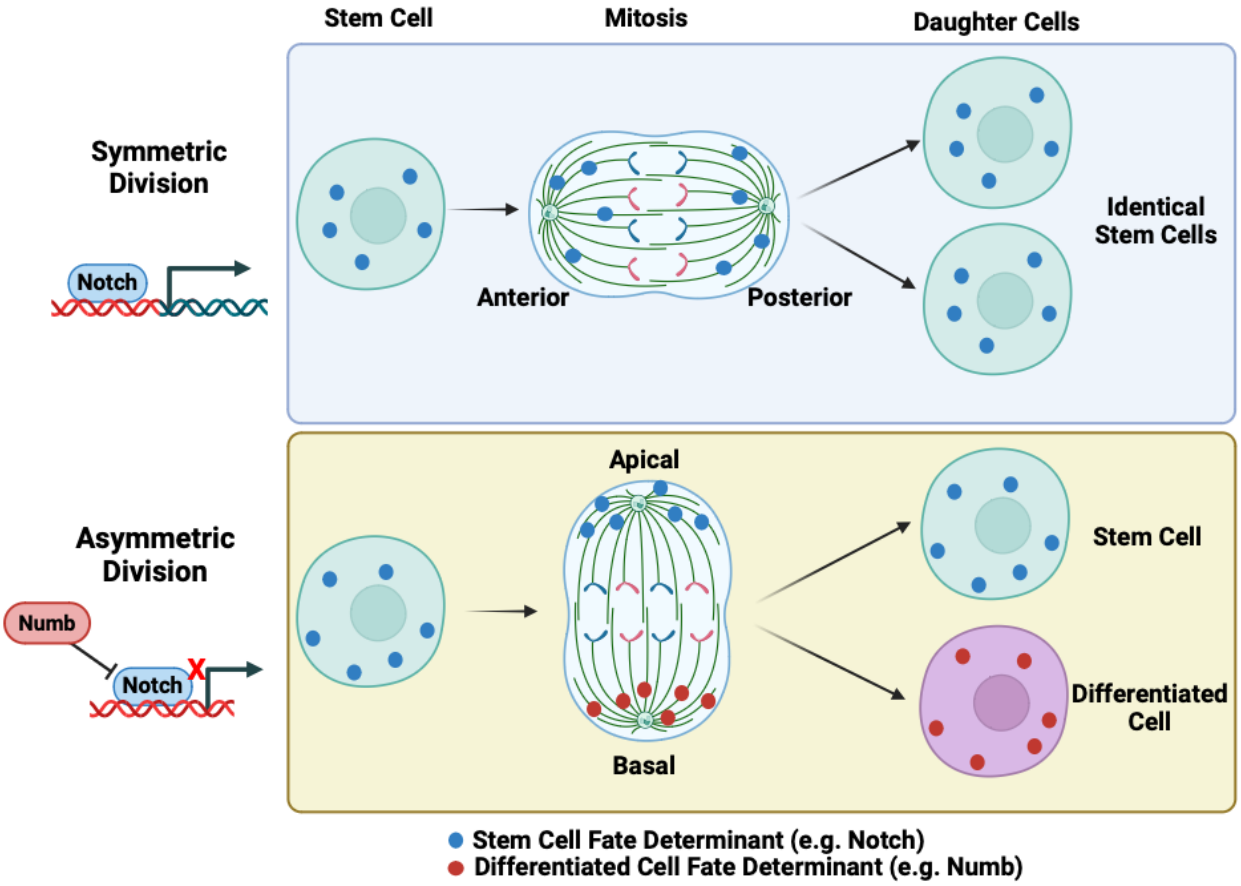

Figure 2: Illustration of symmetric versus asymmetric cell division of stem cells. Cell fate determinates and cell polarity during mitosis will dictate the mechanism of cell division. Adapted from Chhabra & Booth, 2021. Made with BioRender.

1.2.2 Neural stem cell quiescence versus activation

Within the adult brain, the conserved NSC populations are located within the SVZ and subgranular zone (SGZ) (Obernier & Alvarez-Buylla, 2019; Rasool et al., 2022), and are maintained in a balance between quiescence and activation. Quiescence is a state of dormancy that is characterized by a temporary exit from cell cycle (known as G0), wherein NSCs will exist in a reduced metabolic activity state (Cheung & Rando, 2013). This dormancy is important for preventing depletion of the stem cell population and protects NSCs from environmental stressors and DNA damage (Cheung & Rando, 2013; Urbán & Cheung, 2021). The mechanisms governing quiescence are factors in the niche, and intrinsic suppressors of proliferative signalling pathways (Fuchs et al., 2004; Llorens-Bobadilla et al., 2015). On the other hand, stem cell activation is the re-entry of quiescent NSCs into active cell cycle and is catalyzed by environmental factors and physiological needs. An orchestra between niche signalling, such as growth factors, and intrinsic elements, such as transcriptional regulators and epigenetic modifications, work together to regulate the activation process (Codega et al., 2014; Daynac et al., 2016). Importantly, the transition from stem cell dormancy to activation is a crucial step in neurogenesis that enables NSCs to not only proliferate, but to also differentiate into specialized cell types.

NSCs are able to switch between quiescence and activation, an essential function for maintaining the stem cell pool throughout lifetime, as quiescence will protect NSCs from depletion (Urbán & Cheung, 2021). The balance between quiescence and activation is regulated by a network of transcription factors (TFs), which can either promote the maintenance of stemness or encourage entry into the cell cycle. Key TFs in quiescence include HES and RE1-silencing transcription factor (REST). HES1 and HES5 mainly function by repressing the

transcription of genes involved in cell cycle progression, such as Cyclin D1, keeping NSCs in a non-proliferative state (Shimojo et al., 2008). Furthermore, the feedback loop involving Notch signalling, which increases HES1 and HES5 expression, also maintains the quiescent state (Harada et al., 2021; Kageyama et al., 2015). On the other hand, REST maintains quiescence by inhibiting genes associated with neuronal differentiation and synaptic plasticity. By controlling the expression of genes such as BDNF and SYN1, REST prevents neuronal differentiation, thus conserving the NSC pool (Su et al., 2022; Yucebas et al., 2016).

In contrary, the transition from quiescence to activation is driven by TFs such as SOX2 and TLX, which respond to internal and external cues signalling the need for new neurons. SOX2 is known for its role in maintaining the pluripotency of stem cells by upregulating genes essential for proliferation and lineage commitment (Feng & Wen, 2015; Sarkar & Hochedlinger, 2013; Shimozaki, 2014). SOX2's ability to modulate the expression of various cyclins and other cell cycle regulators enables the activation of NSCs, allowing them to respond swiftly to regenerative signals or developmental cues (Metz et al., 2020; Ouyang et al., 2015).

TLX plays a similar role by promoting NSC proliferation and self-renewal via repressing the expression of cell cycle inhibitors such as $p21^{WAF1/CIP1}$, thus allowing the G1/S transition in NSCs (Wu et al., 2015). Additionally, TLX regulates several genes that maintain the undifferentiated state of NSCs and supports their proliferation, highlighting its role as a master regulator of NSC activation (Islam & Zhang, 2015; Niu et al., 2011).

Other factors that influence NSC quiescence and activation include the neurovascular niche as it modulates NSC states through signalling molecules such as betacellulin and neurotrophin 3, which promote proliferation and maintain quiescence, respectively (Delgado et al., 2014; Ding et al., 2020; Gómez-Gaviro et al., 2012). Additionally, the metabolic state of

NSCs, influenced by changes in mitochondrial dynamics and lipid metabolism, has been shown to impact NSC quiescence or activation (Fuchigami et al., 2024; Ito & Suda, 2014; Scandella et al., 2023)

1.2.3.1 The role of signalling pathways in NSC activation and differentiation

The process of neurogenesis is comprised of proliferation and fate specification of NSCs, migration of newborn neurons, and maturation of neurons. The development from pluripotency to specialized cell is regulated through a combination of intrinsic genetic and extrinsic environmental factors (Gil-Ranedo et al., 2019; Shi et al., 2008). Intrinsic factors for differentiation encompass transcription factory activity, regulation via microRNAs (miRNA), and the epigenetic landscape (Shi et al., 2008). Extrinsic factors come from the microenvironment, this includes chemicals secreted by, and contact with, neighbouring cells (Gil-Ranedo et al., 2019; Shi et al., 2008). The differentiation process can be broadly defined as, 1) quiescent NSCs become activated in response to certain cues and re-enter cell cycle, 2) activated NSCs proliferate and generate a pool of transient amplifying progenitor cells, 3) progenitors are then influenced by transcription factors and signalling pathways to commit to specific lineages (neuronal vs glial), 4) finally, lineage-specified progenitors undergo terminal differentiation to develop into the mature cell types of their specified lineages. The mechanisms of differentiation are controlled by a complicated network of signalling pathways, such as Notch, Wnt, and Sonic Hedgehog (Shh), that interact with transcription factors and guide NSC fate (Ahn & Joyner, 2005; Andersen et al., 2014). Transcription factors regulate NSC fate through stem cell maintenance, proliferation, and differentiation, and act as molecular switches to turn genes on or off in order to influence a cells developmental trajectory. For example, the transcription

factor Sox2 maintains NSC pluripotency and inhibits differentiation (Graham et al., 2003), whereas Neurogenin (Ngn) promotes the expression of neuronal genes and suppresses glial differentiation (Bertrand et al., 2002; Schuurmans et al., 2004).

1.2.3.2 Wnt signalling pathway

The canonical Wnt pathway is crucial for promoting the identity of radial glial and neuroepithelial cells (Doe, 2008). When the levels of Wnt ligands are reduced or when key components like β-catenin are removed, there is a noticeable decline in the number of these stem cells, accompanied by an early shift towards neuronal differentiation (Machon et al., 2003; Zechner et al., 2003). Conversely, enhancing Wnt signalling has been shown to expand the stem cell pool (Chenn & Walsh, 2002; Machon et al., 2007; Viti et al., 2003; Woodhead et al., 2006; Zechner et al., 2003), and it also supports the self-renewal of NSCs during postnatal neurogenesis (Machon et al., 2007; Machon et al., 2003; Wexler et al., 2008; Zhou et al., 2004). Additionally, Wnt signalling can increase the proliferation of committed neuronal progenitors (Doe, 2008; Lie et al., 2005). In cells, Wnt triggers the activation of cyclin D along with NSC factors such as Sox2 (Doe, 2008). As cortical development progresses, the role of Wnt signalling switches to a driver of neuronal differentiation, partly through activation of Neurogenin 1 (Ngn1; Neurog1), a proneural gene (Hirabayashi et al., 2004; Israsena et al., 2004; Muroyama et al., 2004; Viti et al., 2003). The expression of stabilized β-catenin on E10 promotes neuroepithelial proliferation and self-renewal, whereas by E14, it encourages neuronal differentiation (Chenn & Walsh, 2002; Doe, 2008; Hirabayashi & Gotoh, 2005).

The Wnt signalling pathway is highly conserved, is active from neural tube formation to the development of midbrain structures, and has been linked to various CNS pathologies

including schizophrenia, mood disorders, autism, and Alzheimer's disease (AD) (Lovestone et al., 2007; Navarro Quiroz et al., 2018). Wnt ligands, a family of glycoproteins that act via autocrine and paracrine mechanisms, initiate a cascade in the absence of Wnt ligands that activates glycogen synthetase kinase-3 beta (GSK-3β) leading to the formation of a degradation complex that phosphorylates and tags β-catenin for degradation by the proteasome (Chen et al., 2000; McMahon & Bradley, 1990; Navarro Quiroz et al., 2018). In the presence of Wnt, this degradation is halted, allowing β-catenin to stabilize, accumulate, and activate Wnt target gene transcription by binding to TCF/LEF transcription factors in the nucleus (Logan & Nusse, 2004; Navarro Quiroz et al., 2018).

Moreover, the transcription factor NeuroD1, downstream of Wnt signalling, is critical for promoting neurogenesis in adult hippocampal progenitors. In the presence of Wnt, β-catenin accumulates extracellularly and forms a complex that activates NeuroD1 transcription, essential for the survival and maturation of neurons in the adult hippocampus (Gao et al., 2009; Kuwabara et al., 2009). While there is evidence for Wnt/β-catenin's role in regulating neurogenesis by promoting neuronal differentiation, some studies suggest that the pathway may also enhance the proliferation of adult NSCs, adding complexity to its role in neural tissue dynamics (Mao et al., 2009; Navarro Quiroz et al., 2018).

1.2.3.3 Notch signalling pathway

Notch signalling plays an important role in both embryonic and adult neurogenesis where it impacts a variety of cellular processes including cell proliferation, differentiation, and apoptosis (Artavanis-Tsakonas et al., 1999; Imayoshi et al., 2010; Louvi & Artavanis-Tsakonas, 2006). Notch receptors, which are transmembrane single-pass heterodimers, become activated

upon binding to ligands such as Delta-1 and Jagged-1 on adjacent cells (Navarro Quiroz et al., 2018). This interaction leads to the gamma-secretase-mediated cleavage of the receptor and the release of the Notch intracellular domain (NICD), which then moves to the nucleus. Once inside the nucleus, NICD pairs with the DNA-binding protein RBPj to act as a transcriptional activator, inducing the expression of bHLH transcription factors and enhancers like HES (Bray & Bernard, 2010; Imayoshi et al., 2010). Expression of Notch signalling components, including various ligands and receptors, as well as effectors such as RBPjk and Hes family transcription factors, is critical in maintaining NSC populations (Doe, 2008). Mutations in these components lead to a loss of radial glia and cause neuronal differentiation in both embryonic and adult neural stem cells (Doe, 2008). In contrary, overexpression or activation of these components, such as Hes1 and Hes5, or activated Notch in the embryonic cortex, inhibits neuronal differentiation (Doe, 2008; Imayoshi et al., 2010).

Notch's impact on neurogenesis is not limited to embryonic stages but extends into postnatal and adult neurogenesis across various brain regions, including the SVZ and hippocampus (Ables et al., 2010; Doe, 2008; Ehm et al., 2010; Hitoshi et al., 2002; Imayoshi et al., 2010; Stump et al., 2002). Conditional deactivation of key components such as RBPj, which subsequently inactivates Notch signalling, prompts the differentiation of progenitor cells into neurons (Imayoshi et al., 2010; Navarro Quiroz et al., 2018). This inactivation leads to a spur of initial neurogenesis followed by rapid depletion of the NSC pool, particularly SOX2+ cells, thereby inhibiting sustained neurogenesis in the adult hippocampus (Ehm et al., 2010).

Furthermore, radial glial cells with mutations in Notch signalling genes like Dll1, Notch1, RBPj, Hes1, and Hes5 exhibit decreased neurosphere-forming capacity, which indicates a reduced capacity for self-renewal (Hitoshi et al., 2002; Ohtsuka et al., 2001; Yoon et al., 2004).

Studies using flow cytometry have further demonstrated that radial glial cells expressing a Notch-induced green fluorescent protein (GFP) reporter can be sorted into high and low Notch activity groups, with the high-activity group showing greater efficacy in generating primary and secondary neurospheres and the ability to differentiate into neurons, astrocytes, and oligodendrocytes in vivo (Mizutani et al., 2007), further emphasizing Notch's role in preserving embryonic and post-natal NSCs (Doe, 2008; Imayoshi et al., 2010).

1.2.3.4 Shh signalling pathway

The Shh pathway plays a role in a number of developmental processes as well as in adult neurogenesis. The Shh signalling cascade includes the Shh ligand, the transmembrane protein Smoothened (Smo), and the nuclear effectors Gli2 and Gli3, among others (Doe, 2008). Shh influences neural development and maintains neural functions in various brain regions, including the hippocampus and dentate gyrus (Ahn & Joyner, 2005; Lai et al., 2003; Machold et al., 2003; Palma et al., 2005). It is also crucial in dorso-ventral patterning and proliferation (Chiang et al., 1996; Rallu et al., 2002; Shi et al., 2008). In the adult brain, Shh signalling is required for proliferation of neuronal progenitors, particularly in the hippocampus and SVZ, where it promotes neurogenesis (Ahn & Joyner, 2004, 2005; Lai et al., 2003; Palma et al., 2005). The pathway's activity is mediated via the complex formed by Patched (Ptc); located in the primary cilia, and its co-receptor Smo (Navarro Quiroz et al., 2018; Rohatgi et al., 2007). In the absence of the Shh ligand, Ptc inhibits Smo, thus preventing the activation of downstream transcriptional responses for cell proliferation and differentiation (Fuccillo et al., 2006; Philipp & Caron, 2009; Rohatgi et al., 2007).

Research has shown that conditional mutants lacking Smo in the postnatal hippocampus

exhibits a significant reduction in neurosphere formation, suggesting a role for Shh in NSC proliferation and stem cell maintenance (Machold et al., 2003). Furthermore, studies using embryonic cortical tissue of Gli2/Gli3 mutant mice supported the importance of Shh in neurosphere viability (Palma & Altaba, 2004), whereas Shh inhibited differentiation in postnatal cerebral granule cells (Argenti et al., 2005; Wechsler-Reya & Scott, 1999), implicating Shh in self-renewal and differentiation.

Additionally, Shh has been identified as a regulator of neuronal migration wherein inhibition or deactivation of Shh signalling in the adult SVZ disrupts neuroblast migration towards the olfactory bulb (Balordi & Fishell, 2007). Furthermore, other studies have shown a role for Shh in transition of NSCs towards a committed progenitor lineage, suggesting a dual role in both maintaining stem cell identity and causing progenitor cell proliferation (Agathocleous et al., 2007). These findings illustrate the multifaceted roles of Shh in both embryonic development and adult neurogenesis, making it a critical focus for understanding fundamental mechanisms of brain function.

1.2.4 Neural stem cell niches

The SVZ of the lateral ventricles (LV) and the SGZ of the dentate gyrus (DG), a structure within the hippocampus, are two areas in the brain where neurogenesis occurs throughout development and into adulthood (Obernier & Alvarez-Buylla, 2019; Rasool et al., 2022). These highly dense areas housing stem cells are distinguishable not only by their location, but also by the unique role they play in brain functioning.

The SGZ houses NSCs, also referred to as radial glial-like cells. These NSCs function to generate granule cells which in turn integrate into the DG to support the functioning of the

hippocampus, a region significant for learning, memory, and emotion. The process of generating neurons from radial glial-like cells in the SGZ is linked to cognitive flexibility and memory formation (Deng et al., 2010; Spalding et al., 2013).

The SVZ is located within the walls of the lateral ventricles and harbours NSCs known as Type B cells, which will give rise to transit-amplifying cells referred to as Type C (Alvarez-Buylla & Lim, 2004). Type C cells then differentiate into neuroblasts, otherwise known as Type A cells. The neuroblasts then migrate along the rostral migratory stream (RMS), making their way to the olfactory bulb where they will then differentiate into interneurons and contribute to olfactory functioning, learning, and memory (Alvarez-Buylla & Lim, 2004; Ming & Song, 2011). In humans, the SVZ is far more complex. A secondary expansion of the SVZ will occur due to an increase in the number of basal progenitor (BP) cell types (Stepien et al., 2021). The "secondary expansion" here implies that after the primary phase of SVZ development, which occurs during early brain development, there is an additional phase of proliferation in this region, leading to further neurogenesis and perhaps establishment of more complex neural networks (Stepien et al., 2021). The human SVZ is thus divided into the inner SVZ (iSVZ), and outer SVZ (oSVZ) (Smart et al., 2002). The iSVZ is similar to the rodent SVZ, however, the oSVZ will grow considerably in human neurogenesis due to the increase proliferative capacities of human BPs (Smart et al., 2002; Stepien et al., 2021).

Introduction part 3 - Intellectual disabilities

1.3.1 Intellectual disability

Intellectual disability (ID) covers a broad range of cognitive and developmental impairments which are characterized by deficits in both intellectual functioning as well as social and practical skills. Most IDs are diagnosed through standardized testing of intelligence quotient (IQ) and adaptive behaviours. An IQ below 70 before the age of 18 is typically the benchmark for ID diagnosis. However, IDs can vary according to severity, and fall under the falling four categories: mild (IQ 50-69), moderate (IQ 35-49), severe (IQ 20-34), and profound (IQ < 20) (Ropers, 2010). IDs are further characterized into two types: syndromic versus non-syndromic. Non-syndromic is used to define a person that only exhibits intellectual impairment, with no other physical or neurological deficits. Syndromic is characterized by IQ impairment as well as other clinical manifestations such as developmental delays, physical phenotypes, or sensory impairments (Bhasin et al., 2006; Kleine-Kohlbrecher et al., 2010; van Bokhoven & Kramer, 2010).

1.3.2 Underlying mechanisms of ID

The causes of ID are diverse and can include genetic factors as well as environmental. 1-3% of Western societies is affected by some form of ID (Leonard & Wen, 2002). In syndromic IDs, genetic factors play a large role in their clinical manifestation as specific gene mutations or chromosomal abnormalities are often the diagnosing factor. Diagnosis includes a combination of family medical history, physical examination, genetic testing, and standardized testing. Only about 50% of moderate to severe cases of ID are pinpointed to a cause, with an even lower

percent for mild ID (Chelly et al., 2006).

25-50% of ID is caused by genetic factors including chromosomal rearrangements which often lead to gene deletions, deregulation of gene or genomic region imprinting, and mutations of single genes (monogenic) (Chelly et al., 2006; McLaren & Bryson, 1987). Sub telomeric rearrangements alone make up 5-7% of syndromic ID (Flint & Knight, 2003) and can be diagnosed with a number of methods (Rooms et al., 2005) but most commonly, multiprobe fluorescent in-situ hybridization (FISH) and multiplex ligation dependent probe amplification (MLPA) (Chelly et al., 2006). A majority of chromosomal-end deletions have been linked to ID. Tip deletion of chromosome 16 in ATR-16 syndrome, and deletion of chromosome 17p tip in Miller-Dieker syndrome (Chelly et al., 2006) are examples representing these cases. Chromosomal deletions, duplications, inversions, or translocations where the end of the chromosome remains intact can also cause a number of IDs, for example, chromosome 22q11 deletion in DiGorges syndrome, or chromosome 7q11.2 deletion in Williams-Beuren syndrome (Chelly et al., 2006). These are often diagnosed by molecular cytogenic techniques such as Microarray-based Comparative Genomic Hybridization (CGH) (Van Esch et al., 2005; Vissers et al., 2003).

Genomic imprinting is a phenomenon where only one allele of a gene is expressed depending on maternal or paternal inheritance, while the other allele is turned off through epigenetic modifications such as DNA-cytosine methylation, histone acetylation, and histone methylation (Chelly et al., 2006). Deregulation of imprinting genes or clusters of genes (genomic regions) are seen in a number of cognitive impairments, most notably Angelman's syndrome and Prader-Willi syndrome, where microdeletions of the genomic region 15q11.2-15q13 occurs (Chelly et al., 2006; Nicholls, 2000; Petersen et al., 1995; Steffenburg et al., 1996; Walter &

Paulsen, 2003).

Macrocephaly, microcephaly, lissencephaly, and heterotopia, among others, are deficits in brain development that can contribute to secondary effects such as intellectual impairments (Chelly et al., 2006; Jean et al., 2020). In cases of ID where there is no abnormal brain phenotype, the mutated genes implicated, often X-linked, would be associated with less extensive cellular abnormalities such as neuronal or glial functioning, changes in cellular morphology, or impaired cell-cell connections (Chelly et al., 2006; Jean et al., 2020). 8-10% of sporadic genetically-caused-ID in males is from monogenic mutations (Chelly et al., 2006). Most monogenic ID-related genes can be classified as transcriptional regulators, chromatin-remodelling factors, transmembrane proteins, actin or microtubule-associated proteins, and regulators or effectors of Rho GTPases (Chelly et al., 2006). The RSK2 gene in Coffin-Lowry, MECP2 in Rett syndrome, and PHF6 in Börjeson–Forssman–Lehmann syndrome (Chelly et al., 2006), are examples representing monogenic ID-related genes.

Environmental causes of ID include pregnancy malnutrition, prematurity, infections during pregnancy, postnatal infections, exposure to environmental toxins, fetal alcohol syndrome, or other complications during birth or early life (Chelly et al., 2006). Many factors may go awry during fetal development specifically that may cause intellectual impairments, for instance, malnutrition in folic acid and iodine during pregnancy can lead to fetal developmental abnormalities (Bleichrodt & Born, 1996; Hibbard & Smithells, 1965; Hoxha et al., 2021; Levine et al., 2018). Furthermore, birth before 37 weeks gestation is considered pre-term as the brain may not be fully developed before this timepoint- leading to a higher risk of intellectual impairments and other cognitive deficits (Allen, 2008; Bhutta et al., 2002; Hirvonen et al., 2017; Soleimani et al., 2014). Infections such as rubella or zika virus during pregnancy may cause

disruptions of normal brain development (Aly et al., 2017; Gordon-Lipkin & Peacock, 2019; Munro et al., 1987; Satterfield-Nash, 2017; Van Nguyen & Abe, 2015; Wheeler et al., 2020), and infections after birth, such as meningitis, that are not treated right away can also cause significant brain damage (Chau et al., 2012). Maternal alcohol consumption during pregnancy can cause fetal alcohol syndrome which has numerous effects on the baby including physical, behavioural, and cognitive deficits (Jones & Smith, 1973; May & Gossage, 2001). Cases of asphyxia during birth have been shown to cause intellectual impairments and cerebral palsy (Pappas & Korzeniewski, 2016). Additionally, the environment a child grows up in where there is severe neglect or trauma (Anda et al., 2006), or exposure to toxins such as lead and arsenic (Grandjean & Landrigan, 2006), can impact the development of the brain negatively.

1.3.3 NSCs deregulated in neurodevelopmental disorders

The study of NSC deregulation in neurodevelopmental disorders (NDDs) has garnered interest in the past decade. Specifically, deregulation of NSC proliferation and differentiation represent a convergence point across many NDDs (Ernst, 2016), regardless of the underlying molecular mechanisms (Sacco et al., 2018). Research utilizing genetic models of a number of NDDs, including 9q34 deletion syndrome (Chen et al., 2014), 2q23.1 and 2q33.1 deletion syndromes (Gigek et al., 2015) indicates that these conditions can profoundly affect the regulation of NSCs. Knockdown (KD) of the genes implicated in each of the aforementioned deletion syndromes (e.g. TCF4, EHMT1, SATB2, and MBD5), led to similar changes in DNA methylation and the transcriptome of human NSCs, suggesting that NSC proliferation and differentiation alterations are the main outcome caused by the deregulation of these genes (Chen et al., 2014; Gigek et al., 2015; Sacco et al., 2018). Aligning with the concept of NDDs often

sharing similar cellular phenotypes, irrespective of the underlying mechanisms, the function of MECP2's mutations which cause the ID, Rett syndrome, lead to impairments in NSC proliferation and differentiation (Li et al., 2014).

Mutations that cause ID and autism spectrum disorder (ASD) have been identified in genes involved at different stages of the Wnt/β-catenin pathway, a key pathway involved in neurogenesis. These mutations affect important upstream ligands, such as WNT1 and WNT2 (Lin et al., 2012; Martin et al., 2013; Marui et al., 2010; Wassink et al., 2001), as well as the downstream transcription factor TCF7L2 (De Rubeis et al., 2014; El Khouri et al., 2021; Iossifov et al., 2014). Additionally, mutations in β-catenin have also been reported (Dubruc et al., 2014; Krumm et al., 2014; Kuechler et al., 2015; O'Roak et al., 2012). Most recently, research using an ID-ASD patient's primary fibroblasts confirmed the impairment of the Wnt/β-catenin pathway (El Khouri et al., 2021). Furthermore, haploinsufficiency of the chromatin remodeler, CHD8, which has also been associated with ASD, has been linked to both upregulated and downregulated Wnt signalling (Durak et al., 2016; Kobayashi et al., 2002; Sacco et al., 2018; Sakamoto et al., 2000). Eliciting KD of CHD8 in the developing mouse brain led to a decrease in the neural progenitor cell (NPC) population (Bernier et al., 2014; Durak et al., 2016; Sacco et al., 2018).

UPF3B, a developmentally regulated gene implicated in ID and ASD, was found to regulate neural proliferation and decrease differentiation of NPCs, while also affecting neurite growth (Jolly et al., 2013). Interestingly, deletions in the PTEN gene initially promoted proliferation of embryonic NSCs (Groszer et al., 2006) as well as adult NSCs, but eventually resulted in depletion of the NSC population and contributed to characteristics of ASD such as seizures, impaired learning, social impairments, and anxiety (Sacco et al., 2018).

In summary, the deregulation of NSC proliferation and differentiation emerges as a common theme across various NDDs, including IDs. Despite the diverse molecular mechanisms underlying these conditions, the impact on NSCs remains a point of convergence across these disorders. Furthermore, the economic and social impacts of IDs are profound, highlighting the urgent need for a deeper understanding of these disorders as there are currently no effective treatments available. Over 100 X-linked genes have been identified with mutations associated with various forms of X-linked intellectual disability (XLIDs), yet only a fraction of these have been extensively researched.

1.3.4 X-linked intellectual disability

Within the realm of ID, there exists another category that is specifically caused by mutations in X-chromosomal genes, referred to as XLID. XLID predominantly affects males, and 16% of males with ID are X-linked (Bustos, Segarra-Fas, Chaugule, Brandenburg, Branigan, Toth, Macartney, Knebel, Hay, & Walden, 2018). XLID is similarly defined as having an IQ below 70 before the age of 18 (van Bokhoven & Kramer, 2010; Vissers et al., 2016), and can have varying levels of severity for both syndromic and non-syndromic types (De Luca et al., 2020). In syndromic XLID, characteristics such as cleft palate, facial and skeletal dysplasia, growth defects, and polydactyly have a 2.4-fold X-chromosome bias (Chelly et al., 2006).

Among the well-studied XLID-associated proteins is the Fragile Mental Retardation Protein (FMRP), linked to Fragile X Syndrome (FXS), the most common form of inherited intellectual disability. Research utilizing FMR1 knockout (KO) mice has shown that the absence of FMRP leads to abnormalities in synaptogenesis, synaptic structure, and function, mirroring the phenotype observed in patients with FXS (Qurashi et al., 2012). Furthermore, FMR1 KO

mice exhibit learning and memory deficits consistent with FXS characteristics, highlighting the critical role of FMRP in cognitive development and functioning (Qurashi et al., 2012). In a number of studies, FXS has been characterized by excessive mGluR5 activity in neurons, outlining it as a disorder of NSC proliferation and differentiation (Callan et al., 2010; Castren et al., 2005; Gong et al., 2013; Jeon et al., 2014; Scotto-Lomassese et al., 2011).

Similarly, investigations into RNF12/RLIM E3 ubiquitin ligase, an XLID gene, in neural development using ESC models found that RNF12's catalytic activity is essential for proper stem cell maintenance and neural differentiation, and these processes are disrupted by mutations associated with XLID (Bustos, Segarra-Fas, Chaugule, Brandenburg, Branigan, Toth, Macartney, Knebel, Hay, & Walden, 2018; Zhang et al., 2012). Mutations in RNF12/RLIM accelerates the expression of neural lineage markers and neurite outgrowths (Bustos, Segarra-Fas, Chaugule, Brandenburg, Branigan, Toth, Macartney, Knebel, Hay, & Walden, 2018). These neural characteristics have been observed in other types of IDs where abnormal neural specialization (Telias & Ben-Yosef, 2014) and irregular dendritic spine arborization (Iwase et al., 2016; Korb et al., 2017) have been observed, suggesting that abnormal neural development and differentiation are key mechanisms in XLID.

Another emerging area of research within XLID pertains to single gene mutations affecting protein function associated with neurodevelopmental disorders in early life. For instance, the study of plant homeodomain zinc-finger protein (PHF6) and its role in Börjeson-Forssman-Lehmann Syndrome (BFLS) offers new insights into the molecular mechanisms underpinning brain development in the context of XLIDs (Cheng et al., 2018; Rasool et al., 2024; Zhang et al., 2013). Given the complexity and diversity of XLID, advancing our understanding of the genetic and molecular basis of these disorders is crucial for the

development of targeted therapies and interventions that could significantly improve the quality of life for individuals affected by these disabilities.

1.3.5 Börjeson-Forssman-Lehmann Syndrome

BFLS was first identified as an XLID in 1962 by Mats Börjeson and colleagues (BÖRJESON et al., 1962), and is the most rare form of XLID. Although BFLS manifestations may not be present at birth, they become apparent as a child reaches their developmental milestones. Diagnosis of BFLS typically relies on family history, physical examination, and sequencing of the PHF6 gene (Lower et al., 2002). The clinical features of BFLS include developmental delays, IQ impairment ranging from mild to severe, small genitalia, and short stature (Gecz et al., 2006; Lower et al., 2002). Additionally, 75% of patients with BFLS experience obesity by late childhood, and seizures are present in 8% of cases (Jahani-Asl et al., 2016).

BFLS diagnosis is further complicated by the overlapping features it shares with other XLID disorders, such as Klinefelter and Prader-Willi syndrome (Carter et al., 2009; Gecz et al., 2006; Turner et al., 2004). Despite these challenges, the phenotypic hallmarks of BFLS—such as tapered fingers and toes, deep-set eyes, broad forehead, and obesity help in its diagnosis (Gecz et al., 2006; Lower et al., 2002). Furthermore, the role of the PHF6 gene and how its mutations cause BFLS, emphasizes the syndrome's genetic basis and highlights the potential for targeted therapeutic strategies in the future (Lower et al., 2002). The ongoing study of BFLS not only contributes to our broader understanding of this very rare syndrome but can also be applied to other forms of IDs.

Introduction part 4 - PHF6 function and regulation

1.4.1 PHF6 structure

Multiple mutations in the *PHF6* gene have been identified in BFLS patients (Berland et al., 2010; Carter et al., 2009; Lower et al., 2002; Turner et al., 2004). Most *PHF6* mutations in BFLS are either missense or lead to the truncation of the PHF6 protein (Jahani-Asl et al., 2016). Located on the X-chromosome at Xq26-27, PHF6 is a member of the plant homeodomain-like family (Jahani-Asl et al., 2016). The *PHF6* gene is made up of 11 exons encoding a 365 amino acid protein (Liu et al., 2014). This protein contains two plant homeodomain (PHD) zinc-like finger domains and consist of two nuclear and one nucleolar localization sequence (Liu et al., 2014) (**Fig 3**). PHF6 is highly conserved in vertebrates where it holds high expression during early fetal life and embryonic tissue- specifically in the developing central nervous system during the early stages of corticogenesis (Voss et al., 2007; Zhang et al., 2013). This expression will diminish to low levels in adult tissues, with the exception of its expression during adult neuronal projection processes, specifically mitral cells, cerebral cortex pyramidal neurons, and cerebellar Purkinje cells (Voss et al., 2007). Outside the CNS, it also has high expression within the spleen, kidney, and thymus (Voss et al., 2007). In the adult brain, localization of PHF6 was only nuclear (Voss et al., 2007). Five *PHF6* mutations in BFLS are recurrent including c.2T>C/p.M1T, c134G>A/p.C45Y, c769A>G/p.R257G, c.999-1001delTGA/p.D333del and c1024C>T/p.R342X, found in 21% of patients (Chao et al., 2010; Crawford et al., 2006; Lower et al., 2004; Lower et al., 2002). The evolutionary conserved PHF6 cysteine residues; C45, C99, and C305, in either the C2HC or PHD-type zinc finger affects the PHF6-ePHD2 domain to thwart accurate protein folding leading to loss of function of the protein in BFLS (Erdős et al.,

2019; Jahani-Asl et al., 2016). The PHF6 protein is 97.5% identical in humans and mice, thus mouse models of PHF6 can be effectively applied to patient cases (McRae et al., 2019).

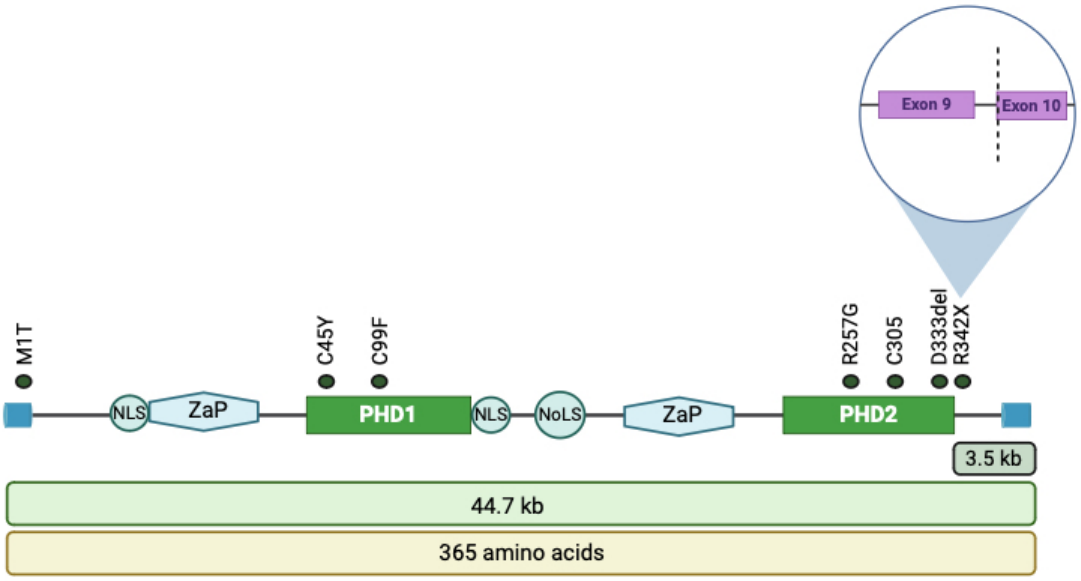

Figure 3: Illustration of the full *Phf6* gene. *Phf6* spans 44.7 kb and is comprised of 365 amino acids and 11 exons. The gene includes two ZaP domains, PHD domains 1 and 2, two nuclear localization sequences (NLS), and one nucleolar localization sequence (NoLS). The five recurrent BFLS mutations are shown; p.M1T, p.C45Y, p.R257G, p.D333del, and p.R342X (truncation at exon 10). The three conserved cysteine residues are C45, C99, and C305. Made with BioRender.

1.4.2 PHF6 binding partners

1.4.2.1 Polymerase Associated Factor 1 (PAF1)

Neuron migration to the cortical layers during embryonic development is fundamental for proper brain development (Kim et al., 2010; Rondón et al., 2004; Shi et al., 1996). An inhibition of PHF6 in the mouse cerebral cortex caused gross stoppage of neuronal migration followed by abnormal migration patterns (Zhang et al., 2013). In this study, neurons were unable to travel to the upper cortical layers but rather amassed in the IZ and lower cortical layers (Zhang et al., 2013). Additionally, there was a boost in the number of migrating neurons that did not have a proper leading process and were not fully developed (Zhang et al., 2013). Polymerase associated factor 1 (PAF1) is a part of the transcriptional elongation complex and PHF6 can associate to PAF1's four subunits to promote neuronal migration in the cerebral cortex (Zhang et al., 2013). This association at E17 concurs with neuronal migration to the upper layers (Kim et al., 2010; Rondón et al., 2004; Shi et al., 1996). A KD of *Paf1* in the cerebral cortex phenocopied *Phf6* KD wherein neuronal migration was affected (Zhang et al., 2013). Furthermore, through gene expression (GE) analyses and ChIP-PCR, Neuroglycan C/Chondroitin sulfate proteoglycan 5 (NGC/CSPG5) was identified as a downstream target of the PHF6/PAF1 complex in the control of cortical neuronal migration (Zhang et al., 2013).

1.4.2.2 Upstream Binding Factor 1 (UBF1)

PHF6, previously reported to localize to the nucleolus, was found to inhibit gene expression as mediated through binding to the promoter region of its targets via enlistment of the repressor complex (Wang et al., 2013). Upstream binding factor (UBF1) is a transcriptional

activator for ribosomal RNA (rRNA) to suppress rRNA transcription and exert control over the cell cycle (Wang et al., 2013). A direct interaction between PHF6 and UBF1 followed by an inhibition of rRNA transcription was observed (Wang et al., 2013). This suppression was done by PHF6's recruitment of a repressor complex that affected the level of UBF1 protein and exhibited cell cycle control through the plant homeodomain 1 (PHD1) (Wang et al., 2013). Furthermore, dysfunctions in cellular proliferation, cell cycle arrest, and DNA damage following a *Phf6* KD suggests a role for PHF6 in regulating rRNA synthesis (Fliedner et al., 2020; Wang et al., 2013; Warmerdam et al., 2020).

