TCF4 mediates PHF6 transcriptional regulation of neural stem cells in the developing brain, a mechanism disrupted in Börjeson-Forssman-Lehmann Syndrome

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Abstract

Intellectual disability (ID) comprises a heterogeneous group of neurodevelopmental disorders characterized by impairments in cognitive function and adaptive behavior. Börjeson–Forssman–Lehmann syndrome (BFLS) is a rare X-linked form of ID caused by mutations in plant homeodomain finger protein 6 (PHF6). Individuals with BFLS exhibit moderate-to-severe ID, characteristic craniofacial abnormalities, and seizure susceptibility. Although PHF6 has been implicated in brain development, the mechanisms through which PHF6 deficiency contributes to BFLS pathogenesis remain poorly understood.

Using integrated chromatin immunoprecipitation sequencing (ChIP-seq), transcriptomic analyses, molecular and functional assays in embryonic and adult neural stem cells (NSCs), this thesis identifies transcription factor 4 (TCF4), a basic helix–loop–helix transcription factor essential for brain development, as a previously unrecognized downstream target of PHF6. PHF6 was found to directly occupy the *Tcf4* regulatory region, promoting *Tcf4* transcription. Consistent with this mechanism, TCF4 expression was significantly reduced in multiple BFLS mouse models. Functional studies demonstrated that *Tcf4* knockdown phenocopies PHF6 deficiency by enhancing NSC self-renewal, increasing neurosphere formation, and elevating expression of the stem cell markers Nestin and Sox2. Importantly, restoration of TCF4 expression rescued the abnormal NSC phenotypes observed following PHF6 loss, demonstrating that TCF4 is a major functional mediator of PHF6 during neurogenesis. Furthermore, this regulatory relationship persisted in adult NSCs, indicating that PHF6-dependent regulation of TCF4 contributes to stem cell homeostasis beyond embryonic development.

Together, these findings establish the PHF6–TCF4 signaling axis as a critical transcriptional pathway regulating NSC fate during corticogenesis and provide a mechanistic insight for how PHF6 mutations disrupt neurodevelopment in BFLS. Beyond identifying TCF4 as a key downstream effector of PHF6, this work expands our understanding of the transcriptional networks governing cortical development and provides a foundation for future studies investigating whether targeting the PHF6–TCF4 pathway may represent a therapeutic strategy for BFLS and other TCF4-associated neurodevelopmental disorders.

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Contributions

The experiments in this thesis were performed under the supervision and consultation from Dr. Jahani-Asl. The sequencing data was analyzed by Dr. Hamed Najafabadi lab.

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List of Abbreviations

AD	Activation Domain
AML	Acute Myeloid Leukemia
aNSC	Adult Neural Stem Cell
APC	Adenomatous Polyposis Coli
ASCL1	Achaete–Scute Family BHLH Transcription Factor 1
bFGF	Basic Fibroblast Growth Factor
BFLS	Börjeson–Forssman–Lehmann Syndrome
bHLH	Basic Helix–Loop–Helix
BSA	Bovine Serum Albumin
CA1	Cornu Ammonis 1
CE	Conserved Element
ChIP	Chromatin Immunoprecipitation
ChIP-qPCR	Chromatin Immunoprecipitation Quantitative Polymerase Chain Reaction
ChIP-seq	Chromatin Immunoprecipitation Sequencing
CK1	Casein Kinase 1
cKO	Conditional Knockout
CMV	Cytomegalovirus
CNS	Central Nervous System
CP	Cortical Plate
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid

dNTP	Deoxyribonucleotide Triphosphate
EGF	Epidermal Growth Factor
eNSC	Embryonic Neural Stem Cell
ePHD	Extended Plant Homeodomain
FISH	Fluorescence In Situ Hybridization
FMR1	Fragile X Messenger Ribonucleoprotein 1
FMRP	Fragile X Mental Retardation Protein
FXS	Fragile X Syndrome
GABA	Gamma-Aminobutyric Acid
Gli	Glioma-Associated Oncogene Homolog
GSK3β	Glycogen Synthase Kinase 3 Beta
HBSS	Hank's Balanced Salt Solution
ID	Intellectual Disability
iPSC	Induced Pluripotent Stem Cell
IQ	Intelligence Quotient
ITF2	Immunoglobulin Transcription Factor 2
IZ	Intermediate Zone
KD	Knockdown
KO	Knockout
LEF	Lymphoid Enhancer-Binding Factor
LRP5/6	Low-Density Lipoprotein Receptor-Related Protein 5/6
MECP2	Methyl-CpG Binding Protein 2

mRNA	Messenger Ribonucleic Acid
NES	Nuclear Export Signal
NICD	Notch Intracellular Domain
NLS	Nuclear Localization Signal
NoLS	Nucleolar Localization Sequence
NPC	Neural Progenitor Cell
NSC	Neural Stem Cell
NuRD	Nucleosome Remodeling and Deacetylase
OE	Overexpression
PAF1	Polymerase-Associated Factor 1
PAX6	Paired Box Protein 6
PCR	Polymerase Chain Reaction
PHD	Plant Homeodomain
PHF6	Plant Homeodomain Finger Protein 6
PTHS	Pitt–Hopkins Syndrome
PWS	Papain Working Solution
qPCR	Quantitative Polymerase Chain Reaction
RGC	Radial Glial Cell
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SCZ	Schizophrenia

SGZ	Subgranular Zone
SHH	Sonic Hedgehog
Smo	Smoothened
SOX2	SRY-Box Transcription Factor 2
SVZ	Subventricular Zone
T-ALL	T-cell Acute Lymphoblastic Leukemia
TCF/LEF	T-cell Factor/Lymphoid Enhancer-Binding Factor
TCF4	Transcription Factor 4
TF	Transcription Factor
UBF	Upstream Binding Factor
VZ	Ventricular Zone
WES	Whole-Exome Sequencing
WGS	Whole-Genome Sequencing
Wnt	Wingless/Integrated Signaling Pathway
WT	Wild Type
XLID	X-Linked Intellectual Disability
ZaP	Zinc Knuckle-Containing Atypical PHD Domain

Introduction

1.1 Intellectual disability

Intellectual disability (ID) is a neurodevelopmental disorder characterized by significant limitations in both intellectual functioning and adaptive behavior. The onset of ID typically occurs during the developmental period, with symptoms emerging from infancy through adolescence (Patel et al., 2020; Shree & Shukla, 2016). The prevalence of ID is estimated to be approximately 1–3% in the general population (Patel et al., 2018). The diagnosis of ID is primarily based on assessments of intellectual functioning and adaptive behavior. Intelligence quotient (IQ) testing is commonly used to assess intellectual functioning, while an individual's ability to perform everyday conceptual skills serves as an additional criterion for evaluation (Patel et al., 2020; Schalock & Luckasson, 2015). Generally, individuals with an IQ score of 50 or below are classified as having severe intellectual disability, whereas those with an IQ above 50 are considered to have mild intellectual disability (Patel et al., 2020; Purugganan, 2018).