1.4.2.3 Nucleosome Remodeling and Deacetylase Chromatin-Remodeling Complex

In the absence of DNA, PHF6 associates with several histones including H3 that are important for long term regulation of genes (Miyagi et al., 2019). Other binding targets include the RBBP4 component of the nucleosome remodeling and deacetylase chromatin-remodeling complex (NURD) (Wang et al., 2013). This complex functions to stimulate nucleosome remodeling in an ATP-dependent fashion, as well as prompting embryogenesis, neurogenesis, oncogenesis, and hematopoiesis at its target genes (Reynolds et al., 2012; Tong et al., 1998; Yamada et al., 2014; Zhang et al., 2011). The p21 gene is controlled by the NURD complex and is an important cell cycle regulator that reduces the effectiveness of drug treatments (e.g. prednisolone) and responses to glucocorticoids in T-cell acute lymphoblastic leukemia (T-ALL) (Xiang et al., 2019). Under normal conditions, P21 is inhibited by PHF6. However, dysfunctions in PHF6, as seen in T-ALL, allows for an increase in the activity of p21 leading to the drug resistance phenotype of lymphoblasts (Wang et al., 2013; Xiang et al., 2019).

1.4.3 Downstream effectors of PHF6; the ephrin receptors

During my studies, my work led to the identification of several members of the ephrin receptors (EphRs) as direct transcriptional targets of PHF6 (Rasool et al., 2024). PHF6 is highly conserved in vertebrates with high expression during early fetal life, specifically during the early stages of corticogenesis (Cheng et al., 2018; Voss et al., 2007; Zhang et al., 2013). Characterization of PHF6 genome-wide binding sites in the mouse developing cortex revealed a panel of genes involved in neurogenesis and CNS development (Rasool et al., 2024). PHF6 is shown to function as either a transcriptional activator or repressor, depending on its genomic binding pattern (Rasool et al., 2024). In the context of EphRs, PHF6 binds to the EphA and EphB gene regulatory regions inducing their upregulation (Rasool et al., 2024). Investigations in two patient-related mouse models of BFLS, R342X and C99F-m, which is caused by mutations in PHF6, have shown significant deregulation of EphRs in a PHF6-dependent manner, as well as impairments in neurogenesis and eNSC fate specification (Rasool et al., 2024). Mechanistically, PHF6's direct impact on altering eNSC processes may be done through directly influencing EphR signalling, particularly through the Eph-A family of receptors, and most significantly via EphA4 (Rasool et al., 2024). This interaction may lead to alterations in the spatial and temporal patterns of eNSC differentiation and maturation, which are essential for proper brain development. Dysregulation of this axis may contribute to the pathogenesis of BFLS and other XLIDs that also exhibit impaired neurogenesis. The role of the EphA receptors, downstream of PHF6, suggests their potential as viable drug targets. Furthermore, targeting the PHF6-EphA4 pathway offers a novel approach for treating BFLS and XLID. Additionally, this research sheds light on the broader implications for cognitive neurodevelopmental disorders, suggesting that

similar mechanisms might be disrupted in other conditions, unveiling promising avenues for investigating impaired neurogenesis in other cognitive neurodevelopmental disorders.

Introduction part 5 - Master regulators of neurogenesis: the dynamic roles of Ephrin receptors across diverse cellular niches (Rasool & Jahani-Asl, 2024, *revised)*

1.5.1 Ephrin receptors

The first ephrin receptor (EphR), was discovered in 1987 as a new class of receptor tyrosine kinases (RTKs) (Hirai et al., 1987). Since then, 13 additional EphRs have been discovered leading to their classification into A- and B-type receptors. Currently, there are 9 EphA and 5 EphB receptors each requiring binding to their corresponding ephrin ligands to initiate signal transduction (Committee, 1997; Darling & Lamb, 2019). The ligands are also grouped into A- and B-type classes. Although EphA receptors typically bind to ephrinA ligands, and EphB receptors bind to ephrinB ligands, cross-talk between the classes can occur (Pasquale, 2008), adding to the complexity of EphR signalling.

EphRs are abundantly expressed during brain development and play important roles in cellular communication, in particular short-range communication (Barquilla & Pasquale, 2015; Darling & Lamb, 2019; Lisabeth et al., 2013), in which the transfer of information between cells in close proximity occurs. Since both EphRs and ephrins are membrane-bound, direct cell-cell contact is often required to induce signalling by these receptors (Committee, 1997). Eph receptors and ligands can also be expressed on the same cell (in cis) (Gerstmann & Zimmer, 2018; Klein, 2012), adding another level of complexity to understanding their mechanisms of action.

EphRs are important in modulating a spectrum of processes during corticogenesis, including proliferation, apoptosis, cell adhesion, cell division, and cell fate specification (Arvanitis et al., 2010; Gerstmann & Zimmer, 2018; North et al., 2009; Qiu et al., 2008;

Wilkinson, 2014). In adulthood, however, EphRs are shown to regulate alternate processes ranging from synaptic remodelling, epithelial differentiation, immune function, and the self-renewal of stem cells (Conover et al., 2000; Durbin et al., 1998; Kullander & Klein, 2002; Sakamoto et al., 2008; Tremblay et al., 2007; Yamaguchi & Pasquale, 2004). Given that dysregulation of these pathways are associated with several neurodevelopmental disorders (NDDs) (Lamprecht & LeDoux, 2004; Liang et al., 2019; Pasquale, 2010; Taylor et al., 2017), a better understanding of signal transduction by EphRs in the developing and adult brain may lay the foundation for EphR targeted therapies. EphR structure and regulation, and their role in cancers, cardiovascular disease, and NDDs have been extensively reviewed (Lamprecht & LeDoux, 2004; Liang et al., 2019; Pasquale, 2010; Taylor et al., 2017).

1.5.2 EphR structure & activation

EphRs are comprised of 7 domains (**Fig 4**) (Dravis, 2010; Taylor et al., 2017), with specificity of receptor-to-ligand binding being regulated by a cysteine-rich domain (CRD) and an epidermal growth factor-like (EGF) domain (Seiradake et al., 2010). The CRD is tethered by a ligand-binding domain (LBD), leading to receptor dimerization and clustering (Sahoo & Buck, 2021). The PSD-95, Dlg1, ZO-1 (PDZ) binding domain located at the C-terminal end, is required for anchoring the receptors from membrane to cytoskeleton, and is of particular importance for cell-cell communication (Liu & Fuentes, 2019). Two fibronectin (FN) 3 domains and a sterile alpha motif (SAM) domain function to control protein-protein interactions, facilitating assembly of protein complexes required for signal transduction (Campbell & Spitzfaden, 1994; Liang et al., 2019). Importantly, a kinase domain present in all RTKs, initiates the phosphorylation of tyrosine residues and is crucial for EphR activation (Kullander & Klein, 2002). At the N-

terminus of EphRs, a juxta membrane (JM) region maintains the neighbouring kinase domain in an inactive form by inhibiting access to ATP (Lisabeth et al., 2013). This inhibition is removed following ligand binding, receptor dimerization, and phosphorylation of key residues, including those within the JM region.

Ephrin ligands bind to EphRs through their receptor binding domain (RBD). EphrinA ligands lack a cytoplasmic domain and their RBD is attached to the cell surface through a glycosylphosphatidylinositol (GPI) linker. EphrinB ligands possess a transmembrane domain (Klein, 2012), and an intracellular PDZ binding motif (**Fig. 4**) (Dravis, 2010; Taylor et al., 2017). Dimerization of ephrin ligands with their receptors is the catalyst for phosphorylation of tyrosine kinase domain (Binns et al., 2000). The EphR and ephrins can function bi-directionally, where the signal comes from either the cell that carries the receptor (forward signal), or the cell that carries the ligand (reverse signal) (**Fig. 4**) (Klein, 2009; Pasquale, 2005). This signalling is often dependent on SRC family of kinases (SFKs), which are non-receptor kinases known to phosphorylate EphRs (Dravis, 2010; Taylor et al., 2017; Wybenga-Groot et al., 2001). For example, binding of ephrinB to the ectodomain of EphR induces phosphorylation of their tyrosine residue and initiates reverse signalling mediated by SFK members, Src and Fyn (Aoto & Chen, 2007; Georgakopoulos et al., 2006; Knöll & Drescher, 2004; Palmer et al., 2002). A better understanding of EphR phosphorylation/activation will be instrumental in developing readouts to assess their biology and therapeutic potential (e.g. high throughout compound screens). This knowledge assists in designing novel pharmacological approaches to activate or block select EphRs in a context dependent manner.

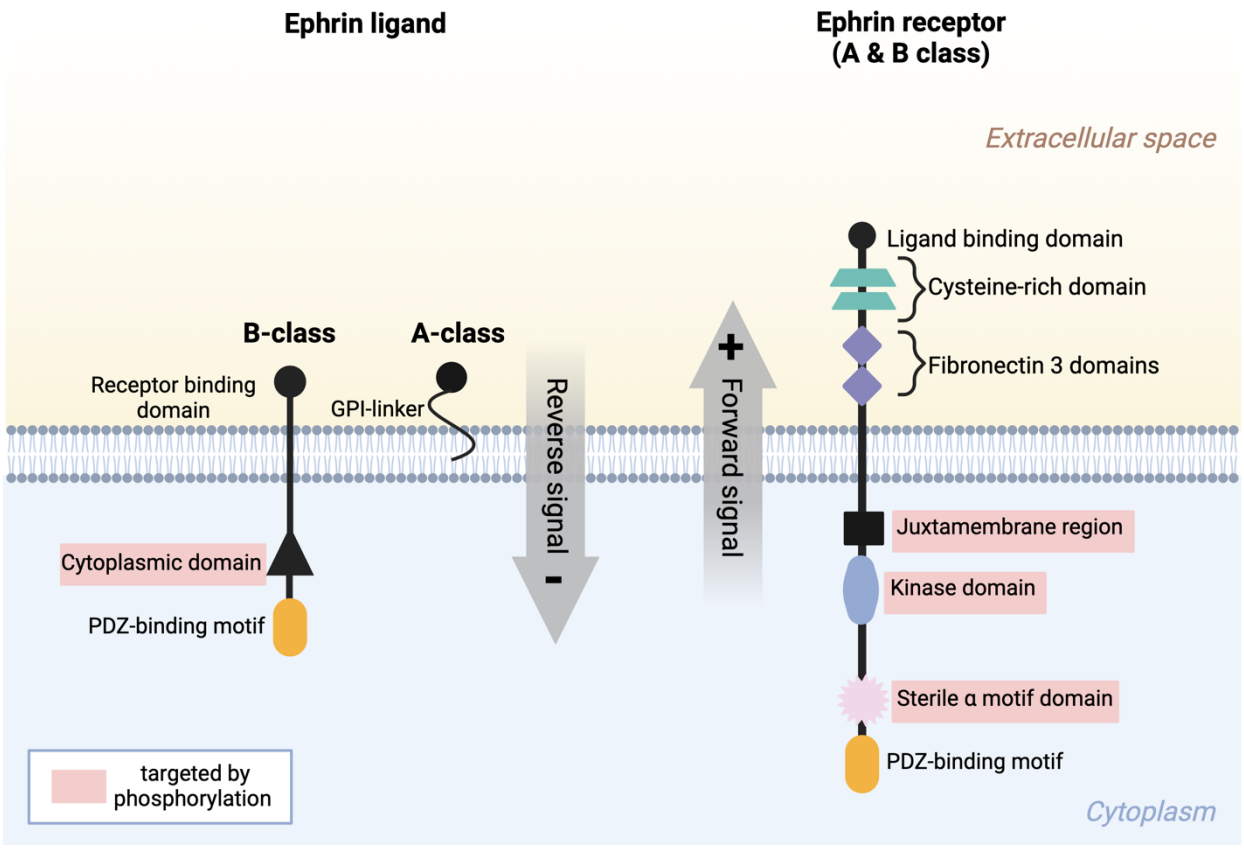

Figure 4: Schematic of ephrin receptors (EphR) and ligands. The ephrin A and B ligands, as well as different domains of EphR are illustrated. Forward and reverse signalling are highlighted in which the receptor-carrying cell, or the ligand carrying cell initiates signal transduction, respectively. Phosphorylation sites for the receptors and ligands are highlighted. Figure made with BioRender.

1.5.3 EphR gene regulation and downstream effectors

EphRs and ephrins operate through modulating members of the Rho GTPase family, including RhoA, Rac1, and Cdc42 (**Fig. 5**), which are known to control components of cytoskeleton dynamics (Herath & Boyd, 2010; Locke, 2018; Noren & Pasquale, 2004; Ségaliny et al., 2015). GTPases go through cycles of inactive (bound by GDP) and active (bound by GTP) forms and upon activation, they bind to their downstream targets. EphRs regulate the GDP-GTP transitions through guanine nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPS) (**Fig. 5**) (Herath & Boyd, 2010; Locke, 2018; Noren & Pasquale, 2004; Ségaliny et al., 2015). An emerging GEF is the Ephexin protein family that activates RhoA, and controls the activation of Cdc42 (Kim et al., 2019; Shamah et al., 2001). Ephexins are able to bind EphA4's kinase domain (Kim et al., 2019; Shamah et al., 2001), an interaction documented in neurons (Sahin et al., 2005). Interestingly, ephrinA-induced activation of EphA upregulates Ephexin activity, which then triggers increased activity of RhoA (Ogita et al., 2003). These findings suggest that RhoA activation by EphAs may require Ephexin. Interestingly, Ephexins have been linked to neurological disorders such as depression, epilepsy, and Alzheimer's disease (AD) (Kim et al., 2019; Sell et al., 2017; Veeramah et al., 2013; Zhang et al., 2017).

GEFs including Intersectin and Kalirin, which activate Rac1 and Cdc42 respectively, are implicated in EphB activity (Irie & Yamaguchi, 2002; Noren & Pasquale, 2004; Penzes et al., 2003). For example, EphB2 influences dendritic spine structuring in hippocampal neurons by binding to Intersectin and Kalirin, thus activating Rac1 and Cdc42 (Irie & Yamaguchi, 2002; Noren & Pasquale, 2004; Penzes et al., 2003). Intersectin can also specifically bind to inactive EphB2 (Irie & Yamaguchi, 2002; Noren & Pasquale, 2004), while Kalirin can only interact with a previously active EphB2 in early-born hippocampal neurons (Penzes et al., 2003). In addition,

ephrinB1 activity upregulates Kalirin in dendritic spines of hippocampal neurons, which leads to subsequent activation of the downstream Rac1 effector, p21-activated kinase (PAK) (Penzes et al., 2003). Thus, within hippocampal neurons, Rac1 activity appears to be regulated by Kalirin.

Rac1 and Cdc42 deregulation has been implicated in intellectual disability (ID) (Govek et al., 2005). For example, mutations in the gene encoding oligophrenin-1 (OPHN1), a Rho-GAP that regulates Rho GTPases, results in X-linked intellectual disability (XLID) characterized by dendritic spine abnormalities and impaired synaptic function (Billuart et al., 1998; Govek et al., 2005). Similarly, mutations in PAK3, an effector for both Rac1 and Cdc42, has been identified in families with non-syndromic XLID. These mutations disrupt the kinase activity of PAK3, leading to deficits in dendritic spine morphology and cognitive impairments (Bienvenu et al., 2000; Govek et al., 2005). Additionally, mutations in ARHGEF6, a GEF for Rac1 and Cdc42, are associated with ID and are thought to impair the regulation of actin dynamics necessary for proper neuronal connectivity (Govek et al., 2005; Kutsche et al., 2000). The involvement of Rac1 and Cdc42 in the maintenance of dendritic spines highlights their significance in diverse cellular processes underlying learning and memory, and thus their dysregulation may underlie the pathogenesis of NDDs (Govek et al., 2005).

EphR gene expression is regulated by numerous transcription factors (TFs). Among these transcriptional regulators are the Homeobox (HOX)-containing TFs including HOXA1/A2/A9/A13, HOXB1, HOXD13, and LIM1 which regulate EphR expression in a tissue-specific manner. For example, in the developing mouse brain, HOXA1 and HOXB1 induce the expression of EphA2 (Chen & Ruley, 1998), while HOXA2 alters EphA4 expression in rhombomeres (Theil et al., 1998), the segmented regions of the developing vertebrate hindbrain that contribute to the formation of cranial nerves and neural circuits. Researchers have

demonstrated that EphA4 is also positively regulated by Twist1, a basic helix-loop-helix TF (Arvanitis & Davy, 2012; Ting et al., 2009), in the context of coronal suture development and by LIM1 (Arvanitis & Davy, 2012; Kania & Jessell, 2003) in motor neurons. Furthermore, in developing mouse limbs, HOXA13 and HOXD13 regulate the expression of EphA7 (Salsi & Zappavigna, 2006).

EphB2/B3 expression during cell migration of the intestinal epithelium is shown to be regulated by transcription factor 4 (TCF4) and ß-catenin (Batlle et al., 2002). EphB1 activity in retinal ganglion cells during the time of optic chiasm divergence is controlled by Zic family member 2 (Zic2) (Arvanitis & Davy, 2012; García-Frigola et al., 2008). Interestingly, HOXA9 regulates EphB4, influencing both endothelial cell migration and endothelial cell tube formation (Bruhl et al., 2004). The regulation of EphB family members in the brain remains poorly understood, although TFs appear to be involved. As an example, EphB4 was shown to be regulated by the Valentino (Val) TF in boundary formation of the zebrafish hindbrain (Arvanitis & Davy, 2012; Cooke et al., 2001).

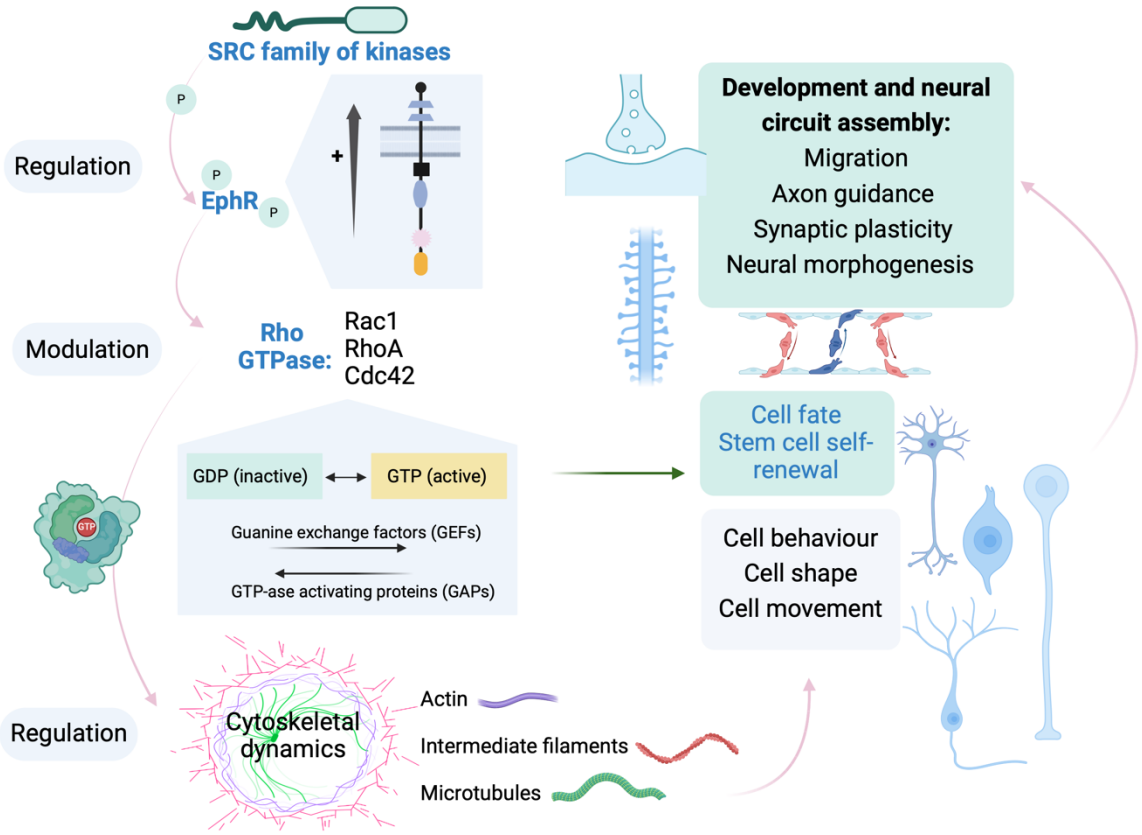

Figure 5: The signalling pathway of EphRs is depicted. SRC family of kinases regulate EphR signalling through phosphorylation. Once active, the EphRs then modulate the activity of the Rho family of GTPases. EphRs modulate the switch between inactive (GDP) and active (GTP) confirmations of the Rho family, or EphRs can cause reversion of the GTP state back to GDP. Influencing cell behaviour, shape, and movement is important for cell migration, axon guidance, synaptic plasticity, tissue morphogenesis, and cancer metastasis. Figure made with BioRender.

1.5.4 EphR regulation of neurogenesis

1.5.4.1 Role of EphR in stem cell niche: SVZ

The subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles are two regions with the highest density of stem cells in both the embryonic and adult brain (Ming & Song, 2011; Obernier & Alvarez-Buylla, 2019; Urbán & Guillemot, 2014) (**Fig 6**). Researchers have investigated the role of EphR and their ligands in these stem cell dense regions, particularly their influence on the proliferation of NPCs within the SVZ (Conover et al., 2000; Genander & Frisén, 2010; Holmberg et al., 2005; Theus et al., 2010). For instance, ephrinA2 expression is detected in neuroblasts and progenitor cells, and EphA7 is expressed in quiescent ependymal cells (Holmberg et al., 2005). The signalling via ephrinA2/EphA7 interaction appears to negatively regulate the self-renewal of adult NPCs (Holmberg et al., 2005).

Similarly, ephrinB ligands are widely expressed in the SVZ, and loss- and gain-of-function studies have revealed that ephrinB/EphB signalling impacts the number of dividing cells within the SVZ (Conover et al., 2000; Holmberg et al., 2005; Ricard et al., 2006). Specifically, genetic ablation of ephrinB3 in a mouse model appears to increase the number of proliferating stem/progenitor cells in the lateral ventricle of adult mice (Ricard et al., 2006). At the same time, EphB3 is shown to suppress progenitor cell proliferation in the SVZ (Theus et al., 2010). In contrast another EphB member, EphB2, which is expressed in NSCs and progenitor cells (Conover et al., 2000), induces cell proliferation in the SVZ through downregulation of neurogenic notch homolog protein 1 (Notch1) (Katakowski et al., 2005). Interestingly, studies on mice lacking the Notch1 ligand, Delta1, showed downregulated EphB2 and ephrinB2 expression in neural crest cells (De Bellard et al., 2002), suggesting a feedforward mechanism between stem

cell-related pathways and EphR. Of note, EphB2 is also shown to enhance neurogenesis following cerebral infarction (He et al., 2005; Xing et al., 2008).

EphA4 is an extensively studied receptor of the ephrin family in the contexts of axon guidance, NSC proliferation during development, and neuroblast migration to the olfactory bulbs (Goldshmit et al., 2004; North et al., 2009; Todd et al., 2017). High expression of EphA4 is detected in hippocampal endothelial cells, mature astrocytes, neurons, and NSCs (Deininger et al., 2008; Goldshmit et al., 2006; Goldshmit et al., 2004; North et al., 2009; Todd et al., 2017; Tremblay et al., 2009). EphA4 has a diverse set of signalling mechanisms compared to other EphRs and is able to bind both A- and B-class ephrin ligands (Lackmann & Boyd, 2003; Qin et al., 2010). Ablation of EphA4 led to the misalignment of neuroblasts and deficits in astrocyte organization in the subventricular zone/rostral migratory stream/olfactory bulb (SVZ/RMS/OB), indicating that EphA4 is an important regulatory factor for proper neuroblast migration and in the spatial organization, orientation, and regulation of the SVZ/RMS/OB (Todd et al., 2017). In summary, recent findings highlight the critical roles of EphRs and their ligands in regulating neurogenesis in SVZ.

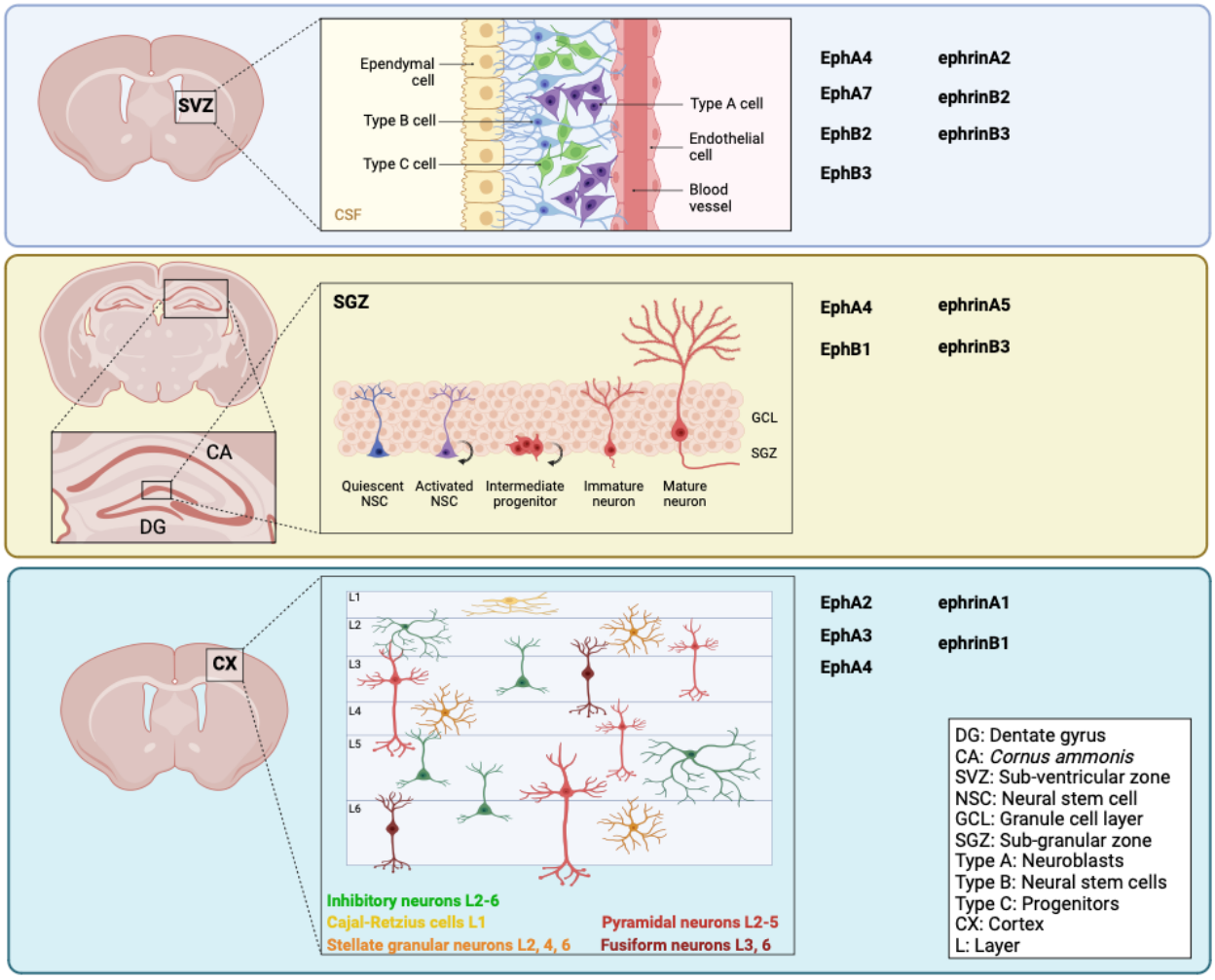

Figure 6: EphR expression across relevant brain regions. The expression of different EphRs and ephrin ligands is shown across the subventricular zone (SVZ), the subgranular zone (SGZ), and the cortex (CX). The cell types that make up each region is also shown. Figure made with BioRender.

1.5.4.2 Role of EphR in the hippocampus: SGZ

The distinct and potentially overlapping roles of EphA and EphB receptors in regulating neural stem/progenitor cells (NSPCs) is also apparent in the dentate gyrus (DG) of the hippocampus (**Fig 6**), specifically in SGZ, where high expression of EphB1 in hippocampal NSPCs has been observed (Chumley et al., 2007). When stimulated by ephrinB3, EphB regulates proliferation and migration of NPCs (Chumley et al., 2007). Further studies on *ephrinA5* knockout (KO) mice, which show downregulation of early neuron proliferation and survival in the DG, suggests a commonality between some roles of EphA and B receptors in the hippocampus (Hara et al., 2010). In particular, EphA4's indirect regulation of hippocampal precursor cell self-renewal within the adult brain is through D-serine regulated NMDAR signalling; important in synaptic plasticity and memory formation (Zhao et al., 2019). Inhibition of EphA4 signalling has also been shown to enhance the proliferation of hippocampal precursor cells (Zhao et al., 2019).

The roles of EphRs in synaptic plasticity and memory formation, highlights their importance in SGZ. It is not surprising that deregulation of these pathways are implicated in NDs such as AD (Jing et al., 2012; Lamprecht & LeDoux, 2004; Taylor et al., 2017; Willi et al., 2012).

1.5.4.3 Role of EphR in the Developing Cortex

In the developing cortex, ephrinB1 has been shown to be highly expressed in NPCs and is required for conserving progenitor fate. Blockade of ephrinB1 resulted in NPC differentiation and cell cycle exit (Laussu et al., 2014; Qiu et al., 2008). Similarly, EphA4 is highly expressed in

NPCs of the developing cortex and EphA4-depleted embryos exhibited decreased cortical wall thickness and reduced proliferation as assessed by BrdU assays (North et al., 2009). These studies led to proposing a model whereby NPC proliferation and maintenance in the embryonic cortex could be positively directed by ephrinB1 binding to EphA4 (North et al., 2009; Qiu et al., 2008). Other studies have reported that inhibition of ephrinA1 and EphA2/A3/A4 interaction resulted in reduced neuron numbers induced by a reduced propensity for differentiation (Aoki et al., 2004). In yet another study, adult mice with *ephrinA2* KO exhibited an abnormal neocortical laminar structure with a significant reduction in neuron density that was similar to what was seen in the neocortex of ASD children (Homman-Ludiye et al., 2017). From these findings, ephrinA2 was proposed to be an important factor for neuronal fate (Homman-Ludiye et al., 2017). Whether EphA-mediated regulation of neuronal fate and density stems directly from deregulation of NSCs/NPCs self-renewal, reprograming of pro-differentiation mechanisms, or both, requires additional investigation. In conclusion, given that alterations in cell identity and NPC fate in developing cortex are associated with various NDDs, such as microcephaly, lissencephaly, and ASD (Ben-Reuven & Reiner, 2021; Des Portes et al., 1998; Xu et al., 2014), understanding the roles of ephrin/EphR signalling in these processes could further our knowledge on the mechanisms underlying the pathogenesis of these diseases.

1.5.5 Role of EphR in other stem cell models: BTSCs

EphRs and ephrins have been studied in the context of glioblastoma (GB) cancer cell invasion, migration, tumorigenicity, and maintenance of the brain tumour stem cell (BTSC) pool (Binda et al., 2012; Day et al., 2013; Nakada et al., 2010; Wykosky et al., 2005). EphA2 was shown to promote BTSC derived xenografts, and its downregulation suppressed tumourigenesis

(Binda et al., 2012). EphA3 expression is also elevated in GB cell lines and functions to preserve the stem cell-like properties of BTSCs (Day et al., 2013). A significant decrease in tumorigenicity of recurrent GB (rGB) cells following EphA2 and EphA3 inhibition has previously been reported to lead to inhibition of the AKT/ERK pathways (Qazi et al., 2018). Co-blockade of EphA2 and EphA3 enhanced differentiation of BTSCs, attenuated the expression of stem cell markers, and reduced the tumorigenicity of rGB (Qazi et al., 2018).

B-class Eph receptors and ligands, are also implicated in several key processes within GB and contribute to tumourigenesis and poor prognosis (Nakada et al., 2006; Nakada et al., 2004; Qiu et al., 2019; Tu et al., 2012; Zhu et al., 2022). Increased expression of EphB2 is detected in human U87 glioma cell line (Nakada et al., 2004). Human glioma samples exhibit elevated ephrinB3 (Nakada et al., 2006), ephrinB2, and EphB4 expression (Tu et al., 2012). Enhanced ephrinB1 signalling in U87 cells induced an increase in migration and invasiveness (Nakada et al., 2004) and its blocking attenuated migration and invasion (Nakada et al., 2006). HIF-2a stabilization of EphB2 during hypoxia is shown to induce phosphorylation of paxillin, and this pathway appears to regulate GB invasion (Qiu et al., 2019). The protein reelin, which binds to EphB receptors, has been identified as a significant contributor to GB and cancer stem cell (CSC) migration (Biamonte et al., 2021). Reelin is a glycoprotein involved in regulating neuronal migration and positioning during brain development, and binds the extracellular domain of EphB, inducing receptor clustering and activation in neurons, consequently controlling neuronal migration during nervous system development (Bouché et al., 2013; Sentürk et al., 2011). Overall, EphBs and ephrinBs appear to facilitate the communication between tumour cells and their microenvironment, promoting tumour growth, invasion, and resistance to therapy (Nakada et al., 2006; Nakada et al., 2004; Qiu et al., 2019; Zhu et al., 2022).

Hypothesis and objectives:

The hypothesis of this research project was that PHF6 plays a role in neurogenesis by altering NSC processes in the developing brain. The major objectives of this thesis were the following:

1) To determine whether PHF6 alters neurogenesis and NSCs in BFLS mouse models

2) To determine whether PHF6 alters neurogenesis via regulation of EphR

3) To determine if EphR can rescue the PHF6-induced mutant NSC phenotype

CHAPTER 2 - Materials & Methods

2. Methods

2.1 Mice generation, housing, and genotyping

All animal experiments were approved by the Animal Care Committee (ACC) at the University of Ottawa in Ottawa, Ontario, Canada, and McGill University in Montreal, Quebec, Canada. Mice were maintained in regular housing conditions with standard access to food and drink in a pathogen-free facility. The R342X mouse model was generated using CRISPR/Cas9 and functions as a truncated PHF6 protein (Chao et al., 2010; Crawford et al., 2006; Gecz et al., 2006; Jahani-Asl et al., 2016; Lower et al., 2004; Lower et al., 2002; Todd et al., 2015). This strain was generated through the breeding of R342X female heterozygous (HET) mice with C57BL6/J WT (B6 WT) male mice. Hemizygous (HEMI) males were used as experimental mice, and B6 WT males were used as a control. The C99F-m mouse model was generated using CRISPR/Cas9 where cysteine-99 is replaced with phenylalanine (C99F) at nt.296G>T (Cheng et al., 2018). This strain was generated through breeding C99F-m female HET mice with B6 WT male mice. HEMI males were used as experimental mice, and B6 WT males were used as control.

The *Phf6*$^{-/Y}$ / *Nestin-CreERT2*$^{+}$ mouse strain (KO) is generated by a brain-specific deletion of *Phf6* via breeding *Phf6*$^{fl/fl}$ female mice (McRae et al., 2019) with Nestin-CreERT2^{+} male mice and inducing the Cre recombinase via oral gavage of Tamoxifen (Sigma-Aldrich, T5648) in pregnant dams at E14 and embryos collected 24-48 hours later. *Phf6*$^{-/Y}$ / *Nestin-CreERT2*$^{+}$ -were characterized and compared to *Phf6*$^{loxP/Y}$ / *Nestin-CreERT2*$^{-}$ control mice subjected to tamoxifen administration and used as control in all analyses. Male mice were used throughout.

The *Phf6*$^{-/Y}$ / *Nestin-Cre*$^{+}$ mouse strain was generated by breeding *Phf6*$^{fl/fl}$ female mice with *Nestin-Cre*$^{+}$ male mice to generate *Phf6*$^{-/Y}$/*Nestin-Cre*$^{+}$ KO males and *Phf6*$^{-/Y}$/ *Nestin-Cre*$^{-}$ control littermates. Here, the *Phf6* gene was deleted from the mouse central and peripheral nervous system from E11.5, which is the onset of *Nestin* gene expression (Tronche et al., 1999).

For genotyping, mouse tissue (tail or ear clipping) was first lysed in alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA pH 12) and then placed in a heat block at 95 °C for 30 minutes. The samples were then neutralized using an equal volume of neutralization buffer (40 mM Tris-HCl pH 5.0).

For genotyping of C99F-m and R342x, the PCR reaction mixture was set up as follows using Klentaq Thermostable DNA Polymerase Thermus aquaticus, recombinant, E. coli (Jena Bioscience, #PCR-217L); 2.5 µL 10x PCR buffer, 0.2 µL 10 mM dNTP, 6.5 µL Betaine, 1 µL 10 µM forward primer, 1 µL 10 µM reverse/mutation primer, 0.2 µL Klentaq enzyme, 12.6 µL RNAse-free H_2O, 2 µL DNA for a total mix of 26 µL per PCR tube. For genotyping of *Phf6*$^{fl/fl}$, the PCR reaction mixture was set up as follows using a 2x Green PCR Master-Mix high performing (ZmTech Scientific, #S2100G); 7.5 µL 2x Green PCR Master-Mix, 0.4 µL 10 µM forward primer, 0.4 µL 10 µM reverse primer, 5.7 µL RNAse-free H_2O, 2 µL DNA for a total mix of 16 µL per PCR tube.

For genotyping of Nestin-CreERT2, the PCR reaction mixture was set up as follows using a 2x Green PCR Master-Mix high performing (ZmTech Scientific, #S2100G); 7.5 µL 2x Green PCR Master-Mix, 1.5 µL 0.5 µM oIMR1084 primer, 1.5 µL 0.5 µM oIMR1085 primer, 1.5 µL 0.5 µM oIMR7338 primer, 1.5 µL 0.5 µM oIMR7339 primer, 0.975 µL 6.5% glycerol, 2 µL DNA for a total mix of 16.5 µL per PCR tube.

The genotyping samples were PCR amplified in a Bio-Rad T100 Thermal Cycler using

the following program for C99F-m, R342X, *Phf6*$^{Loxp/Loxp}$: 1. 95°C for 2 minutes, 2. 95°C for 30 seconds, 3. 60°C for 30 seconds, 4. 72°C for 30 seconds, 5. repeat steps 2-4 33x, and 6. 72°C for 4 minutes.

The Nestin-CreERT2 genotyping samples were PCR amplified using the following program: 1. 94°C for 2 minutes, 2. 94°C for 20 seconds, 3. 65°C for 15 seconds (-0.5°C per cycle), 4. 68°C for 10 seconds, 5. Repeat steps 2-4 10 times, 6. 94°C for 15 seconds, 7. 60°C for 15 seconds, 8. 72°C for 10 seconds, 9. Repeat steps 6-8 28 times, and 10. 72°C for 2 minutes. The Nestin-Cre genotyping samples were PCR amplified using the following program: 1. 94°C for 2 minutes, 2. 94°C for 20 seconds, 3. 60°C for 20 seconds, 4. 72°C for 25 seconds, 5. Repeat steps 2-4 35 times, and 6. 72°C for 2 minutes.

The PCR-amplified products were run on a 3% agarose gel at 100 V for 40 minutes for C99F-m, R342X, Nestin-CreERT2, and Nestin-Cre. The *Phf6*$^{fl/fl}$ PCR amplified products were run on a 3% agarose gel at 100 V for 60 minutes. See primers listed in Table 1.

2.2 Induction of Cre Recombinase in *Phf6*$^{fl/fl}$*/Nestin-CreERT2* mice

Pregnant dames (gestation day E12) were given an oral gavage of one 0.1 mL dose of Tamoxifen (Sigma-Aldrich, T5648) at a concentration of 20 mg/mL using a 1 mL syringe and a 22-gauge feeding needle (Instech Solomon, #FTP-22-25-5).

2.3 Immunoblotting

Protein lysates were created from whole brain tissue harvested in RIPA lysis buffer containing protease and phosphatase inhibitors (ThermoFisher Scientific, A32959). The

concentration of proteins was analyzed by the Bradford Assay (Bio-Rad) with BSA standard. PVDF membranes were activated in Methanol for 5 minutes and then blocked in 5% BSA in TBST. Membranes were probed with anti-PHF6 (NOVUS, NB100-68262, 1:1000), anti-EphA4 (ThermoFisher, 37-1600, 1:500) or (Santa Cruz, sc-365503, 1:100), anti-EphA7 (ThermoFisher, BS-7034R, 1:500) or (R&D Systems, MAB1495, 1:100), anti-EphB1 (Abcam, ab129103, 1:1000), anti-EphB2 (Abcam, ab252935, 1:500), anti-SOX2 (Abcam, ab97959, 1:250), anti-NESTIN (Santa Cruz, sc-23927, 1:100) or (R&D Systems, MAB2736, 1:500), anti-GFP (Abcam, ab1218, 1:1000), anti–GAPDH (Cell Signaling, 2118S, 1:5000), anti-beta-Actin (Sigma-Aldrich, a5316, 1:2000), anti-beta3-Tubulin (Cell Signaling, 5568T, 1:5000), overnight at 4°C, followed by HRP-conjugated secondary antibody, anti-rabbit IgG HRP (Bio-Rad, 1706515) or anti-mouse IgG HRP (Bio-Rad, 1706516) for 2 hours at room temperature. Proteins were visualized with ECL (Bio-Rad), and signals were detected with a Chemidoc imaging system (Bio-Rad).