Current evidence suggests more than 1,500 genes have been implicated in the pathogenesis of ID (Jansen et al., 2023; van Bokhoven, 2011). Approximately 25–50% of cases of ID are attributed to genetic factors, including chromosomal abnormalities, dysregulation of gene expression and single-gene mutations. Among chromosomal abnormalities, deletions, duplications, inversions, or translocations have been linked to ID (Hou & Zheng, 2024; Jansen et al., 2023; van Bokhoven, 2011). Down syndrome (Trisomy 21) is the most common chromosomal cause, characterized by an extra copy of chromosome 21, particularly involving the 21q22 region (Antonarakis et al., 2020; Shapiro, 1999). Williams syndrome is caused by microdeletion at chromosome 7q11.23 (Hobart et al., 2010; Kozel et al., 2021). These

chromosomal abnormalities are commonly diagnosed using cytogenetic and molecular genetic techniques, including karyotyping, fluorescence in situ hybridization (FISH), and chromosomal microarray analysis (Mefford et al., 2012; Milani et al., 2015). Mutations in genes involved in neurodevelopment can cause permanent alterations in DNA sequence that disrupt normal gene function and protein production, while dysregulation of epigenetic mechanisms, such as DNA methylation, histone modification, and chromatin remodeling, can ultimately contribute to ID (Jakovcevski & Akbarian, 2012). For example, mutations in the MECP2 gene cause Rett Syndrome; mutations in the CSNK2A1 gene cause Okur-Chung Neurodevelopmental Syndrome (Bienvenu et al., 2000; Okur et al., 2016; Rett, 1966). Gene mutations are commonly identified using molecular genetic techniques such as whole-exome sequencing (WES), whole-genome sequencing (WGS), and targeted gene panels (Brancato et al., 2025; Jansen et al., 2023).

Environmental factors contribute significantly to the development of ID and may act independently or in combination with underlying genetic susceptibility to impair fetal and early postnatal brain development. Several risk factors have been identified, including lower socioeconomic status, parental factors, prematurity, and complication before or after birth. Lower socioeconomic status is strongly associated with a higher prevalence of intellectual disability and is often linked to limited access to healthcare resources, poor maternal nutrition, inadequate antenatal care, and reduced availability of prenatal genetic screening methods (Durkin & Yeargin-Allsopp, 2018; Maulik et al., 2011). Maternal malnutrition during gestation, particularly deficiencies in essential nutrients such as folic acid and iodine, can impair fetal brain development and increase the risk of developmental abnormalities and cognitive deficits (Bath et al., 2013; Cao & O'Brien, 2013; Freedman et al., 2018; Veena et al., 2016; Velasco et al., 2018).

In addition, maternal alcohol consumption, smoking, and substance abuse during pregnancy are closely related to neurodevelopmental impairment (Chen et al., 2024; O'Leary et al., 2013; Wozniak et al., 2019). On top of that, maternal health conditions during pregnancy have been associated with an increased risk of ID in offspring. Children born to mothers with renal or urinary tract disorders, asthma, diabetes, hypertension, or other chronic medical conditions have shown a higher risk of mild-to-moderate intellectual disability (Leonard et al., 2006). These maternal conditions may alter placental function, oxygen supply, inflammatory signaling, or metabolic homeostasis during fetal development. Premature birth before 37 weeks of gestation is associated with a higher risk of intellectual impairment, as the fetal brain may not yet be fully developed (Heuvelman et al., 2018; Mitha et al., 2024; Yin et al., 2022). Infections can also severely affect fetal brain development. Viral infections that can occur congenitally, such as cytomegalovirus (CMV), rubella, or Zika virus are known to disrupt neurogenesis and neuronal migration, potentially resulting in microcephaly, cortical malformations, and intellectual impairment (Al Beloushi et al., 2024; Brynge et al., 2022; Cordeiro et al., 2015; Egilmezer et al., 2024; Gordon-Lipkin & Peacock, 2019). Complications during or after birth may further contribute to intellectual disability. Perinatal hypoxia, neonatal seizures and intracranial hemorrhage can cause damage to the developing nervous system and impair cognitive function (Almli et al., 2000; Cheung et al., 2014; Shehzad et al., 2023). Postnatal trauma, including severe head injury or prolonged neglect during early childhood, may also interfere with normal neural circuit development and contribute to long-term intellectual and behavioral impairments (Cloitre et al., 2009; Danese & McEwen, 2012; Hoover, 2020; Kira et al., 2011) (**Figure 1**).

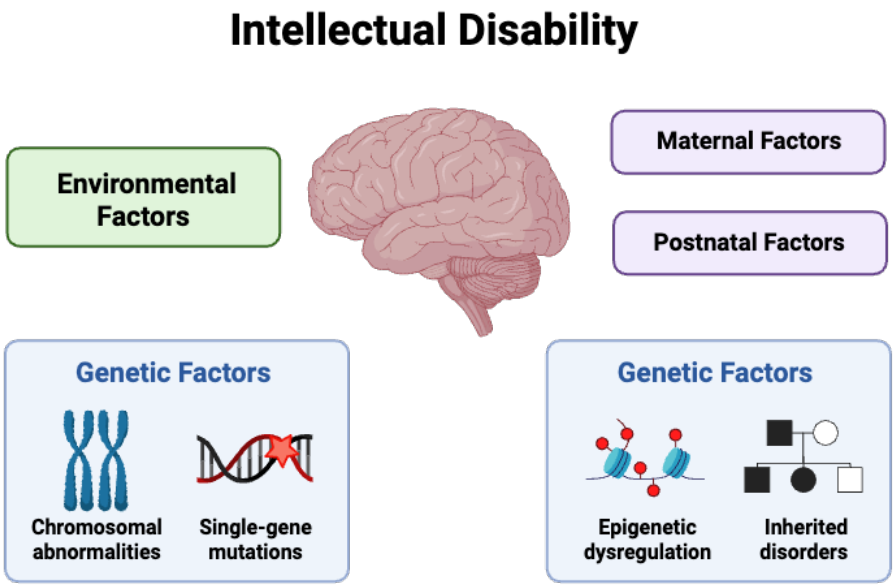

Figure 1. Factors contributing to intellectual disability. Schematic overview illustrating the factors of intellectual disability (ID). ID can arise from a combination of genetic and non-genetic influences that disrupt normal brain development. Genetic factors include chromosomal abnormalities, single-gene mutations, epigenetic dysregulation, and inherited disorders. Non-genetic factors encompass environmental exposures, maternal factors during pregnancy, and postnatal factors. Figure created with BioRender.