2.4 Immunoprecipitation (IP)

80 µg of total cell extracts from *Phf6*$^{loxP/Y}$ */ Nestin-CreERT2*$^{-}$ or *Phf6*$^{-/Y}$ */ Nestin-CreERT2*$^{+}$ eNSCs were employed for immunoprecipitation (IP), using either 1 µg of IgG or PHF6 antibody (NOVUS, NB100-68262, 1:1000). For input, 4 µg of total cell lysates from both *Phf6*$^{loxP/Y}$ */ Nestin-CreERT2*$^{-}$ and *Phf6*$^{-/Y}$ */ Nestin-CreERT2*$^{+}$ eNSCs were utilized.

2.5 Quantitative Real-Time PCR

RNA was isolated from cells and whole brain tissue with Trizol (Invitrogen) according to the manufacturer's instructions. Reverse transcription of RNA was performed using 5x All-In-One RT MasterMix cDNA synthesis (Abm, G492). Quantitative real-time PCR was performed using SsoAdvanced™ Universal SYBR®Green Supermix (Bio-Rad, 1725271). Samples were incubated at 25°C for 10 minutes, followed by incubation at 42°C for 15 minutes, and finally 85°C for 5 minutes to inactivate the reaction. See primers listed in Table 1.

2.6 Immunofluorescence staining of tissue

Mouse brains were fixed in 4% paraformaldehyde (PFA) for 24 hours, followed by 24 hours of 15% sucrose fixation, and another 24 hours of 30% sucrose fixation before being snap frozen in OCT on dry ice. 8 μm frozen sections were cut using a cryostat. Antigen retrieval was performed on sections prior to blocking by submerging slides in a slide holder with Dako Target Retrieval Solution (Agilent, S1699) and heating in a beaker of water for 20 minutes at 95-98°C. Sections were then cooled for 15 minutes and blocked in 20% donkey serum, 0.1% Triton-X, 0.1% Tween in PBS, for 20 minutes at room temperature. We applied the SOX2 (1:250) antibody (Abcam, ab97959) and the TBR2 antibody (1:50) (ThermoFisher, 14-4875-82) overnight at 4°C in a humid chamber. Secondary antibodies (1:500); Anti-rabbit IgG, Alexa Fluor® 647 Conjugate (Cell Signaling, 4414S), Anti-rat IgG Alexa Fluor® 488 Conjugate (Cell Signaling, 4416S), and DAPI (1:1000 of 1 μg/ml) (ThermoFisher, D1306) were applied for 45 minutes at room temperature in a humid chamber. Slides were mounted with ProLong Gold Antifade Mountant (ThermoFisher, P36934) with a #1.5 coverslip. Images were obtained with a laser scanning confocal microscope (ZEISS LSM 800) at 20x objective. Detection wavelengths were as follows: DAPI detection 400-605, TBR2 (AF488) 400-650, SOX2 (AF647) 645-700,

and all with a detector gain of 650V.

For PHF6 and coronal layer marker immunofluorescent brain section staining, brains were fixed with 4% PFA and equilibrated in 30% sucrose solution at 4°C until the brains sank to the bottom of the vail. Brains were immersed in 50% OCT (VWR) solution diluted by 30% sucrose for overnight at 4°C. Brains were transferred to the cryomold (VWR) filled with 50% OCT and flash frozen in liquid nitrogen. Frozen brains were stored at -80°C. Brain blocks were subjected to cryosection at the thickness of 12 µm and mounted onto SuperFrost slides (Fisher Scientific). Sections were then washed three times with PBS and 0.1% Tween-20 detergent (PBST), then antigen retrieval in Citrate buffer (0.1M, pH 6.0) by microwave boiling for 10 minutes and blocked in 10% horse serum/PBST for 30 minutes at room temperature. After blocking, sections were subjected to the following primary antibody for PHF6 immunofluorescence (overnight at 4°C): rabbit anti-PHF6 (1:150, Sigma-HPA001023), or for coronal layer markers; mouse anti-SATB2 (1:200, Abcam-ab51502), rat anti-CTIP2 (1:200, Abcam-ab18465), and rabbit anti-TBR1 (1:200, Abcam-ab31940). The next day, after washing three times in PBST, sections were incubated in secondary antibody for 1 hour at room temperature: anti-rabbit 555 Alex Fluor (1:500, Invitrogen A21206), anti-mouse 488 Alexa Fluor (1:500, Invitrogen A21202), or anti-rat 647 Alexa Fluor (1:500, Invitrogen A21247). Nuclei were counterstained by incubating sections in Hoechst 33342 dye (ThermoFisher Scientific) for 15 minutes at room temperature. Finally, slides were mounted onto coverslips (Fisher Scientific) in DAKO Fluorescence Mounting Medium (Agilent Technologies).

2.7 Histology staining

For Nissl staining, brain sections were rehydrated by 10 minutes submersions in 95% ethanol, followed by 1 minute submersion in 70% ethanol and 1 minute submersion in 50% ethanol. Sections were rinsed in tap water and then in distilled water. After washing, sections were stained in 0.25% cresyl violet stain solution in distilled water for 5 minutes, followed by a quick wash in distilled water. Sections were quickly differentiated in 70% ethanol with 1% acetic acid for 10 seconds to 1 minute and checked under the microscope. Sections were then dehydrated via two 5-minute submersions in 100% ethanol. Finally, slides were cleared by three 5-minute submersions in xylene and mounted onto coverslips (Fisher Scientific) with Permount Mounting Medium (Fisher Chemical). Stained slides were air-dried overnight in the fume hood at room temperature. Immunofluorescent images were acquired by using Zeiss Axiovert Observer Z1 epifluorescent/light microscope equipped with an AxioCam cooled-colour camera (Zeiss) or SP8 confocal microscope (Leica). Nissl-stained slides were scanned by a Zeiss AxioScan Z1.

2.8 Embryonic neural stem cell culture

Embryonic NSCs (eNSCs) were obtained by whole brain culturing of E14 mice (Azari et al., 2010; Burban & Jahani-Asl, 2022; Nasser et al., 2018) (excluding cerebellum). Pregnant mice were euthanized, uterine horns were removed, and embryos were placed in cold 1x HBSS. Brain tissue was cut into small pieces and placed in 15 mL falcon tubes containing 1 mL cold 1x HBSS. Tissue was allowed to settle to the bottom, HBSS was replaced with 1 mL fresh HBSS for washing, and then replaced once more with 1 mL stem cell media (SCM) containing 1:1 DMEM-F12 (Wisent, 319-005-CL) (ThermoFisher, 31765035), 50 units/mL penicillin-streptomycin (Wisent, 450-201-EL), 1X B-27 supplement (Invitrogen, 17504044), 2 µg/mL

Heparin (Stemcell Technologies, 07980), 20 ng/mL mEGF (Cell Signaling, 5331SC), 12.5 ng/mL bFGF (Abbiotec, 600182). The tissue was mechanically dissociated 15x with P1000 then an additional 15x with P200. The lysate was then plated in 6 mL of SCM and left in the incubator for 6-7 days until spheres grew to 40-200 μm in size, replenishing with 2 mL SCM media at day 4.

2.9 Analysis of self-renewal and proliferation

For the limiting dilution assay (LDA), NSCs were dissociated to single-cell suspension using Accumax. Single cells were counted and plated in a 96-well plate at different cell doses per well, in triplicates. Spheres were counted 7 days post-plating.

For the extreme limiting dilution assay (ELDA), NSCs were dissociated to single-cell suspension using Accumax. Single cells were counted and plated in a 96-well plate at different cell doses per well with a minimum of 12 wells/cell dose (Rasool et al., 2022). 7 days post-plating, the presence or absence of spheres in each well was recorded and analyzed with http://bioinf.wehi.edu.au/software/elda/33 (Hu & Smyth, 2009).

For cell viability, NSCs were dissociated to single-cell suspension using Accumax. Single cells were counted and seeded at a density of 200 cells/well, in a 96-well plate. Cell viability was evaluated 7 days post-plating using alamarBlue (Thermo Fisher Scientific, #DAL1100) according to the manufacturer's protocol. 10% resazurin was added to the cells in each well and incubated for 4 h at 37 °C. Fluorescence was read using a fluorescence excitation wavelength of 560 nm and an emission of 590 nm.

Representative images of spheres were taken with the 10X objective lens of an Olympus

IX83 microscope with an X-Cite 120 LED from Lumen Dynamics, and an Olympus DP80 camera.

2.10 5-ethynyl-2´-deoxyuridine (EdU) proliferation assay

eNSCs were dissociated into single-cell suspension using Accumax, counted and plated at a density of $1x10^6$ cells. Cells were incubated with 10 μM EdU upon plating. Following 22 hours in culture, eNSCs were fixed, permeabilized, and stained using the Click-iT EdU proliferation kit (Thermo Fisher Scientific, #C10337) according to the manufacturer's protocol. Fluorescence was analyzed by flow cytometry (BD FACS CantoII & Sony SH800). Data was analyzed using the FlowJo software. The number of cells that had incorporated EdU was defined as the ratio of EdU-positive cells over total number of cells and following dead cell removal with propidium iodide (FxCycle PI/RNase staining solution, Invitrogen, F10797).

2.11 Chromatin immunoprecipitation (ChIP)

PBS containing protease inhibitors (Thermo Fisher Scientific, #A32959) was used as cell washing buffer prior to fixation. Cross-linking was done via 1% formaldehyde in PBS for 10 minutes and quenched with 0.125 M glycine in PBS for 5 minutes at room temperature (RT). Washing, fixation, and quenching was done in 15 mL tubes while rotating at RT. Post-quenching, cells were washed twice with PBS containing protease inhibitors. Cells were then pelleted by spinning at 150 g for 10 minutes at 4ºC. Cell pellets were dissolved in ChIP lysis buffer (40 mM Tris-HCl, pH 8.0, 1.0% Triton X-100, 4 mM EDTA, 300 mM NaCl) containing protease inhibitors. Chromatin fragmentation was performed through water bath sonication

(BioRuptor) at 4ºC, creating an average length of 500 base pairs (bp) of product. Cell lysates were spun down at 12,000G for 15 minutes, followed by dilution of supernatant (1:1) in ChIP dilution buffer (40 mM Tris-HCl, pH 8.0, 4 mM EDTA, protease inhibitors). Immunoprecipitation (IP) was done using a PHF6 antibody (Novus Biological, NB100-68262), rabbit IgG antibody (Cell Signaling, #3900S). Antibody-protein-DNA complexes were collected, washed, and then eluted. Reverse cross-linking was done as described in Soleimani et al., 2013 (Soleimani et al., 2013). Immunoprecipitated DNA was analyzed by qPCR, and the binding enrichment was expressed as a percentage of the input.

2.12 Dual-luciferase reporter assay

The PHF6 binding regions (based on ChIP-seq peaks) were cloned into the pGL4.23 (Promega) vector to generate the *EphA4*, *EphA7* and *EphB1* luciferase reporter genes by digesting the plasmid and the annealed primer pair using EcoRV (NEB, #R0195L) and KpnI (NEB, #R3142) then ligating them with T4 DNA ligase (NEB, #M0202L). The constructs were confirmed by DNA sequencing. Cells were electroporated with the *EphA4*-pGL4.23, *EphA7*-pGL4.23, *EphB1*-pGL4.23 or the empty pGL4.23. Luciferase assays were performed 48 hours after transfection with the Dual-Luciferase Reporter Assay system (Promega, #E1910) with a GloMax Luminometer (Promega). In all experiments, cells were electroporated with a Renilla firefly reporter control and the firefly luminescence signal was normalized to the Renilla luminescence signal. See primers listed in Table 1.

2.13 siRNA

Transient KD of *Phf6* and *EphA4/A7/B1/B2* using an siRNA approach was performed with ON TARGET-plus SMART pool mouse *Phf6* siRNA (Dharmacon, #L-058690-01-0005), mouse *EphA4* siRNA (Sino Biological, #MG50575-M), mouse *EphA7* siRNA (Sino Biological, #MG50587-M, mouse *EphB1* siRNA (Sino Biological, #MG50479-M), mouse *EphB2* siRNA (Santa Cruz, #sc-39950), and ON TARGET-plus non-targeting pool (Santa Cruz, #sc-36869). siRNA (100 nM) were nucleofected into eNSCs (10^6 cells) and cultured in eNSC media at 37ºC in a humidified atmosphere of 5% CO2.

2.14 Leveraging published sequencing datasets

Single cell RNA-seq data from the mouse cerebral cortex was obtained [Data ref: (Di Bella et al., 2021)]. Log normalized counts, cell type annotation and UMAP coordinates were retrieved from the original publication and used to generate UMAP plots. For the correlation analysis, MAGIC (Van Dijk et al., 2018) was applied to obtain imputed gene expression. Correlation values were obtained on the imputed gene expression after applying MAGIC. Normalized RPKM (Reads per Kilobase Million) values of RNA-seq data were obtained from the Allen Brain Atlas BrainSpan dataset [Data ref:(BrainSpan, 2013)] and data from the ventral frontal cortex (VFC) was taken. The average RPKM values was calculated per developmental time. All plots were generated using R (version 4.0.0).

2.15 ChIP-seq data processing

ChIP-seq was performed by pooling the cortex of three mice (n=3) prior to sequencing. ChIP-seq data were processed as previously described (Hernandez-Corchado & Najafabadi,

2022). Briefly, raw reads were aligned to the mouse genome assembly version mm10 with bowtie2 (version 2.3.4.1) using the "--very-sensitive-local" mode. Duplicate reads were removed using samtools (version 1.9) (Danecek et al., 2021). ChIP-seq peaks were identified using MACS (version 1.4) (Feng et al., 2012; Zhang et al., 2008) with a permissive p-value threshold of 0.001, using "--nomodel" option. Fragment size was specified using "--shiftsize" argument, with the fragment length obtained by cross-correlation analysis using phantompeakqualtools (Landt et al., 2012). Peak- transcription start site (TSS) distances were calculated using bedtools (Quinlan & Hall, 2010) only for peaks that passed p-value threshold of 10^{-5}, with TSS coordinates obtained from GENCODE (Frankish et al., 2019) (release M9).

2.16 Identifying Pol II occupancy and its intersection with PHF6 data

Pol II occupancy data were obtained from GEO (accession number GSM2442441) (Liu et al., 2017). The bedGraph file representing Pol II occupancy was directly downloaded from GEO, converted to bigWig, and overlayed on gene TSS coordinates using bwtool (Pohl & Beato, 2014).

2.17 mRNA-seq

Cortical progenitors were established from the cortex of wild type E14 mice and subjected to electroporation with *Phf6* siRNAs (n=3) and non-targeting control siRNA (n=3). Cell were subjected to mRNA-Seq analysis following 5 days in culture. mRNA-seq raw reads were mapped to mm10 genome using HISAT2 (Kim et al., 2015), followed by duplicate read removal using samtools. Gene-level read counts were obtained by HTSeq (Anders et al., 2015),

using gene annotations from GENCODE (release M9). Genes with a minimum of 150 reads in at least one sample were retained. Gene set analysis was performed using ConsensusPathDB (Kamburov et al., 2011).

2.18 Quantification and statistical analysis

Statistical analysis was performed with the aid of GraphPad software 7. Two-tailed unpaired student t-tests were used to compare two conditions (normal distribution). One-way ANOVA was used for analyzing multiple groups (normal distribution). Data are shown as mean with standard error of mean (mean ± SEM). p-values of equal or less than 0.05 were considered significant and were marked with one asterisk (*). p-values of less than 0.01 are denoted by **, and p values of less than 0.001 are denoted by ***. All data presented are from 3 or more independent biological (n) replicates ($n \geq 3$), unless otherwise noted in corresponding figure legends, thus no additional statistical methods were used to predetermine sample size. Randomization was used to allocate animals to experimental groups, following genotyping. The researchers were blind to treatment groups for all quantifications as well as imaging analysis. Only male mice were included in this study. Methods of statistical analysis and p-values employed are reported in corresponding figure legends.

CHAPTER 3 - PHF6-mediated transcriptional control of NSC via Ephrin receptors is impaired in the intellectual disability syndrome BFLS

3.1 Preface

The plant homeodomain zinc-finger protein, PHF6, is a transcriptional regulator, with its germline mutations causing the X-linked intellectual disability (XLID), Börjeson-Forssman-Lehmann syndrome (BFLS). The precise mechanisms by which PHF6 regulates transcription and how its mutations cause BFLS remain poorly characterized. Here, we show genome-wide binding of PHF6 in the developing cortex, in the vicinity of genes involved in central nervous system development and neurogenesis. Characterization of BFLS mice reveals an increase in embryonic neural stem cells (eNSC) self-renewal and a significant attenuation of neural progenitors in mice harbouring *Phf6* patient mutations. We report a panel of Ephrin receptors (*EphRs*) as direct transcriptional targets of PHF6. Mechanistically, we show that PHF6 regulation of *EphR* is impaired in BFLS mice and in conditional *Phf6* knock-out mice. Importantly, knockdown of *EphR*-A phenocopies the PHF6 loss-of-function defects in altering eNSCs, and their forced expression rescues the eNSC defects in BFLS mice. Our results suggest that PHF6 regulates Ephrin receptors to alter NSCs in the developing brain, and this pathway is impaired in BFLS.

3.2 Introduction

The plant homeodomain zinc finger protein, PHF6, is a transcriptional regulator (Liu et al., 2014) that is highly conserved in vertebrates with high expression during the early stages of corticogenesis (Cheng et al., 2018; Voss et al., 2007). PHF6 is found in a complex with different components of the Polymerase associated factor 1 (PAF1) complex to promote neuronal migration in the developing cerebral cortex (Jahani-Asl et al., 2016; Zhang et al., 2013) suggesting a role for PHF6 in transcriptional elongation. The PAF1 complex, has also been shown to regulate promotor proximal pausing of RNA polymerase II (Chen et al., 2015). Whether and how PHF6 may be involved in transcriptional elongation and polymerase pausing has remained to be investigated.

Germline mutations in *Phf6* causes the X-linked intellectual disability (XLID), Börjeson-Forssman-Lehmann syndrome (BFLS), characterized by impairments in cognitive function, epileptic-like seizures, and behavioural disturbances (Lower et al., 2002), in addition to endocrine defects (McRae et al., 2020). Multiple mutations on the *Phf6* gene within the X chromosome have been identified in BFLS patients (Berland et al., 2010; Carter et al., 2009; Lower et al., 2002; Turner et al., 2004). Although prior research has established that loss of PHF6 function impairs the migration of newly born neurons, the involvement of PHF6 in the regulation of different aspects of neural development remains unexplored.

Neurogenesis is outlined as a process in which new neurons are generated from neural stem cells (NSCs). This process is comprised of proliferation and fate specification of NSCs, migration of newborn neurons, and maturation of these neurons (Ming & Song, 2005). A number of XLID genes appear to impair neurogenesis via altering NSC fate (Bustos, Segarra-Fas, Chaugule, Brandenburg, Branigan, Toth, Macartney, Knebel, Hay, Walden, et al., 2018; Kim et

al., 2016; Luo et al., 2010; May et al., 2015; Selvan et al., 2018; Telias et al., 2015), raising the question of whether *Phf6* mutations impact the NSC pool in the developing brain.

Ephrin receptors (EphR), the largest family of receptor tyrosine kinases (RTK) (Becker et al., 2000), are highly expressed in the developing brain and play crucial roles in the regulation of proliferation, apoptosis, cell adhesion, cell fate specification, and neurogenesis (Gerstmann & Zimmer, 2018; Kullander & Klein, 2002; Park, 2013). EphRs are classified as either A- or B-type of receptors according to sequence homology, and require binding to membrane-bound ephrin ligands for signal transduction (Committee, 1997). EphA members have been studied in the contexts of axon guidance, neural stem cell proliferation during development, embryogenesis, and neuroblast migration to the olfactory bulbs via forward signalling mechanisms (North et al., 2009; Park, 2013; Todd et al., 2017). EphB members have also been reported to alter hippocampal progenitor cells and cell proliferation (Calò et al., 2005; Genander & Frisén, 2010; He et al., 2005).

In the present study, we characterize global PHF6 regulation of the genome in the developing cortex and show a position-dependent role for PHF6 in the regulation of transcription as an activator or repressor. We employ several genetic mouse models including BFLS patient mouse models and *Phf6* knock-out models to establish a role for PHF6 in altering eNSCs. Importantly, we report several members of EphRs as direct transcriptional targets of PHF6, with the EphA family members involved in the regulation of neurogenic processes. Our data suggests that these receptors could represent a therapeutically exploitable target for BFLS and other XLID disorders with impaired neurogenesis.

3.3 Results

3.3.1 Genome-wide analysis of PHF6 targets in the developing brain

To begin to examine the function of PHF6 as a transcriptional regulator in the embryonic brain, we performed ChIP-seq analysis of PHF6 in the developing cortex of mouse embryos at embryonic day 17 to 18 (E17-18). We identified 2467 PHF6 binding sites at P-value < 10^{-5} (**Dataset 1, Fig 7-8**). These binding sites occurred in various genomic regions, including the proximal region of TSS', gene bodies, and intergenic regions (**Fig 7A**). Compared to what would be expected from the random distribution of binding sites across the genome, we observed significant enrichment upstream of the TSS as well as in the 5' untranslated region (UTR) of protein-coding genes (**Fig 7A**). Particularly, PHF6 sites are strongly enriched in the 1kb region around the TSS, with the highest density immediately downstream of the TSS (**Fig 7B**). This pattern suggests a role of PHF6 in regulating gene expression.

Follow up analysis revealed that PHF6-bound regions significantly overlap $(CA)_n$-microsatellite repeats, as revealed by motif analysis of the top 1000 PHF6 sites (**Fig 7C, 8B**). These microsatellites are specifically located at the center of PHF6 sites (**Fig 7D, 8B**), suggesting that they are associated with PHF6 binding. Among the top 1000 PHF6 peaks, 609 overlap a $(CA)_n$ repeat on either DNA strand. In comparison, we observed an overlap of only 67 between $(CA)_n$ repeats and shuffled peak coordinates (Fisher's exact test P < 2.2e–16) (**Dataset 2**). An unbiased analysis of the distribution of all genomic $(CA)_n$ repeats revealed that they are largely enriched near genes involved in developmental processes, including central nervous system development, neurogenesis, and neuron differentiation (**Fig 7E**, **Dataset 3**). Function enrichment analysis of PHF6 sites also revealed the same trend (**Dataset 4**), with many Gene Ontology (GO) terms such as forebrain development and regulation of neurogenesis commonly found among the

most enriched terms for both PHF6 sites and $(CA)_n$ microsatellite repeats (**Fig 7F**, **Dataset 5**). These results suggest that $(CA)_n$ repeats are specifically enriched near neural development genes and are bound by PHF6.

Next, we profiled the genome-wide pattern of gene deregulation by analysis of *Phf6* knockdown (KD) and control cortical progenitors following their isolation at embryonic day 14 (E14) and expansion for 5 days in culture. RNA-seq analysis (**Dataset 6**) revealed that PHF6 functions as a transcriptional activator or repressor (**Fig 9A**). In addition, enrichment analysis, performed separately on upregulated and downregulated genes, revealed that a large panel of genes involved in nervous system development are downregulated in the *Phf6* KD group (FDR < 0.02) (**Fig 9B**). A number of significant PHF6-differentially expressed genes were found to have peaks within the +/- 2kb vicinity of the TSS (**Fig 9C-D**).

To further understand the role of PHF6 in the regulation of transcription, we employed Pol II occupancy data (Liu et al., 2017), and examined the association between PHF6 binding and Pol II occupancy in neural progenitor cells. Interestingly, we observed that TSS' with a PHF6 site within 300 bp tend to be depleted of Pol II, compared to genes with a PHF6 site between 300–1000 bp of the TSS (**Fig 9E**). This pattern suggests that the binding of PHF6 within the immediate vicinity of TSS might have a negative effect on the recruitment of Pol II to the TSS. To examine this prediction, we analyzed the association of PHF6 peaks and PHF6 differentially regulated genes. We found that PHF6 inhibition led to an overall increase in the expression of 65% of genes with a PHF6 site at or immediately downstream of the TSS (**Fig 9D, F**). These observations suggest a position-dependent role for PHF6 in regulating transcription which may provide mechanistic insight into the dual role of PHF6 as a transcriptional activator and repressor.

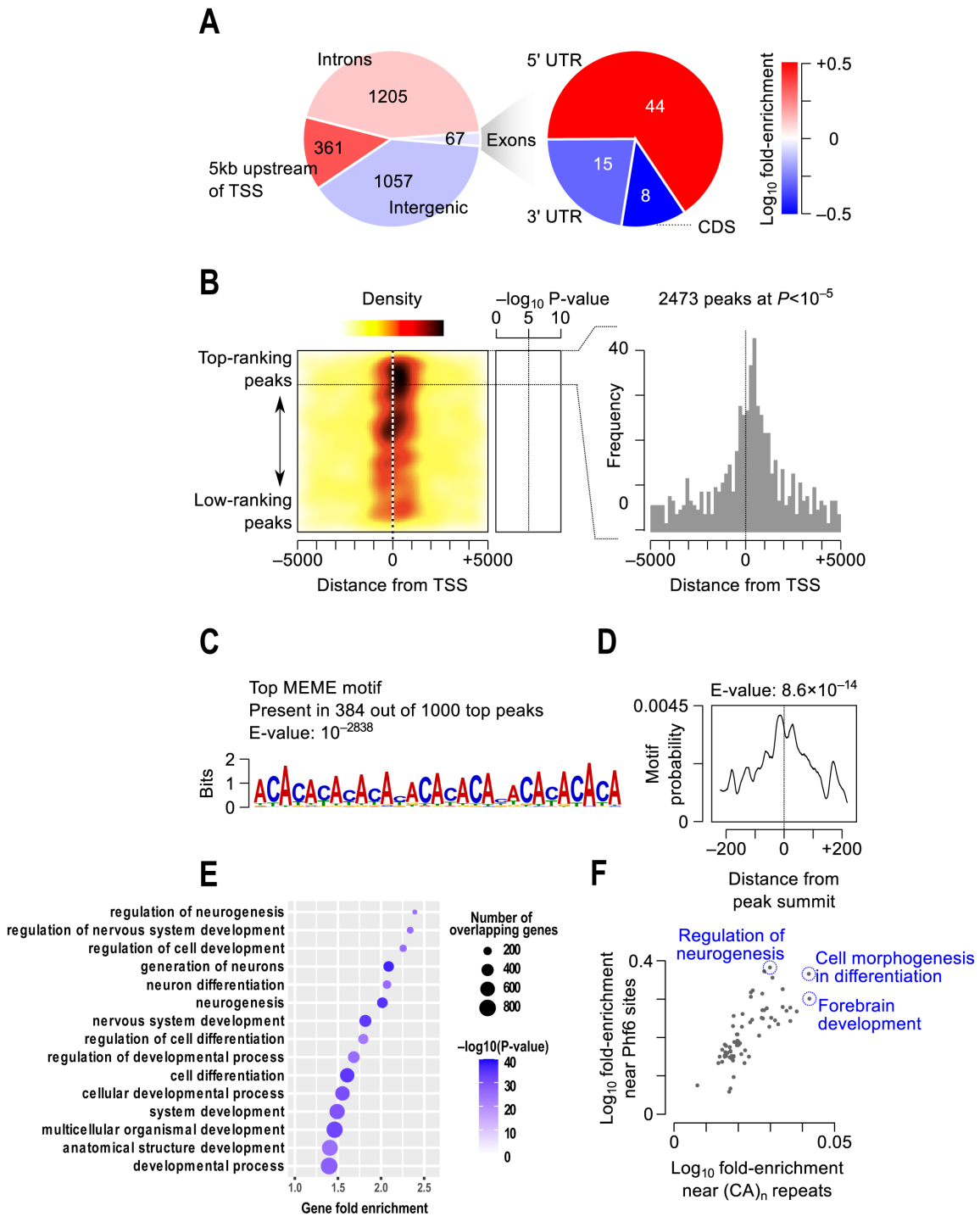

Figure 7: Genomic distribution of PHF6 binding sites in the developing cortex. (A) The numbers of PHF6 sites that overlap different genomic regions are shown in the pie chart. The right pie chart shows a breakdown of sites that overlap exonic regions. The colour gradient, shown on

the right, represents the logarithm of enrichment of PHF6 sites in each region relative to random expectation. Only PHF6 sites with $P < 10^{-5}$ are included in the charts. (B) The heatmap on the left shows the distribution of PHF6 sites relative to TSS'. The peaks are sorted by ascending order of their *P*-values (shown in the middle) from the top to the bottom. The colour gradient depicts the frequency of PHF6 sites relative to the position of the nearest TSS, also shown for top-ranking PHF6 sites using the histogram on the right. (C–F) PHF6 binds to $(CA)_n$-microsatellite repeats. (C) The sequence logo depicts the top motif identified by MEME-ChIP [PMID: 21486936]. (D) The distribution of the $(CA)_n$ motif relative to the peak summits is shown, as revealed by CentriMo [PMID: 22610855]. (E) Dot plot representation of the GO terms that are enriched near PHF6 sites. Only the top 15 terms with the most significant *p*-values are shown. The *x*-axis shows the fold-enrichment of the term, while the dot size and colour represent the number of PHF6 targets that overlap the GO term and the hypergeometric p-value, respectively. (F) Each dot in the scatterplot represents a GO term that is significantly enriched in both the GREAT analysis of $(CA)_n$ simple repeats and the GREAT analysis of PHF6 sites. The *x*- and *y*-axes reflect the logarithm of the hypergeometric fold-enrichment of the terms. The GO terms with the largest enrichment are highlighted. $n = 6$ mouse cortices were pooled for each PHF6 ChIP and IgG control ChIP, where *n* represents an independent biological sample.

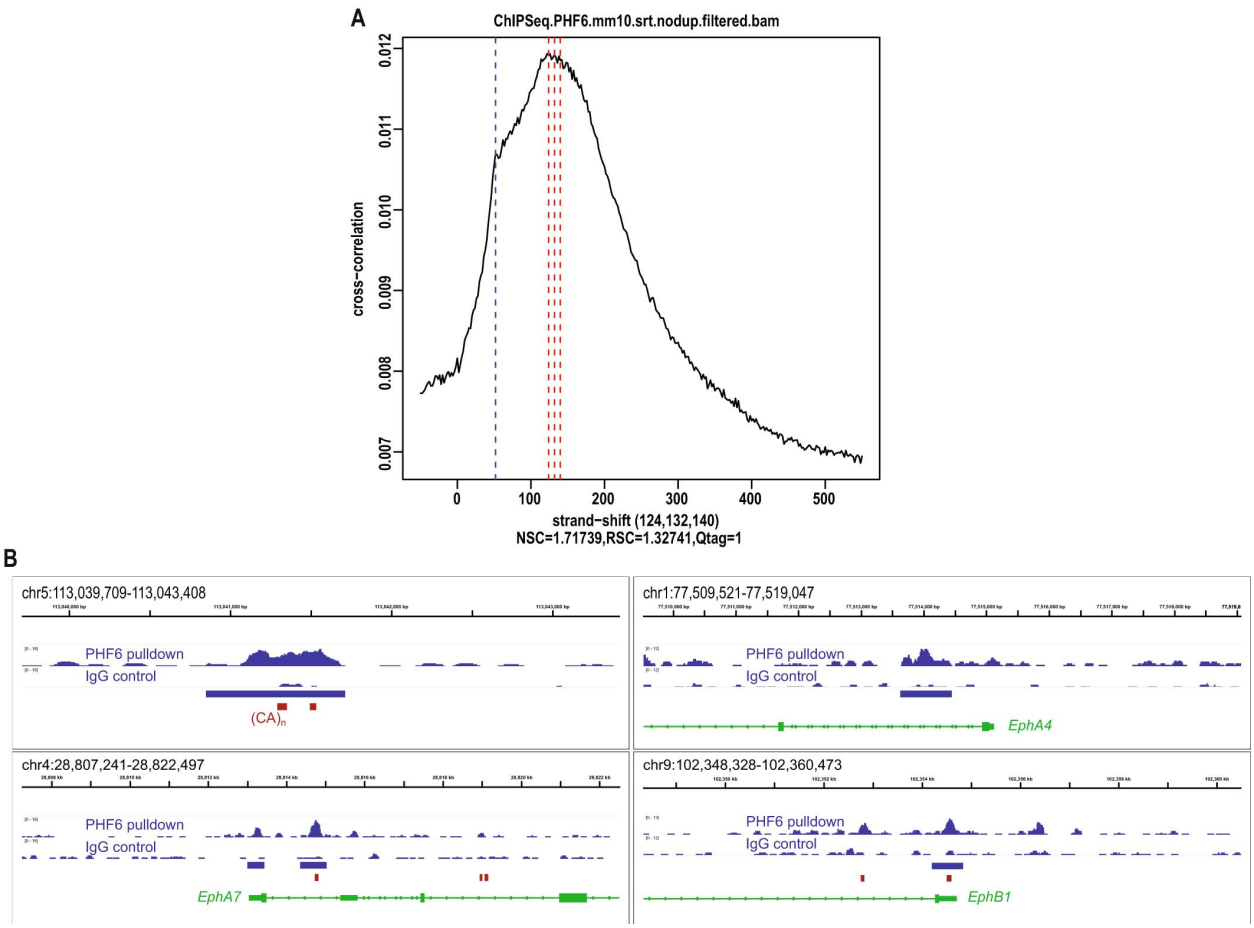

Figure 8: PHF6 ChIP-Seq analysis. (A) PHF6 ChIP-seq cross-correlation analysis was conducted using cross-correlation metrics as described in Landt et al, (Landt et al., 2012). (B) Example ChIP-seq tracks for PHF6 pull-down and IgG control. (CA)n repeats are demarcated with red boxes, while the blue boxes represent the identified PHF6 peak.

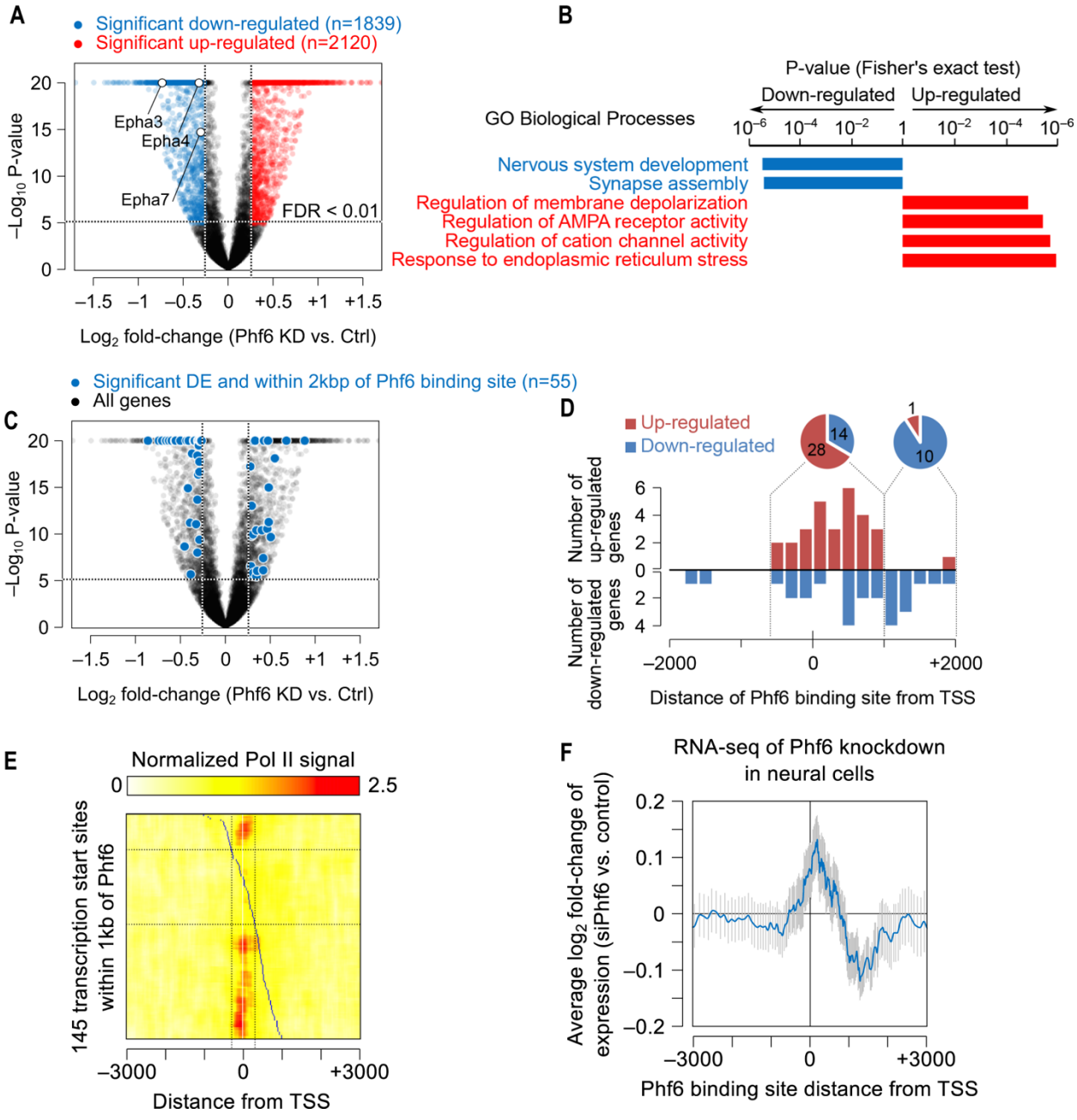

Figure 9: Position-dependent effect of PHF6 on transcription. (A,B) *Phf6* KD and control cortical progenitors were subjected to mRNA-seq analysis ($n = 3$). Plots represent differentially regulated candidate target genes (A), and functional annotation of downregulated versus upregulated genes (B). GO term enrichment analysis was performed using CPDB (Kamburov et al., 2011). (C,D) PHF6 peak-gene associations within +/− 2Kb of TSS and the effect of *Phf6* KD

($n = 3$) on expression is presented. (E) PolII signal near the TSS of the PHF6-bound genes is shown using the colour gradient in the heatmap. The rows represent the genes, sorted based on the position of the PHF6 site. The PHF6 binding sites are depicted in blue. The vertical dotted lines delineate the +/−300 bp region around the TSS'. The horizontal dotted lines delineate the genes with a PHF6 site within this +/−300 bp region. (F) The expression changes in *Phf6* KD cells as a function of the binding position of PHF6. Each data point shows the average for 50 genes that have PHF6 binding, with the binding site location relative to the TSS shown on the *x*-axis. Data information: Error bars represent ± SEM. mRNA-seq raw reads were mapped to mm10 genome using HISAT2 (Kim et al., 2015), followed by duplicate read removal using samtools. Gene-level read counts were obtained by HTSeq (Anders et al., 2015), using gene annotations from GENCODE (release M9). Genes with a minimum of 150 reads in at least one sample were retained. Gene set analysis was performed using ConsensusPathDB (Kamburov et al., 2011). *n* represents an independent biological sample.

3.3.2 *Phf6* knockdown in primary eNSC cultures alters eNSC expansion

Our data on functional annotation of PHF6 binding sites suggest that PHF6 regulates neurogenesis (**Fig 7F**). Interestingly, previous studies in hematopoietic stem cells (HSCs) showed that PHF6 can restrict the self-renewal capacity of HSCs (McRae et al., 2019; Miyagi et al., 2019). These findings led us to investigate whether PHF6 regulates cell proliferation or self-renewal. To begin with, we subjected PHF6-GFP or control GFP-expressing neuroblastoma (N2A) cell lines to KI67 staining and found that PHF6 significantly suppressed the proliferation of these cells (**Fig 10A-C**). Next, we induced the KD of *Phf6* via a pool of siRNA in primary E14 eNSC cultures followed by limiting dilution assay (LDA). Compared with eNSCs transfected with non-targeting siRNA control, we found a significant increase in eNSC neurosphere numbers upon KD of *Phf6* (**Fig 11A-B**). Consistent with this observation, immunoblotting analyses of neurospheres following 7 days in culture, revealed upregulation of stem cell markers, SOX2 and NESTIN in *Phf6* KD relative to the control cells (**Fig 11C**). Our data suggest that PHF6 restricts stem cell self-renewal in primary neurosphere cultures.

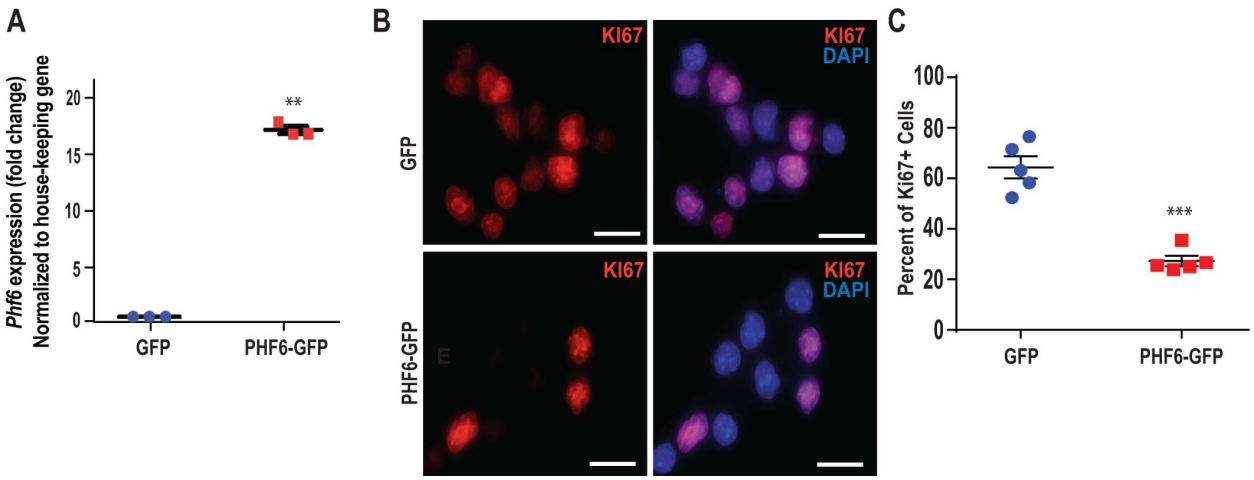

Figure 10: PHF6 regulation of proliferation in neuroblastoma (N2A) cells. (A–C) N2A cells were transfected with *Phf6 (*PHF6-GFP) or GFP-expressing control (GFP) constructs. (A) Gene expression was assessed by RT-qPCR ($n = 3$). (B) Samples were subjected to KI67 staining for assessment of proliferation ($n > 3$, representative image shown). Scale bar represents 20 µm. (C) Quantification of percent KI67 positive cells are shown ($n > 3$). Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (two-tailed unpaired student *t*-test). *n* represents an independent biological sample.

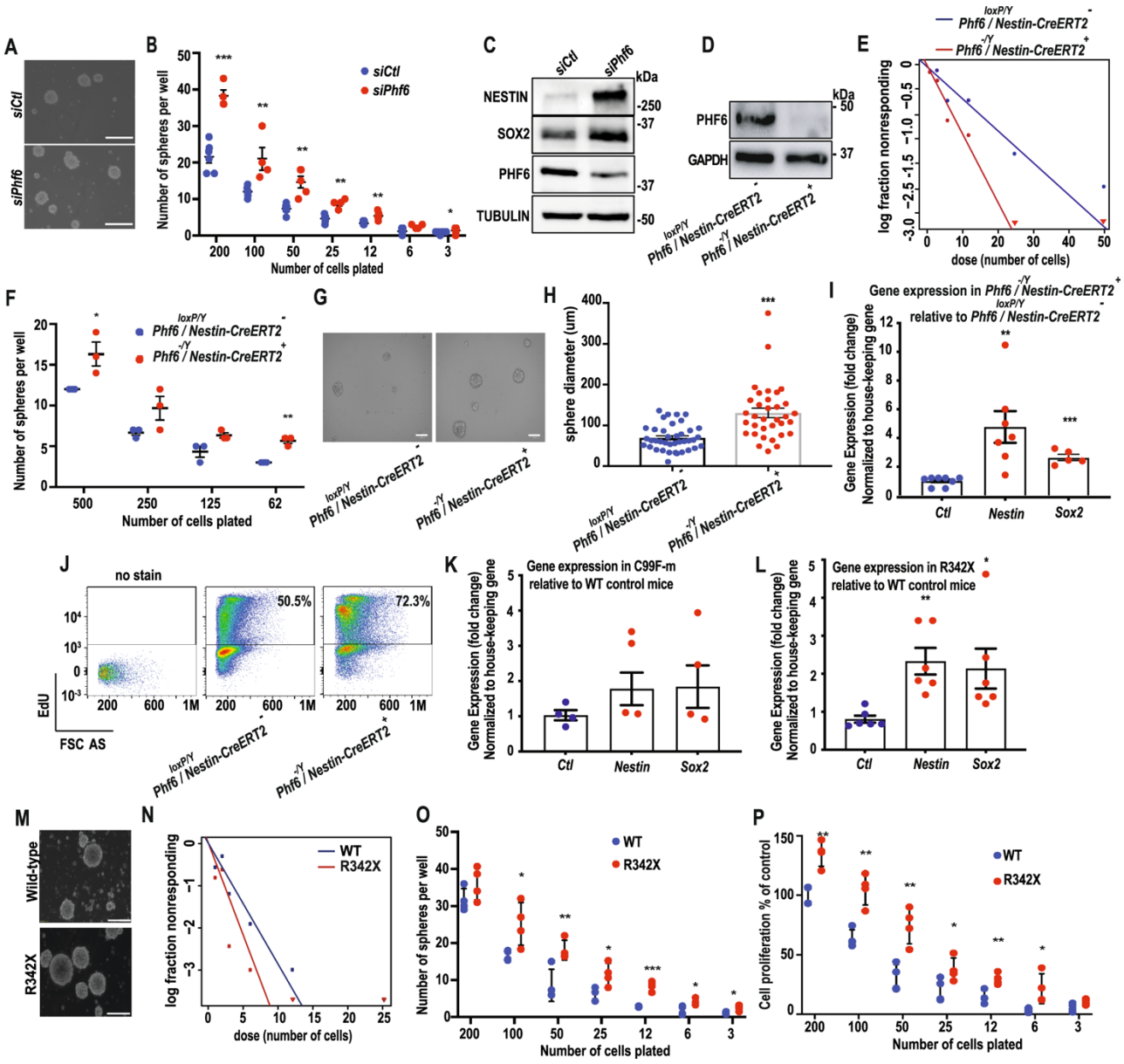

Figure 11: PHF6 suppresses self-renewal of eNSCs. (A–C) eNSC were isolated and cultured from WT mice at E14 and *Phf6* KD was induced using an siRNA approach. Samples were analyzed using a limiting dilution assay (LDA) (A,B) and immunoblotting (C) using antibodies indicated on the blot. (D–J) eNSCs were cultured from *Phf6*$^{-/Y}$ / *Nestin-CreERT2*$^{+}$ and control *Phf6*$^{loxP/Y}$ / *Nestin-CreERT2*$^{-}$ mouse brains at ~E15 and were subjected to immunoblotting

analysis (D), ELDA (E) ($p = 0.00686$), LDA (F), sphere diameter (G,H) ($p < 0.0001$), RT-qPCR analysis using *Nestin* and *Sox2* primers (I), and 5-ethynyl-2'-deoxyuridine (EdU) analysis (J). (K,L) eNSCs were cultured from C99F (K), R342X (L) and corresponding wild-type control mice. mRNA expression of *Nestin* and *Sox2* were analyzed by RT-qPCR. (M–P) eNSC were cultured from R342X mice and wild-type control mice and were subjected to ELDA (M,N) ($p = 0.0211$), LDA (O), and alamarBlue analysis (P) 7 days post-plating. Scale bar represents 100 μm. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (two-tailed unpaired student *t*-test). Representative plots of $n > 3$ independent replicates are shown in (A,C–E,G,J,M,N), data in panels (B,F,H,I,K,L,O,P) are plotted with $n > 3$ mean +/− SEM. *n* represents an independent biological sample.

3.3.3 *Phf6* conditional knock-out mice exhibit alterations in eNSC processes

We next set out to characterize the role of PHF6 in stemness using a genetic mouse model in which we induced genetic deletion of *Phf6* via breeding $Phf6^{loxP/loxP}$ with *Nestin-CreERT2*$^{+}$ mice followed by tamoxifen administration at E14 for 24-48 hours to delete *Phf6* exons 4 and 5 in the Nestin expressing cells (**Fig 11D**). Extreme limiting dilution assay (ELDA) (Hu & Smyth, 2009; Rasool et al., 2022) and LDA analyses of eNSCs obtained from $Phf6^{-/Y}$ / *Nestin-CreERT2*$^{+}$ *(Phf6* KO*)* and $Phf6^{loxP/Y}$ / *Nestin-CreERT2*$^{-}$ (control) mice revealed a significant increase in self-renewal (**Fig 11E**), sphere number (**Fig 11F**), and sphere diameter (**Fig 11G-H**) in *Phf6* KO eNSCs. Importantly, a significant increase in the expression of the stemness markers, *Nestin* and *Sox2,* (**Fig 11I**) and an increase in EdU incorporation (**Fig 11J**) was observed in the eNSCs of $Phf6^{-/Y}$ / *Nestin-CreERT2*$^{+}$ mice. Our data shows that the genetic deletion of *Phf6* promotes the self-renewal of eNSCs, suggesting that PHF6 loss of function may restrict eNSC commitment to differentiated progenies in the developing brain.

In parallel, we employed a second *Phf6* KO mouse model wherein $Phf6^{loxP/loxP}$ were bred with *Nestin-Cre*$^{+}$ mice to induce deletion of *Phf6* from the mouse central and peripheral nervous system at E11.5, the onset of *Nestin* gene expression (Tronche et al., 1999), thus producing a highly efficient KO model (**Fig 12A-B**). We subjected the brain sections from $Phf6^{-/Y}$ / *Nestin-Cre*$^{+}$ (KO) and $Phf6^{loxP/Y}$ / *Nestin-Cre*$^{-}$ (Ctl) mice at post-natal day 0 (P0) to Nissl staining and found a notable decline in neuron density within the forebrain and midbrain sections of $Phf6^{-/Y}$ / *Nestin-Cre*$^{+}$ brains compared to $Phf6^{loxP/Y}$ / *Nestin-Cre*$^{-}$ controls (**Fig 12C**). Our data suggest that the deletion of *Phf6* induces a decline in neuron density. Taken together, these results support a model whereby PHF6 may restrict eNSC self-renewal and promotes eNSC commitment to newly born neurons.

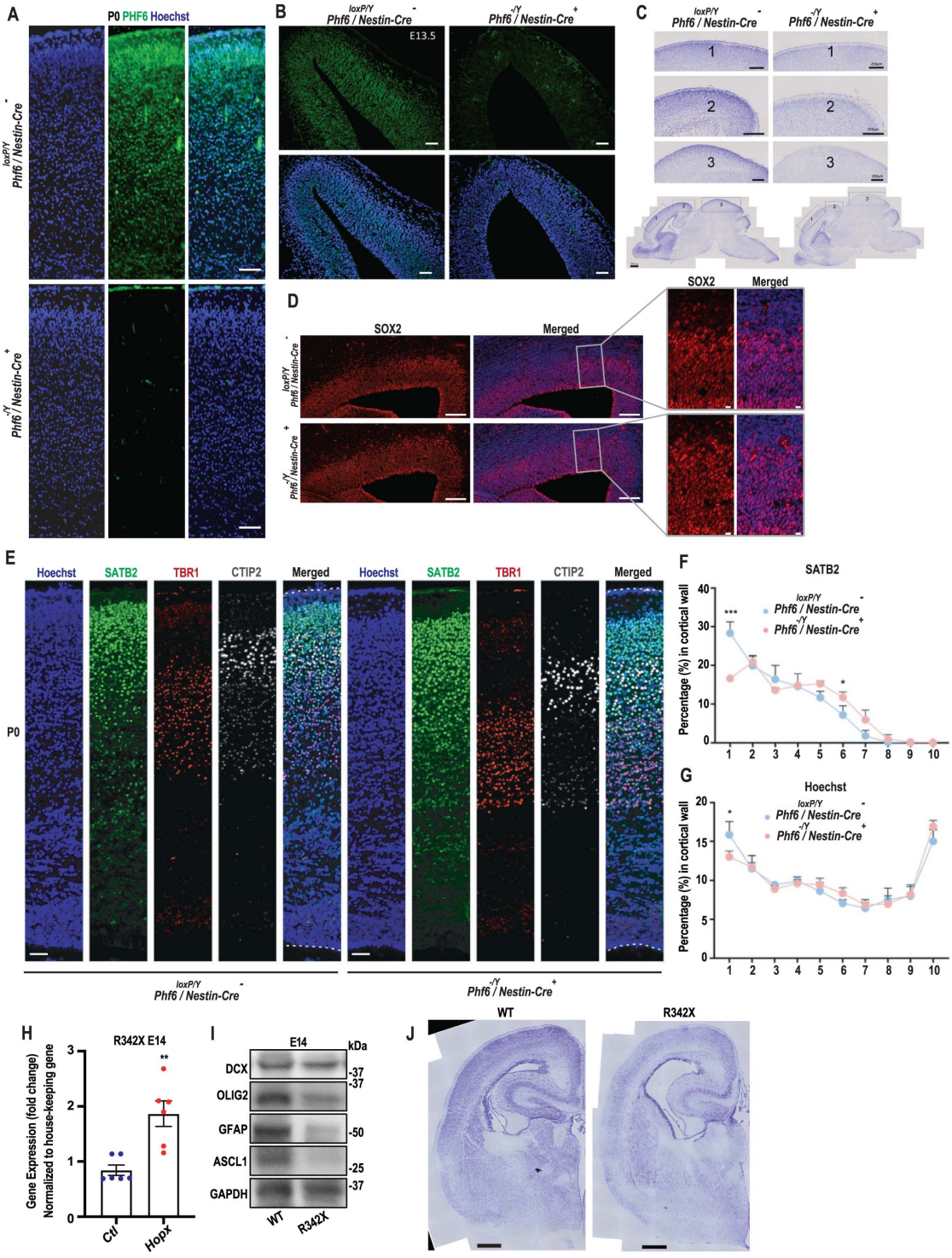

Figure 12: Characterization of *Phf6/Nestin-Cre* and BFLS mouse brain development. (A,B) Immunofluorescence (IF) staining of coronal sections from P0 (A) and E13.5 (B) for *Phf6*$^{-/Y}$ /

Nestin-Cre^{+} and *Phf6*$^{loxp/Y}$ */ Nestin-Cre*$^{-}$ male mice using a PHF6 antibody (green) in the cerebral cortex. Nuclei were counterstained by Hoechst. Scale bars represent 50 μm. (C) *Phf6*$^{-/Y}$ */ Nestin-Cre*$^{+}$ and *Phf6*$^{loxp/Y}$ */ Nestin-Cre*$^{-}$ male mice were collected at P0 and subjected to Nissl staining with sagittal sections shown. Scale bars represent 500 μm in lower magnification and 250 μm in higher magnification photomicrographs. (D) IF staining of coronal sections from ~E15 male mice using a SOX2 antibody is shown. Scale bar represents 100 μm at lower magnification and 10 μm at higher magnification. (E) IF staining of coronal sections from P0 using cortical layer markers: SATB2 (green, layer II-V), TBR1 (red, layer VI), and CTIP2 (grey, layer V). Nuclei were counterstained by Hoechst. The cortical wall spanning from the basal of ventricle zone to the pial surface was equally divided into ten bins, the bin 1 covers the most superficial layer and bin 10 covers the deepest layer. (F) Comparative analysis of SATB2+ neurons in each segment of P0 male mice ($n = 3$). (G) Comparative analysis of Hoechst+ nuclei in each segment of P0 male mice (n=3). Scale bars represent 50 μm. (H,I) mRNA and protein of E14 R342X and wild-type control mice were subjected to RT-qPCR for *Hopx* expression ($n > 3$) (H) ($p = 0.0021$), and immunoblotting analysis of cell type-specific markers (I) ($n = 3$, representative blots shown). (J) R342X and WT mice were collected at P0 and subjected to Nissl staining ($n = 2$, representative image shown). Coronal sections are shown. Scale bars represent 500 μm. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. two-tailed unpaired student *t*-test (H). two-way ANOVA with multiple comparisons (F,G). *n* represents an independent biological sample.

3.3.4 BFLS patient mouse models exhibit alterations in stemness markers and eNSC self-renewal

The R324X mutation is the most recurrent BFLS patient-mutation occurring at exon 10 (C.1024C>T). This mutation impairs the ePHD2 domain, whereby PHF6 is proposed to function as a truncated protein (Ahmed, Sarwar, Hu, Cardin, Qiu, Zapata, Vandeleur, Yan, Lerch, Corbett, et al., 2021; Chao et al., 2010; Crawford et al., 2006; Gecz et al., 2006; Jahani-Asl et al., 2016; Lower et al., 2004; Lower et al., 2002; Todd et al., 2015). Another BFLS patient point mutation (m) in *Phf6* is wherein cysteine-99 is replaced with phenylalanine (C99F) at nt.296G>T impairing the function of the PHD1 domain. To investigate whether impairment in eNSC fate specification may underlie BFLS pathogenesis, we employed both BFLS mouse models, R342X and C99F-m. Analysis of mRNA expression in E14 cerebral cortices revealed a consistent increase in the expression of both *Nestin* and *Sox2* in BFLS relative to wild-type control mice (**Fig 11K-L**). We thus conducted additional analysis in eNSCs of R342X mice and found a significant increase in their self-renewal, neurosphere number, and proliferation relative to eNSCs of the wild-type control mice (**Fig 11M-P**). Taken together, our findings demonstrate that similar to *Phf6*$^{-/Y}$ / *Nestin-CreERT2*$^{+}$ mice, BFLS patient mouse models exhibit alterations in eNSC expansion.

3.3.5 PHF6 target analysis: Identification of Ephrin Receptors (EphRs)

To identify downstream effectors of PHF6 function in the regulation of neurogenesis, we first analyzed the candidate target genes with their expression significantly deregulated based on the RNA-seq analysis with particular focus on druggable targets (e.g., Receptors, Kinases).

These analyses revealed a host of candidate genes that could serve as PHF6 targets to regulate neurogenesis (**Dataset 6**). We focused on members of the ephrin receptors (EphRs) family (*EphA4/7*, and *EphB1/2)* given that EphRs are the largest family of RTKs highly expressed in the developing brain (Barquilla & Pasquale, 2015; Darling & Lamb, 2019; Lisabeth et al., 2013). Importantly, EphRs have been shown to play different roles in regulating neuronal development (Aoki et al., 2004; del Valle et al., 2011; Stuckmann et al., 2001; Wilkinson, 2014). Prior to validation of EphRs as viable targets of PHF6 in the context of BFLS, we conducted additional gene expression analysis using public databases. First, via querying single-cell RNA-seq data [Data ref: (Di Bella et al., 2021)] of the developing mouse brain, we found that *Phf6*, *EphA4/7*, and *EphB1/2* are expressed in the developing brain of mice ranging from embryonic day 10 (E10) to postnatal mice at day 4 (P4) (**Fig 13A-F, 14A-E**). Furthermore, Pearson correlation analysis between *Phf6*, *EphA4/7*, and *EphB1/2* revealed a positive correlation between *Phf6* and *EphR* expression in different cell types, in particular progenitors and migrating neurons (**Fig 13G-H, 14A-E**). Second, we analyzed the RNA-seq data of the human ventral frontal cortex (VFC) [Data ref:(BrainSpan, 2013)] and found a similar trend in the expression of *EPHR* genes across development, and their correlation with *PHF6* expression (**Fig 14F-J**). We, thus, asked if EphR expression levels are altered in *Phf6* KO and BFLS mice. Via subjecting eNSCs from *Phf6*$^{loxP/Y}$ */ Nestin-CreERT2*$^{-}$ and *Phf6*$^{-/Y}$ */ Nestin-CreERT2*$^{+}$ mice to RT-qPCR and immunoblotting analyses, we observed a significant decrease in both mRNA and protein expression of each of the identified EphR upon genetic deletion of *Phf6* (**Fig 15A-B**). Independently, we also subjected E14 brain tissue from R342X and C99F-m mice to RT-qPCR and immunoblotting analyses. The results revealed downregulation of *EphR* mRNA and protein expression in both C99F and R342X mice with a more profound impact in R342X mice (**Fig**

15C-D, Fig 16A-B), confirming that the expression of *EphRs* is altered in BFLS mice harbouring PHF6 patient mutations.

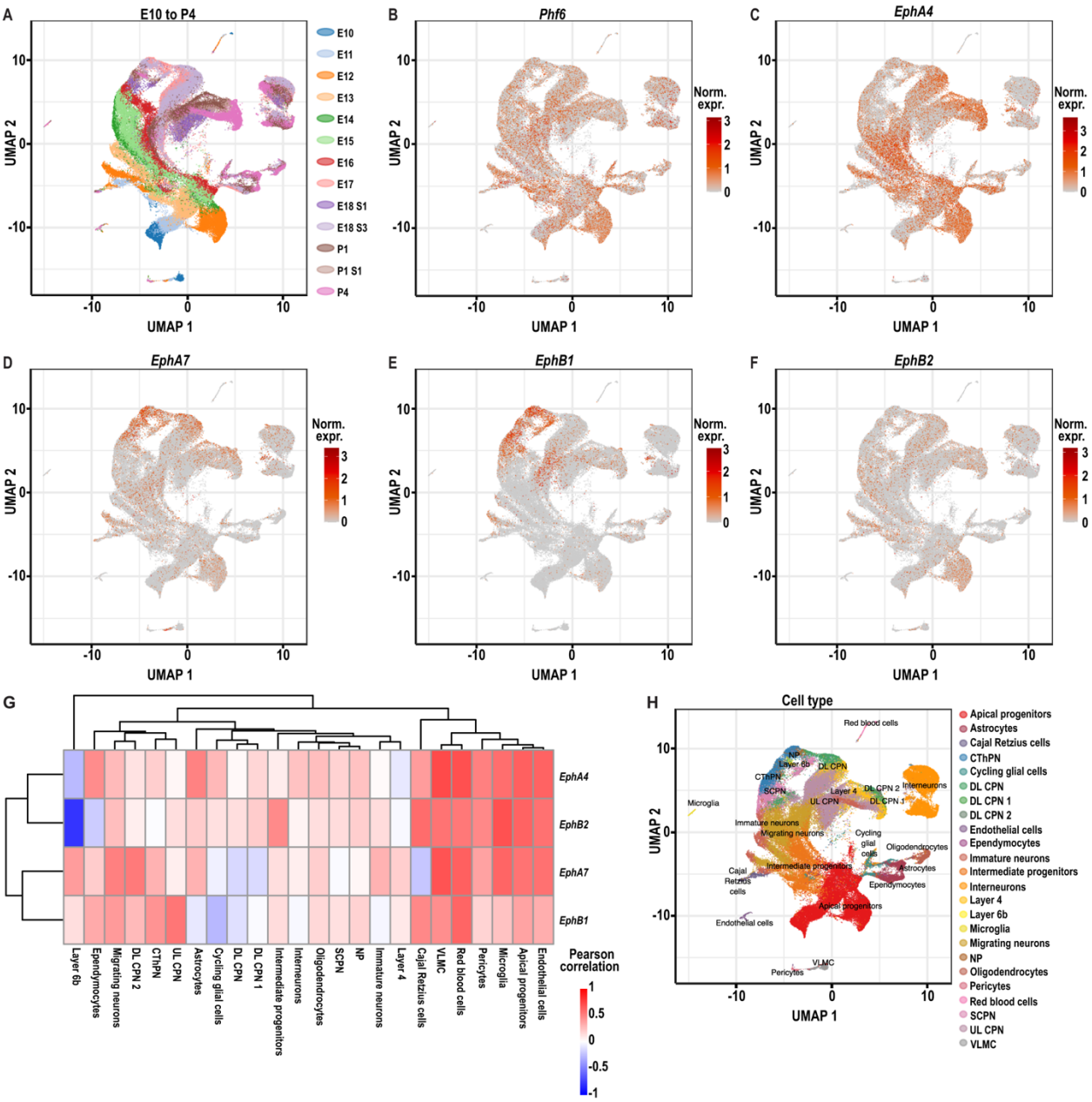

Figure 13: Cell type specific co-expression analysis of *EphR* and *Phf6* in mouse cerebral cortex. (A–F) Low-dimensional representation of single cells from mouse cerebral cortex, based on UMAP embedding of single-cell RNA-seq data [Data ref: (Di Bella et al., 2021)] are shown. Cells are coloured based on animal age (A), or the expression of *Phf6* (B), *EphA4* (C),

EphA7 (D), *EphB1* (E), or *EphB2* (F). (G) Heatmap representation of the Pearson correlation coefficients between *Phf6* and *EphR* across various cell types are shown. Correlation values were calculated using imputed gene expression profiles after applying MAGIC (Van Dijk et al., 2018). (H) UMAP embedding of cells are coloured by cell type. UMAP coordinates and cell type annotations are from [Data ref: (Di Bella et al., 2021) (GEO GSE153164)].

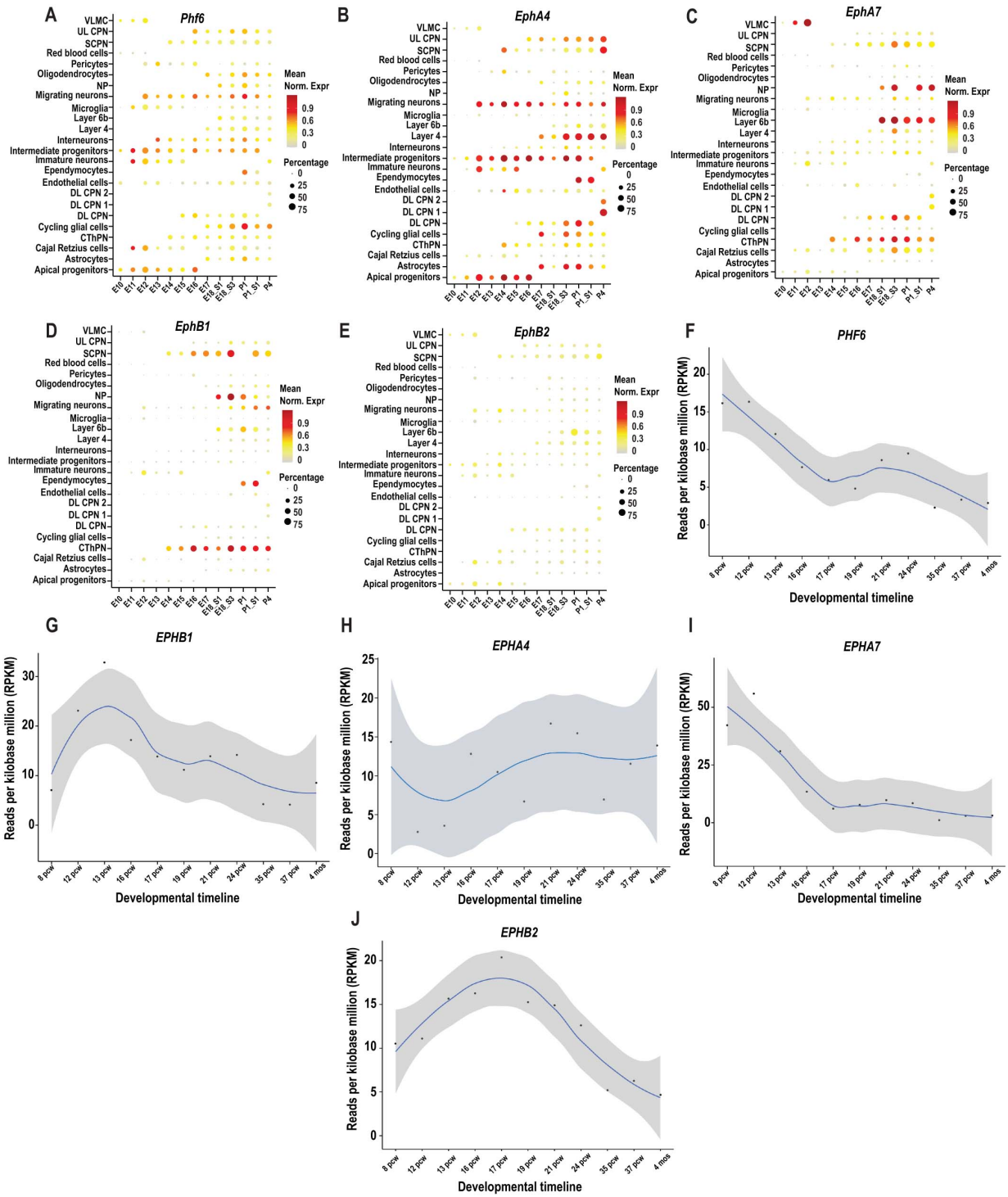

Figure 14: Analysis of *Phf6* and *EphR* mRNA expression across development. (A–E) Dot plots showing expression of *Phf6* (A), *EphA4* (B), *EphA7* (C), *EphB1* (D), and *EphB2* (E) in the

mouse cerebral cortex during development where the colour of each dot represents the mean normalized expression values per cell type for a given timepoint. The size of the circle represents the percentage of cells expressing each gene. Single cell mouse RNA-seq data was obtained from GEO GSE153164 [Data ref: (Di Bella et al., 2021)]. (F–J) Analysis of *PHF6* and *EPHR* expression in the human cortex. Average reads per kilobase million (RPKM) values over human developmental time (post-conceptual weeks; pcw) for gene analysis of *PHF6* (F), *EPHB1* (G), *EPHA4* (H), *EPHA7* (I), and *EPHB2* (J) are shown. Gene analysis was taken from publicly available RNA-seq data taken from the human ventral frontal cortex (VFC) of the Allen Brain Atlas BrainSpan dataset [Data ref: (BrainSpan, 2013)]

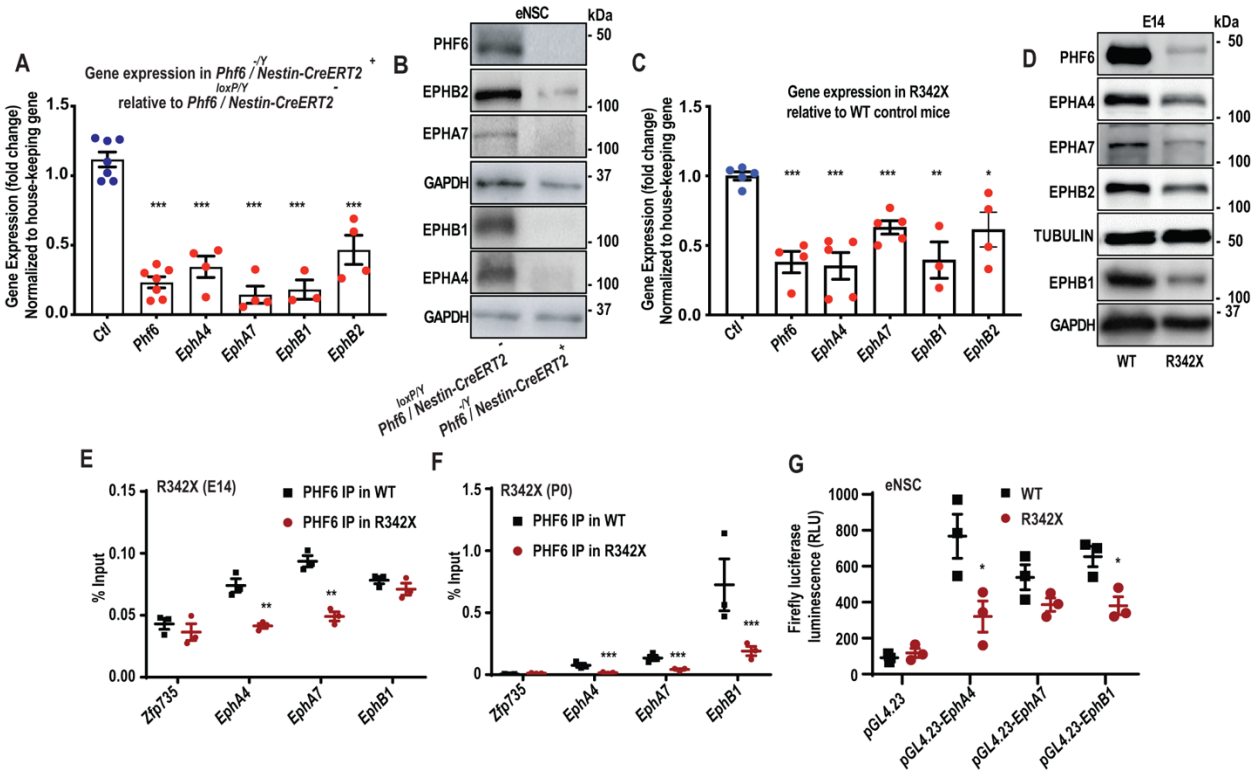

Figure 15: EphR are direct PHF6 targets. (A,B) eNSCs were cultured from *Phf6*$^{-/Y}$ / *Nestin-CreERT2*$^{+}$ and control *Phf6*$^{loxP/Y}$ / *Nestin-CreERT2*$^{-}$ at ~E15 and mRNA and protein expression of EphR were analyzed by RT-qPCR (A) and immunoblotting (B). (C,D) mRNA and protein of brain tissue obtained from E14 R342X and wild-type control mice were analyzed as described in (A,B). (E,F) Cerebral cortical tissues were isolated from WT and R342X mice at E14 (E) or at P0 (F). Samples were subjected to ChIP-qPCR using a PHF6 antibody. *Zfp735* loci was used as negative control for the PCR. (G) Dual luciferase reporter assay was performed in WT or R342X eNSC cultures 48 h following electroporation with pGL4.23-*EphA4*, pGL4.23-*EphA7*, pGL4.23-*EphB1* or pGL4.23-basic reporter plasmids. RLU Relative luminescence units. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Two-tailed unpaired student *t*-test (A,C,G), one-way ANOVA (E,F). Representative data of $n > 3$

independent replicates are shown in panels (B,D). Data in panels (A,C,E–G) are plotted with $n > 3$ mean ± SEM. *n* represents an independent biological sample.

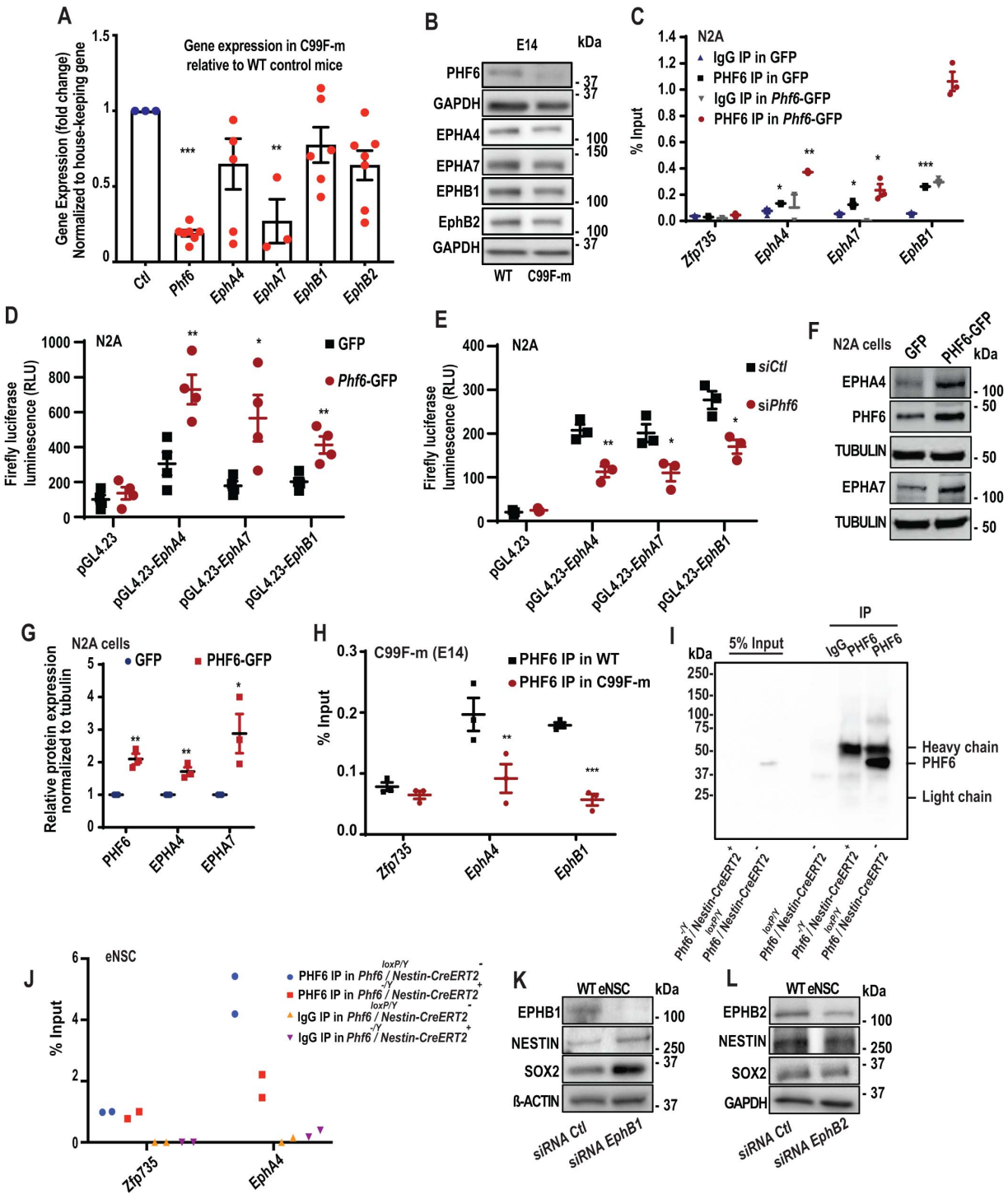

Figure 16: Analysis of PHF6 and EphR expression in BFLS mice. (A,B) mRNA and protein of E14 C99F-m and wild-type control mice were subjected to RT-qPCR and immunoblotting

analysis ($n \geq 3$). (C) GFP or PHF6-GFP expressing N2A cells were subjected to ChIP using an antibody to PHF6 or IgG control followed by PCR analysis using primers to *EphA4*, *EphA7* and *EphB1*. *Zfp* locus was used as control ($n = 3$). (D) GFP or PHF6-GFP-expressing cells were electroplated with a luciferase reporter plasmid driven by a promoter containing 583 bp of the *EphA4*gene (pGL4.23-*EphA4*), 550 bp of the *EphA7* gene (pGL4.23-*EphA7*) or 709 bp of the *EphB1* gene (pGL4.23-*EphB1*). The pGL4.23-basic reporter plasmid (pGL4.23) was used as a control. Renilla expression plasmid was used as an internal control for all samples. RLU Relative luminescence unit. Dual luciferase reporter assay was performed 48 h following electroporation ($n = 3$). (E) N2A cells were electroporated with siRNA against *Phf6* (si*Phf6*) or control siRNA (siCtl) followed by dual luciferase reporter assay at 48 h ($n = 3$). (F) EPHA4, EPHA7 and PHF6 levels were analyzed by immunoblotting in PHF6-GFP-expressing N2A cells. TUBULIN was used as a loading control. (G) Densitometric quantification of PHF6, EPHA4 and EPHA7 protein level normalized to TUBULIN is shown ($n = 3$). (H) E14-Cerebral cortical tissues from WT and C99F-m mice were subjected to ChIP-PCR analysis, as described in panel (C). (I) eNSCs cultured from *Phf6*$^{-/Y}$ / *Nestin-CreERT2*$^{+}$ and control *Phf6*$^{loxP/Y}$ / *Nestin-CreERT2*$^{-}$ ~E15 mouse brains were subjected to immunoprecipitation (IP) using PHF6 antibody or IgG as control followed by immunoblotting analysis using a PHF6 antibody. (J) eNSCs from *Phf6*$^{-/Y}$ / *Nestin-CreERT2*$^{+}$ and control *Phf6*$^{loxP/Y}$ / *Nestin-CreERT2*$^{-}$ mouse brains at ~E15, were subjected to ChIP-PCR using a PHF6 antibody. *Zfp735* loci was used as control for the PCR ($n = 2$). (K,L) Protein expression of EPHB1 (K), EPHB2 (L), SOX2 and NESTIN were analyzed by immunoblotting in *EphB1*and *EphB2* knockdown (KD) cells. Loading controls of ß-ACTIN and GAPDH were used ($n = 2$). Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$,

***$p < 0.001$. [(C,H) one-way ANOVA, (A,D,E,G) two-tailed unpaired student *t*-test]. *n* represents an independent biological sample.