1.1.2 X-Linked Intellectual Disability

X-Linked Intellectual Disability (XLID) refers to a group of neurodevelopmental disorders caused by mutations in genes located on the X chromosome. XLID accounts for 5–10% of intellectual disability cases in males and is most commonly inherited in an X-linked recessive pattern, which predominantly affects males (Lubs et al., 2012; Schwartz et al., 2026; Skuse, 2005; Stevenson & Schwartz, 2009). The XLID genes encode proteins that are highly expressed during early brain development, particularly within neural stem and progenitor cell populations of the developing cortex (Leitão et al., 2022; Ma et al., 2024). These genes play essential roles in regulating neural stem cell (NSC) proliferation, self-renewal, and neuronal differentiation (Bertin et al., 2026; Bustos et al., 2018; Rasool et al., 2024).

Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability, it is caused by a deficiency of fragile X mental retardation protein (FMRP), resulting from mutation-induced silencing of the FMR1 gene (Bassani et al., 2013; Richter & Zhao, 2021). FMRP is an RNA-binding protein associated with approximately 4% of all mRNAs expressed in the mammalian brain (Adinolfi et al., 2003; Bassani et al., 2013). FMRP has been shown to regulate mRNA translation during neurodevelopment and plays critical roles in NSC proliferation, neuronal differentiation, neuron maturation and synaptic development. A study using FMRP-deficient mouse and human neural stem cells demonstrated abnormal spontaneous Ca^{2+} oscillations and altered intracellular Ca^{2+} signaling dynamics, suggesting that disruption of calcium-dependent signaling pathways impairs NSC proliferation and neuronal differentiation during early brain development (Castrén et al., 2005). Similarly, mutations in the MECP2 gene cause Rett syndrome (Amir et al., 1999; Bienvenu et al., 2000; Rett, 1966). Mechanistically,

MECP2 deficiency results in premature neuronal differentiation, accompanied by impaired dendritic maturation and reduced synaptic integration of newly generated neurons (Feng et al., 2024; Smrt et al., 2007). Overall, genes involved in XLID are closely associated with neurodevelopmental processes; therefore, investigating the underlying mechanisms of XLID is important for developing targeted therapies and interventions that may improve the quality of life of individuals affected by ID.

1.1.3 Börjeson–Forssman–Lehmann Syndrome (BFLS)

BFLS is a rare X-linked recessive intellectual disability caused by mutations in the PHF6 gene (Gécz et al., 2006; Jain et al., 2023). It was first described in 1962 by Mats Börjeson and colleagues (Borjeson et al., 1962). Clinically, patients with BFLS present with several features that overlap with other forms of ID, including an IQ below 70 and impaired adaptive functioning (Gécz et al., 2006; Lower et al., 2002). Neurological manifestations additionally include seizures, hearing impairment, reduced coordination, and abnormalities in motor development. Distinctive features of BFLS include deep-set eyes, prominent supraorbital ridges, large fleshy ears, thick lips, a broad nasal bridge, coarse facial appearance, and a long narrow face (Gécz et al., 2006; Lower et al., 2002). Endocrine and metabolic abnormalities are also characteristic of the syndrome and contribute significantly to its clinical recognition. Many affected males develop truncal obesity during late childhood or adolescence, accompanied by hypogonadism, gynecomastia, delayed puberty, and reduced secondary sexual characteristics (Birrell et al., 2003; Gécz et al., 2006; Lower et al., 2002; Turner et al., 2004). Craniofacial dysmorphism is a hallmark feature of BFLS and often becomes more pronounced with age. Neuroimaging studies

in some patients have identified structural brain abnormalities, including cortical atrophy, delayed myelination, and corpus callosum abnormalities (Kasper et al., 2017). Mutations in PHF6 associated with BFLS include missense, nonsense, frameshift, duplication, and deletion. Five recurrent PHF6 mutations were identified in 21% of patients with BFLS: c.2T > C/p.M1T, c134G > A/p.C45Y, c769A > G/p.R257G, c.999-1001delTGA/p.D333del, and c1024C > T/ p.R342X (Chao et al., 2010; Crawford et al., 2006; Gécz et al., 2006; Jahani-Asl et al., 2016; Lower et al., 2004; Lower et al., 2002). A patient-derived BFLS mouse model was created by replacing cysteine 99 with phenylalanine (C99F) (Cheng et al., 2018). Another mouse model was generated carrying the most common mutation in the PHF6 gene, a nonsense mutation (c.1024C > T; p.R342X) (Ahmed et al., 2021). The PHF6 protein is 97.5% identical between humans and mice (Voss et al., 2007). Therefore, these mouse models of PHF6 can be effectively used to investigate the role of PHF6 in BFLS and to develop potential therapeutic strategies.

Diagnosis of BFLS is based on a combination of clinical evaluation and molecular genetic testing. Initial clinical suspicion is typically raised in males presenting with syndromic ID accompanied by obesity, hypogonadism, hypotonia, and characteristic facial dysmorphism. However, because many clinical features overlap with other neurodevelopmental and chromatin-associated disorders, definitive diagnosis requires identification of pathogenic PHF6 variants through molecular testing (Gécz et al., 2006; Turner et al., 2004). Although the clinical features of BFLS are well characterized, the developmental mechanisms through which PHF6 mutations disrupt corticogenesis remain poorly understood.

1.1.4 Plant Homeodomain Finger protein 6 (PHF6)

The PHF6 gene is located on chromosome Xq26–27 and is composed of 11 exons that are transcribed into a 4.5 kb mRNA and translated into a 41 kDa protein consisting of 365 amino acids (Jahani-Asl et al., 2016; Liu et al., 2014). Structurally, the PHF6 protein is characterized by two atypical extended plant homeodomain (ePHD)-like zinc finger domains, ePHD1 and ePHD2, located at amino acid positions 14–134 and 209–332 (Liu et al., 2014). Each ePHD domain consists of a zinc knuckle (C2HC) and an atypical PHD zinc finger (ZaP) (Eisa et al., 2023). Furthermore, PHF6 contains two nuclear localization sequences (NLSs) located at amino acids 13–16 (NLS1) and 129–133 (NLS2), as well as a nucleolar localization sequence (NoLS) at amino acids 157–169 of the protein (Liu et al., 2014; Lower et al., 2002) (**Figure 2**). Collectively, these features suggest that PHF6 functions as a transcriptional regulator. PHF6 is highly conserved in vertebrates and is most highly expressed during early fetal and embryonic development, particularly in the developing central nervous system during the early stages of corticogenesis (Voss et al., 2007; Zhang et al., 2013). Its expression gradually declines to low levels in adulthood, although expression is maintained in projection neurons (Voss et al., 2007). Outside the CNS, PHF6 is also highly expressed in the spleen, kidney, and thymus (Voss et al., 2007).

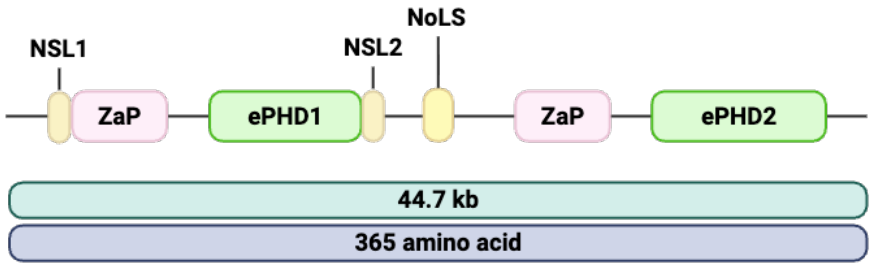

Figure 2. The Structure of PHF6 gene. The PHF6 gene spans 44.7 kb on the X chromosome and encodes a 365 amino acid. PHF6 contains two ePHD domains, two ZaP, two nuclear localization signals (NLS) and a nucleolar localization signal (NoLS). Figure created with BioRender.

In addition to the dysregulation of PHF6 in BFLS, PHF6 mutations have been identified in hematological malignancies, particularly T-cell acute lymphoblastic leukemia (T-ALL) and acute myeloid leukemia (AML) (Kubota et al., 2024; Kurzer & Weinberg, 2021; Pinton et al., 2024; Van Vlierberghe, et al., 2010). Unlike BFLS which results from germline PHF6 modifications, leukemia-associated mutations are somatic and include nonsense, frameshift, splice-site, and deletion mutations, supporting a tumour-suppressive role for PHF6 in hematopoietic cells (Eisa et al., 2023; McRae et al., 2019). BFLS-associated variants include both missense and truncating mutations distributed throughout the protein, whereas leukemia-associated mutations are distributed throughout the coding region, with truncating variants occurring across the gene and missense mutations showing a relative enrichment within the C-terminal ePHD2 domain (Kubota et al., 2024).

1.2 Neurogenesis

Neurogenesis is the process where NSCs generate neurons during CNS development. This process requires a precisely regulated balance between stem cell self-renewal, proliferation, neuronal differentiation, migration, circuit integration, and maturation.

In the developing cerebral cortex, neurogenesis begins shortly after neural tube closure. The earliest neural progenitors are neuroepithelial cells, which are highly polarized epithelial

cells with apical ends lining the ventricular surface (Götz & Huttner, 2005; Huttner & Kosodo, 2005). As development proceeds, neuroepithelial cells differentiate into radial glial cells, which become the primary NSCs of the developing cortex. Radial glial cells (RGCs) are located in the ventricular zone (VZ), with their cell bodies lining the ventricular surface and basal processes extending toward the pial surface (Götz & Huttner, 2005). RGCs also function as scaffolding structures during corticogenesis, with their long radial processes serving as guides for migrating neurons (Kim et al., 2022; Rakic, 1972). RGCs undergo asymmetric cell divisions, generating two distinct daughter cells: one daughter cell retains progenitor characteristics and maintains the progenitor pool, while the other can either differentiates into an intermediate progenitor cell (IPC) or directly into a neuron (Miyata et al., 2001; Noctor et al., 2004). IPCs predominantly reside in the subventricular zone (SVZ), where they further proliferate and differentiate into neurons (Javaherian & Kriegstein, 2009; Pontious et al., 2008).

Following that, newly born neurons will migrate away from the germinal VZ to their final positions in the developing cortex. Neurons born earlier form the deeper cortical layers (VI and V), while later-born neurons migrate past these layers to establish the upper cortical layers (IV–II). The cerebral cortex is organized into six distinct layers, each characterized by specific neuronal populations (Noctor et al., 2004; Stepien et al., 2021). Thus, the sequential production of deeper layer followed by upper layer neurons is closely coordinated with neuronal migration to establish the mature laminar architecture of the cortex. Cortical neurons can be broadly classified into two major categories: interneurons, which form local connections within the cortex, and projection neurons, which extend axons to transmit information between different regions of the neocortex and to other areas of the brain (Greig et al., 2013; Stepien et al., 2021).

As corticogenesis progresses, cortical progenitors gradually shift from producing neurons to glial cells. During this transition, RGCs progressively lose their neurogenic potential and acquire gliogenic competence, which give rise to glial progenitors that generate astrocytes and oligodendrocyte-lineage cells (Clavreul et al., 2022; Di Bella et al., 2024; Toma & Hanashima., 2015; Li et al., 2021). Cortical development is generally considered complete once cortical neurogenesis and neuronal migration have ceased, the six-layered neocortical architecture has been established, and both excitatory projection neurons and inhibitory interneurons have reached their final positions (Rakic, 2009). Nevertheless, postnatal maturation processes, including synaptogenesis, synaptic pruning, and myelination continue to develop after birth (**Figure 3**).

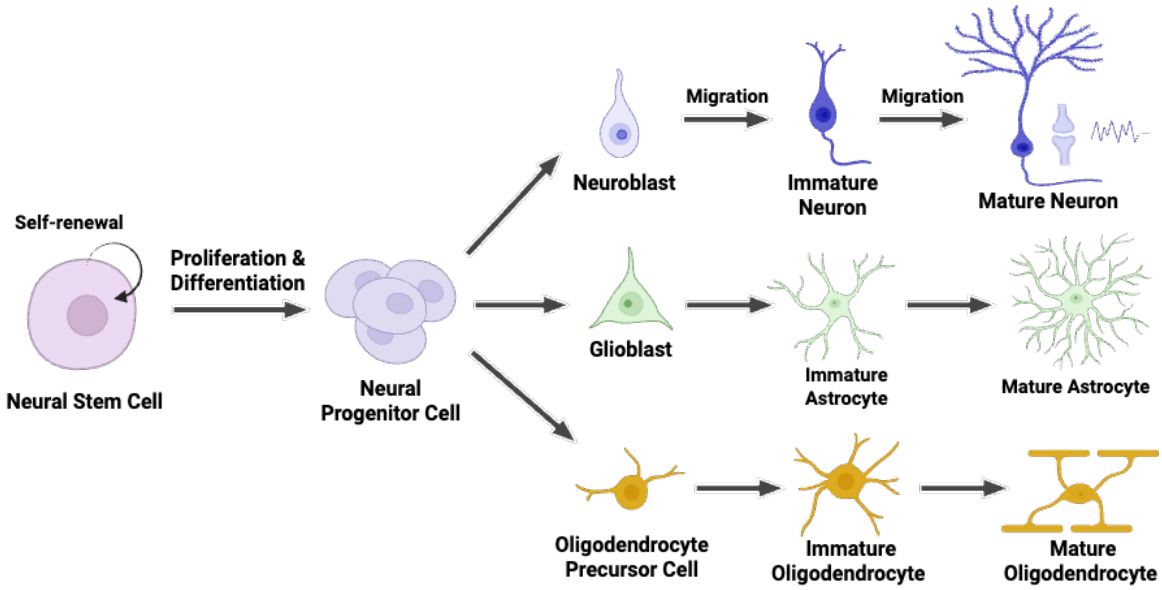

Figure 3. Illustration of the process of neurogenesis. NSCs undergo self-renewal to maintain the stem cell pool while generating NPCs through proliferation and differentiation. NPCs

subsequently give rise to three lineages: (i) the neuronal lineage; (ii) the astrocytic lineage; (iii) the oligodendroglial lineage. Figure created with BioRender.