3.3.6 PHF6 directly occupies the gene regulatory regions of EphR to alter their expression

We next set out to investigate if the identified *EphRs* are direct PHF6 targets. Our ChIP-seq data revealed robust and significant binding of PHF6 to the promoter of *EphA4* with a p-value of 1.8E-08 (**Dataset 1**). ChIP-seq data also revealed peaks associated with the TSS of *EphA7* and *EphB1* although the p-values did not reach the cut off values for significance (p-value for *EphA7*: 7.2E-04; p-value for *EphB1*, 1.4E-04) (**Dataset 1, Fig 8**). We designed ChIP-qPCR experiments to specifically investigate the possibility of PHF6 binding to *EphA4* but also examined *EphA7/EphB1* (**Fig 8**) due to their significant deregulated expression in a PHF6-dependent manner (**Dataset 6**). To begin with, ChIP-qPCR experiments were conducted in PHF6-overexpressing N2A cell lines using a ChIP-grade PHF6 antibody. We established PHF6 enrichment on the *EphR* genes that we examined in N2A cells expressing PHF6-GFP relative to GFP control (**Fig 16C**). We further assessed the functional consequences of PHF6 binding to *EphR* via loss- and gain-of-function studies. We conducted a firefly luciferase assay in N2A cell lines expressing PHF6-GFP or GFP control (**Fig 16D**). The cells were electroporated with either the control pGL4.23-basic reporter plasmid (pGL4.23), or the luciferase reporter plasmids harbouring the promoters of different *EphR* genes, including pGL4.23-*EphA4*, pGL4.23-*EphA7*, and pGL4.23-*EphB1*, together with a Renilla expression plasmid, and were subjected to a dual luciferase assay after 48 hours. Cells expressing PHF6-GFP showed increased reporter activity for EphR regulatory regions (**Fig 16D**). Second, we induced the KD of *Phf6* via a pool of siRNA (**Fig 16E**) and subjected the cells to a firefly luciferase assay. Our data revealed significant downregulation of *EphR* promoter activity in *Phf6* KD cells. Importantly, parallel immunoblotting and RT-qPCR analyses revealed significant deregulation of the EphR protein and mRNA expression levels in a PHF6-dependent manner (**Fig 16F-G**).

To further assess if PHF6 direct regulation of *EphR* might be perturbed in the patient mouse models, we conducted ChIP assays in either E14 or P0 whole brain tissue of R342X, as well as luciferase assay in primary eNSCs and found that PHF6 regulation of *EphR* is consistently impaired in R342X mice (**Fig 15E-G**). Similarly, the ChIP assay revealed that the binding of PHF6 to *EphA4* and *EphB1* promoters were significantly attenuated in whole brain tissue of E14 C99F mice relative to the wild-type control (**Fig 16H**). The specificity of the PHF6 antibody used for ChIP was also confirmed in IP and ChIP-PCR experiments using $Phf6^{loxP/Y}$ / *Nestin-CreERT2*$^{-}$ and $Phf6^{-/Y}$ / *Nestin-CreERT2*$^{+}$ eNSCs (**Fig 16I-J**).

3.3.7 Knockdown of EphA phenocopies PHF6 loss-of-function

We have established that PHF6 directly binds to gene regulatory elements of *EphR* to upregulate their expression. Mice harbouring *Phf6* deletion, or BFLS patient mutations exhibit altered NSC self-renewal and deregulated *EphR* expression, raising the question of whether knockdown of either *EphA4/7* or *EphB1* can phenocopy the PHF6 mutant induced eNSC phenotype in BFLS.

EphA4 and EphA7 are involved in NSC regulation and neural development. EphA4 has been studied in axon guidance and neural circuit formation, whereas EphA7 plays a key role in apoptosis and cortical patterning (Depaepe et al., 2005; Kania & Klein, 2016; Klein, 2012). We employed an siRNA approach in primary E14 WT eNSCs to induce the KD of each of these receptors followed by ELDA analysis to assess eNSC self-renewal (**Fig 17A-D**). Our results revealed a significant increase in eNSC self-renewal in both *EphA4* and *EphA7* KD cells with the most profound impact in the *EphA4* KD cells (**Fig 17A-B**). Although there was a similar trend with *EphB* KD in eNSCs, no significant changes in self-renewal were induced upon the

knockdown of *EphB* family of receptors (**Fig 17C-D**). To investigate the impact of *EphR* KD on stemness, we also subjected whole protein lysates to immunoblotting using SOX2 and NESTIN antibodies (**Fig 17E-F, Fig 16K-L**). We found that KD of *EphA* members and *EphB1*, but not *EphB2*, induced an increase in the protein expression levels of SOX2 and NESTIN (**Fig 17E-F**, **Fig 16K-L**). Our studies demonstrate that although PHF6 regulates the gene expression of several *EphR* family members, KD of *EphA4* induces the most significant phenotype on eNSC self-renewal.

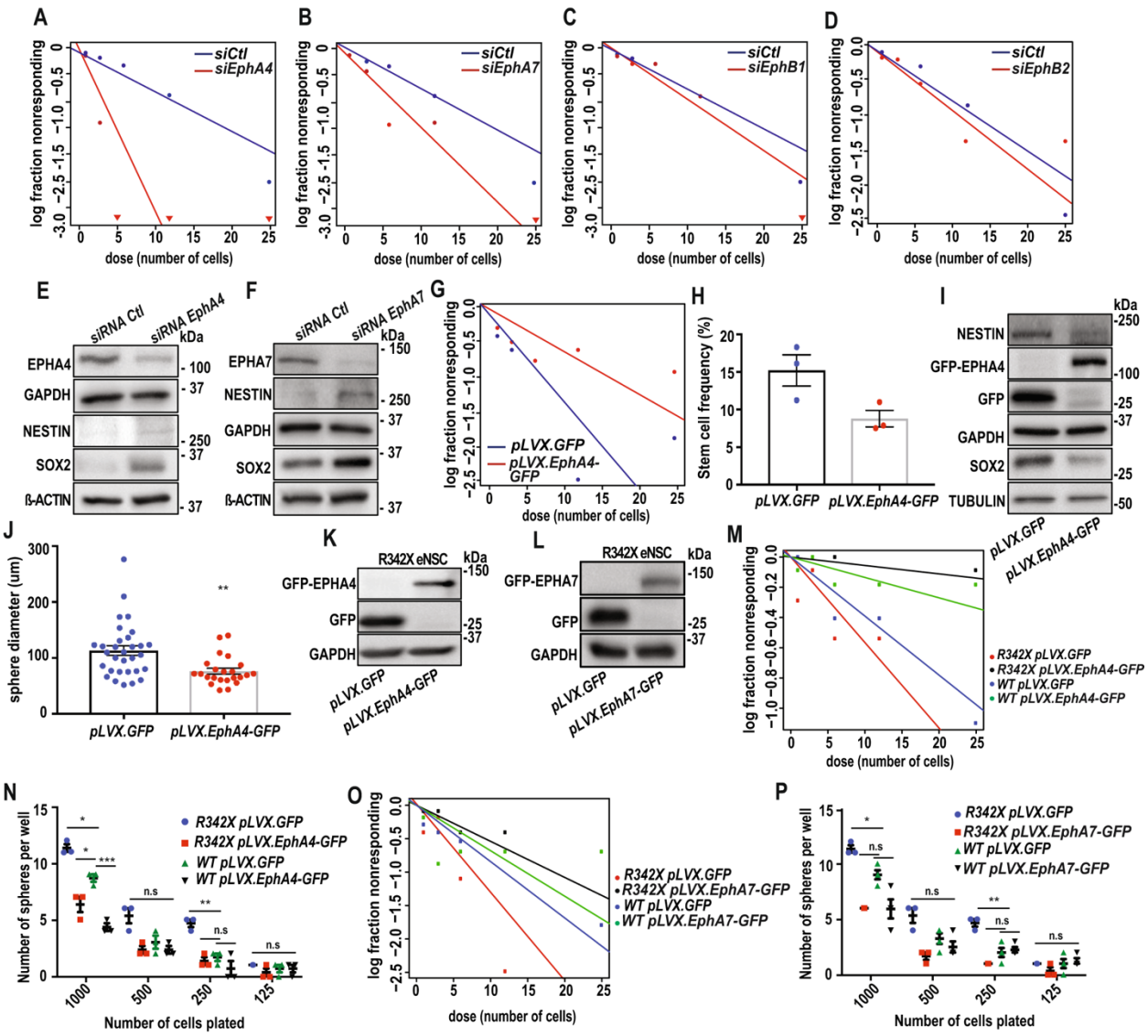

Figure 17: EphA-family of receptors rescues the eNSC phenotype in R342X mice. (A–D) WT eNSCs cultured at E14 were electroporated with siRNA targeting each of the *EphR* followed by self-renewal analysis. ELDA plots are presented for *EphA4* (A) ($p > 0.00001$), *EphA7* (B) ($p = 0.0219$), *EphB1* (C) ($p = 0.426$), and *EphB2* (D) ($p = 0.569$). (E,F) Protein expression of each EPHR, SOX2 and NESTIN were analyzed by immunoblotting. B-ACTIN was used as loading control. (G–J) E14 WT eNSCs were electroporated with pLVX.GFP and pLVX.*EphA4*-GFP constructs followed by ELDA (G) ($p = 0.00355$), and stem cell frequency analysis (H)

($p = 0.0527$), immunoblotting using EPHA4, NESTIN, SOX2, and GFP antibodies (I), and sphere diameter analysis (J) ($p = 0.0017$). (K–P) R342X and WT eNSCs cultured at E14 were electroporated with pLVX.GFP, pLVX.*EphA4*-GFP, and pLVX.*EphA7*-GFP and samples were subjected to immunoblotting analysis with EPHA4, EPHA7, and GFP antibodies (K,L), ELDA (M,O), and sphere analysis (N,P) following 7 days in culture [$p = 0.00264$ (M) and $p = 0.00255$ (O)]. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (H,J) two-tailed unpaired student *t*-test, (N,P) One-way ANOVA with Tukey's multiple comparisons test. Representative data of $n > 3$ independent replicates are shown in panels (A–G,I,K–M,O). Data in panels (H,J,N,P) are plotted with $n > 3$ mean +/− SEM. n represents an independent biological sample.

3.3.8 EphA- family of receptors rescues the R342X induced eNSC alterations

In view of our observations that *EphA4* KD most closely phenocopies the *Phf6* mutant-induced eNSC phenotype, we next assessed if forced expression of EphA4 alters eNSC expansion. We generated an EphA4 plasmid fused with a GFP tag on the C-terminus. E14 WT eNSCs were cultured and electroporated with *EphA4*-GFP (pLVX.*EphA4*-GFP), or control GFP plasmid (pLVX.GFP) followed by ELDA and immunoblotting analysis (**Fig 17G-J**). Our results showed that the expression of *EphA4* induced a significant decline in eNSC self-renewal (**Fig 17G**), the protein expression of both SOX2 and NESTIN (**Fig 17I**), stem cell frequency (SCF) (**Fig 17H**), and eNSC sphere size (**Fig 17J**). We thus aimed to examine if the EphA- family of receptors can rescue the PHF6- mutant induced phenotype using the R342X mouse model (**Fig 17K-P**). Forced expression of *EphA4*-GFP and *EphA7*-GFP was induced in eNSC cultures from the R342X mouse brain, and efficient electroporation of EphA4 and EphA7-GFP plasmids were confirmed by immunoblotting (**Fig 17K-L**). LDA and ELDA analysis revealed that both *EphA4* (**Fig 17M-N**) and *EphA7* (**Fig 17O-P**) rescue the R342X induced eNSC phenotype. In particular, EphA4 more profoundly decreased eNSC self-renewal and SCF in R342X eNSC (**Fig 17M-N**). These findings assert the potential for the EphA- family of receptors, specifically EphA4, in ameliorating the PHF6-mutant induced eNSC phenotype.

3.3.9 BFLS and PHF6-mutant mouse brains display imbalances in stem cell population

We have established that PHF6 patient mutations alter eNSC fate in BFLS, prompting us to characterize the eNSCs, in their niche, in the developing brain. We analyzed the whole-brain lysates of C99F-m (**Fig 18A**) and R342X (**Fig 18B**) at E14 by immunoblotting analysis. Similar

to results from primary eNSCs, we found a marked increase in the expression of stem cell markers, in BFLS mouse brains (**Fig 18A-B, 12H**), and a decrease in protein expression of mature cell-type markers including oligodendrocytes (OLIG2), astrocytes (GFAP), as well as progenitor cells (ASCL1) (**Fig 12I**). We next analyzed the stem cell marker, SOX2 (**Fig 18C**), and the progenitor cell marker, TBR2 (**Fig 18D**), via immunohistochemical analysis of R342X E14 coronal brain sections (**Fig 18F**). Percent population of both cell types were imaged and quantified in the ventricular zone (VZ) and subventricular zone (SVZ), which are regions of high stem cell density. We observed a reverse correlation between SOX2 positive (SOX2+) and TBR2 positive (TBR2+) cells in their neurogenic niches, whereby BFLS mice exhibited a higher percentage of SOX2+ cells and an attenuated number of TBR2+ cells (**Fig 18C-D, F**), with no significant differences observed in the percentage of merged SOX2+/TBR2+ cells (**Fig 18E-F**). A similar trend of increased SOX2+ cells was noted in the *Phf6*$^{-/Y}$ / *Nestin-Cre*$^{+}$ brains (**Fig 12D**).

The changes in the proportion of SOX2+ and TBR2+ cells suggest altered cell populations manifesting a disproportionate number of neural stem versus progenitor cells in BFLS, which may contribute to disease pathogenesis. In parallel studies, Nissl staining analyses revealed that similar to *Phf6* KO mice, R342X brain sections exhibited a decrease in neuronal density throughout the cortex (**Fig 12J**), suggesting the possibility of impaired neuronal migration. We thus set out to analyze the impact of *Phf6* deletion on cortical layer neurons via subjecting *Phf6*$^{loxP/Y}$ / *Nestin-Cre*$^{-}$ and *Phf6*$^{-/Y}$ / *Nestin-Cre*$^{+}$ brain sections at P0 to immunohistochemical analysis using antibodies to SATB2^{+}, CTIP2^{+}, and TBR1^{+} to quantify neuronal numbers in cortical layers II-VI, layer V, and layer VI, respectively (**Fig 12E-F**). Our results revealed a shift of SATB2+ neurons away from the apical cerebral cortex plate in *Phf6*$^{-/Y}$ /

Nestin-Cre^{+} mice, with no significant changes in the number of SATB2+, CTIP2+, and TBR1+ neurons. To quantify the migration patterns of SATB2+ neurons in the cerebral cortex influenced by loss of *Phf6*, a grid consisting of 10 equivalent bins was applied to the image of P0 cerebral cortex to equally divide the cortical wall spanning from the basal of ventricle zone to the pial surface into ten bins. The ten bins were marked sequentially from apical to basal, with bin 1 covering the most superficial (i.e., apical) layer, and bin 10 covering the deepest (i.e., basal) layer. Neurons within each bin were counted and a significant decline in SATB2+ neurons in bin 1 of the cerebral cortex was observed in *Phf6*$^{-/Y}$ */ Nestin-Cre*$^{+}$ mice (**Fig 12E-F**), suggesting impairment in the ability of SATB2+ neurons to migrate to superficial layers of the developing cerebral cortex in *Phf6* KO mice. This finding is consistent with the attenuation of neuron density (**Fig 12C**) and suggests that PHF6 is involved in regulating the process of radial neuronal migration during the establishment of cortical lamination.

Together, we report that PHF6 alters the mechanisms that regulate NSC fate in the developing brain, and that loss-of-function of PHF6 in BFLS results in an imbalance in the number of uncommitted stem cells and neural progenitors which may contribute to BFLS pathogenesis.

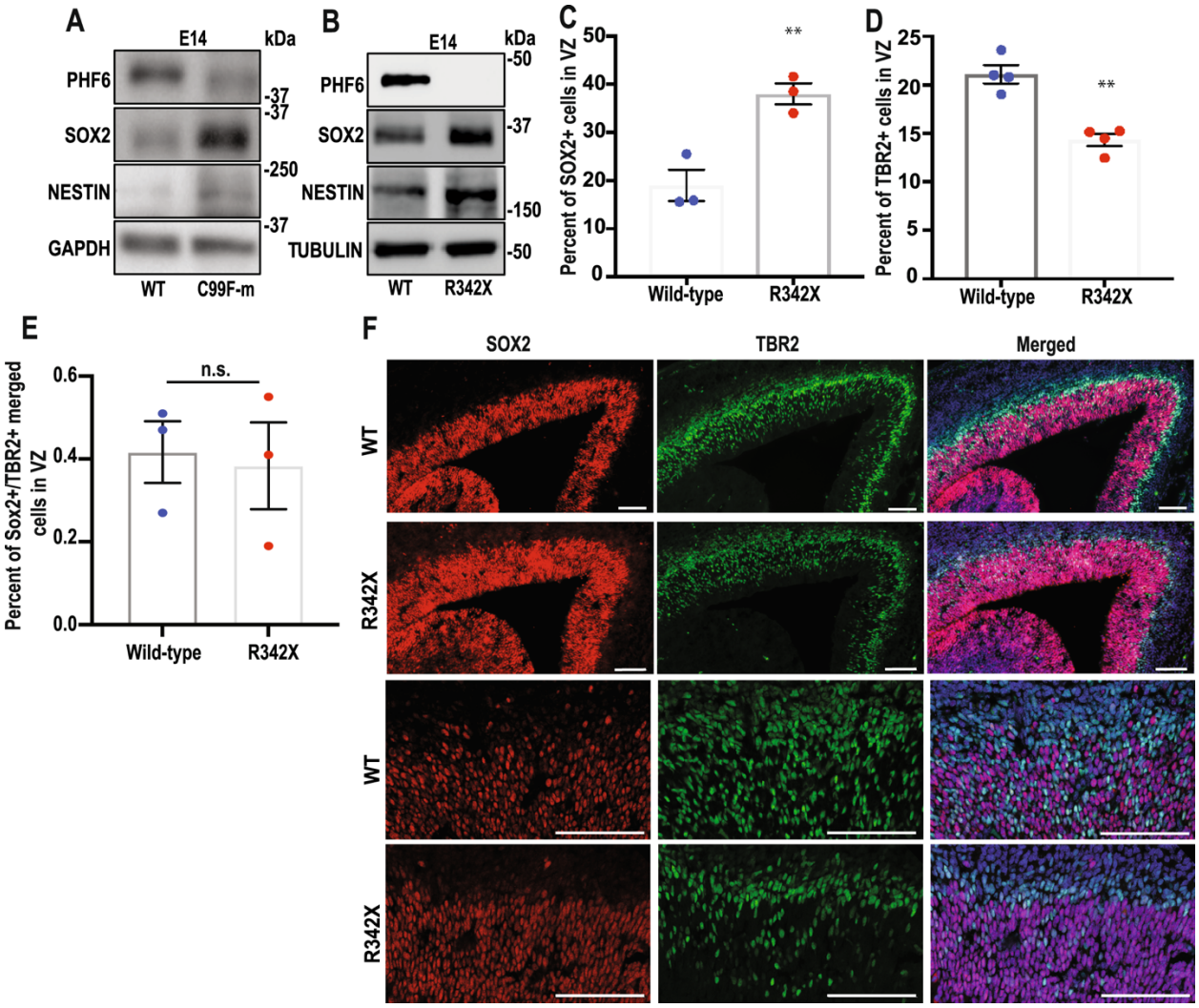

Figure 18: BFLS patient mouse models exhibit imbalance in the percent population of stem cell and neural progenitors. (A,B) Protein expression of PHF6, SOX2, and NESTIN in C99F-m (A) or R342X (B) E14 brains were analyzed with immunoblotting. GAPDH or TUBULIN were used as loading controls. (C–F) E14 brains were sectioned at a thickness of 8 μm and were subjected to staining using SOX2 and TBR2 antibodies. DAPI was used as a nuclei marker. Percentage of SOX2+ (C) ($p = 0.0084$), TBR2+ (D) ($p = 0.001$), and SOX2+/TBR2+ merged (E) cells were quantified using FIJI software. Representative images are shown (F). Scale bar

represents 100 μm. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for panels (C–E), two-tailed unpaired student *t*-test ($n > 3$ independent replicates). Data in (A,B) represents 3 biological replicates ($n = 3$ mice).

3.4 Discussion

In the present study, we report the discovery of a PHF6/Ephrin receptor transcriptional pathway in the regulation of neural stem cells in the developing brain. To begin with, mapping PHF6 sites of occupancy in the developing mouse cortex led to the identification of PHF6-bound regions, enriched near genes involved in central nervous system development and neurogenesis. Through a combination of gene expression profiling and PHF6 sites of occupancy, we established a dual function for PHF6 as both a transcriptional activator and repressor, depending on its binding pattern to the genome. Importantly, we established that PHF6 regulates neurogenesis via altering eNSC fate. Mechanistically, we report that members of EphRs including *EphA4, EphA7, EphB1*, and *EphB2* serve as downstream targets of PHF6. EphRs play crucial roles in the proper formation of the brain (Gerstmann & Zimmer, 2018; Kullander & Klein, 2002; Park, 2013). We show that PHF6 directly binds the gene regulatory regions of the identified EphRs to upregulate their expression. Importantly, characterization of BFLS mice including R342X and C99F revealed that EphRs are significantly impacted in BFLS. Furthermore, we generated a conditional *Phf6* KO mouse and confirmed our observations from the BFLS mice whereby impaired NSC pool and deregulation of EphRs resulted from *Phf6* genetic deletion. Finally, we report that although EphA and EphB members function downstream of PHF6, EphA members play the most profound roles in altering eNSC fate. Our results suggest that EphA-receptors could serve as a potential therapeutic target for BFLS. These studies not only shed mechanistic insights on BFLS and XLID but opens up new avenues of research for impaired NSC processes in other neurodevelopmental disorders of cognition.

There are contradicting reports on the binding of PHF6 to either histones or double-stranded DNA (dsDNA) (Liu et al., 2014; Soto-Feliciano et al., 2017; Todd & Picketts, 2012;

Xiang et al., 2019). Our study suggests that PHF6 directly binds DNA to regulate transcription in the developing brain. In particular, we find enrichment of $(CA)_n$ repeats in PHF6 peak summits, consistent with a previous study in T-cell acute lymphoblastic leukaemia (T-ALL) where PHF6 was also shown to bind $(CA)_n$ repeats (Binhassan, 2020). However, whether PHF6 regulation of the genome could also be epigenetically encoded in the context of BFLS pathogenesis remains a subject for future studies. In investigating the pattern of PHF6 binding to the genome, we found enrichment in the 5' UTR and TSS consistent with previous studies in B-cell leukemia where PHF6 was shown to bind to the TSS, the 5' UTR (Soto-Feliciano et al., 2017), and enhancer regions in a model of acute myeloid leukemia (AML) (Pawar et al., 2021). Notably, consistent with our findings in stem cell regulation, other groups have also reported a role for PHF6 in cell differentiation (Pawar et al., 2021) and lineage specification (Soto-Feliciano et al., 2017) in leukemia myeloid cell models.

Our analyses suggesting that PHF6 functions as a transcriptional activator or repressor depending on its binding pattern, could also describe the association of PHF6 with the PAF1 complex (Jahani-Asl et al., 2016; Zhang et al., 2013), as the PAF1 complex can either occupy the promoter and gene body of actively transcribed genes and associates with Pol II to promote transcriptional elongation (Pokholok et al., 2002; Wood et al., 2003), or PAF1 also appears to regulate promoter-proximal pausing of Pol II in mammalian cells (Chen et al., 2015). Mechanistically, we present a model that can help explain the dual role of PHF6 in the regulation of gene expression as an activator or repressor, depending on its binding pattern to the gene bodies downstream of the TSS to promote transcriptional elongation, or to the TSS to halt Pol II recruitment and transcription. However, we found that this pattern applies to 65% of candidate genes identified in our screen. How other factors or co-factors enhance or suppress PHF6's role

in the regulation of gene expression requires further investigation.

In the present study, we employed a combination of genome-wide studies, conditional *Phf6* KO mice, and BFLS patient mouse models to characterize the mechanisms by which PHF6 regulates gene expression and NSCs in the developing brain. We report a role for PHF6 in the regulation of eNSC fate in the developing brain whereby PHF6 loss-of-function leads to an imbalance of proper fate commitment of NSCs. However, GO term analysis also revealed the upregulation of cation channels (**Fig 3B**). Cation channels are vital for action potential generation and propagation, synaptic transmission, and overall neuronal communication and functioning (Chen & Lui, 2019). The upregulation of cation channel activities might represent a compensatory mechanism to enhance neuronal function or to accelerate certain aspects of neuronal maturation given the developmental delays observed in BFLS.

EphA4 is of particular importance amongst the EphRs in the context of stem cell processes and is a widely studied receptor of the ephrin family. High expression of EphA4 is present in hippocampal endothelial cells, mature astrocytes, neurons, and neural progenitor cells (Deininger et al., 2008; Goldshmit et al., 2006; North et al., 2009; Todd et al., 2017; Tremblay et al., 2009). Single-cell studies further proved that EphA4 is expressed in neuroblasts (Todd et al., 2017). Previously, overexpression of *EphA4* in neural progenitor cells in the cortex was shown to cause a decrease in stem cell frequency (North et al., 2009), specifically through ephrinB1-initiated signalling. However, another recent study showed that inhibition of *EphA4* via an antagonist that blocks EphA4 forward signalling, increased proliferation of hippocampal precursor cells (Zhao et al., 2019). In yet another recent study, EphA4 activity via ephrinA1 and VEGFR2 was shown to play a role in neural stem and progenitor cell (NSPC) differentiation (Chen, Liu, et al., 2020). These results suggest that EphA4 functions in a cell-type and stimuli-

dependent manner to confer different outcomes.

Previous studies suggest that EphRs play important roles in cell fate specification (Aoki et al., 2004; Vazin et al., 2009; Wilkinson, 2014). The upstream regulators of EphR remain largely unknown. Here we identify PHF6 as a key upstream regulator of EphR expression and function. Specifically, our data suggest that EphA family members profoundly alter the fate of NSCs suggesting its potential as a therapeutic target to rescue PHF6 loss-of-function in BFLS. Although the EphB family members also appear to serve as PHF6 targets, we did not observe a significant phenotype in the regulation of eNSC with EphB1 or EphB2. It remains to be investigated whether the EphB family members are involved in the regulation of other aspects of neural development such as neuronal morphogenesis and migration in the context of BFLS.

CHAPTER 4 - DISCUSSION

Discussion

In this thesis, I present my investigations on the molecular mechanisms underlying BFLS pathogenesis with particular focus on the PHF6 transcriptome and interacting networks. I report the discovery of a transcriptional pathway involving PHF6 and EphRs that plays an important role in regulating NSCs in the developing brain. To begin with, we mapped genome-wide PHF6 sites of occupancy in the developing mouse cortex, uncovering PHF6-binding sites that are predominantly located near genes associated with CNS development and neurogenesis. Through a combination of gene expression profiling and the analysis of PHF6 binding sites, we determined that PHF6 functions both as a transcriptional activator and repressor, depending on its binding pattern within the genome.

Importantly, we established that PHF6 regulates neurogenesis via altering eNSC fate. Mechanistically, we report that members of EphRs including *EphA4, EphA7, EphB1*, and *EphB2* serve as downstream targets of PHF6. EphRs play crucial roles in the proper formation of the brain (Gerstmann & Zimmer, 2018; Kullander & Klein, 2002; Park, 2013). We demonstrate that PHF6 directly binds to the regulatory regions of the identified EphRs, thereby upregulating their expression. Notably, the characterization of BFLS mice, including mice harbouring R342X and C99F mutations, revealed significant deregulation of EphRs in BFLS. Additionally, we generated a conditional *Phf6* KO mouse and confirmed our observations from the BFLS mice, showing that genetic deletion of *Phf6* leads to an impaired NSC pool and deregulation of EphRs. Our findings show that while both EphA and EphB members function downstream of PHF6, EphA members play the most critical roles in altering eNSC fate. Consequently, our results suggest that EphA receptors could be potential therapeutic targets for BFLS. These findings not only provides mechanistic insights into BFLS and XLID pathogenesis, but also opens new

research avenues for understanding impaired NSC processes in other neurodevelopmental disorders of cognition.

4.1 PHF6 binding to dsDNA in the developing cortex

We present ChIP-seq data suggesting that PHF6 binds dsDNA, in particular the microsatellite repeats, in the developing brain. We identified 2467 PHF6 binding sites and using de novo motif analysis of the top 1000 peaks, we demonstrated that the peak summits are enriched for $(CA)_n$ repeats (Rasool et al., 2024) (**Fig 19**). In follow up analyses we showed that these microsatellites were mainly found near genes involved in neural development and neurogenesis (Rasool et al., 2024) (**Fig 19**).

Genomic repetitive regions are made up of tandem repeats (TRs) and transposable elements (TE) (Vieira et al., 2016). Microsatellite repeats are a type of TR, also referred to as short tandem repeats (STRs) and are characterized by repeated short DNA motifs that are 1-6 base pairs in length. STRs are found mainly in non-coding regions, are highly polymorphic, and contribute to genomic instability or altering gene expression, which can impact diseases (Fan & Chu, 2007; Vieira et al., 2016). As an example, Fragile X Syndrome (FXS) is caused by an expansion of the CGG trinucleotide repeat in the FMR1 gene (Garber et al., 2008).

TEs are also sequences of DNA that can alter their position in the genome to affect genome structure and function. Discovered in 1950, they have since been recognized as important players in genomic evolution as they can create new mutations and genetic variations (McClintock, 1950). TEs are grouped into two types: retrotransposons and DNA transposons. Retrotransposons move via an RNA intermediate, whereas DNA transposons move via a “cut-and-paste” mechanism, being excised from one site and inserted into another (Fueyo et al., 2022;

Hickman & Dyda, 2016). TEs encourage insertions, deletions, and chromosome rearrangements in the genome to contribute to genomic plasticity. Additionally, TEs may provide novel binding sites for TFs, or can change chromatin structure through interactions with histone modifications such as H3K27me3 and H3K9me3 (Trizzino et al., 2018). Importantly, TEs are not randomly distributed, and they are abundantly found in the genome (Bourque et al., 2018). For example, the TEs Alu and LINE account for approximately 30% of the human genome (Es, 2001).

Proper regulation of TRs and TEs is important in order to prevent genomic instability and is done by multiple factors such as DNA methylation, histone modifications, and regulatory proteins (Fueyo et al., 2022). Dysregulation in genomic repetitive regions have been linked to numerous neurological disorders, for example, in both Rett syndrome (RTT) and autism there is an activation of the retrotransposon LINE-1 (Muotri et al., 2010; Shpyleva et al., 2018). Given the important roles for genomic repetitive regions as outlined above, the significance of CA repeat occurrences in the summits of PHF6 peaks merits further investigation. PHF6 has been linked to gene expression regulation via its interaction with chromatin remodelers (Alvarez et al., 2022; Jahani-Asl et al., 2016; Liu et al., 2014; Todd et al., 2015; Todd & Picketts, 2012; Yamada et al., 2014). As previously mentioned, TEs often reside in regions marked by specific histone modifications such as H3K27me3 and H3K9me3, which are associated with repressive chromatin states (Trizzino et al., 2018). Given PHF6's role in chromatin dynamics, it may contribute to the maintenance of these repressive marks or may affect the accessibility of genomic regions where TEs are located, thereby regulating the activity of TEs.

Our data suggesting PHF6 binding to DNA within $(CA)_n$ repeats is consistent with a previous study in T-cell acute lymphoblastic leukaemia (T-ALL) where PHF6 was also shown to bind $(CA)_n$ repeats (Binhassan, 2020). In addition, we found enrichment of PHF6 peaks in the 5’

UTR and TSS relative to percent genome. This data is consistent with previous reports in B-cell leukemia where PHF6 was shown to exhibit binding to the TSS, the 5' UTR (Soto-Feliciano et al., 2017), and enhancer regions in a model of acute myeloid leukemia (AML) (Pawar et al., 2021).

In support of our findings, other studies have shown PHF6 binding to dsDNA (Liu et al., 2014; Xiang et al., 2019). A subunit of the NuRD complex has been widely studied as a binding target of PHF6 (Liu et al., 2014; Xiang et al., 2019). NuRD displays control of the p21 gene, a cell cycle regulator that has been shown to decrease efficacy of drug treatment in leukemia (Xiang et al., 2019). Under normal conditions, p21 expression is suppressed by PHF6 (Liu et al., 2014; Xiang et al., 2019) through direct binding and recruitment of a NuRD complex component and histone-binding protein; RBPP4, to the promoter region of its repressed genes, including p21 (Liu et al., 2014). Ablation of PHF6 led to less PHF6 and RBBP4 being present on the p21 promoter region, leading to drug resistance (Xiang et al., 2019). While the study confirms the binding of PHF6 to RBPP4, it's important to note that the researchers did not investigate potential interactions with other nucleolar proteins, raising the possible interaction of PHF6 with other proteins beyond RBPP4.

Although our findings of PHF6 binding to DNA is supported by several studies, there are opposing findings in literature regarding the binding of PHF6 to either dsDNA or histones (Liu et al., 2014; Soto-Feliciano et al., 2017; Todd & Picketts, 2012; Xiang et al., 2019). Histones are important long-term regulators of genes found in the nucleus of eukaryotic cells. Researchers have studied the PHF6 binding pattern in murine B-ALL cells transfected with a PHF6 plasmid to generate *Phf6* KO B-ALL cells (Soto-Feliciano et al., 2017). Through PHF6 ChIP-seq signal correlation with histone marks and Co-IP experiments, researchers revealed that PHF6 controls

transcription of its target genes through protein-protein interactions with histones, specifically histone-3 (H3) (Soto-Feliciano et al., 2017). This interaction was found to be independent of the presence of DNA (Soto-Feliciano et al., 2017). Although we employed ChIP-seq, ChIP-PCR, and luciferase assays to establish a model for PHF6 binding to DNA, the possibility that PHF6 can also bind histones in the developing brain remains a possibility for future investigations.

Histone ChIP assays and histone-tail array can be employed to screen for potential PHF6-histone interactions. Additionally, employing the Assay for Transposase-Accessible Chromatin Using Sequencing (ATAC-seq) in wild type or null PHF6 cells provides mechanistic insights into the potential role of PHF6 as an epigenetic modifier.

4.2. PHF6 regulation of cell fate

4.2.1 PHF6 regulation of cell fate through the cell cycle

I have established that PHF6 alters stem cell behaviour, self-renewal, and proliferation in several mouse models and in primary NSC cultures using loss- and gain-of-function studies. PHF6 loss-of-function and BFLS point mutation studies revealed that PHF6 suppresses proliferation and self-renewal. These findings support a role for PHF6 in cell cycle regulation consistent with previous studies on PHF6 regulation of cell cycle whereby it can disable checkpoint recovery in the G2 phase of irradiated human U2OS cells (Warmerdam et al., 2020) (**Fig 19**). It will therefore be important to conduct cell cycle analysis in BFLS mice using EdU tracing to delineate the specific phases of the cell cycle that are affected by loss of *Phf6*. This analysis will help to determine whether the suppression of proliferation and self-renewal observed in *Phf6*-deficient cells is due to disruptions at specific cell cycle checkpoints or a

general impairment in cell cycle progression. Additionally, exploring the interplay between PHF6 and other cell cycle regulators in these models will provide deeper insights into the molecular mechanisms underlying BFLS pathology.

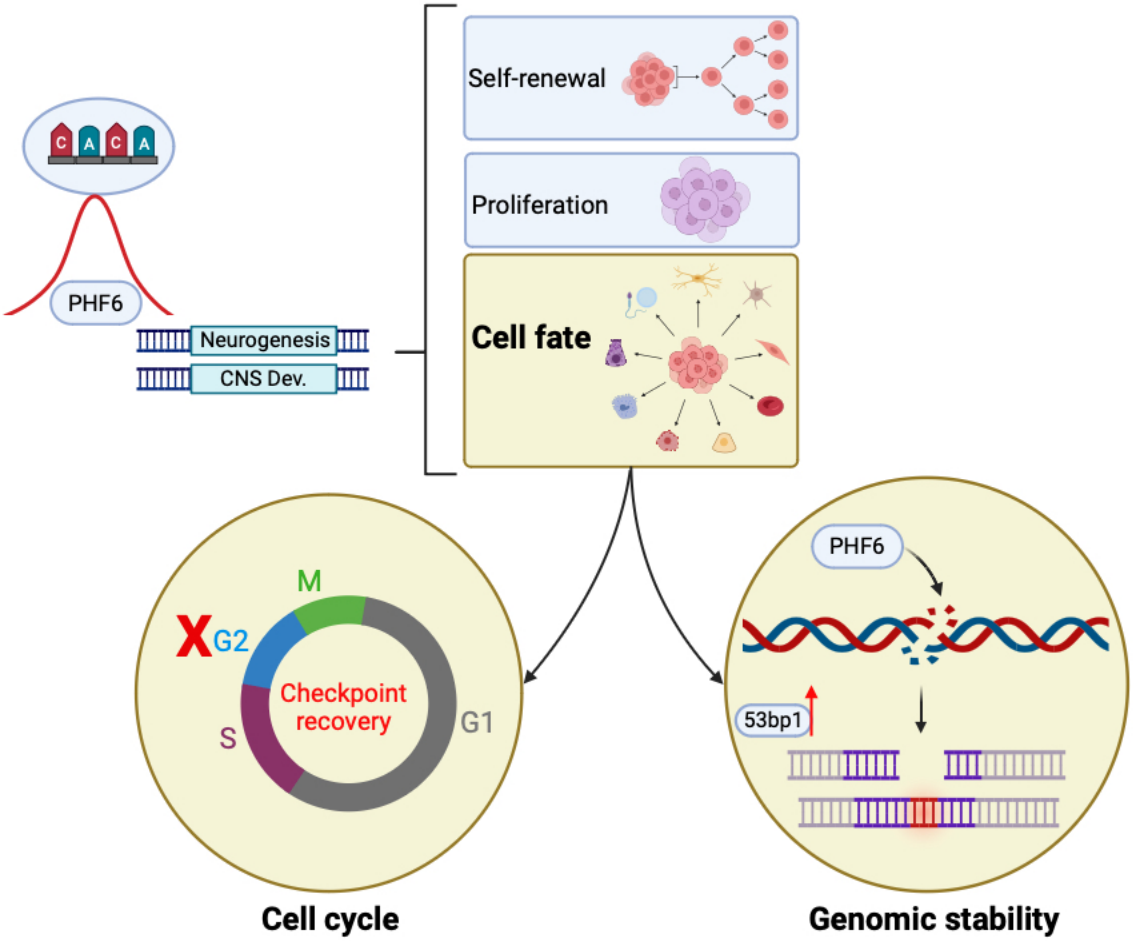

Figure 19: PHF6 regulation of cell fate. $CA_{(n)}$ microsatellite repeats were found in the peak summit of PHF6 and enriched near genes involved in neurogenesis and CNS development. PHF6 regulation of cell fate may be via cell cycle control or maintaining genomic stability. Made with BioRender.

4.2.2 PHF6 regulation of cell fate through genomic stability

My thesis findings have established a role for PHF6 in suppression of NSC proliferation and self-renewal which could be due to altering cell cycle processes as outlined above. However, it is possible that PHF6's alterations in cell fate may be caused by its role in genomic stability and maintenance (**Fig 19**). In support of this, a study showed that PHF6 is recruited to DNA damage sites for DNA repair through non-homologous end joining (NHEJ), a process by which sister chromatids are used to repair double-stranded breaks (DSB) via direct ligation of the broken DNA ends (Warmerdam et al., 2020; Warmerdam & Kanaar, 2010) (**Fig 19**). NHEJ is most predominant in the G1 cell cycle phase (Branzei & Foiani, 2008). Following repair, cell cycle progression is resumed through checkpoint recovery wherein many kinases work to re-activate cell cycle factors (Macůrek et al., 2008; Vugt & Medema, 2004). Researchers have revealed a new role for PHF6 in DNA damage repair, where employment of an siRNA-based library screen in human U2OS cells was used to targeted several hundred genes related to the structure, maintenance, and modification of chromatin (Warmerdam et al., 2020). Additional screening identified eight genes, including PHF6, that were labelled as regulators of DNA damage recovery (Warmerdam et al., 2020). To confirm PHF6's role in checkpoint recovery, *PHF6* KO in the human U2OS cell line through CRISPR/Cas9 technology was used to elicit a decrease in checkpoint recovery that was then rescued through exogenous expression of GFP-*PHF6* (Warmerdam et al., 2020). *PHF6* KO was able to reduce the accumulation of 53BP1, a DNA damage response factor, at the site of double-stranded breaks (DSBs) (Warmerdam et al., 2020). These studies suggest that PHF6 is an essential modulator for the recruitment of 53BP1 at the site of DSBs rather than directly causing defects in cell cycle progression.