1.2.1 Neural Stem Cell Self-Renewal and Differentiation

The balance between NSC self-renewal and differentiation is tightly regulated by molecular signals during embryonic neurogenesis. During early neurogenesis, NSCs are symmetrically divided to expand the uncommitted pool while asymmetrically divided to generate neurons that form the brain (Casas Gimeno & Paridaen, 2022; Dwyer et al., 2016). Both genetic and molecular factors have been identified to support the stem cell niche and control stem cell identity.

NSCs are highly polarized along their apical-basal axis, and play a critical role in regulating symmetric and asymmetric cell division (Götz & Huttner, 2005). During cell division, the Par complex (Par3, Par6, aPKC) localizes to the apical membrane, where it establishes apical–basal polarity and regulates mitotic spindle orientation (Knoblich, 2008). During symmetric divisions, the mitotic spindle is oriented perpendicular to the apical surface, allowing equal inheritance of apical components by both daughter cells and promoting expansion of the progenitor pool. In contrast, asymmetric divisions result in the unequal segregation of apical determinants. One daughter cell retains apical contact and stem cell identity, whereas the other loses apical attachment and differentiates into either an intermediate progenitor cell or a neuron (Homem et al., 2015; Knoblich, 2008).

Canonical Wnt signaling mediated by β-catenin is important in preserving neuroepithelial and radial glial identity. Studies have shown increased β-catenin enhance Wnt signaling to expand the stem cell pool while reduced β-catenin leads to premature differentiation and a decrease of the progenitor pool (Chenn & Walsh, 2002; Machon et al., 2007; Woodhead et al., 2006; Zechner et al., 2003). In the canonical pathway, Wnt ligands bind to Frizzled receptors together with the co-receptors LRP5/6 (Bem et al., 2019). In the absence of Wnt ligand, β-catenin is phosphorylated by the destruction complex, which includes APC, Axin, CK1, and GSK3β, leading to β-catenin degradation. When Wnt ligands activate Frizzled-LRP5/6 receptor complexes, this destruction complex is inhibited, allowing β-catenin to accumulate in the cytoplasm and enter the nucleus. Once in the nucleus, β-catenin acts as a transcriptional co-activator by binding TCF/LEF transcription factors, converting them from transcriptional repressors into activators of Wnt target genes involved in progenitor maintenance, cell-cycle progression, and neural fate regulation (Alkailani et al., 2022; Chenn, 2008; van de Wetering et al., 1997).

Notch signaling is one of the central pathways regulating stem cell identity during embryonic neurogenesis. Notch signaling is activated when a Notch receptor on one cell interacts with Notch ligands, such as Delta-like (Dll1, Dll3, and Dll4) or Jagged proteins expressed on an adjacent cell (Lasky & Wu, 2005). Through this cell-cell communication, Notch signaling helps maintain the balance between NSC self-renewal and differentiation (McLaren & Butts, 2025; Nian & Hou, 2022). One of the primary mechanisms of Notch regulates stem cell fate is through lateral inhibition. During neurogenesis, differentiating progenitors upregulate Delta ligands, which activate Notch receptors in surrounding cells. As a result, Notch-active cells maintain

progenitor characteristics, while Delta-expressing cells are permitted to exit the cell cycle and differentiate (Artavanis-Tsakonas et al., 1999; Lasky & Wu, 2005). In addition to ligand-mediated regulation, Numb functions as an intrinsic antagonist of Notch signaling and is a major regulator of asymmetric stem cell division. Numb is asymmetrically inherited by one daughter cell during divisions that generate distinct progeny. The Numb-positive daughter cell exhibits reduced Notch activity, promoting neuronal differentiation, whereas the daughter cell lacking Numb maintains active Notch signaling and retains stem cell characteristics (Guo et al., 1996; Spana & Doe, 1996).

Sonic Hedgehog signaling is a highly conserved pathway that is involved in multiple neurodevelopmental processes. Signaling is initiated when Shh binds to Patched (Ptc) at the cell surface, mitigating inhibition of the transmembrane protein Smoothened (Smo) and activating the Gli (Gli2 and Gli3) transcription factors (Ho & Scott, 2002; Murone et al., 1999). During early embryogenesis, Shh is secreted from the notochord and floor plate, establishing a ventral-to-dorsal signaling gradient within the developing neural tube. Exposure to different Shh concentrations activates unique transcriptional programs, allowing progenitor cells to acquire specific regional identities (Briscoe & Novitch, 2008). Shh signaling plays a major role in regulating NSC proliferation and maintaining progenitor multipotency. Activation of the Shh pathway promotes cell cycle progression through the induction of proliferative genes (Cyclin D1, Cyclin D2, Mycn), which expand neural progenitor populations during embryonic neurogenesis (Li et al., 2021; Rowitch et al., 1999). In addition to stimulating proliferation, Shh signaling supports the maintenance of NSC characteristics and delays premature differentiation, allowing progenitor cells to retain the capacity to generate multiple neuronal and glial lineages (Chai et

al., 2013; Oh et al., 2009). Shh signaling also contributes to the regulation of neuronal migration and tissue organization. In the ventral telencephalon, Shh promotes the generation of GABAergic interneuron progenitors within the medial and lateral ganglionic eminences (Belgacem & Borodinsky, 2011). These interneurons subsequently undergo long-distance tangential migration into the developing cerebral cortex, where they integrate into cortical circuits.

Transcription factors (TFs) act as master regulators that integrate developmental signaling pathways into gene expression programs controlling self-renewal and differentiation. Among these TFs, members of the SOX family, particularly SOX2 play an essential role in maintaining NSC identity. SOX2 promotes stem cell multipotency by activating genes associated with self-renewal while repressing premature neuronal differentiation. Loss of SOX2 results in early cell cycle exit, depletion of the progenitor pool, and impaired cortical development (Favaro et al., 2009; Sarkar & Hochedlinger, 2013). PAX6 is another key transcription factor that regulates NSC fate and lineage progression. PAX6 is expressed predominantly in radial glial cells of the developing cortex and promotes progenitor proliferation while simultaneously preparing NSCs for neuronal differentiation. PAX6 regulates the transition from neuroepithelial cells to radial glial cells and controls the production of intermediate progenitor cells through downstream targets such as *Tbr2*. Consequently, PAX6 serves as an important regulator linking progenitor maintenance with the onset of cortical neurogenesis (Götz et al., 1998; Sansom et al., 2009). Neuronal differentiation is initiated by basic helix-loop-helix (bHLH) TFs, including ASCL1, NEUROG1, and NEUROG2. These factors promote cell cycle exit and activate transcriptional programs required for neuronal specification, migration, and maturation.