These insights into PHF6's function in genomic maintenance provide a connection to its regulatory roles in NSCs. The integrity and stability of the genome are critical for the proper functioning and survival of NSCs, which are highly proliferative and sensitive to DNA damage. PHF6's role in facilitating DNA repair and maintaining genomic stability is crucial in preventing mutations that could lead to aberrant cell behaviour, including unchecked proliferation or unbalanced differentiation, as observed in our results. Therefore, PHF6's involvement in DNA damage repair and checkpoint recovery may influence its regulatory role in NSCs, supporting the suppression of their proliferative and self-renewal capacities while safeguarding against genomic instability.

4.3 Insights into the function of PHF6 in other model systems

4.3.1 PHF6 regulation of Leukemia

PHF6 deregulation has not only been studied in the developing brain but has been widely investigated in the context of leukemia and hematopoiesis. The current field of research into PHF6's role in hematopoiesis, cell cycle regulation, and leukemia development provides a strong foundation for understanding its broader implications in neurodevelopment as parallels may be drawn between the brain and blood model systems.

Acute lymphoblastic leukemia (ALL) is propagated by either B- or T- lineage lymphoblasts (Soto-Feliciano et al., 2017). Interestingly, PHF6 may act as either a tumour suppressor or activator in leukemia depending on the cell lineage that causes the malignancy. PHF6 targets nucleosomes at the TSS of lineage-specific genes, activating B-ALL cell line genes by recruiting B-cell specific transcription factors and inhibiting T-cell specific gene expression

through chromatin compaction (Soto-Feliciano et al., 2017). Thus, in B-ALL PHF6 acts as a tumour promoting factor, whereas in T-ALL, it is a tumour suppressor (Soto-Feliciano et al., 2017). From this, we can gather that the role of PHF6 differs between cell types and is thus context dependent.

Researchers have demonstrated that the second PHD domain of PHF6, encoded by exons 4 and 5, plays a key role in the function of PHF6 whereby deletion of these exons resulted in deactivation of PHF6 which led to enhanced resilience of hematopoietic stem cells (HSCs) to cytokines (Miyagi et al., 2019). This mutation allowed *Phf6* KO cells to more effectively rebuild the hematopoietic system compared to WTs, specifically under conditions of stress, as the reconstitution levels between WT and KO adult mice did not significantly differ in the absence of stress. Most importantly, PHF6 mutant HSCs showed a decrease in apoptotic pathways, specifically tumour necrosis factor-alpha (TNFa) and transforming growth factor-beta (TGFb), while an increase in the cell cycle controller E2 factor (E2F) was observed (Miyagi et al., 2019). This explains the proliferative advantage of HSCs lacking PHF6, and these findings can be used to delineate the role of PHF6 in the BFLS brain. Similar mechanisms of regulating cell cycle and apoptotic pathways as seen in HSCs may underlie the increased proliferation observed in our own findings in *Phf6* KO and BFLS NSCs. Thus, the study of PHF6 in the context of leukemia provides insights into its role in cell identity and maintenance of lineage-specific gene expression patterns. Similarly, our study of PHF6 in the context of BFLS provides insights on how PHF6 may govern cell identity via altering gene expression programs.

4.3.2 The Role of PHF6 in hormone regulation and hunger response: A genome-wide approach

A hallmark feature of BFLS is truncal/abdominal obesity, affecting 75% of patients by late childhood (Jahani-Asl et al., 2016). Although we did not directly study how PHF6 patient mutations impact obesity, literature supports a role for PHF6 in endocrine regulation and hunger response. Researchers highlighted PHF6's involvement in hunger responses by demonstrating its enrichment in agouti-related peptide (AgRP) neurons, which are crucial for increasing appetite and reducing metabolism (Gan et al., 2020). ChIP-Seq analysis showed that PHF6's chromatin binding in these neurons is regulated by hunger state, which affects the promotor regions of immediate-early genes (IEGs) and suggests that PHF6 acts as a transcriptional regulator (Gan et al., 2020). Interestingly, PHF6's influence extends beyond hunger responses, as a recent study uncovered PHF6 as a regulator of the growth hormone-releasing hormone (GHRH)/GH/IGF-1 axis (McRae et al., 2020). In a BFLS mouse model, deletion of PHF6 resulted in a proportional decrease in body size, tied to diminished GH and IGF-1 levels, illustrating PHF6's role in growth through neuroendocrine regulation (McRae et al., 2020). Additionally, it was shown that a BFLS patient-related mutant mouse model showed a smaller pituitary gland with reduced PHF6 expression, especially in the anterior and intermediate lobes (Ahmed, Sarwar, Hu, Cardin, Qiu, Zapata, Vandeleur, Yan, Lerch, & Corbett, 2021). This reduction was linked to decreased expression of growth hormone (GH) and prolactin (Prl), with Prl+ cells being fewer in number (Ahmed, Sarwar, Hu, Cardin, Qiu, Zapata, Vandeleur, Yan, Lerch, & Corbett, 2021). These pituitary defects mirror those observed in BFLS patients, providing a valuable model for understanding the endocrine aspects of the syndrome, as it can be tied to impacts on NSCs.

Neuroendocrine regulation significantly impacts NSCs in the brain, influencing their proliferation, differentiation, and overall function. Studies have shown that maternal thyroid hormone (TH) deficiency impairs neurogenesis by disrupting cell cycle kinetics and diminishing the progenitor pool (Mohan et al., 2012). TH replacement can partly reinstate the rate of neurogenesis, highlighting the importance of maternal TH in early brain development, before onset of fetal thyroid function (Mohan et al., 2012). Furthermore, THs are important for establishing corticogenesis through their action on genes, such as PAX6 involved in cortico-development (Mohan et al., 2012), with deficiencies leading to severe brain defects as observed in conditions such as Allan–Herndon–Dudley syndrome (Vancamp et al., 2017). In mice, it has been shown that maternal hypothyroidism during pregnancy hinders neurogenesis in the embryonic telencephalon, leading to impairments in learning and memory in offspring (Chen et al., 2012), indicating that eNSCs are key targets for TH in regulating early brain development. Moreover, the GH/Prl axis was found to play a significant role in neuroprotection by promoting the proliferation and migration of NSCs (Pathipati et al., 2011). Exogenous application of GH and Prl in NSCs from fetal human forebrains was found to enhance proliferation of NSCs and neuronal progenitors, however Prl specifically promoted the proliferation of only glial progenitors (Pathipati et al., 2011). These findings suggest that PHF6's regulation of NSC proliferation and cell fate may be partly mediated through its effects on neuroendocrine signalling.

The relationship between PHF6 and various hormonal pathways highlights the importance of this protein in maintaining metabolic and growth homeostasis. As outlined above, literature is mounting on the role of neuroendocrine signalling in modulating NSC behaviour, and the role of PHF6 in endocrine regulation. In view of these findings, our discovery of PHF6

regulation of NSC behaviour suggests a new model: PHF6's role in endocrine regulation and its deregulation in BLFS may underlie defects in NSC fate specification and impaired neurogenesis.

4.4 Significance of EphA4: a direct PHF6 transcriptional target

I have identified EphA4 as a promising direct transcriptional target of PHF6 (Rasool et al., 2024). EphA4 is one of the most widely studied EphRs, with its well-known binding partners consisting of a number of growth factors (Chen, Liu, et al., 2020; Fukai et al., 2008; Yokote et al., 2005). Interestingly, the proliferation and differentiation of neural progenitor cells (NPCs) are influenced by both EphR and angiogenic growth factor receptors (Chen, Liu, et al., 2020). Understanding the binding partners of EphA4 can model other potential molecules involved in the PHF6/EphA4 pathway that act on the NSC processes studied in this thesis.

A number of growth factors have been identified as EphA4 binding partners. Previously, immunoprecipitation studies have shown EphA4 to directly interact with PDGFRβ in NPCs (Chen, Song, et al., 2020). Ephrin-A1 and platelet-derived growth factors BB (PDGF-BB) promote NPC proliferation and neuronal differentiation, and this effect is enhanced when both ligands are co-treated (Chen, Song, et al., 2020). Additionally, an inhibition of EphA4, or PDGFR, blocked ligand-dependent proliferation and differentiation of NPCs. However, injection of ephrin-A1 and/or PDGF-BB increased hippocampal NPC proliferation in an AD mouse model, demonstrating an EphA4-PDGFRβ-mediated function in neurogenesis via ERK signalling (Chen, Song, et al., 2020). In another set of studies in mouse embryonic neural stem and progenitor cells (NSPCs), researchers found an interaction between EphA4 and the vascular endothelial growth factor receptor 2 (VEGFR2) which was shown to be involved in NSPC differentiation (Chen, Liu, et al., 2020). Treatment of Ephrin-A1 in combination with, and

independently of $VEGF_{165}$ (one of the most abundant isoforms of VEGF), caused increased neuronal differentiation, suggesting the EphA4-VEGFR2 interaction may be mediated by the Ephrin-A1 ligand (Chen, Liu, et al., 2020). Additionally, the fibroblast growth factors (FGF) have also been shown to be binding partners of EphA4 (Fukai et al., 2008; Yokote et al., 2005). Direct interaction of the cytoplasmic domain of EphA4 and FGF receptor causes trans-activation of downstream signalling pathways that promotes cell migration and axon guidance (Yokote et al., 2005). Furthermore, the role of EphA4 in cell migration and proliferation was tested in a human glioma (U251) cell line where activated EphA4 was found to phosphorylate the FGF receptor 1 (FGFR1) and this pathway was shown to modulate proliferation and migration of human glioma cells (Fukai et al., 2008).

Aside from growth factors, EphA4 has previously been shown as a binding partner of growth hormone receptor (GHR), Janus kinase 2 (JAK2), and signal transducer and activator of transcription 5B (STAT5B) (Sawada et al., 2017). As previously mentioned, PHF6 regulates the GHRH/GH/IGF-1 axis, influencing growth and metabolic pathways. Interestingly, EphA4 can phosphorylate GHR, while it can bind JAK2 and STAT5B, allowing their activation and increasing activity of the canonical GH-IGF1 axis (Sawada et al., 2017). Together, these studies furthermore highlight the importance of a PHF6/EphR regulatory pathway in GH/IGF1 signalling.

EphA4's ability to phosphorylate it's binding partners was further demonstrated in a study of a mouse model for temporal lobe epilepsy (TLE), wherein the importance of ephrin-A5 and EphA4 interaction on neurogenesis and angiogenesis was shown. This interaction involved the activation of phosphorylated Akt (p-Akt) and ERK (p-ERK) signalling pathways and was shown to decrease the number of newborn neurons in the TLE model (Shu et al., 2016). The

connection between EphA4 signalling and neurogenesis in epilepsy models provides insights into similar mechanisms that might be at play in BFLS, which is often associated with neurological issues, including epilepsy (Jahani-Asl et al., 2016). In our own findings, we observed a reduced number of neural progenitors in BFLS mouse brains (Rasool et al., 2024). This highlights the potential overlap between the pathways involved in EphA4 phosphorylation of p-Akt and p-ERK in TLE, with the epileptic seizure phenotype observed in BFLS, further emphasizing the critical role of PHF6/EphA4 pathway in regulating neural progenitor cell dynamics.

The study of additional EphA4 binding partners aligns well with the work in this thesis by providing potential models for the molecular mechanisms through which PHF6 may regulate NSCs via EphA4. Our research supports the notion that PHF6 directly regulates the expression of EphA4, and that this regulation is crucial for maintaining the balance between NSC self-renewal and differentiation in the developing brain (Rasool et al., 2024). By influencing key signalling pathways, such as those mediated by PDGFR, VEGFR2, FGFR, the GH-IGF1 axis, and phosphorylation of p-Akt and p-Erk, the PHF6/EphA4 pathway may impact important aspects of cell fate determination. Understanding these interactions not only elucidates the pathways involved in BFLS pathogenesis, but also identifies potential therapeutic targets of PHF6/EphA4 for treating NDDs associated with impaired neurogenesis.

4.5 EphB family of receptors: Where do they stand in regulation of neurogenesis?

Unlike the EphA family of receptors, we did not observe a role for EphB1/2 in regulation of eNSCs in our BFLS or *Phf6* KO models. Although the role of EphB in BFLS remains a subject for future studies, literature suggest that the EphB family of receptors have been

implicated in alternate aspects of neurogenesis, including dendrite and neural morphogenesis and migration. Dendrite morphogenesis ensures proper neural circuit assembly and usually starts after neuronal migration is completed (Meltzer & Chen, 2016). EphBs also appear to regulate synapse differentiation, spine morphogenesis, and dendritic filopodia motility allowing for the formation of new synapses between neurons (Kayser et al., 2008). These findings highlight the importance of EphB in neural circuit plasticity.

In adults, EphB receptors are shown to be expressed in NSPCs in the hippocampus, and interactions between EphB and their ephrin-B ligands have been shown to regulate the migration of neural progenitors (Chumley et al., 2007), as well as taking part in dendritic processes within the hippocampus (i.e., dendritic pruning and spine maturation) (Hoogenraad et al., 2005; Ledda & Paratcha, 2017; Xu & Henkemeyer, 2012; Xu et al., 2011). The importance of EphB in the regulation of axon guidance during spinal cord development was previously shown, where the expression pattern of EphB and its ephrin-B ligand in the ventral midline of the spinal cord facilitated axon guidance towards their respective targets (Kadison et al., 2006). EphB regulation of axon guidance and synapse formation can also be shown in the olfactory system where a shift in localization of EphB to the dendritic arborization of mitral cells in the postnatal olfactory bulb was noted (St John & Key, 2001). These findings suggest that EphB receptors are integral to various aspects of neurogenesis that extend beyond eNSC regulation, and their involvement in dendritic and neural morphogenesis, synapse differentiation, and migration highlights their versatility and importance in neural development.

Interestingly, our ChIP-Seq data in the developing cortex suggests that PHF6 mutations may alter neurogenesis through different mechanisms. We have established that PHF6 mutations enhances NSC self-renewal which may in turn result in their reduced commitment to neuronal

progenitors. Importantly however, another GO term highly enriched in our ChIP-seq data has been dendrite morphogenesis (Rasool et al., 2024), suggesting that PHF6 point mutations may impair neural circuit assembly by impairing proper maturation of neurons. Having shown that PHF6 directly regulates the expression of EphB1/2 (Rasool et al., 2024), and EphB receptors have been implicated in the regulation of dendrite morphogenesis and migration (Hoogenraad et al., 2005; Ledda & Paratcha, 2017; Xu & Henkemeyer, 2012; Xu et al., 2011), suggests that PHF6 mutations could disrupt dendritic development and neuronal connectivity by altering EphB-mediated signalling pathways, thus contributing to the neurological deficits observed in BFLS.

4.6 Future Directions

The role of PHF6 in regulating the transcriptome and epigenome of the developing cortex, and mechanisms by which PHF6 patient mutations alter gene regulatory networks in vivo, remains key subjects for future investigation. Examining chromatin state and its role in BFLS pathogenesis can shed light on the possibility that PHF6-mediated transcriptional reprogramming occurs at the chromatin level. This notion is supported by previous evidence of PHF6 functioning as an epigenetic modifier (Oh et al., 2020).

ATAC-seq can determine whether PHF6-altered genes or specific gene clusters exhibit changes in chromatin accessibility. Observing such changes would support the hypothesis that PHF6 regulates gene clusters through interactions with histones. Conversely, if altered transcriptome profiles do not correspond with changes in chromatin accessibility, this would suggest that the associated changes with BFLS patient mutations are not epigenetically encoded. This line of inquiry can redirect our focus towards other regulatory mechanisms influencing

transcriptional changes. Additionally, performing ATAC-seq analyses on the cerebral cortex of BFLS embryos electroporated with select EphRs can help determine whether these EphRs can restore genetically encoded transcriptional alterations, providing further insight into the interplay between PHF6 and EphR signalling pathways in neurogenesis and BFLS pathogenesis.

By exploring these avenues, we can deepen our understanding of PHF6's role in transcriptional regulation and its potential interactions with chromatin, ultimately contributing to the development of targeted therapeutic strategies for BFLS.

4.7 Potential Therapeutic Implications of Thesis Findings

The findings from this thesis provide a foundation for exploring therapeutic strategies aimed at mitigating the effects of PHF6 mutations in BFLS and potentially other types of NDDs. One of the main discoveries in this thesis is the identification of EphRs as direct transcriptional targets of PHF6, implicating the PHF6/EphR pathway in the regulation of eNSCs and neurogenesis. This novel insight suggests that modulating the activity of EphRs, particularly EphA receptors, could be a promising therapeutic approach for BFLS.

PHF6 functions as a transcriptional regulator by binding to gene regulatory regions of EphRs and modulating their expression, this is deregulated in BFLS mouse models. The upregulation of EphA4 and EphA7 expression by PHF6 suggests that enhancing the activity of these receptors could potentially restore normal eNSC dynamics and neurogenesis- since the KD of *EphA4* and *EphA7* was able to phenocopy the PHF6 loss-of-function effects. Thus, pharmacological agents that specifically enhance EphA4 and EphA7 activity could be developed for BFLS.

Finally, the insights gained from this thesis show the importance of further research into the molecular mechanisms underlying the PHF6/EphR pathway and its role in NDDs. Future studies should focus on characterizations of how PHF6 mutations affect EphR-mediated signalling and the downstream effects on neurogenesis and NSC dynamics. This could lead to the identification of additional therapeutic targets within this pathway and the development of treatment strategies that address multiple aspects of the syndrome. This thesis provides a strong rationale for exploring EphA4 as a therapeutic target in BFLS. By enhancing our understanding of the PHF6/EphA4 pathway, we can develop targeted therapies that may significantly improve the neurodevelopmental outcomes for individuals affected by BFLS and related IDs.

4.8 Limitations of the Thesis

While this thesis has made significant contributions to the field of PHF6, BFLS, and NSC regulation, some limitations should be acknowledged. The mouse models used in this project, R342X and C99F, while valuable for studying mammalian genetics and brain development, have inherent differences with human neurogenesis, cortical development, and the overall architecture of the brain (as outlined in Chapter 1 - Introduction). Furthermore, the conditional *Phf6* KO models used do not mimic the human pathology of BFLS, as the extent of gene deletion is not recapitulated in human patients.

Next, neurogenesis is regulated by a complex network of genes and signalling pathways that may interact with one another. The dual role of PHF6 as both a transcriptional activator and repressor adds another layer of complexity that might not be fully captured in the experimental models used. The mechanisms by which PHF6 switches roles based on its binding pattern are not entirely understood and require further investigation.

The thesis predominantly focuses on genetic aspects of BFLS and PHF6 regulation. However, NDDs often result from a combination of genetic predispositions and environmental influences. Factors such as human maternal nutrition, exposure to toxins, and other environmental stressors were not considered in this study but could impact the findings.

Furthermore, the phenotypic variability observed in BFLS patients is not fully addressed. While the mouse models provided valuable insights, they may not capture the full spectrum of clinical manifestations seen in humans. Although the study provides a strong foundation using animal models, integrating human clinical data could enhance the relevance and applicability of the findings. Future studies incorporating patient-derived cells (i.e., IPSCs) and correlating genetic findings with clinical phenotypes would be valuable.

Finally, the therapeutic potential of targeting EphR in BFLS, while promising, remains speculative. Extensive clinical trials are necessary to validate EphR as a viable therapeutic target. The safety, efficacy, and long-term effects of over-expressing EphRs in human patients will require substantial testing. Treatment of NDDs is challenging for several reasons. First, a significant challenge in treating NDDs is that these disorders originate from early prenatal stages but manifest after birth and throughout early childhood. Promising but challenging approaches may include brain-delivered CRISPR vectors to correct DNA mutations directly in cells (Ernst, 2016). However, these treatments face large issues with vector delivery and require precise identification of the molecular readouts that go awry in each specific NDD. Second, effective treatments for NDDs in most cases needs to target a single gene, even a single mutation in a gene. However, gene mutations can vary across individuals diagnosed with the same disorder, as seen in PHF6 mutations causing BFLS. By understanding and targeting the convergence points of NDDs (i.e., common NSC defects across different NDDs), we may be able to overcome the

challenge of varying gene mutations. Finally, deficits in proliferation and differentiation of neural progenitors during early corticogenesis cannot be treated in postmitotic neurons (Ernst, 2016). Yet, CRISPR-based epigenetic editing may be a tool to alter gene expression without changing the DNA sequence. This method can potentially reactivate developmental genes or silence inhibitory pathways in mature neurons, enhancing their function and plasticity. Despite advancements in diagnosing NDDs, effective therapies for IDs remain limited and obsolete. Furthermore, in most conditions, there are multiple clinical phenotypes that challenges prognosis. This highlights the importance of a multidisciplinary approach to managing and increasing quality of patient and caregiver life in these complex disorders.

While this study has provided significant insights into the role of PHF6 in neurogenesis and its implications in BFLS, addressing these limitations through more integrative approaches will advance our understanding and help develop therapeutic strategies for XLIDs such as BFLS.

4.9 Final Conclusion and Summary

The hypothesis of this research project was that PHF6 regulates neurogenesis and cell fate via Ephrin receptors and impairment of PHF6/EphR signalling underlies the pathogenesis of BFLS (**Fig 20**). The major objectives of this thesis were to determine whether PHF6 alters neurogenesis in BFLS mouse models, to determine whether PHF6 alters neurogenesis by regulation of EphRs, and to determine if EphR forced expression can ameliorate the PHF6-induced NSC phenotype. All three objectives were achieved in Chapter 3, where this study revealed genome-wide binding of PHF6 in the developing cortex and showed that it altered NSCs via regulation of EphR. Importantly, we found that the PHF6/EphR pathway was impaired in BFLS patient-related mutant and *Phf6* KO mouse models. We also found that PHF6 regulates

NSC fate in the developing brain of BFLS mice, and that PHF6 binds the gene regulatory elements of its target EphRs to upregulate their expression and alter neurogenesis. Additionally, we showed that BFLS mice exhibit altered NSC and progenitor populations, and deregulation of EphRs. Finally, EphR-A family of receptors rescues PHF6 loss-of-function defects in BFLS mice-derived eNSCs.

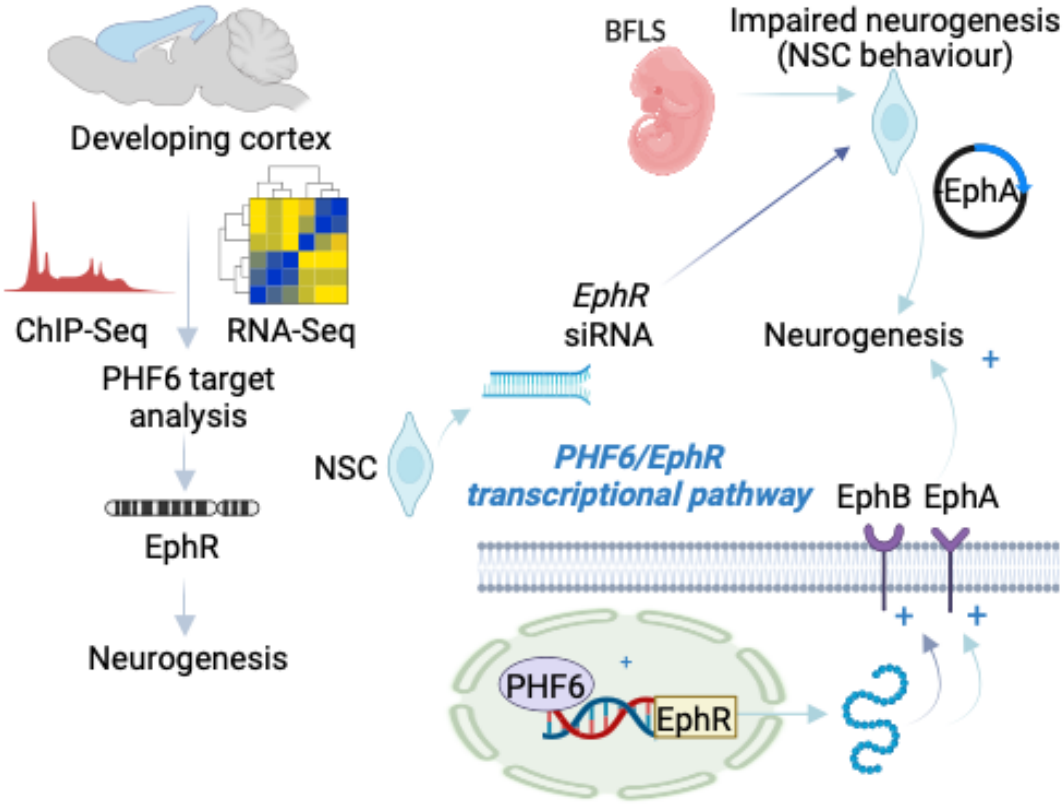

Figure 20: Summary of findings. This thesis revealed genome wide binding of PHF6 in the developing cortex and shows that it alters neural stem cells via regulation of Ephrin receptors. PHF6/EphR pathway is impaired in the rare X-linked intellectual disability, BFLS. Made with BioRender.

References

Ables, J. L., DeCarolis, N. A., Johnson, M. A., Rivera, P. D., Gao, Z., Cooper, D. C., . . . Eisch, A. J. (2010). Notch1 is required for maintenance of the reservoir of adult hippocampal stem cells. *Journal of Neuroscience, 30*(31), 10484-10492.

Agathocleous, M., Locker, M., Harris, W. A., & Perron, M. (2007). A general role of hedgehog in the regulation of proliferation. *Cell Cycle, 6*(2), 156-159.

Ahmed, R., Sarwar, S., Hu, J., Cardin, V., Qiu, L. R., Zapata, G., . . . Corbett, M. A. (2021). Transgenic mice with an R342X mutation in Phf6 display clinical features of Börjeson–Forssman–Lehmann Syndrome. *Human molecular genetics, 30*(7), 575-594.

Ahmed, R., Sarwar, S., Hu, J., Cardin, V., Qiu, L. R., Zapata, G., . . . Picketts, D. J. (2021). Transgenic mice with an R342X mutation in Phf6 display clinical features of Borjeson-Forssman-Lehmann Syndrome (BFLS). *Hum Mol Genet*. doi:10.1093/hmg/ddab081

Ahn, S., & Joyner, A. L. (2004). Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. *Cell, 118*(4), 505-516.

Ahn, S., & Joyner, A. L. (2005). In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. *Nature, 437*(7060), 894-897.

Allen, M. C. (2008). Neurodevelopmental outcomes of preterm infants. *Current opinion in neurology, 21*(2), 123-128.

Alvarez, S., da Silva Almeida, A. C., Albero, R., Biswas, M., Barreto-Galvez, A., Gunning, T. S., . . . Piovan, E. (2022). Functional mapping of PHF6 complexes in chromatin remodeling, replication dynamics, and DNA repair. *Blood, The Journal of the American Society of Hematology, 139*(23), 3418-3429.

Alvarez-Buylla, A., & Lim, D. A. (2004). For the long run: maintaining germinal niches in the adult brain. *Neuron, 41*(5), 683-686.

Aly, G. S., Abdel-Hady, H., & Ibrahim, M. Z. (2017). Congenital viral infections. *Viral Infections in Children, Volume I*, 1-46.

Anda, R. F., Felitti, V. J., Bremner, J. D., Walker, J. D., Whitfield, C., Perry, B. D., . . . Giles, W. H. (2006). The enduring effects of abuse and related adverse experiences in childhood: A convergence of evidence from neurobiology and epidemiology. *European archives of psychiatry and clinical neuroscience, 256*, 174-186.

Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics, 31*(2), 166-169. doi:10.1093/bioinformatics/btu638

Andersen, J., Urbán, N., Achimastou, A., Ito, A., Simic, M., Ullom, K., . . . Frisén, J. (2014). A transcriptional mechanism integrating inputs from extracellular signals to activate hippocampal stem cells. *Neuron, 83*(5), 1085-1097.

Anderson, S., Eisenstat, D., Shi, L., & Rubenstein, J. (1997). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science, 278*(5337), 474-476.

Aoki, M., Yamashita, T., & Tohyama, M. (2004). EphA receptors direct the differentiation of mammalian neural precursor cells through a mitogen-activated protein kinase-dependent pathway. *Journal of Biological Chemistry, 279*(31), 32643-32650.

Aoto, J., & Chen, L. (2007). Bidirectional ephrin/Eph signaling in synaptic functions. *Brain research, 1184*, 72-80.

Argenti, B., Gallo, R., Di Marcotullio, L., Ferretti, E., Napolitano, M., Canterini, S., . . . Maroder, M. (2005). Hedgehog antagonist RENKCTD11 regulates proliferation and

apoptosis of developing granule cell progenitors. *Journal of Neuroscience, 25*(36), 8338-8346.

Artavanis-Tsakonas, S., Rand, M. D., & Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science, 284*(5415), 770-776.

Arvanitis, D. N., & Davy, A. (2012). Regulation and misregulation of Eph/ephrin expression. *Cell adhesion & migration, 6*(2), 131-137.

Arvanitis, D. N., Jungas, T., Behar, A., & Davy, A. (2010). Ephrin-B1 reverse signaling controls a posttranscriptional feedback mechanism via miR-124. *Molecular and cellular biology, 30*(10), 2508-2517.

Azari, H., Rahman, M., Sharififar, S., & Reynolds, B. A. (2010). Isolation and expansion of the adult mouse neural stem cells using the neurosphere assay. *JoVE (Journal of Visualized Experiments)*(45), e2393.

Balordi, F., & Fishell, G. (2007). Hedgehog signaling in the subventricular zone is required for both the maintenance of stem cells and the migration of newborn neurons. *Journal of Neuroscience, 27*(22), 5936-5947.

Barquilla, A., & Pasquale, E. B. (2015). Eph receptors and ephrins: therapeutic opportunities. *Annual review of pharmacology and toxicology, 55*, 465-487.

Batlle, E., Henderson, J. T., Beghtel, H., van den Born, M. M., Sancho, E., Huls, G., . . . Pawson, T. (2002). β-Catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell, 111*(2), 251-263.

Beachy, P. A., Karhadkar, S. S., & Berman, D. M. (2004). Tissue repair and stem cell renewal in carcinogenesis. *Nature, 432*(7015), 324-331.

Becker, E., Huynh-Do, U., Holland, S., Pawson, T., Daniel, T. O., & Skolnik, E. Y. (2000). Nck-interacting Ste20 kinase couples Eph receptors to c-Jun N-terminal kinase and integrin activation. *Molecular and cellular biology, 20*(5), 1537-1545.

Ben-Reuven, L., & Reiner, O. (2021). Dynamics of cortical progenitors and production of subcerebral neurons are altered in embryos of a maternal inflammation model for autism. *Molecular psychiatry, 26*(5), 1535-1550.

Berland, S., Alme, K., Brendehaug, A., Houge, G., & Hovland, R. (2010). PHF6 deletions may cause Borjeson-Forssman-Lehmann syndrome in females. *Molecular syndromology, 1*(6), 294-300.

Bernier, R., Golzio, C., Xiong, B., Stessman, H. A., Coe, B. P., Penn, O., . . . Vulto-van Silfhout, A. T. (2014). Disruptive CHD8 mutations define a subtype of autism early in development. *Cell, 158*(2), 263-276.

Bertrand, N., Castro, D. S., & Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nature Reviews Neuroscience, 3*(7), 517-530.

Bhasin, T. K., Brocksen, S., Avchen, R. N., & Van Naarden Braun, K. (2006). Prevalence of four developmental disabilities among children aged 8 years; Metropolitan Atlanta Developmental Disabilities Surveillance Program, 1996 and 2000.

Bhutta, A. T., Cleves, M. A., Casey, P. H., Cradock, M. M., & Anand, K. J. (2002). Cognitive and behavioral outcomes of school-aged children who were born preterm: a meta-analysis. *Jama, 288*(6), 728-737.

Biamonte, F., Sica, G., Filippini, A., & D'Alessio, A. (2021). Evidence of reelin signaling in GBM and its derived cancer stem cells. *Brain Sciences, 11*(6), 745.

Bienvenu, T., Des Portes, V., McDonell, N., Carrié, A., Zemni, R., Couvert, P., . . . Fryns, J. P. (2000). Missense mutation in PAK3, R67C, causes X-linked nonspecific mental retardation. *American journal of medical genetics, 93*(4), 294-298.

Billuart, P., Bienvenu, T., Ronce, N., Des Portes, V., Vinet, M. C., Zemni, R., . . . Cherry, M. (1998). Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. *Nature, 392*(6679), 923-926.

Binda, E., Visioli, A., Giani, F., Lamorte, G., Copetti, M., Pitter, K. L., . . . DiMeco, F. (2012). The EphA2 receptor drives self-renewal and tumorigenicity in stem-like tumor-propagating cells from human glioblastomas. *Cancer cell, 22*(6), 765-780.

Binhassan, S. (2020). *PHF6 as a novel interactor of the transcriptional co-regulator LMO2 in T-Cell acute lymphoblastic leukaemia (T-ALL) and myeloid differentiation.* University of Birmingham,

Binns, K. L., Taylor, P. P., Sicheri, F., Pawson, T., & Holland, S. J. (2000). Phosphorylation of tyrosine residues in the kinase domain and juxtamembrane region regulates the biological and catalytic activities of Eph receptors. *Molecular and cellular biology, 20*(13), 4791-4805.

Bleichrodt, N., & Born, M. P. (1996). A metaanalysis of research on iodine and its relationship to cognitive development. In *The damaged brain of iodine deficiency.* (pp. 195-200): Cognizant Communication Corporation.

BÖRJESON, M., FORSSMAN, H., & LEHMANN, O. (1962). An X-linked, recessively inherited syndrome characterized by grave mental deficiency, epilepsy, and endocrine disorder. *Acta medica Scandinavica, 171*(1), 13-22.

Bouché, E., Romero-Ortega, M. I., Henkemeyer, M., Catchpole, T., Leemhuis, J., Frotscher, M., . . . Bock, H. H. (2013). Reelin induces EphB activation. *Cell research, 23*(4), 473-490.

Bourque, G., Burns, K. H., Gehring, M., Gorbunova, V., Seluanov, A., Hammell, M., . . . Macfarlan, T. S. (2018). Ten things you should know about transposable elements. *Genome biology, 19*, 1-12.

BrainSpan. (2013). BrainSpan: Atlas of the Developing Human Brain. Retrieved from http://www.brainspan.org. http://www.brainspan.org

Branzei, D., & Foiani, M. (2008). Regulation of DNA repair throughout the cell cycle. *Nature reviews Molecular cell biology, 9*(4), 297-308.

Bray, S., & Bernard, F. (2010). Current Topics in Developmental Biology.

Bruhl, T., Urbich, C., Aicher, D., Acker-Palmer, A., Zeiher, A. M., & Dimmeler, S. (2004). Homeobox A9 transcriptionally regulates the EphB4 receptor to modulate endothelial cell migration and tube formation. *Circulation research, 94*(6), 743-751.

Burban, A., & Jahani-Asl, A. (2022). Isolation of Mouse Embryonic Neural Stem Cells and Characterization of Neural Stem Markers by Flow Cytometry. In *Neuronal Cell Death: Methods and Protocols* (pp. 297-308): Springer.

Bustos, F., Segarra-Fas, A., Chaugule, V. K., Brandenburg, L., Branigan, E., Toth, R., . . . Walden, H. (2018). RNF12 X-linked intellectual disability mutations disrupt E3 ligase activity and neural differentiation. *Cell reports, 23*(6), 1599-1611.

Bustos, F., Segarra-Fas, A., Chaugule, V. K., Brandenburg, L., Branigan, E., Toth, R., . . . Findlay, G. M. (2018). RNF12 X-Linked Intellectual Disability Mutations Disrupt E3 Ligase Activity and Neural Differentiation. *Cell Rep, 23*(6), 1599-1611. doi:10.1016/j.celrep.2018.04.022

Butt, S. J., Fuccillo, M., Nery, S., Noctor, S., Kriegstein, A., Corbin, J. G., & Fishell, G. (2005). The temporal and spatial origins of cortical interneurons predict their physiological subtype. *Neuron, 48*(4), 591-604.

Callan, M. A., Cabernard, C., Heck, J., Luois, S., Doe, C. Q., & Zarnescu, D. C. (2010). Fragile X protein controls neural stem cell proliferation in the Drosophila brain. *Human molecular genetics, 19*(15), 3068-3079.

Calò, L., Spillantini, M., Nicoletti, F., & Allen, N. D. (2005). Nurr1 co-localizes with EphB1 receptors in the developing ventral midbrain, and its expression is enhanced by the EphB1 ligand, ephrinB2. *Journal of neurochemistry, 92*(2), 235-245.

Campbell, I. D., & Spitzfaden, C. (1994). Building proteins with fibronectin type III modules. *Structure, 2*(5), 333-337.

Carter, M. T., Picketts, D. J., Hunter, A. G., & Graham, G. E. (2009). Further clinical delineation of the Borjeson-Forssman-Lehmann syndrome in patients with PHF6 mutations. *Am J Med Genet A, 149a*(2), 246-250. doi:10.1002/ajmg.a.32624

Castren, M., Tervonen, T., Kärkkäinen, V., Heinonen, S., Castrén, E., Larsson, K., . . . Åkerman, K. (2005). Altered differentiation of neural stem cells in fragile X syndrome. *Proceedings of the National Academy of Sciences, 102*(49), 17834-17839.

Chao, M. M., Todd, M. A., Kontny, U., Neas, K., Sullivan, M. J., Hunter, A. G., . . . Kratz, C. P. (2010). T-cell acute lymphoblastic leukemia in association with Borjeson-Forssman-Lehmann syndrome due to a mutation in PHF6. *Pediatr Blood Cancer, 55*(4), 722-724. doi:10.1002/pbc.22574

Chau, V., Brant, R., Poskitt, K. J., Tam, E. W., Synnes, A., & Miller, S. P. (2012). Postnatal infection is associated with widespread abnormalities of brain development in premature newborns. *Pediatric research, 71*(3), 274-279.

Chelly, J., Khelfaoui, M., Francis, F., Chérif, B., & Bienvenu, T. (2006). Genetics and pathophysiology of mental retardation. *European Journal of Human Genetics, 14*(6), 701-713.

Chen, C., Zhou, Z., Zhong, M., Zhang, Y., Li, M., Zhang, L., . . . Yu, Z. (2012). Thyroid hormone promotes neuronal differentiation of embryonic neural stem cells by inhibiting STAT3 signaling through TRα1. *Stem cells and development, 21*(14), 2667-2681.

Chen, E. S., Gigek, C. O., Rosenfeld, J. A., Maussion, G., Chen, G. G., Vaillancourt, K., . . . Shaffer, L. G. (2014). Molecular convergence of neurodevelopmental disorders. *The American Journal of Human Genetics, 95*(5), 490-508.

Chen, F. X., Woodfin, A. R., Gardini, A., Rickels, R. A., Marshall, S. A., Smith, E. R., . . . Shilatifard, A. (2015). PAF1, a molecular regulator of promoter-proximal pausing by RNA polymerase II. *Cell, 162*(5), 1003-1015.

Chen, I., & Lui, F. (2019). Neuroanatomy, neuron action potential.

Chen, J., & Ruley, H. E. (1998). An enhancer element in the EphA2 (Eck) gene sufficient for rhombomere-specific expression is activated by HOXA1 and HOXB1 homeobox proteins. *Journal of Biological Chemistry, 273*(38), 24670-24675.

Chen, Q., Liu, J., Sawada, T., Wei, C., Wu, S., & Han, F. (2020). Possible role of EphA4 and VEGFR2 interactions in neural stem and progenitor cell differentiation. *Experimental and Therapeutic Medicine, 19*(3), 1789-1796.

Chen, Q., Song, H., Liu, C., Xu, J., Wei, C., Wang, W., & Han, F. (2020). The Interaction of EphA4 With PDGFRβ Regulates Proliferation and Neuronal Differentiation of Neural Progenitor Cells in vitro and Promotes Neurogenesis in vivo. *Frontiers in aging neuroscience, 12*, 7.

Chen, R.-H., Ding, W. V., & McCormick, F. (2000). Wnt signaling to β-catenin involves two interactive components: glycogen synthase kinase-3β inhibition and activation of protein kinase C. *Journal of Biological Chemistry, 275*(23), 17894-17899.

Cheng, C., Deng, P. Y., Ikeuchi, Y., Yuede, C., Li, D., Rensing, N., . . . Bonni, A. (2018). Characterization of a Mouse Model of Borjeson-Forssman-Lehmann Syndrome. *Cell Rep, 25*(6), 1404-1414 e1406. doi:10.1016/j.celrep.2018.10.043

Cheng, C., Deng, P. Y., Ikeuchi, Y., Yuede, C., Li, D., Rensing, N., . . . Bonni, A. (2018). Characterization of a Mouse Model of Borjeson-Forssman-Lehmann Syndrome. *Cell Rep, 25*(6), 1404-1414.e1406. doi:10.1016/j.celrep.2018.10.043

Chenn, A., & Walsh, C. A. (2002). Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science, 297*(5580), 365-369.

Cheung, T. H., & Rando, T. A. (2013). Molecular regulation of stem cell quiescence. *Nature reviews Molecular cell biology, 14*(6), 329-340.

Chhabra, S. N., & Booth, B. W. (2021). Asymmetric cell division of mammary stem cells. *Cell Division, 16*(1), 5.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., & Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature, 383*(6599), 407-413.

Chumley, M. J., Catchpole, T., Silvany, R. E., Kernie, S. G., & Henkemeyer, M. (2007). EphB receptors regulate stem/progenitor cell proliferation, migration, and polarity during hippocampal neurogenesis. *Journal of Neuroscience, 27*(49), 13481-13490.

Codega, P., Silva-Vargas, V., Paul, A., Maldonado-Soto, A. R., DeLeo, A. M., Pastrana, E., & Doetsch, F. (2014). Prospective identification and purification of quiescent adult neural stem cells from their in vivo niche. *Neuron, 82*(3), 545-559.

Committee, E. N. (1997). Unified nomenclature for Eph family receptors and their ligands, the ephrins. *Cell, 90*(3), 403-404.

Conover, J. C., Doetsch, F., Garcia-Verdugo, J.-M., Gale, N. W., Yancopoulos, G. D., & Alvarez-Buylla, A. (2000). Disruption of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone. *Nature neuroscience, 3*(11), 1091-1097.

Cooke, J., Moens, C., Roth, L., Durbin, L., Shiomi, K., Brennan, C., . . . Holder, N. (2001). Eph signalling functions downstream of Val to regulate cell sorting and boundary formation in the caudal hindbrain. *Development, 128*(4), 571-580.

Crawford, J., Lower, K. M., Hennekam, R. C., Van Esch, H., Megarbane, A., Lynch, S. A., . . . Gecz, J. (2006). Mutation screening in Borjeson-Forssman-Lehmann syndrome: identification of a novel de novo PHF6 mutation in a female patient. *J Med Genet, 43*(3), 238-243. doi:10.1136/jmg.2005.033084

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., . . . Li, H. (2021). Twelve years of SAMtools and BCFtools. *Gigascience, 10*(2). doi:10.1093/gigascience/giab008

Darling, T. K., & Lamb, T. J. (2019). Emerging roles for Eph receptors and ephrin ligands in immunity. *Frontiers in Immunology, 10*, 1473.

Day, B. W., Stringer, B. W., Al-Ejeh, F., Ting, M. J., Wilson, J., Ensbey, K. S., . . . Offenhäuser, C. (2013). EphA3 maintains tumorigenicity and is a therapeutic target in glioblastoma multiforme. *Cancer cell, 23*(2), 238-248.

Daynac, M., Morizur, L., Chicheportiche, A., Mouthon, M.-A., & Boussin, F. D. (2016). Age-related neurogenesis decline in the subventricular zone is associated with specific cell cycle regulation changes in activated neural stem cells. *Scientific reports, 6*(1), 21505.

De Bellard, M. E., Ching, W., Gossler, A., & Bronner-Fraser, M. (2002). Disruption of segmental neural crest migration and ephrin expression in delta-1 null mice. *Developmental biology, 249*(1), 121-130.

De Luca, C., Race, V., Keldermans, L., Bauters, M., & Van Esch, H. (2020). Challenges in molecular diagnosis of X-linked Intellectual disability. *British Medical Bulletin, 133*(1), 36-48.

De Rubeis, S., He, X., Goldberg, A. P., Poultney, C. S., Samocha, K., Ercument Cicek, A., . . . Walker, S. (2014). Synaptic, transcriptional and chromatin genes disrupted in autism. *Nature, 515*(7526), 209-215.

Deininger, K., Eder, M., Kramer, E. R., Zieglgänsberger, W., Dodt, H.-U., Dornmair, K., . . . Klein, R. (2008). The Rab5 guanylate exchange factor Rin1 regulates endocytosis of the EphA4 receptor in mature excitatory neurons. *Proceedings of the National Academy of Sciences, 105*(34), 12539-12544.

del Valle, K., Theus, M. H., Bethea, J. R., Liebl, D. J., & Ricard, J. (2011). Neural progenitors proliferation is inhibited by EphB3 in the developing subventricular zone. *International journal of developmental neuroscience, 29*(1), 9-14.

Delgado, A. C., Ferrón, S. R., Vicente, D., Porlan, E., Perez-Villalba, A., Trujillo, C. M., . . . Fariñas, I. (2014). Endothelial NT-3 delivered by vasculature and CSF promotes quiescence of subependymal neural stem cells through nitric oxide induction. *Neuron, 83*(3), 572-585.

Deng, W., Aimone, J. B., & Gage, F. H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nature Reviews Neuroscience, 11*(5), 339-350.

Depaepe, V., Suarez-Gonzalez, N., Dufour, A., Passante, L., Gorski, J. A., Jones, K. R., . . . Vanderhaeghen, P. (2005). Ephrin signalling controls brain size by regulating apoptosis of neural progenitors. *Nature, 435*(7046), 1244-1250.

Des Portes, V., Pinard, J. M., Billuart, P., Vinet, M. C., Koulakoff, A., Carrié, A., . . . Berwald-Netter, Y. (1998). A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. *Cell, 92*(1), 51-61.

Di Bella, D. J., Habibi, E., Stickels, R. R., Scalia, G., Brown, J., Yadollahpour, P., . . . Arlotta, P. (2021). Molecular logic of cellular diversification in the mouse cerebral cortex. *Nature, 595*(7868), 554-559. doi:10.1038/s41586-021-03670-5

Ding, W. Y., Huang, J., & Wang, H. (2020). Waking up quiescent neural stem cells: molecular mechanisms and implications in neurodevelopmental disorders. *PLoS genetics, 16*(4), e1008653.

Doe, C. Q. (2008). Neural stem cells: balancing self-renewal with differentiation.

Douglas, R. J., & Martin, K. A. (2004). Neuronal circuits of the neocortex. *Annu. Rev. Neurosci., 27*, 419-451.

Dravis, C. (2010). Ephs, ephrins, and bidirectional signaling. *Nat Educ, 3*, 22.

Dubois, J., Benders, M., Borradori-Tolsa, C., Cachia, A., Lazeyras, F., Ha-Vinh Leuchter, R., . . . Hüppi, P. (2008). Primary cortical folding in the human newborn: an early marker of later functional development. *Brain, 131*(8), 2028-2041.

Dubruc, E., Putoux, A., Labalme, A., Rougeot, C., Sanlaville, D., & Edery, P. (2014). A new intellectual disability syndrome caused by CTNNB1 haploinsufficiency. *American journal of medical genetics Part A, 164*(6), 1571-1575.

Durak, O., Gao, F., Kaeser-Woo, Y. J., Rueda, R., Martorell, A. J., Nott, A., . . . Tsai, L.-H. (2016). Chd8 mediates cortical neurogenesis via transcriptional regulation of cell cycle and Wnt signaling. *Nature neuroscience, 19*(11), 1477-1488.

Durbin, L., Brennan, C., Shiomi, K., Cooke, J., Barrios, A., Shanmugalingam, S., . . . Holder, N. (1998). Eph signaling is required for segmentation and differentiation of the somites. *Genes & development, 12*(19), 3096-3109.

Ehm, O., Göritz, C., Covic, M., Schäffner, I., Schwarz, T. J., Karaca, E., . . . Espinosa, L. (2010). RBPJκ-dependent signaling is essential for long-term maintenance of neural stem cells in the adult hippocampus. *Journal of Neuroscience, 30*(41), 13794-13807.

El Khouri, E., Ghoumid, J., Haye, D., Giuliano, F., Drevillon, L., Briand-Suleau, A., . . . Bienvenu, T. (2021). Wnt/β-catenin pathway and cell adhesion deregulation in CSDE1-related intellectual disability and autism spectrum disorders. *Molecular psychiatry, 26*(7), 3572-3585.

Erdős, G., Mészáros, B., Reichmann, D., & Dosztányi, Z. (2019). Large-Scale Analysis of Redox-Sensitive Conditionally Disordered Protein Regions Reveals Their Widespread Nature and Key Roles in High-Level Eukaryotic Processes. *Proteomics, 19*(6), 1800070.

Ernst, C. (2016). Proliferation and differentiation deficits are a major convergence point for neurodevelopmental disorders. *Trends in neurosciences, 39*(5), 290-299.

Es, L. (2001). Initial sequencing and analysis of the human genome. *Nature, 409*, 860-921.

Fan, H., & Chu, J.-Y. (2007). A brief review of short tandem repeat mutation. *Genomics, Proteomics and Bioinformatics, 5*(1), 7-14.

Feng, J., Liu, T., Qin, B., Zhang, Y., & Liu, X. S. (2012). Identifying ChIP-seq enrichment using MACS. *Nat Protoc, 7*(9), 1728-1740. doi:10.1038/nprot.2012.101

Feng, R., & Wen, J. (2015). Overview of the roles of Sox2 in stem cell and development. *Biological Chemistry, 396*(8), 883-891.

Fliedner, A., Gregor, A., Ferrazzi, F., Ekici, A. B., Sticht, H., & Zweier, C. (2020). Loss of PHF6 leads to aberrant development of human neuron-like cells. *Scientific reports, 10*(1), 1-15.

Flint, J., & Knight, S. (2003). The use of telomere probes to investigate submicroscopic rearrangements associated with mental retardation. *Current opinion in genetics & development, 13*(3), 310-316.

Frankish, A., Diekhans, M., Ferreira, A. M., Johnson, R., Jungreis, I., Loveland, J., . . . Flicek, P. (2019). GENCODE reference annotation for the human and mouse genomes. *Nucleic Acids Res, 47*(D1), D766-D773. doi:10.1093/nar/gky955

Fuccillo, M., Joyner, A. L., & Fishell, G. (2006). Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development. *Nature Reviews Neuroscience, 7*(10), 772-783.

Fuchigami, T., Itokazu, Y., & Yu, R. K. (2024). Ganglioside GD3 regulates neural stem cell quiescence and controls postnatal neurogenesis. *Glia, 72*(1), 167-183.

Fuchs, E., Tumbar, T., & Guasch, G. (2004). Socializing with the neighbors: stem cells and their niche. *Cell, 116*(6), 769-778.

Fueyo, R., Judd, J., Feschotte, C., & Wysocka, J. (2022). Roles of transposable elements in the regulation of mammalian transcription. *Nature reviews Molecular cell biology, 23*(7), 481-497.

Fukai, J., Yokote, H., Yamanaka, R., Arao, T., Nishio, K., & Itakura, T. (2008). EphA4 promotes cell proliferation and migration through a novel EphA4-FGFR1 signaling pathway in the human glioma U251 cell line. *Molecular cancer therapeutics, 7*(9), 2768-2778.

Gan, L., Sun, J., Yang, S., Zhang, X., Chen, W., Sun, Y., . . . Li, A. (2020). Chromatin-binding protein PHF6 regulates activity-dependent transcriptional networks to promote hunger response. *Cell reports, 30*(11), 3717-3728. e3716.

Gao, Z., Ure, K., Ables, J. L., Lagace, D. C., Nave, K.-A., Goebbels, S., . . . Hsieh, J. (2009). Neurod1 is essential for the survival and maturation of adult-born neurons. *Nature neuroscience, 12*(9), 1090-1092.

Garber, K. B., Visootsak, J., & Warren, S. T. (2008). Fragile X syndrome. *European journal of human genetics, 16*(6), 666-672.

García-Frigola, C., Carreres, M. I., Vegar, C., Mason, C., & Herrera, E. (2008). Zic2 promotes axonal divergence at the optic chiasm midline by EphB1-dependent and-independent mechanisms.

Gaspard, N., & Vanderhaeghen, P. (2010). Mechanisms of neural specification from embryonic stem cells. *Current opinion in neurobiology, 20*(1), 37-43.

Gecz, J., Turner, G., Nelson, J., & Partington, M. (2006). The Borjeson-Forssman-Lehman syndrome (BFLS, MIM #301900). *Eur J Hum Genet, 14*(12), 1233-1237. doi:10.1038/sj.ejhg.5201639

Genander, M., & Frisén, J. (2010). Ephrins and Eph receptors in stem cells and cancer. *Current opinion in cell biology, 22*(5), 611-616.

Georgakopoulos, A., Litterst, C., Ghersi, E., Baki, L., Xu, C., Serban, G., & Robakis, N. K. (2006). Metalloproteinase/Presenilin1 processing of ephrinB regulates EphB-induced Src phosphorylation and signaling. *The EMBO journal, 25*(6), 1242-1252.

Gerstmann, K., & Zimmer, G. (2018). The role of the Eph/ephrin family during cortical development and cerebral malformations. *Medical Research Archives, 6*(3).

Gigek, C., Chen, E., Ota, V., Maussion, G., Peng, H., Vaillancourt, K., . . . Vasuta, C. (2015). A molecular model for neurodevelopmental disorders. *Translational Psychiatry, 5*(5), e565-e565.

Gil-Ranedo, J., Gonzaga, E., Jaworek, K. J., Berger, C., Bossing, T., & Barros, C. S. (2019). STRIPAK Members Orchestrate Hippo and Insulin Receptor Signaling to Promote Neural Stem Cell Reactivation. *Cell reports, 27*(10), 2921-2933. e2925.

Goldshmit, Y., Galea, M. P., Bartlett, P. F., & Turnley, A. M. (2006). EphA4 regulates central nervous system vascular formation. *Journal of Comparative Neurology, 497*(6), 864-875.

Goldshmit, Y., Galea, M. P., Wise, G., Bartlett, P. F., & Turnley, A. M. (2004). Axonal regeneration and lack of astrocytic gliosis in EphA4-deficient mice. *Journal of Neuroscience, 24*(45), 10064-10073.

Gómez-Gaviro, M. V., Scott, C. E., Sesay, A. K., Matheu, A., Booth, S., Galichet, C., & Lovell-Badge, R. (2012). Betacellulin promotes cell proliferation in the neural stem cell niche

and stimulates neurogenesis. *Proceedings of the National Academy of Sciences, 109*(4), 1317-1322.

Gönczy, P. (2008). Mechanisms of asymmetric cell division: flies and worms pave the way. *Nature reviews Molecular cell biology, 9*(5), 355-366.

Gong, X., Zhang, K., Wang, Y., Wang, J., Cui, Y., Li, S., & Luo, Y. (2013). MicroRNA-130b targets Fmr1 and regulates embryonic neural progenitor cell proliferation and differentiation. *Biochemical and biophysical research communications, 439*(4), 493-500.

Gordon-Lipkin, E., & Peacock, G. (2019). The spectrum of developmental disability with Zika exposure: what is known, what is unknown, and implications for clinicians. *Journal of Developmental & Behavioral Pediatrics, 40*(5), 387-395.

Götz, M., & Huttner, W. B. (2005). The cell biology of neurogenesis. *Nature reviews Molecular cell biology, 6*(10), 777-788.

Govek, E.-E., Newey, S. E., & Van Aelst, L. (2005). The role of the Rho GTPases in neuronal development. *Genes & development, 19*(1), 1-49.

Graham, V., Khudyakov, J., Ellis, P., & Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron, 39*(5), 749-765.

Grandjean, P., & Landrigan, P. J. (2006). Developmental neurotoxicity of industrial chemicals. *The Lancet, 368*(9553), 2167-2178.

Greig, L. C., Woodworth, M. B., Galazo, M. J., Padmanabhan, H., & Macklis, J. D. (2013). Molecular logic of neocortical projection neuron specification, development and diversity. *Nature Reviews Neuroscience, 14*(11), 755-769.

Groszer, M., Erickson, R., Scripture-Adams, D. D., Dougherty, J. D., Le Belle, J., Zack, J. A., . . . Wu, H. (2006). PTEN negatively regulates neural stem cell self-renewal by modulating

G0-G1 cell cycle entry. *Proceedings of the National Academy of Sciences, 103*(1), 111-116.

Guillemot, F. (2007). Cell fate specification in the mammalian telencephalon. *Progress in neurobiology, 83*(1), 37-52.

Guo, M., Jan, L. Y., & Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron, 17*(1), 27-41.

Hara, Y., Nomura, T., Yoshizaki, K., Frisén, J., & Osumi, N. (2010). Impaired hippocampal neurogenesis and vascular formation in ephrin-A5-deficient mice. *Stem cells, 28*(5), 974-983.

Harada, Y., Yamada, M., Imayoshi, I., Kageyama, R., Suzuki, Y., Kuniya, T., . . . Gotoh, Y. (2021). Cell cycle arrest determines adult neural stem cell ontogeny by an embryonic Notch-nonoscillatory Hey1 module. *Nature communications, 12*(1), 6562.

Hatten, M. E. (1999). Central nervous system neuronal migration. *Annual review of neuroscience, 22*(1), 511-539.

Haubensak, W., Attardo, A., Denk, W., & Huttner, W. B. (2004). Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proceedings of the National Academy of Sciences, 101*(9), 3196-3201.

He, Y., Zeng, J., Yu, J., He, M., Cui, C., Zhao, Z., . . . Fang, Y. (2005). EphB2-Fc promotes activation of endogenous neural stem cells after cerebral cortex infarction: experimental with hypertensive rats. *Zhonghua yi xue za zhi, 85*(34), 2395-2399.

Herath, N. I., & Boyd, A. W. (2010). The role of Eph receptors and ephrin ligands in colorectal cancer. *International Journal of Cancer, 126*(9), 2003-2011.

Hernandez-Corchado, A., & Najafabadi, H. S. (2022). Toward a base-resolution panorama of the in vivo impact of cytosine methylation on transcription factor binding. *Genome Biol, 23*(1), 151. doi:10.1186/s13059-022-02713-y

Hibbard, E. D., & Smithells, R. (1965). Folio acid metabolism and human embryopathy.

Hickman, A. B., & Dyda, F. (2016). DNA transposition at work. *Chemical reviews, 116*(20), 12758-12784.

Hinnell, C., Hurt, C. S., Landau, S., Brown, R. G., Samuel, M., & Group, P. P. S. (2012). Nonmotor versus motor symptoms: how much do they matter to health status in Parkinson's disease? *Movement Disorders, 27*(2), 236-241.

Hirabayashi, Y., & Gotoh, Y. (2005). Stage-dependent fate determination of neural precursor cells in mouse forebrain. *Neuroscience research, 51*(4), 331-336.

Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N., & Gotoh, Y. (2004). The Wnt/β-catenin pathway directs neuronal differentiation of cortical neural precursor cells.

Hirai, H., Maru, Y., Hagiwara, K., Nishida, J., & Takaku, F. (1987). A novel putative tyrosine kinase receptor encoded by the eph gene. *Science, 238*(4834), 1717-1720.

Hirvonen, M., Ojala, R., Korhonen, P., Haataja, P., Eriksson, K., Rantanen, K., . . . Tammela, O. (2017). Intellectual disability in children aged less than seven years born moderately and late preterm compared with very preterm and term-born children–a nationwide birth cohort study. *Journal of Intellectual Disability Research, 61*(11), 1034-1054.

Hitoshi, S., Alexson, T., Tropepe, V., Donoviel, D., Elia, A. J., Nye, J. S., . . . Van Der Kooy, D. (2002). Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes & development, 16*(7), 846-858.

Holmberg, J., Armulik, A., Senti, K.-A., Edoff, K., Spalding, K., Momma, S., . . . Frisén, J. (2005). Ephrin-A2 reverse signaling negatively regulates neural progenitor proliferation and neurogenesis. *Genes & development, 19*(4), 462-471.

Homman-Ludiye, J., Kwan, W. C., De Souza, M. J., Rodger, J., & Bourne, J. A. (2017). Ephrin-A2 regulates excitatory neuron differentiation and interneuron migration in the developing neocortex. *Scientific reports, 7*(1), 11813.

Hoogenraad, C. C., Milstein, A. D., Ethell, I. M., Henkemeyer, M., & Sheng, M. (2005). GRIP1 controls dendrite morphogenesis by regulating EphB receptor trafficking. *Nature neuroscience, 8*(7), 906-915.

Hoxha, B., Hoxha, M., Domi, E., Gervasoni, J., Persichilli, S., Malaj, V., & Zappacosta, B. (2021). Folic acid and autism: a systematic review of the current state of knowledge. *Cells, 10*(8), 1976.

Hu, Y., & Smyth, G. K. (2009). ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *Journal of immunological methods, 347*(1-2), 70-78.

Imayoshi, I., Sakamoto, M., Yamaguchi, M., Mori, K., & Kageyama, R. (2010). Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. *Journal of Neuroscience, 30*(9), 3489-3498.

Inaba, M., & Yamashita, Y. M. (2012). Asymmetric stem cell division: precision for robustness. *Cell Stem Cell, 11*(4), 461-469.

Iossifov, I., O'roak, B. J., Sanders, S. J., Ronemus, M., Krumm, N., Levy, D., . . . Patterson, K. E. (2014). The contribution of de novo coding mutations to autism spectrum disorder. *Nature, 515*(7526), 216-221.

Irie, F., & Yamaguchi, Y. (2002). EphB receptors regulate dendritic spine development via intersectin, Cdc42 and N-WASP. *Nature neuroscience, 5*(11), 1117-1118.

Islam, M. M., & Zhang, C.-L. (2015). TLX: A master regulator for neural stem cell maintenance and neurogenesis. *Biochimica Et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 1849*(2), 210-216.

Israsena, N., Hu, M., Fu, W., Kan, L., & Kessler, J. A. (2004). The presence of FGF2 signaling determines whether β-catenin exerts effects on proliferation or neuronal differentiation of neural stem cells. *Developmental biology, 268*(1), 220-231.

Ito, K., & Suda, T. (2014). Metabolic requirements for the maintenance of self-renewing stem cells. *Nature reviews Molecular cell biology, 15*(4), 243-256.

Iwase, S., Brookes, E., Agarwal, S., Badeaux, A. I., Ito, H., Vallianatos, C. N., . . . Thompson, A. (2016). A mouse model of X-linked intellectual disability associated with impaired removal of histone methylation. *Cell reports, 14*(5), 1000-1009.

Jahani-Asl, A., Cheng, C., Zhang, C., & Bonni, A. (2016). Pathogenesis of Borjeson-Forssman-Lehmann syndrome: Insights from PHF6 function. *Neurobiol Dis, 96*, 227-235. doi:10.1016/j.nbd.2016.09.011

Jean, F., Stuart, A., & Tarailo-Graovac, M. (2020). Dissecting the genetic and etiological causes of primary microcephaly. *Frontiers in Neurology, 11*, 570830.

Jeon, S. J., Kim, J.-W., Kim, K. C., Han, S. M., Go, H. S., Seo, J. E., . . . Song, M.-R. (2014). Translational regulation of NeuroD1 expression by FMRP: involvement in glutamatergic neuronal differentiation of cultured rat primary neural progenitor cells. *Cellular and molecular neurobiology, 34*, 297-305.

Jing, X., Miwa, H., Sawada, T., Nakanishi, I., Kondo, T., Miyajima, M., & Sakaguchi, K. (2012). Ephrin-A1-mediated dopaminergic neurogenesis and angiogenesis in a rat model of Parkinson's disease. *PloS one, 7*(2), e32019.

Jolly, L. A., Homan, C. C., Jacob, R., Barry, S., & Gecz, J. (2013). The UPF3B gene, implicated in intellectual disability, autism, ADHD and childhood onset schizophrenia regulates neural progenitor cell behaviour and neuronal outgrowth. *Human molecular genetics, 22*(23), 4673-4687.

Jones, K., & Smith, D. (1973). Recognition of the fetal alcohol syndrome in early infancy. *The Lancet, 302*(7836), 999-1001.

Kadison, S. R., Mäkinen, T., Klein, R., Henkemeyer, M., & Kaprielian, Z. (2006). EphB receptors and ephrin-B3 regulate axon guidance at the ventral midline of the embryonic mouse spinal cord. *Journal of Neuroscience, 26*(35), 8909-8914.

Kageyama, R., Shimojo, H., & Imayoshi, I. (2015). Dynamic expression and roles of Hes factors in neural development. *Cell and tissue research, 359*, 125-133.

Kamburov, A., Pentchev, K., Galicka, H., Wierling, C., Lehrach, H., & Herwig, R. (2011). ConsensusPathDB: toward a more complete picture of cell biology. *Nucleic Acids Res, 39*(Database issue), D712-717. doi:10.1093/nar/gkq1156

Kamelska-Sadowska, A. M., Wojtkiewicz, J., & Kowalski, I. M. (2019). Review of the Current Knowledge on the Role of Stem Cell Transplantation in Neurorehabilitation. *BioMed research international, 2019*.

Kania, A., & Jessell, T. M. (2003). Topographic motor projections in the limb imposed by LIM homeodomain protein regulation of ephrin-A: EphA interactions. *Neuron, 38*(4), 581-596.

Kania, A., & Klein, R. (2016). Mechanisms of ephrin–Eph signalling in development, physiology and disease. *Nature reviews Molecular cell biology, 17*(4), 240-256.

Kasper, E. M., Larkman, A. U., Lübke, J., & Blakemore, C. (1994). Pyramidal neurons in layer 5 of the rat visual cortex. II. Development of electrophysiological properties. *Journal of Comparative Neurology, 339*(4), 475-494.

Katakowski, M., Zhang, Z., decarvalho, A. C., & Chopp, M. (2005). EphB2 induces proliferation and promotes a neuronal fate in adult subventricular neural precursor cells. *Neuroscience letters, 385*(3), 204-209.

Kayser, M. S., Nolt, M. J., & Dalva, M. B. (2008). EphB receptors couple dendritic filopodia motility to synapse formation. *Neuron, 59*(1), 56-69.

Kim, D., Langmead, B., & Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nat Methods, 12*(4), 357-360. doi:10.1038/nmeth.3317

Kim, J., Guermah, M., & Roeder, R. G. (2010). The human PAF1 complex acts in chromatin transcription elongation both independently and cooperatively with SII/TFIIS. *Cell, 140*(4), 491-503.

Kim, K., Lee, S.-A., & Park, D. (2019). Emerging roles of ephexins in physiology and disease. *Cells, 8*(2), 87.

Kim, N. H., Livi, C. B., Yew, P. R., & Boyer, T. G. (2016). Mediator subunit Med12 contributes to the maintenance of neural stem cell identity. *BMC Dev Biol, 16*(1), 17. doi:10.1186/s12861-016-0114-0

Klein, R. (2009). Bidirectional modulation of synaptic functions by Eph/ephrin signaling. *Nature neuroscience, 12*(1), 15-20.

Klein, R. (2012). Eph/ephrin signalling during development. *Development, 139*(22), 4105-4109.

Kleine-Kohlbrecher, D., Christensen, J., Vandamme, J., Abarrategui, I., Bak, M., Tommerup, N., . . . Salcini, A. E. (2010). A functional link between the histone demethylase PHF8 and the transcription factor ZNF711 in X-linked mental retardation. *Molecular cell, 38*(2), 165-178.

Knöll, B., & Drescher, U. (2004). Src family kinases are involved in EphA receptor-mediated retinal axon guidance. *Journal of Neuroscience, 24*(28), 6248-6257.

Kobayashi, M., Kishida, S., Fukui, A., Michiue, T., Miyamoto, Y., Okamoto, T., . . . Kikuchi, A. (2002). Nuclear localization of Duplin, a β-catenin-binding protein, is essential for its inhibitory activity on the Wnt signaling pathway. *Journal of Biological Chemistry, 277*(8), 5816-5822.

Korb, E., Herre, M., Zucker-Scharff, I., Gresack, J., Allis, C. D., & Darnell, R. B. (2017). Excess translation of epigenetic regulators contributes to fragile X syndrome and is alleviated by Brd4 inhibition. *Cell, 170*(6), 1209-1223. e1220.

Krumm, N., O'Roak, B. J., Shendure, J., & Eichler, E. E. (2014). A de novo convergence of autism genetics and molecular neuroscience. *Trends in neurosciences, 37*(2), 95-105.

Kuechler, A., Willemsen, M. H., Albrecht, B., Bacino, C. A., Bartholomew, D. W., van Bokhoven, H., . . . Cremer, K. (2015). De novo mutations in beta-catenin (CTNNB1) appear to be a frequent cause of intellectual disability: expanding the mutational and clinical spectrum. *Human genetics, 134*, 97-109.

Kullander, K., & Klein, R. (2002). Mechanisms and functions of Eph and ephrin signalling. *Nature reviews Molecular cell biology, 3*(7), 475-486.

Kutsche, K., Yntema, H., Brandt, A., Jantke, I., Gerd Nothwang, H., Orth, U., . . . Fryns, J.-P. (2000). Mutations in ARHGEF6, encoding a guanine nucleotide exchange factor for Rho GTPases, in patients with X-linked mental retardation. *Nature genetics, 26*(2), 247-250.

Kuwabara, T., Hsieh, J., Muotri, A., Yeo, G., Warashina, M., Lie, D. C., . . . Gage, F. H. (2009). Wnt-mediated activation of NeuroD1 and retro-elements during adult neurogenesis. *Nature neuroscience, 12*(9), 1097-1105.

Lackmann, M., & Boyd, A. W. (2003). Ephrins and their receptors.

Lai, K., Kaspar, B. K., Gage, F. H., & Schaffer, D. V. (2003). Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. *Nature neuroscience, 6*(1), 21-27.

Lamprecht, R., & LeDoux, J. (2004). Structural plasticity and memory. *Nature Reviews Neuroscience, 5*(1), 45-54.

Landt, S. G., Marinov, G. K., Kundaje, A., Kheradpour, P., Pauli, F., Batzoglou, S., . . . Snyder, M. (2012). ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. *Genome Res, 22*(9), 1813-1831. doi:10.1101/gr.136184.111

Laussu, J., Khuong, A., Gautrais, J., & Davy, A. (2014). Beyond boundaries—Eph: ephrin signaling in neurogenesis. *Cell adhesion & migration, 8*(4), 349-359.

Ledda, F., & Paratcha, G. (2017). Mechanisms regulating dendritic arbor patterning. *Cellular and Molecular Life Sciences, 74*, 4511-4537.

Leonard, H., & Wen, X. (2002). The epidemiology of mental retardation: challenges and opportunities in the new millennium. *Mental retardation and developmental disabilities research reviews, 8*(3), 117-134.

Levine, S. Z., Kodesh, A., Viktorin, A., Smith, L., Uher, R., Reichenberg, A., & Sandin, S. (2018). Association of maternal use of folic acid and multivitamin supplements in the

periods before and during pregnancy with the risk of autism spectrum disorder in offspring. *JAMA psychiatry, 75*(2), 176-184.

Levitt, P., Cooper, M. L., & Rakic, P. (1983). Early divergence and changing proportions of neuronal and glial precursor cells in the primate cerebral ventricular zone. *Developmental biology, 96*(2), 472-484.

Li, H., Zhong, X., Chau, K. F., Santistevan, N. J., Guo, W., Kong, G., . . . Chi, J. (2014). Cell cycle-linked MeCP2 phosphorylation modulates adult neurogenesis involving the Notch signalling pathway. *Nature communications, 5*(1), 5601.

Liang, L.-Y., Patel, O., Janes, P. W., Murphy, J. M., & Lucet, I. S. (2019). Eph receptor signalling: from catalytic to non-catalytic functions. *Oncogene, 38*(39), 6567-6584.

Lie, D.-C., Colamarino, S. A., Song, H.-J., Désiré, L., Mira, H., Consiglio, A., . . . Dearie, A. R. (2005). Wnt signalling regulates adult hippocampal neurogenesis. *Nature, 437*(7063), 1370-1375.

Lin, P.-I., Chien, Y.-L., Wu, Y.-Y., Chen, C.-H., Gau, S. S.-F., Huang, Y.-S., . . . Chiu, Y.-N. (2012). The WNT2 gene polymorphism associated with speech delay inherent to autism. *Research in developmental disabilities, 33*(5), 1533-1540.

Lisabeth, E. M., Falivelli, G., & Pasquale, E. B. (2013). Eph receptor signaling and ephrins. *Cold Spring Harbor perspectives in biology, 5*(9), a009159.

Liu, J., Wu, X., Zhang, H., Pfeifer, G. P., & Lu, Q. (2017). Dynamics of RNA polymerase II pausing and bivalent histone H3 methylation during neuronal differentiation in brain development. *Cell reports, 20*(6), 1307-1318.

Liu, X., & Fuentes, E. J. (2019). Emerging themes in PDZ domain signaling: structure, function, and inhibition. *International review of cell and molecular biology, 343*, 129-218.

Liu, Z., Li, F., Ruan, K., Zhang, J., Mei, Y., Wu, J., & Shi, Y. (2014). Structural and functional insights into the human Börjeson-Forssman-Lehmann syndrome-associated protein PHF6. *Journal of Biological Chemistry, 289*(14), 10069-10083.

Llorens-Bobadilla, E., Zhao, S., Baser, A., Saiz-Castro, G., Zwadlo, K., & Martin-Villalba, A. (2015). Single-cell transcriptomics reveals a population of dormant neural stem cells that become activated upon brain injury. *Cell Stem Cell, 17*(3), 329-340.

Locke, C. (2018). Effects of Localized EphB2 Stimulation on Dendritic Filopodia of Hippocampal Neurons.

Logan, C. Y., & Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol., 20*, 781-810.

Louvi, A., & Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nature Reviews Neuroscience, 7*(2), 93-102.

Lovestone, S., Killick, R., Di Forti, M., & Murray, R. (2007). Schizophrenia as a GSK-3 dysregulation disorder. *Trends in neurosciences, 30*(4), 142-149.

Lower, K. M., Solders, G., Bondeson, M. L., Nelson, J., Brun, A., Crawford, J., . . . Gecz, J. (2004). 1024C> T (R342X) is a recurrent PHF6 mutation also found in the original Borjeson-Forssman-Lehmann syndrome family. *Eur J Hum Genet, 12*(10), 787-789. doi:10.1038/sj.ejhg.5201228

Lower, K. M., Turner, G., Kerr, B. A., Mathews, K. D., Shaw, M. A., Gedeon, A. K., . . . Gecz, J. (2002). Mutations in PHF6 are associated with Borjeson-Forssman-Lehmann syndrome. *Nat Genet, 32*(4), 661-665. doi:10.1038/ng1040

Luo, L., & O'Leary, D. D. (2005). Axon retraction and degeneration in development and disease. *Annu. Rev. Neurosci., 28*, 127-156.

Luo, Y., Shan, G., Guo, W., Smrt, R. D., Johnson, E. B., Li, X., . . . Barkho, B. Z. (2010). Fragile x mental retardation protein regulates proliferation and differentiation of adult neural stem/progenitor cells. *PLoS genetics, 6*(4).

Machold, R., Hayashi, S., Rutlin, M., Muzumdar, M. D., Nery, S., Corbin, J. G., . . . Rubin, L. L. (2003). Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. *Neuron, 39*(6), 937-950.

Machon, O., Backman, M., Machonova, O., Kozmik, Z., Vacik, T., Andersen, L., & Krauss, S. (2007). A dynamic gradient of Wnt signaling controls initiation of neurogenesis in the mammalian cortex and cellular specification in the hippocampus. *Developmental biology, 311*(1), 223-237.

Machon, O., Van Den Bout, C., Backman, M., Kemler, R., & Krauss, S. (2003). Role of β-catenin in the developing cortical and hippocampal neuroepithelium. *Neuroscience, 122*(1), 129-143.

Macůrek, L., Lindqvist, A., Lim, D., Lampson, M. A., Klompmaker, R., Freire, R., . . . Medema, R. H. (2008). Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. *Nature, 455*(7209), 119-123.

Malatesta, P., Hartfuss, E., & Götz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development, 127*(24), 5253-5263.

Mao, Y., Ge, X., Frank, C. L., Madison, J. M., Koehler, A. N., Doud, M. K., . . . Singh, K. K. (2009). Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3β/β-catenin signaling. *Cell, 136*(6), 1017-1031.

Martin, P., Yang, X., Robin, N., Lam, E., Rabinowitz, J., Erdman, C., . . . Kwok, P. (2013). A rare WNT1 missense variant overrepresented in ASD leads to increased Wnt signal pathway activation. *Translational psychiatry, 3*(9), e301-e301.

Marui, T., Funatogawa, I., Koishi, S., Yamamoto, K., Matsumoto, H., Hashimoto, O., . . . Kasai, K. (2010). Association between autism and variants in the wingless-type MMTV integration site family member 2 (WNT2) gene. *International Journal of Neuropsychopharmacology, 13*(4), 443-449.

May, M., Hwang, K. S., Miles, J., Williams, C., Niranjan, T., Kahler, S. G., . . . Kim, C. H. (2015). ZC4H2, an XLID gene, is required for the generation of a specific subset of CNS interneurons. *Hum Mol Genet, 24*(17), 4848-4861. doi:10.1093/hmg/ddv208

May, P. A., & Gossage, J. P. (2001). Estimating the prevalence of fetal alcohol syndrome: A summary. *Alcohol research & health, 25*(3), 159.

McClintock, B. (1950). The origin and behavior of mutable loci in maize. *Proceedings of the National Academy of Sciences, 36*(6), 344-355.

McLaren, J., & Bryson, S. E. (1987). Review of recent epidemiological studies of mental retardation: prevalence, associated disorders, and etiology. *American journal of mental retardation: AJMR, 92*(3), 243-254.

McMahon, A. P., & Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell, 62*(6), 1073-1085.

McRae, H. M., Eccles, S., Whitehead, L., Alexander, W. S., Gécz, J., Thomas, T., & Voss, A. K. (2020). Downregulation of the GHRH/GH/IGF1 axis in a mouse model of Börjeson-Forssman-Lehman syndrome. *Development, 147*(21), dev187021.

McRae, H. M., Garnham, A. L., Hu, Y., Witkowski, M. T., Corbett, M. A., Dixon, M. P., . . . Thomas, T. (2019). PHF6 regulates hematopoietic stem and progenitor cells and its loss synergizes with expression of TLX3 to cause leukemia. *Blood, 133*(16), 1729-1741. doi:10.1182/blood-2018-07-860726

Meltzer, S., & Chen, C. (2016). Balancing dendrite morphogenesis and neuronal migration during cortical development. *Journal of Neuroscience, 36*(42), 10726-10728.

Metz, E. P., Wuebben, E. L., Wilder, P. J., Cox, J. L., Datta, K., Coulter, D., & Rizzino, A. (2020). Tumor quiescence: elevating SOX2 in diverse tumor cell types downregulates a broad spectrum of the cell cycle machinery and inhibits tumor growth. *BMC cancer, 20*, 1-14.

Ming, G.-l., & Song, H. (2005). Adult neurogenesis in the mammalian central nervous system. *Annu. Rev. Neurosci., 28*, 223-250.

Ming, G.-l., & Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron, 70*(4), 687-702.

Miyagi, S., Sroczynska, P., Kato, Y., Nakajima-Takagi, Y., Oshima, M., Rizq, O., . . . Iwama, A. (2019). The chromatin-binding protein Phf6 restricts the self-renewal of hematopoietic stem cells. *Blood, 133*(23), 2495-2506. doi:10.1182/blood.2019000468

Mizutani, K.-i., Yoon, K., Dang, L., Tokunaga, A., & Gaiano, N. (2007). Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. *Nature, 449*(7160), 351-355.

Mohan, V., Sinha, R. A., Pathak, A., Rastogi, L., Kumar, P., Pal, A., & Godbole, M. M. (2012). Maternal thyroid hormone deficiency affects the fetal neocorticogenesis by reducing the

proliferating pool, rate of neurogenesis and indirect neurogenesis. *Experimental neurology, 237*(2), 477-488.

Molyneaux, B. J., Arlotta, P., Menezes, J. R., & Macklis, J. D. (2007). Neuronal subtype specification in the cerebral cortex. *Nature Reviews Neuroscience, 8*(6), 427-437.

Morrison, S. J., & Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature, 441*(7097), 1068-1074.

Munro, N., Smithells, R., Sheppard, S., Holzel, H., & Jones, G. (1987). Temporal relations between maternal rubella and congenital defects. *The Lancet, 330*(8552), 201-204.

Muotri, A. R., Marchetto, M. C., Coufal, N. G., Oefner, R., Yeo, G., Nakashima, K., & Gage, F. H. (2010). L1 retrotransposition in neurons is modulated by MeCP2. *Nature, 468*(7322), 443-446.

Muroyama, Y., Kondoh, H., & Takada, S. (2004). Wnt proteins promote neuronal differentiation in neural stem cell culture. *Biochemical and biophysical research communications, 313*(4), 915-921.

Nakada, M., Anderson, E. M., Demuth, T., Nakada, S., Reavie, L. B., Drake, K. L., . . . Berens, M. E. (2010). The phosphorylation of ephrin-B2 ligand promotes glioma cell migration and invasion. *International Journal of Cancer, 126*(5), 1155-1165.

Nakada, M., Drake, K. L., Nakada, S., Niska, J. A., & Berens, M. E. (2006). Ephrin-B3 ligand promotes glioma invasion through activation of Rac1. *Cancer research, 66*(17), 8492-8500.