Increased expression of bHLH TFs shifts NSCs from a proliferative state towards neuronal commitment, promoting embryonic neurogenesis (Bertrand et al., 2002; Guillemot, 2007).

Overall, dysregulation of these pathways can lead to developmental abnormalities, including microcephaly, megalencephaly, and cortical malformations. Therefore, precise regulation of NSC fate is essential for neurodevelopment **(Figure 4)**.

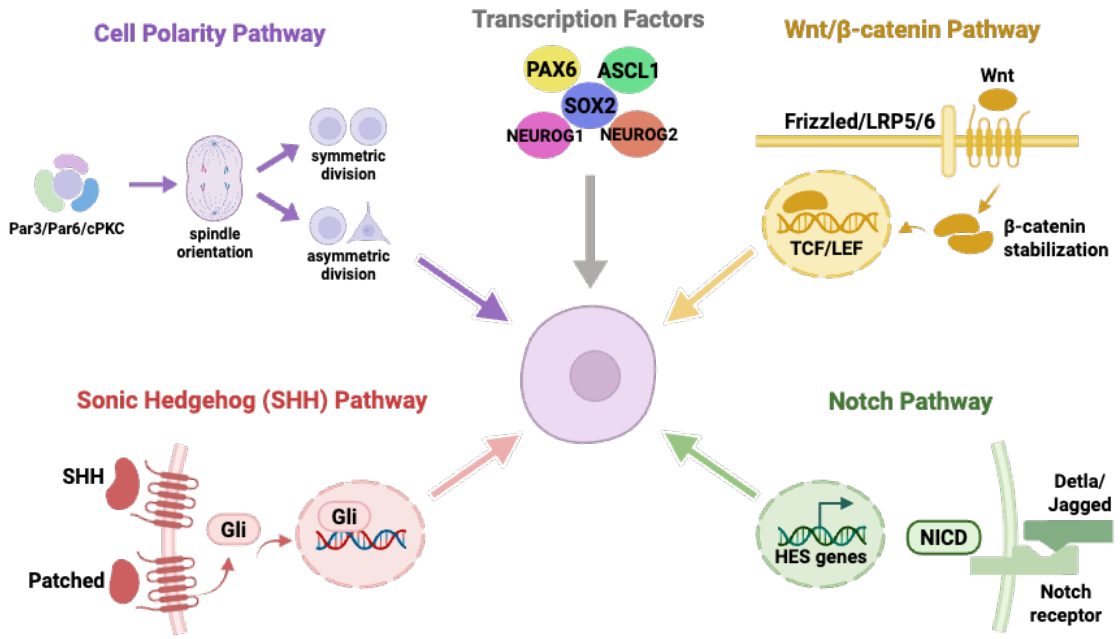

Figure 4. Signaling pathways regulating neural stem cell fate during embryonic neurogenesis. NSC maintenance, proliferation, and differentiation are tightly controlled by multiple signaling pathways and transcriptional regulators. These extrinsic signaling pathways and intrinsic transcriptional networks integrate to regulate NSC fate decisions and ensure proper cortical development. Figure created with BioRender.

1.3 PHF6 in Neurogenesis

PHF6 is highly expressed during embryonic corticogenesis, with expression enriched throughout embryonic neurogenesis and subsequently declining during postnatal brain maturation. During cortical development, PHF6 is expressed in neural stem cells, neural progenitor cells, and immature neurons, consistent with its important role in early neurodevelopment (Franzoni et al., 2015; Jahani-Asl et al., 2016; Rasool et al., 2024). PHF6 acts as a chromatin-associated transcriptional regulator through interactions with several nuclear protein complexes. These include the nucleosome remodelling and deacetylase (NuRD) complex, the RNA polymerase II-associated PAF1 transcription elongation complex, and the nucleolar upstream binding factor (UBF), which regulates ribosomal DNA transcription (Todd & Picketts, 2012; Wang et al., 2013; Zhang et al., 2013). Through these interactions, PHF6 controls the transcriptional events involved in cell proliferation, cell-cycle progression, and neuronal differentiation. PHF6 also contributes to neuronal maturation and cortical organization. PHF6 deficiency alters the expression of genes involved in neuronal differentiation, axon guidance, and synapse formation, ultimately leading to reduced neuronal density and abnormalities in cortical architecture (Fliedner et al., 2020; Franzoni et al., 2015; Zhang et al., 2013).

1.3.1 PHF6 Regulation of Neural Stem Cell Fate

Recent work from our lab established PHF6 as a critical regulator of eNSC fate during corticogenesis (Rasool et al., 2024). Genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) identified 2,467 PHF6 binding sites in the developing mouse cortex, with significant enrichment near transcription start sites and genes associated with CNS development,

neurogenesis and neuronal differentiation. Complementary RNA sequencing further demonstrated that PHF6 functions as both a transcriptional activator and repressor depending on its genomic binding position, indicating that PHF6 regulates broad transcriptional programmes controlling neural development rather than acting on individual genes (Rasool et al., 2024).

Furthermore, functional studies demonstrated that loss of PHF6 profoundly alters eNSC behaviour. Knockdown (KD) or conditional knockout (cKO) of *Phf6* significantly increased NSC self-renewal, neurosphere number, neurosphere diameter and stem cell frequency, accompanied by increased expression of the stem cell markers SOX2 and Nestin. Similar phenotypes were observed in two independent BFLS patient-derived mouse models (C99F-m and R342X), indicating that disease-causing PHF6 mutations impair normal regulation of NSC fate. Furthermore, *Phf6* cKO reduced neuronal density within the developing forebrain, suggesting that excessive maintenance of the progenitor pool occurs at the expense of neuronal differentiation (Rasool et al., 2024). Together, these findings demonstrate that PHF6 restricts eNSC self-renewal and promotes commitment towards neuronal lineages during corticogenesis.

1.3.2 Downstream target of PHF6 — Transcription Factor 4 (TCF4)

As a transcriptional regulator, PHF6 directly associates with chromatin to regulate gene expression during corticogenesis. Previous ChIP-seq studies suggested that PHF6 occupies regulatory regions throughout the developing cortex, with enrichment near transcription start sites and 5′ untranslated regions of genes involved in brain development and neurogenesis. *De novo* motif analysis further revealed a strong enrichment of CA microsatellite repeats at PHF6

binding sites, suggesting that PHF6 recognizes specific genomic regulatory regions involved in developmental gene expression (Rasool et al., 2024).