Nakada, M., Niska, J. A., Miyamori, H., McDonough, W. S., Wu, J., Sato, H., & Berens, M. E. (2004). The phosphorylation of EphB2 receptor regulates migration and invasion of human glioma cells. *Cancer research, 64*(9), 3179-3185.

Nasser, M., Ballout, N., Mantash, S., Bejjani, F., Najdi, F., Ramadan, N., . . . Kobeissy, F. (2018). Transplantation of embryonic neural stem cells and differentiated cells in a controlled cortical impact (CCI) model of adult mouse somatosensory cortex. *Frontiers in Neurology, 9*, 895.

Navarro Quiroz, E., Navarro Quiroz, R., Ahmad, M., Gomez Escorcia, L., Villarreal, J. L., Fernandez Ponce, C., & Aroca Martinez, G. (2018). Cell signaling in neuronal stem cells. *Cells, 7*(7), 75.

Nicholls, R. D. (2000). The impact of genomic imprinting for neurobehavioral and developmental disorders. *The Journal of clinical investigation, 105*(4), 413-418.

Niklison-Chirou, M. V., Agostini, M., Amelio, I., & Melino, G. (2020). Regulation of adult neurogenesis in mammalian brain. *International Journal of Molecular Sciences, 21*(14), 4869.

Niu, W., Zou, Y., Shen, C., & Zhang, C.-L. (2011). Activation of postnatal neural stem cells requires nuclear receptor TLX. *Journal of Neuroscience, 31*(39), 13816-13828.

Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S., & Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature, 409*(6821), 714-720.

Noctor, S. C., Martínez-Cerdeño, V., & Kriegstein, A. R. (2007). Contribution of intermediate progenitor cells to cortical histogenesis. *Archives of neurology, 64*(5), 639-642.

Noren, N. K., & Pasquale, E. B. (2004). Eph receptor–ephrin bidirectional signals that target Ras and Rho proteins. *Cellular signalling, 16*(6), 655-666.

North, H. A., Zhao, X., Kolk, S. M., Clifford, M. A., Ziskind, D. M., & Donoghue, M. J. (2009). Promotion of proliferation in the developing cerebral cortex by EphA4 forward signaling. *Development, 136*(14), 2467-2476.

O'Roak, B. J., Vives, L., Girirajan, S., Karakoc, E., Krumm, N., Coe, B. P., . . . Smith, J. D. (2012). Sporadic autism exomes reveal a highly interconnected protein network of de novo mutations. *Nature, 485*(7397), 246-250.

Obernier, K., & Alvarez-Buylla, A. (2019). Neural stem cells: origin, heterogeneity and regulation in the adult mammalian brain. *Development, 146*(4), dev156059.

Ogita, H., Kunimoto, S., Kamioka, Y., Sawa, H., Masuda, M., & Mochizuki, N. (2003). EphA4-mediated Rho activation via Vsm-RhoGEF expressed specifically in vascular smooth muscle cells. *Circulation research, 93*(1), 23-31.

Oh, S., Boo, K., Kim, J., Baek, S. A., Jeon, Y., You, J., . . . Lee, J. M. (2020). The chromatin-binding protein PHF6 functions as an E3 ubiquitin ligase of H2BK120 via H2BK12Ac recognition for activation of trophectodermal genes. *Nucleic acids research, 48*(16), 9037-9052.

Ohtsuka, T., Sakamoto, M., Guillemot, F., & Kageyama, R. (2001). Roles of the basic helix-loop-helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain. *Journal of Biological Chemistry, 276*(32), 30467-30474.

Ouyang, J., Yu, W., Liu, J., Zhang, N., Florens, L., Chen, J., . . . Xie, T. (2015). Cyclin-dependent kinase-mediated Sox2 phosphorylation enhances the ability of Sox2 to establish the pluripotent state. *Journal of Biological Chemistry, 290*(37), 22782-22794.

Palma, V., & Altaba, A. R. i. (2004). Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex.

Palma, V., Lim, D. A., Dahmane, N., Sánchez, P., Brionne, T. C., Herzberg, C. D., . . . Altaba, A. R. i. (2005). Sonic hedgehog controls stem cell behavior in the postnatal and adult brain.

Palmer, A., Zimmer, M., Erdmann, K. S., Eulenburg, V., Porthin, A., Heumann, R., . . . Klein, R. (2002). EphrinB phosphorylation and reverse signaling: regulation by Src kinases and PTP-BL phosphatase. *Molecular cell, 9*(4), 725-737.

Pappas, A., & Korzeniewski, S. J. (2016). Long-term cognitive outcomes of birth asphyxia and the contribution of identified perinatal asphyxia to cerebral palsy. *Clinics in perinatology, 43*(3), 559-572.

Park, S. (2013). Brain-region specific apoptosis triggered by Eph/ephrin signaling. *Experimental neurobiology, 22*(3), 143-148.

Park, S. (2013). Brain-region specific apoptosis triggered by Eph/ephrin signaling. *Experimental neurobiology, 22*(3), 143.

Pasquale, E. B. (2005). Eph receptor signalling casts a wide net on cell behaviour. *Nature reviews Molecular cell biology, 6*(6), 462-475.

Pasquale, E. B. (2008). Eph-ephrin bidirectional signaling in physiology and disease. *Cell, 133*(1), 38-52.

Pasquale, E. B. (2010). Eph receptors and ephrins in cancer: bidirectional signalling and beyond. *Nature Reviews Cancer, 10*(3), 165-180.

Pathipati, P., Gorba, T., Scheepens, A., Goffin, V., Sun, Y., & Fraser, M. (2011). Growth hormone and prolactin regulate human neural stem cell regenerative activity. *Neuroscience, 190*, 409-427.

Pawar, A., Somers, P., Verner, R., Antony, C., George, S. S., Pimkin, M., & Paralkar, V. R. (2021). PHF6 Positively Regulates Transcription of Myeloid Differentiation Genes By Binding at Enhancer Regions. *Blood, 138*, 3303.

Penzes, P., Beeser, A., Chernoff, J., Schiller, M. R., Eipper, B. A., Mains, R. E., & Huganir, R. L. (2003). Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. *Neuron, 37*(2), 263-274.

Petersen, M. B., Brondum-Nielsen, K., Hansen, L. K. r. r., & Wulff, K. (1995). Clinical, cytogenetic, and molecular diagnosis of Angelman syndrome: estimated prevalence rate in a Danish country. *American journal of medical genetics, 60*(3).

Philipp, M., & Caron, M. G. (2009). Hedgehog signaling: is Smo a G protein-coupled receptor? *Current biology, 19*(3), R125-R127.

Pohl, A., & Beato, M. (2014). bwtool: a tool for bigWig files. *Bioinformatics, 30*(11), 1618-1619.

Pokholok, D. K., Hannett, N. M., & Young, R. A. (2002). Exchange of RNA polymerase II initiation and elongation factors during gene expression in vivo. *Molecular cell, 9*(4), 799-809.

Qazi, M. A., Vora, P., Venugopal, C., Adams, J., Singh, M., Hu, A., . . . Yang, J. (2018). Cotargeting ephrin receptor tyrosine kinases A2 and A3 in cancer stem cells reduces growth of recurrent glioblastoma. *Cancer research, 78*(17), 5023-5037.

Qin, H., Noberini, R., Huan, X., Shi, J., Pasquale, E. B., & Song, J. (2010). Structural characterization of the EphA4-Ephrin-B2 complex reveals new features enabling Eph-ephrin binding promiscuity. *Journal of Biological Chemistry, 285*(1), 644-654.

Qiu, R., Wang, X., Davy, A., Wu, C., Murai, K., Zhang, H., . . . Lu, Q. (2008). Regulation of neural progenitor cell state by ephrin-B. *The Journal of cell biology, 181*(6), 973-983.

Qiu, W., Song, S., Chen, W., Zhang, J., Yang, H., & Chen, Y. (2019). Hypoxia-induced EPHB2 promotes invasive potential of glioblastoma. *International journal of clinical and experimental pathology, 12*(2), 539.

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics, 26*(6), 841-842. doi:10.1093/bioinformatics/btq033

Qurashi, A., Li, X., & Jin, P. (2012). Fragile X mental retardation protein and stem cells. In *Modeling Fragile X Syndrome* (pp. 157-164): Springer.

Rakic, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *Journal of Comparative Neurology, 145*(1), 61-83.

Rakic, P. (1995). Radial versus tangential migration of neuronal clones in the developing cerebral cortex. *Proceedings of the National Academy of Sciences, 92*(25), 11323-11327.

Rakic, P. (1995). A small step for the cell, a giant leap for mankind: a hypothesis of neocortical expansion during evolution. *Trends in neurosciences, 18*(9), 383-388.

Rallu, M., Machold, R., Gaiano, N., Corbin, J. G., McMahon, A. P., & Fishell, G. (2002). Dorsoventral patterning is established in the telencephalon of mutants lacking both Gli3 and Hedgehog signaling.

Rasool, D., Burban, A., Sharanek, A., Madrigal, A., Hu, J., Yan, K., . . . Thomas, T. (2024). PHF6-mediated transcriptional control of NSC via Ephrin receptors is impaired in the intellectual disability syndrome BFLS. *EMBO reports*, 1-26.

Rasool, D., Soleimani, V. D., & Jahani-Asl, A. (2022). Isolation of Adult Mouse Neural Stem Cells and Assessment of Self-Renewal by ELDA. In *Neuronal Cell Death: Methods and Protocols* (pp. 343-354): Springer.

Reynolds, N., Latos, P., Hynes-Allen, A., Loos, R., Leaford, D., O'Shaughnessy, A., . . . Kalkan, T. (2012). NuRD suppresses pluripotency gene expression to promote transcriptional heterogeneity and lineage commitment. *Cell Stem Cell, 10*(5), 583-594.

Ricard, J., Salinas, J., Garcia, L., & Liebl, D. J. (2006). EphrinB3 regulates cell proliferation and survival in adult neurogenesis. *Molecular and Cellular Neuroscience, 31*(4), 713-722.

Rohatgi, R., Milenkovic, L., & Scott, M. P. (2007). Patched1 regulates hedgehog signaling at the primary cilium. *Science, 317*(5836), 372-376.

Rondón, A. G., Gallardo, M., García-Rubio, M., & Aguilera, A. (2004). Molecular evidence indicating that the yeast PAF complex is required for transcription elongation. *EMBO reports, 5*(1), 47-53.

Rooms, L., Reyniers, E., & Kooy, R. F. (2005). Subtelomeric rearrangements in the mentally retarded: a comparison of detection methods. *Human mutation, 25*(6), 513-524.

Ropers, H. H. (2010). Genetics of early onset cognitive impairment. *Annual review of genomics and human genetics, 11*, 161-187.

Sacco, R., Cacci, E., & Novarino, G. (2018). Neural stem cells in neuropsychiatric disorders. *Current opinion in neurobiology, 48*, 131-138.

Sahin, M., Greer, P. L., Lin, M. Z., Poucher, H., Eberhart, J., Schmidt, S., . . . Cowan, C. W. (2005). Eph-dependent tyrosine phosphorylation of ephexin1 modulates growth cone collapse. *Neuron, 46*(2), 191-204.

Sahoo, A. R., & Buck, M. (2021). Structural and functional insights into the transmembrane domain association of eph receptors. *International Journal of Molecular Sciences, 22*(16), 8593.

Sakamoto, A., Ishibashi-Ueda, H., Sugamoto, Y., Higashikata, T., Miyamoto, S., Kawashiri, M.-a., . . . Fujino, N. (2008). Expression and function of ephrin-B1 and its cognate receptor EphB2 in human atherosclerosis: from an aspect of chemotaxis. *Clinical science, 114*(10), 643-650.

Sakamoto, I., Kishida, S., Fukui, A., Kishida, M., Yamamoto, H., Hino, S.-i., . . . Kikuchi, A. (2000). A novel β-catenin-binding protein inhibits β-catenin-dependent Tcf activation and axis formation. *Journal of Biological Chemistry, 275*(42), 32871-32878.

Salsi, V., & Zappavigna, V. (2006). Hoxd13 and Hoxa13 directly control the expression of the EphA7 Ephrin tyrosine kinase receptor in developing limbs. *Journal of Biological Chemistry, 281*(4), 1992-1999.

Sarkar, A., & Hochedlinger, K. (2013). The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. *Cell Stem Cell, 12*(1), 15-30.

Satterfield-Nash, A. (2017). Health and development at age 19–24 months of 19 children who were born with microcephaly and laboratory evidence of congenital Zika virus infection during the 2015 Zika virus outbreak—Brazil, 2017. *MMWR. Morbidity and mortality weekly report, 66*.

Sawada, T., Arai, D., Jing, X., Miyajima, M., Frank, S. J., & Sakaguchi, K. (2017). Molecular interactions of EphA4, growth hormone receptor, Janus kinase 2, and signal transducer and activator of transcription 5B. *PloS one, 12*(7), e0180785.

Scandella, V., Petrelli, F., Moore, D. L., Braun, S. M., & Knobloch, M. (2023). Neural stem cell metabolism revisited: a critical role for mitochondria. *Trends in Endocrinology & Metabolism*.

Schuurmans, C., Armant, O., Nieto, M., Stenman, J. M., Britz, O., Klenin, N., . . . Tang, H. (2004). Sequential phases of cortical specification involve Neurogenin-dependent and-independent pathways. *The EMBO journal, 23*(14), 2892-2902.

Scotto-Lomassese, S., Nissant, A., Mota, T., Néant-Féry, M., Oostra, B. A., Greer, C. A., . . . Caillé, I. (2011). Fragile X mental retardation protein regulates new neuron differentiation in the adult olfactory bulb. *Journal of Neuroscience, 31*(6), 2205-2215.

Ségaliny, A. I., Tellez-Gabriel, M., Heymann, M.-F., & Heymann, D. (2015). Receptor tyrosine kinases: Characterisation, mechanism of action and therapeutic interests for bone cancers. *Journal of bone oncology, 4*(1), 1-12.

Seiradake, E., Harlos, K., Sutton, G., Aricescu, A. R., & Jones, E. Y. (2010). An extracellular steric seeding mechanism for Eph-ephrin signaling platform assembly. *Nature structural & molecular biology, 17*(4), 398-402.

Sell, G. L., Schaffer, T. B., & Margolis, S. S. (2017). Reducing expression of synapse-restricting protein Ephexin5 ameliorates Alzheimer's-like impairment in mice. *The Journal of Clinical Investigation, 127*(5), 1646-1650.

Selvan, N., George, S., Serajee, F. J., Shaw, M., Hobson, L., Kalscheuer, V., . . . Wells, L. (2018). O-GlcNAc transferase missense mutations linked to X-linked intellectual disability deregulate genes involved in cell fate determination and signaling. *J Biol Chem, 293*(27), 10810-10824. doi:10.1074/jbc.RA118.002583

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., & Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Progress in neurobiology, 106*, 1-16.

Sentürk, A., Pfennig, S., Weiss, A., Burk, K., & Acker-Palmer, A. (2011). Ephrin Bs are essential components of the Reelin pathway to regulate neuronal migration. *Nature, 472*(7343), 356-360.

Shamah, S. M., Lin, M. Z., Goldberg, J. L., Estrach, S., Sahin, M., Hu, L., . . . Debant, A. (2001). EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. *Cell, 105*(2), 233-244.

Shi, X., Finkelstein, A., Wolf, A. J., Wade, P. A., Burton, Z. F., & Jaehning, J. A. (1996). Paf1p, an RNA polymerase II-associated factor in Saccharomyces cerevisiae, may have both positive and negative roles in transcription. *Molecular and cellular biology, 16*(2), 669-676.

Shi, Y., Sun, G., Zhao, C., & Stewart, R. (2008). Neural stem cell self-renewal. *Critical reviews in oncology/hematology, 65*(1), 43-53.

Shimojo, H., Ohtsuka, T., & Kageyama, R. (2008). Oscillations in notch signaling regulate maintenance of neural progenitors. *Neuron, 58*(1), 52-64.

Shimozaki, K. (2014). Sox2 transcription network acts as a molecular switch to regulate properties of neural stem cells. *World journal of stem cells, 6*(4), 485.

Shpyleva, S., Melnyk, S., Pavliv, O., Pogribny, I., & Jill James, S. (2018). Overexpression of LINE-1 retrotransposons in autism brain. *Molecular neurobiology, 55*, 1740-1749.

Shu, Y., Xiao, B., Wu, Q., Liu, T., Du, Y., Tang, H., . . . Li, Y. (2016). The Ephrin-A5/EphA4 interaction modulates neurogenesis and angiogenesis by the p-Akt and p-ERK pathways in a mouse model of TLE. *Molecular neurobiology, 53*(1), 561-576.

Silva-Vargas, V., Delgado, A. C., & Doetsch, F. (2018). Symmetric stem cell division at the heart of adult neurogenesis. *Neuron, 98*(2), 246-248.

Simuni, T., & Sethi, K. (2008). Nonmotor manifestations of Parkinson's disease. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 64*(S2), S65-S80.

Smart, I. H., Dehay, C., Giroud, P., Berland, M., & Kennedy, H. (2002). Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extrastriate cortex in the monkey. *Cerebral cortex, 12*(1), 37-53.

Soleimani, F., Zaheri, F., & Abdi, F. (2014). Long-term neurodevelopmental outcomes after preterm birth. *Iranian Red Crescent Medical Journal, 16*(6).

Soleimani, V. D., Palidwor, G. A., Ramachandran, P., Perkins, T. J., & Rudnicki, M. A. (2013). Chromatin tandem affinity purification sequencing. *Nature protocols, 8*(8), 1525-1534.

Soto-Feliciano, Y. M., Bartlebaugh, J. M. E., Liu, Y., Sanchez-Rivera, F. J., Bhutkar, A., Weintraub, A. S., . . . Hemann, M. T. (2017). PHF6 regulates phenotypic plasticity through chromatin organization within lineage-specific genes. *Genes Dev, 31*(10), 973-989. doi:10.1101/gad.295857.117

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., . . . Buchholz, B. A. (2013). Dynamics of hippocampal neurogenesis in adult humans. *Cell, 153*(6), 1219-1227.

Spana, E. P., & Doe, C. Q. (1996). Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron, 17*(1), 21-26.

St John, J. A., & Key, B. (2001). EphB2 and two of its ligands have dynamic protein expression patterns in the developing olfactory system. *Developmental Brain Research, 126*(1), 43-56.

Steffenburg, S., Gillberg, C. L., Steffenburg, U., & Kyllerman, M. (1996). Autism in Angelman syndrome: a population-based study. *Pediatric neurology, 14*(2), 131-136.

Stepien, B. K., Vaid, S., & Huttner, W. B. (2021). Length of the neurogenic period—a key determinant for the generation of upper-layer neurons during neocortex development and evolution. *Frontiers in cell and developmental biology, 9*, 676911.

Stiles, J., & Jernigan, T. L. (2010). The basics of brain development. *Neuropsychology review, 20*(4), 327-348.

Stuckmann, I., Weigmann, A., Shevchenko, A., Mann, M., & Huttner, W. B. (2001). Ephrin B1 is expressed on neuroepithelial cells in correlation with neocortical neurogenesis. *Journal of Neuroscience, 21*(8), 2726-2737.

Stump, G., Durrer, A., Klein, A.-L., Lütolf, S., Suter, U., & Taylor, V. (2002). Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain. *Mechanisms of development, 114*(1-2), 153-159.

Su, X.-J., Shen, B.-D., Wang, K., Song, Q.-X., Yang, X., Wu, D.-S., . . . Zhu, C. (2022). Roles of the neuron-restrictive silencer factor in the pathophysiological process of the central nervous system. *Frontiers in cell and developmental biology, 10*, 834620.

Sun, T., & Hevner, R. F. (2014). Growth and folding of the mammalian cerebral cortex: from molecules to malformations. *Nature Reviews Neuroscience, 15*(4), 217-232.

Tang, G., Gudsnuk, K., Kuo, S.-H., Cotrina, M. L., Rosoklija, G., Sosunov, A., . . . Yamamoto, A. (2014). Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits. *Neuron, 83*(5), 1131-1143.

Taylor, H., Campbell, J., & Nobes, C. D. (2017). Ephs and ephrins. *Current biology, 27*(3), R90-R95.

Telias, M., & Ben-Yosef, D. (2014). Modeling neurodevelopmental disorders using human pluripotent stem cells. *Stem Cell Reviews and Reports, 10*, 494-511.

Telias, M., Mayshar, Y., Amit, A., & Ben-Yosef, D. (2015). Molecular mechanisms regulating impaired neurogenesis of fragile X syndrome human embryonic stem cells. *Stem cells and development, 24*(20), 2353-2365.

Theil, T., Frain, M., Gilardi-Hebenstreit, P., Flenniken, A., Charnay, P., & Wilkinson, D. G. (1998). Segmental expression of the EphA4 (Sek-1) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of Krox-20. *Development, 125*(3), 443-452.

Theus, M. H., Ricard, J., Bethea, J. R., & Liebl, D. J. (2010). EphB3 limits the expansion of neural progenitor cells in the subventricular zone by regulating p53 during homeostasis and following traumatic brain injury. *Stem cells, 28*(7), 1231-1242.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science, 282*(5391), 1145-1147.

Ting, M.-C., Wu, N. L., Roybal, P. G., Sun, J., Liu, L., Yen, Y., & Maxson Jr, R. E. (2009). EphA4 as an effector of Twist1 in the guidance of osteogenic precursor cells during calvarial bone growth and in craniosynostosis.

Todd, K. L., Baker, K. L., Eastman, M. B., Kolling, F. W., Trausch, A. G., Nelson, C. E., & Conover, J. C. (2017). EphA4 regulates neuroblast and astrocyte organization in a neurogenic niche. *Journal of Neuroscience, 37*(12), 3331-3341.

Todd, M. A., Ivanochko, D., & Picketts, D. J. (2015). PHF6 Degrees of Separation: The Multifaceted Roles of a Chromatin Adaptor Protein. *Genes (Basel), 6*(2), 325-352. doi:10.3390/genes6020325

Todd, M. A., & Picketts, D. J. (2012). PHF6 interacts with the nucleosome remodeling and deacetylation (NuRD) complex. *Journal of proteome research, 11*(8), 4326-4337.

Tong, J. K., Hassig, C. A., Schnitzler, G. R., Kingston, R. E., & Schreiber, S. L. (1998). Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. *Nature, 395*(6705), 917-921.

Tremblay, M. è., Riad, M., Bouvier, D., Murai, K. K., Pasquale, E. B., Descarries, L., & Doucet, G. (2007). Localization of EphA4 in axon terminals and dendritic spines of adult rat hippocampus. *Journal of Comparative Neurology, 501*(5), 691-702.

Tremblay, M. È., Riad, M., Chierzi, S., Murai, K. K., Pasquale, E. B., & Doucet, G. (2009). Developmental course of EphA4 cellular and subcellular localization in the postnatal rat hippocampus. *Journal of Comparative Neurology, 512*(6), 798-813.

Trizzino, M., Kapusta, A., & Brown, C. D. (2018). Transposable elements generate regulatory novelty in a tissue-specific fashion. *BMC genomics, 19*, 1-12.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P. C., . . . Schütz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nature genetics, 23*(1), 99-103.

Tu, Y., He, S., Fu, J., Li, G., Xu, R., Lu, H., & Deng, J. (2012). Expression of EphrinB2 and EphB4 in glioma tissues correlated to the progression of glioma and the prognosis of glioblastoma patients. *Clinical and Translational Oncology, 14*, 214-220.

Turner, G., Lower, K. M., White, S. M., Delatycki, M., Lampe, A. K., Wright, M., . . . Partington, M. (2004). The clinical picture of the Borjeson-Forssman-Lehmann syndrome in males and heterozygous females with PHF6 mutations. *Clin Genet, 65*(3), 226-232. doi:10.1111/j.0009-9163.2004.00215.x

Urbán, N., & Cheung, T. H. (2021). Stem cell quiescence: the challenging path to activation. *Development, 148*(3), dev165084.

Urbán, N., & Guillemot, F. (2014). Neurogenesis in the embryonic and adult brain: same regulators, different roles. *Frontiers in cellular neuroscience, 8*, 396.

van Bokhoven, H., & Kramer, J. M. (2010). Disruption of the epigenetic code: an emerging mechanism in mental retardation. *Neurobiology of disease, 39*(1), 3-12.

Van Dijk, D., Sharma, R., Nainys, J., Yim, K., Kathail, P., Carr, A. J., . . . Pattabiraman, D. (2018). Recovering gene interactions from single-cell data using data diffusion. *Cell, 174*(3), 716-729. e727.

Van Esch, H., Bauters, M., Ignatius, J., Jansen, M., Raynaud, M., Hollanders, K., . . . Gécz, J. (2005). Duplication of the MECP2 region is a frequent cause of severe mental retardation and progressive neurological symptoms in males. *The American Journal of Human Genetics, 77*(3), 442-453.

Van Nguyen, T., & Abe, K. (2015). Pathogenesis of congenital rubella virus infection in human fetuses: viral infection in the ciliary body could play an important role in cataractogenesis. *EBioMedicine, 2*(1), 59-63.

Vancamp, P., Deprez, M.-A., Remmerie, M., & Darras, V. M. (2017). Deficiency of the thyroid hormone transporter monocarboxylate transporter 8 in neural progenitors impairs cellular processes crucial for early corticogenesis. *Journal of Neuroscience, 37*(48), 11616-11631.

Vazin, T., Becker, K. G., Chen, J., Spivak, C. E., Lupica, C. R., Zhang, Y., . . . Freed, W. J. (2009). A novel combination of factors, termed SPIE, which promotes dopaminergic neuron differentiation from human embryonic stem cells. *PloS one, 4*(8), e6606.

Veeramah, K. R., Johnstone, L., Karafet, T. M., Wolf, D., Sprissler, R., Salogiannis, J., . . . Weinert, S. (2013). Exome sequencing reveals new causal mutations in children with epileptic encephalopathies. *Epilepsia, 54*(7), 1270-1281.

Vieira, M. L. C., Santini, L., Diniz, A. L., & Munhoz, C. d. F. (2016). Microsatellite markers: what they mean and why they are so useful. *Genetics and molecular biology, 39*, 312-328.

Vissers, L. E., de Vries, B. B., Osoegawa, K., Janssen, I. M., Feuth, T., Choy, C. O., . . . van Rijk, A. (2003). Array-based comparative genomic hybridization for the genomewide detection of submicroscopic chromosomal abnormalities. *The American Journal of Human Genetics, 73*(6), 1261-1270.

Vissers, L. E., Gilissen, C., & Veltman, J. A. (2016). Genetic studies in intellectual disability and related disorders. *Nature Reviews Genetics, 17*(1), 9-18.

Viti, J., Gulacsi, A., & Lillien, L. (2003). Wnt regulation of progenitor maturation in the cortex depends on Shh or fibroblast growth factor 2. *Journal of Neuroscience, 23*(13), 5919-5927.

Volpe, J. J. (2003). Cerebral white matter injury of the premature infant—more common than you think. *Pediatrics, 112*(1), 176-180.

Voss, A. K., Gamble, R., Collin, C., Shoubridge, C., Corbett, M., Gecz, J., & Thomas, T. (2007). Protein and gene expression analysis of Phf6, the gene mutated in the Borjeson-Forssman-Lehmann Syndrome of intellectual disability and obesity. *Gene Expr Patterns, 7*(8), 858-871. doi:10.1016/j.modgep.2007.06.007

Vugt, M. A. v., & Medema, R. H. (2004). Checkpoint adaptation and recovery: back with Polo after the break. *Cell Cycle, 3*(11), 1383-1386.

Walter, J., & Paulsen, M. (2003). *Imprinting and disease.* Paper presented at the Seminars in cell & developmental biology.

Wang, J., Leung, J. W.-c., Gong, Z., Feng, L., Shi, X., & Chen, J. (2013). PHF6 regulates cell cycle progression by suppressing ribosomal RNA synthesis. *Journal of Biological Chemistry, 288*(5), 3174-3183.

Warmerdam, D. O., Alonso-de Vega, I., Wiegant, W. W., van den Broek, B., Rother, M. B., Wolthuis, R. M., . . . Smits, V. A. (2020). PHF6 promotes non-homologous end joining and G2 checkpoint recovery. *EMBO reports, 21*(1).

Warmerdam, D. O., & Kanaar, R. (2010). Dealing with DNA damage: relationships between checkpoint and repair pathways. *Mutation Research/Reviews in Mutation Research, 704*(1-3), 2-11.

Wassink, T. H., Piven, J., Vieland, V. J., Huang, J., Swiderski, R. E., Pietila, J., . . . Haines, J. L. (2001). Evidence supporting WNT2 as an autism susceptibility gene. *American journal of medical genetics, 105*(5), 406-413.

Wechsler-Reya, R. J., & Scott, M. P. (1999). Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron, 22*(1), 103-114.

Wexler, E., Geschwind, D., & Palmer, T. (2008). Lithium regulates adult hippocampal progenitor development through canonical Wnt pathway activation. *Molecular psychiatry, 13*(3), 285-292.

Wheeler, A. C., Toth, D., Ridenour, T., Nóbrega, L. L., Firmino, R. B., da Silva, C. M., . . . Ventura, L. O. (2020). Developmental outcomes among young children with congenital Zika syndrome in Brazil. *JAMA network open, 3*(5), e204096-e204096.

Wilkinson, D. G. (2014). Regulation of cell differentiation by Eph receptor and ephrin signaling. *Cell adhesion & migration, 8*(4), 339-348.

Willi, R., Winter, C., Wieske, F., Kempf, A., Yee, B., Schwab, M., & Singer, P. (2012). Loss of EphA4 impairs short-term spatial recognition memory performance and locomotor habituation. *Genes, Brain and Behavior, 11*(8), 1020-1031.

Winner, B., & Winkler, J. (2015). Adult neurogenesis in neurodegenerative diseases. *Cold Spring Harbor perspectives in biology, 7*(4), a021287.

Wood, A., Schneider, J., Dover, J., Johnston, M., & Shilatifard, A. (2003). The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. *Journal of Biological Chemistry, 278*(37), 34739-34742.

Woodhead, G. J., Mutch, C. A., Olson, E. C., & Chenn, A. (2006). Cell-autonomous β-catenin signaling regulates cortical precursor proliferation. *Journal of Neuroscience, 26*(48), 12620-12630.

Wu, D., Yu, S., Jia, L., Zou, C., Xu, Z., Xiao, L., . . . Chan, F. L. (2015). Orphan nuclear receptor TLX functions as a potent suppressor of oncogene-induced senescence in prostate cancer via its transcriptional co-regulation of the CDKN1A (p21WAF1/CIP1) and SIRT1 genes. *The Journal of Pathology, 236*(1), 103-115.

Wybenga-Groot, L. E., Baskin, B., Ong, S. H., Tong, J., Pawson, T., & Sicheri, F. (2001). Structural basis for autoinhibition of the Ephb2 receptor tyrosine kinase by the unphosphorylated juxtamembrane region. *Cell, 106*(6), 745-757.

Wykosky, J., Gibo, D. M., Stanton, C., & Debinski, W. (2005). EphA2 as a novel molecular marker and target in glioblastoma multiforme. *Molecular Cancer Research, 3*(10), 541-551.

Xiang, J., Wang, G., Xia, T., & Chen, Z. (2019). The depletion of PHF6 decreases the drug sensitivity of T-cell acute lymphoblastic leukemia to prednisolone. *Biomed Pharmacother, 109*, 2210-2217. doi:10.1016/j.biopha.2018.11.083

Xing, S., He, Y., Ling, L., Hou, Q., Yu, J., Zeng, J., & Pei, Z. (2008). Blockade of EphB2 enhances neurogenesis in the subventricular zone and improves neurological function after cerebral cortical infarction in hypertensive rats. *Brain research, 1230*, 237-246.

Xu, D., Zhang, F., Wang, Y., Sun, Y., & Xu, Z. (2014). Microcephaly-associated protein WDR62 regulates neurogenesis through JNK1 in the developing neocortex. *Cell reports, 6*(1), 104-116.

Xu, N.-J., & Henkemeyer, M. (2012). *Ephrin reverse signaling in axon guidance and synaptogenesis.* Paper presented at the Seminars in cell & developmental biology.

Xu, N.-J., Sun, S., Gibson, J. R., & Henkemeyer, M. (2011). A dual shaping mechanism for postsynaptic ephrin-B3 as a receptor that sculpts dendrites and synapses. *Nature neuroscience, 14*(11), 1421-1429.

Yamada, T., Yang, Y., Hemberg, M., Yoshida, T., Cho, H. Y., Murphy, J. P., . . . Georgopoulos, K. (2014). Promoter decommissioning by the NuRD chromatin remodeling complex triggers synaptic connectivity in the mammalian brain. *Neuron, 83*(1), 122-134.

Yamaguchi, Y., & Pasquale, E. B. (2004). Eph receptors in the adult brain. *Current opinion in neurobiology, 14*(3), 288-296.

Yamashita, Y. M., Yuan, H., Cheng, J., & Hunt, A. J. (2010). Polarity in stem cell division: asymmetric stem cell division in tissue homeostasis. *Cold Spring Harbor perspectives in biology, 2*(1), a001313.

Yokote, H., Fujita, K., Jing, X., Sawada, T., Liang, S., Yao, L., . . . Sakaguchi, K. (2005). Trans-activation of EphA4 and FGF receptors mediated by direct interactions between their cytoplasmic domains. *Proceedings of the National Academy of Sciences, 102*(52), 18866-18871.

Yoon, K., Nery, S., Rutlin, M. L., Radtke, F., Fishell, G., & Gaiano, N. (2004). Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. *Journal of Neuroscience, 24*(43), 9497-9506.

Yucebas, M., Yilmaz Susluer, S., Onur Caglar, H., Balci, T., Dogan Sigva, Z., Akalin, T., . . . Gunduz, C. (2016). Expression profiling of RE1-silencing transcription factor (REST), REST corepressor 1 (RCOR1), and Synapsin 1 (SYN1) genes in human gliomas. *J BUON, 21*(4), 964-972.

Zechner, D., Fujita, Y., Hülsken, J., Müller, T., Walther, I., Taketo, M. M., . . . Birchmeier, C. (2003). β-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. *Developmental biology, 258*(2), 406-418.

Zeiss, C. J. (2021). Comparative milestones in rodent and human postnatal central nervous system development. *Toxicologic Pathology, 49*(8), 1368-1373.

Zhang, C., Mejia, L. A., Huang, J., Valnegri, P., Bennett, E. J., Anckar, J., . . . Yamada, T. (2013). The X-linked intellectual disability protein PHF6 associates with the PAF1 complex and regulates neuronal migration in the mammalian brain. *Neuron, 78*(6), 986-993.

Zhang, J.-c., Yao, W., Qu, Y., Nakamura, M., Dong, C., Yang, C., . . . Shirayama, Y. (2017). Increased EphA4-ephexin1 signaling in the medial prefrontal cortex plays a role in depression-like phenotype. *Scientific reports, 7*(1), 7133.

Zhang, L., Huang, H., Zhou, F., Schimmel, J., Pardo, C. G., Zhang, T., . . . Porter, J. A. (2012). RNF12 controls embryonic stem cell fate and morphogenesis in zebrafish embryos by targeting Smad7 for degradation. *Molecular cell, 46*(5), 650-661.

Zhang, Y., Liu, T., Meyer, C. A., Eeckhoute, J., Johnson, D. S., Bernstein, B. E., . . . Liu, X. S. (2008). Model-based analysis of ChIP-Seq (MACS). *Genome Biol, 9*(9), R137. doi:10.1186/gb-2008-9-9-r137

Zhang, Y., Wei, H., & Wen, W. (2021). Phase separation and mechanical forces in regulating asymmetric cell division of neural stem cells. *International Journal of Molecular Sciences, 22*(19), 10267.

Zhang, Y., Wu, Y., Zhu, M., Wang, C., Wang, J., Zhang, Y., . . . Jiang, T. (2011). Reduced cortical thickness in mental retardation. *PloS one, 6*(12), e29673.

Zhao, C., Deng, W., & Gage, F. H. (2008). Mechanisms and functional implications of adult neurogenesis. *Cell, 132*(4), 645-660.

Zhao, J., Taylor, C. J., Newcombe, E. A., Spanevello, M. D., O'Keeffe, I., Cooper, L. T., . . . Bartlett, P. F. (2019). EphA4 regulates hippocampal neural precursor proliferation in the adult mouse brain by d-serine modulation of N-Methyl-d-Aspartate receptor signaling. *Cerebral cortex, 29*(10), 4381-4397.

Zhou, C.-J., Zhao, C., & Pleasure, S. J. (2004). Wnt signaling mutants have decreased dentate granule cell production and radial glial scaffolding abnormalities. *Journal of Neuroscience, 24*(1), 121-126.

Zhu, B., Li, Y., & Mao, X. (2022). A review on the role of different ephrins in glioma. *European Journal of Pharmacology, 917*, 174588.

Zhu, J., Wen, W., Zheng, Z., Shang, Y., Wei, Z., Xiao, Z., . . . Zhang, M. (2011). Structures of the LGN/mInsc and LGN/NuMA complexes suggest distinct functions of the Par3/mInsc/LGN and Gαi/LGN/NuMA pathways in asymmetric cell division. *Molecular cell, 43*(3), 418.

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Figure 1 has been adapted from Zeiss, C.J, 2021.

Figure 2 has been adapted from Chhabra & Booth, 2021.

Tables

RT-qPCR primers	**Sequence 5'➔3'**
mEphA4-F	AGCAACTTGGTCTGCAAGGT
mEphA4-R	CTCCAGACATCACTGGCTGA
mEphA7-F	CAGAAAGATCGGGCGGAAAG
mEphA7-R	AAGGCAGTGAAGTCAGGAGT
mEphB1-F	CCAACATCATTCGCCTGGAG
mEphB1-R	GGTCCCGGTGCACATAATTC
mEphB2-F	ATCGTCATGTGGGAGGTGAT
mEphB2-R	TGGGCGGAGGTAGTCTGTAG
PHF6-F	TGAAATATGAGCTGGTCAATCAC
PHF6-R	TACAGTATTTTGGGGAAGCTGG
Genotyping PCR primers	**Sequence 5'➔3'**
PHF6(C99F)-F	CAGTTGTATCTAGCTCAGCTC
PHF6(C99F)-R-wt	TGGTAGTGGTATGTCCTGTGGC
PHF6(C99F)-R-m	TGGTAGTGGTATGTCCTGTGGA
PHF6 R342X F	GCATGGTGTACAAGTGGAGATC
PHF6 R342X R-wt	CCACGGCTTTTACTCTCTCG
PHF6 R342X R-mutant	CCACGGCTTTTACTCTCTCA
PHF6(LoxP)-F	TGAAATATGAGCTGGTCAATCAC
PHF6(LoxP)-R	TACAGTATTTTGGGGAAGCTGG
Nestin-CreERT2 (oIMR1084)	GCGGTCTGGCAGTAAAAACTATC

Nestin-CreERT2 (oIMR1085)	GTGAAACAGCATTGCTGTCACTT
Nestin-CreERT2 (oIMR7338)	CTAGGCCACAGAATTGAAAGATCT
Nestin-CreERT2 (oIMR7339)	GTAGGTGGAAATTCTAGCATCATCC
Nestin-Cre-F	ATGCTTCTGTCCGTTTGCCG
Nestin-Cre-R	CCTGTTTTGCACGTTCACCG
Luciferase primers	**Sequence 5'➔3'**
EphA4-F	TAATCTCGGTACCCCCTACCCCAGATCCTTAGC
EphA4-R	TAATTCCGATATCGGATGTACGGAGTGGGAAGA RC(ATCTATCCATCCAGCCAGCC)
EphA7-F	TAATCTCGGTACCCGTTGATTGGCTCCTGCT
EphA7-R	TAATTCCGATATCTCAGAACAAACTTTGCTTTCCTC RC(GAGGAAAGCAAAGTTTGTTCTGA)
EphB1-F	TAATCTCGGTACCCTTCCTAACCCTCCCACACA
EphB1-R	TAATTCCGATATCTCTCTCTCCAGCACCAGGAT RC(ATCCTGGTGCTGGAGAGAGA)
ChIP-qPCR primers	**Sequence 5'➔3'**
EphA4-F	GGAGGGGGAGAGAGACAGAC
EphA4-R	TTTTTCTGTCCGAGGTGAGG
EphA7-F	AAACGTGCCTCTGAGCTGAT
EphA7-R	CGGTTTCTGGTCACCAAAGT

EphB1-F	CAGGGAGAAAACCAAAGCAA
EphB1-R	CCCGTTTCTTCTCACACTCC
ZFP735-F	TGGTCCATCCTTTTGACACA
ZFP735-R	ACTTTGCCCCTTCGAATTTT

Table 1: Primer sequences. 5' to 3', forward and reverse, are listed for all genes assayed in RT-qPCR analysis, genotyping PCRs, luciferase construct design, and ChIP-qPCR.