Preliminary work from our lab identified TCF4 as a potential downstream transcriptional target of PHF6 (Rasool et al., 2024). ChIP-seq analysis suggested PHF6 occupancy at regulatory regions of the TCF4 locus, indicating that PHF6 may regulate TCF4 expression during cortical development. As TCF4 dysfunction has been implicated in several neurodevelopmental disorders, including Pitt-Hopkins Syndrome (PTHS) and autism spectrum disorders, TCF4 may have a convergent molecular pathway underlying neurodevelopmental disorders. In this study, I aim to investigate this regulatory pathway and provide broader insight into the pathogenesis of these neurodevelopmental disorders.

1.4 Transcription Factor 4 (TCF4)

Transcription factor 4 (TCF4, also known as ITF2 or E2-2) is located on chromosome 18q21.2. TCF4 encodes a transcription factor belonging to the E-protein family (class I basic helix–loop–helix [bHLH] proteins), which regulate gene expression through recognition of E-box DNA sequences (CANNTG) located within promoter and enhancer regions of target genes (Forrest et al., 2012; Schoof et al., 2020; Sepp et al., 2011). Structurally, TCF4 contains the bHLH domain, three transcriptional activation domains (AD1, AD2, and AD3), conserved element and repression intramolecular regulatory domains, nuclear localization signals (NLS-1 and NLS-2), nuclear export signals (NES-1 and NES-2), and an RSRS motif composed of four amino acid residues involved in transcriptional regulation and subcellular localization (Forrest et al., 2012; Savchenko & Skryabin, 2024; Teixeira et al., 2021) (**Figure 5**). The TCF4 gene

consists of 41 exons, including 20 alternative 5′ non-coding exons, 20 internal protein-coding exons, and one 3′ non-coding exon (Sepp et al., 2011). Given the complex structure and alternative splicing, at least 18 distinct TCF4 isoforms exist (Teixeira et al., 2021). Although these isoforms share a core structure, they differ primarily within their N-terminal regions, which contribute to differential transcriptional regulatory activity.

Figure 5. The Structure of TCF4 gene. TCF4 spans 437 kb on chromosome 18 and encodes multiple protein isoforms. The longest canonical TCF4 protein consists of 671 amino acids and contains several conserved functional domains. Including three transcriptional activation domains (AD1–AD3), a conserved element (CE), a repression domain (Rep), two nuclear localization signals (NLS1 and NLS2), two nuclear export signals (NES1 and NES2), and a highly conserved C-terminal basic helix–loop–helix (bHLH) DNA-binding domain. Figure created with BioRender.

TCF4 is highly expressed during embryonic and fetal brain development, with its expression reaches peak levels before birth, declines during the perinatal period, and persists in the forebrain and cerebellum during adulthood (Chen et al., 2023; Li et al., 2018). Beyond the

nervous system, TCF4 is expressed in almost all tissues of the organism, and has been implicated in the regulation of hematopoiesis, myogenesis, osteogenesis, and differentiation of endothelial, mammary, and Sertoli cells (Savchenko & Skryabin, 2024; Teixeira et al., 2021).

Importantly, the TCF4 discussed here is not immune regulator transcription factor 7-like 2 (TCF7L2), a member of the TCF/LEF family that has also historically been referred to as T-cell factor 4 (TCF4).

1.4.1 TCF4 in Neurodevelopment

Studies have shown that TCF4 functions as a critical transcription factor that regulates neural progenitor maintenance, neuronal differentiation, and proper cortical development, while TCF4 deficiency during embryonic neurogenesis disrupts cortical organization and impairs neurodevelopment.

In human, the highest expression of TCF4 was detected in the brain, it peaked during embryonic development and decreased after birth (Li et al., 2018; Sirp et al., 2022). Similarly, in mice, studies have detected *Tcf4* expression in the developing CNS starting at E11.5, followed by robust upregulation from E15.5 (Matthias Jung et al., 2018; Sirp et al., 2022). TCF4 expression remains high through late embryonic and early neonatal stages before gradually declining postnatally (Li et al., 2018; Sirp et al., 2022).

TCF4 supports neurogenesis by maintaining the neural progenitor pool while promoting progression toward neuronal maturation. Human patient-derived induced pluripotent stem cell (iPSC) models, neural progenitor cultures, cortical organoids, and embryonic mouse studies demonstrate that TCF4 loss-of-function significantly impairs neural progenitor proliferation and

neurogenic progression, resulting in smaller organoids, fewer cortical neurons and delayed cortical development. Mechanistically, TCF4 deficiency reduces canonical Wnt/β-catenin signaling and decreased expression of SOX-family genes that are required for progenitor maintenance and differentiation (Papes et al., 2022).

TCF4 also contributes to neuronal migration during embryonic corticogenesis. TCF4 is highly expressed in the VZ during early corticogenesis and subsequently detected in immature and post-migratory neurons within the cortical plate (Jung et al., 2018). Upon TCF4 deficiency, neuronal migration is disrupted primarily through impaired radial migration from the VZ toward the cortical plate (CP). Consequently, *Tcf4*-deficient neurons become abnormally retained within the VZ and intermediate zone (IZ), indicating a severe migration delay or arrest. Consistent with these findings, TCF4 haploinsufficiency has been associated with altered cortical lamination and an increased number of upper-layer neurons (Li et al., 2019). Mechanistically, TCF4 deficiency alters neuronal morphology required for migration. *Tcf4* KD neurons in the IZ exhibit defective leading process formation, with many cells lacking the elongated bipolar morphology necessary for radial migration along glial fibers. Instead, neurons adopt abnormal morphologies and fail to efficiently transition through the IZ into the CP (Chen et al., 2016; Mesman et al., 2020).

Furthermore, TCF4 is required for proper synapse formation and maturation of cortical neurons during neurodevelopment. Studies using *Tcf4* haploinsufficient and KO mouse models demonstrated that reduced TCF4 expression impairs dendritic development, producing cortical neurons with shortened, less branched basal dendrites and simplified apical dendritic arborization, structural abnormalities that limit synaptic connectivity. Consistent with this, primary cortical neurons from *Tcf4*-deficient mice exhibited reduced density of synaptophysin-

positive puncta along dendrites, indicating decreased synapse formation (Li et al., 2019). Functional studies in patient-derived cortical organoids and neurons from individuals with PTHS further showed reduced neuronal firing rates and lower expression of activity-dependent markers, suggesting that TCF4 deficiency not only reduces synaptic development but also impairs neuronal network activity and functional connectivity (Papes et al., 2022). Together, these findings indicate that TCF4 functions as a key regulator of neurodevelopment by coordinating neural progenitor maintenance, neuronal differentiation, migration, dendritic maturation, and synapse formation to ensure proper cortical organization and functional neural circuit development.

1.4.2 TCF4 in Adult Neurogenesis

TCF4 has been shown to be expressed in adult neurogenic niches, including the SGZ of the hippocampus, as well as in other brain regions, including the cortex, the Purkinje cell layer of the cerebellum, and the amygdala (Braun et al., 2020; M. Jung et al., 2018; Kim et al., 2020; Shariq et al., 2021).

Studies have indicated that TCF4 is required for aNSC-mediated hippocampal neurogenesis. TCF4 has been shown to co-localize with radial glia-like stem cells, proliferating precursor cells, as well as immature and mature neurons (Braun et al., 2020; Shariq et al., 2021), indicating that TCF4 is expressed throughout the stages of adult hippocampal neurogenesis. Loss of *Tcf4* in adult progenitors results in marked reductions in proliferative capacity, depletion of the neural precursor pool, impaired neuronal differentiation, and reduced production of new neurons (Braun et al., 2020; Sarkar et al., 2021; Shariq et al., 2021). Furthermore, deletion of Tcf4 in

neural progenitors causes their transformation into a myeloid-like inflammatory cell state and induces inflammation in the DG (Shariq et al., 2021). In addition, *Tcf4* deletion in adult brain excitatory neurons alters membrane properties and results in hyperexcitability of CA1 neurons, with *Tcf4* KO brains exhibiting increased action potential firing and reduced spike amplitude (Sarkar et al., 2021).

TCF4 expression is broadly distributed across multiple mature neuronal and glial populations in other brain regions. In the cortex, *Tcf4* is expressed in both excitatory glutamatergic and inhibitory GABAergic neurons, as well as in astrocytes and oligodendrocytes (Kim et al., 2020; Sarkar et al., 2021). In contrast, within the striatum (caudate putamen), Tcf4 expression is predominantly enriched in inhibitory neurons, including somatostatin and parvalbumin expressing interneurons (Kim et al., 2020). In the cerebellum, TCF4 expression is enriched primarily in the molecular layer and granule cell layer, with the strongest expression observed in mature granule neurons rather than Purkinje cells (Kim et al., 2020). Immunohistochemical analyses of the adult mouse brain demonstrated persistent TCF4 expression long after development, particularly in mature neuronal populations of the cortex and cerebellum, supporting a role in maintaining neuronal identity, structural integrity, and circuit plasticity rather than regulating adult neural progenitors (M. Jung et al., 2018; Kim et al., 2020; Sarkar et al., 2021). However, despite sustained expression in mature brain regions, the precise mechanisms through which TCF4 regulates neuronal maintenance and function in the adult brain require further investigation.

1.4.3 TCF4-Associated Disorders

PTHS is a rare autosomal dominant neurodevelopmental disorder caused by mutations in the TCF4 gene (Pitt & Hopkins, 1978). More than 200 pathogenic TCF4 variants have been identified in individuals with PTHS. The majority of the mutations are de novo loss-of-function variants that result in TCF4 haploinsufficiency. Approximately 30% of patients carry whole-gene deletions, whereas the remaining cases are predominantly caused by protein-truncating sequence variants, including nonsense and frameshift mutations, with missense and splice-site variants occurring less frequently. Most pathogenic variants cluster within exons 9–19, which encode the conserved bHLH DNA-binding domain and other critical functional regions of TCF4. (Amiel et al., 2007; Brockschmidt et al., 2007; de Pontual et al., 2009; Marangi & Zollino, 2015; Pitt & Hopkins, 1978; Zweier et al., 2007). Individuals with PTHS typically present with severe ID, profound speech impairment or complete absence of expressive language, developmental delay, motor dysfunction, seizures and autism-like behaviours. One of the most distinctive clinical hallmarks of PTHS is episodic hyperventilation followed by apnea while awake, an autonomic breathing abnormality that is uncommon in most other neurodevelopmental disorders, and therefore serves as an important diagnostic feature (Marangi et al., 2011; Van Balkom et al., 1998; Whalen et al., 2012). Patients also exhibit a characteristic facial gestalt, including deep-set eyes, a broad and prominent nasal bridge, flared nasal alae, a wide mouth with a cupid's bow upper lip, a full everted lower lip, and a prominent chin, with these craniofacial features becoming more recognizable with age (Rosenfeld et al., 2009; Whalen et al., 2012; Zweier et al., 2007).

Genome-wide association studies have linked TCF4 with schizophrenia (SCZ) and other psychiatric conditions (Ripke et al., 2014; Stefansson et al., 2009). Unlike PTHS, schizophrenia is predominantly associated with common genetic variants that subtly alter TCF4 expression or regulation. These variants are located within non-coding regulatory regions of the gene and influence the timing, level, or spatial pattern of TCF4 expression during brain development.

1.4.4 Clinical Comparison Between PTHS and BFLS

Both PTHS and BFLS are characterized by ID, global developmental delay, impaired language development, and deficits in learning and cognition, suggesting that disruption of PHF6 or TCF4 affects overlapping developmental pathways during embryonic brain development. However, the two disorders also exhibit distinct clinical phenotypes. PTHS is distinguished by episodic respiratory abnormalities, including hyperventilation and breath-holding, together with characteristic craniofacial features. In contrast, patients with BFLS typically present with hypogonadism, gynecomastia and progressive obesity. Overall, the shared neurological manifestations of PTHS and BFLS, despite their distinct syndromic features, raise the possibility that PHF6 and TCF4 converge on shared molecular pathways regulating embryonic neurogenesis.

1.5 Hypothesis and Objectives

Hypothesis: PHF6 cooperates with TCF4 at different timelines to regulate neurogenesis and defects in PHF6 signaling in BFLS reprogram early neurodevelopmental programs in a cell type specific manner.

Aim 1: To determine whether TCF4 is a direct transcriptional target of PHF6 during cortical neurogenesis.

Aim 2: To investigate the PHF6 regulation of TCF4 and its implication in BFLS.

2. Materials and methods

2.1 Mouse Models

All animal experiments received approval from the Animal Care Committee (ACC) at the University of Ottawa, Ottawa, Ontario, Canada. Mice were housed under standard conditions in a pathogen-free facility with ad libitum access to food and water.

The R342X mouse line was created via CRISPR/Cas9 technology, producing a truncated PHF6 protein (Ahmed et al., 2021; Crawford et al., 2006; Gécz et al., 2006; Jahani-Asl et al., 2016; Lower et al., 2004; Lower et al., 2002). This line was maintained by crossing R342X female heterozygous (HET) mice with C57BL/6J WT (B6 WT) male mice. Hemizygous (HEMI) males served as experimental subjects, while B6 WT males were used as controls. The C99F-m mouse model was generated using CRISPR/Cas9, where cysteine at position 99 is substituted with phenylalanine (C99F) through a nucleotide change at nt.29G>T (Cheng et al., 2018). This line was propagated by breeding C99F-m female HET mice with B6 WT male mice. HEMI males were designated as experimental animals, with B6 WT males as controls.

The *Phf6* knockout (*Phf6*$^{-/Y}$*/Nestin-CreERT2*$^{+}$) strain was generated by crossing Phf6$^{fl/fl}$ female mice with Nestin-CreERT2^{+} male mice (McRae et al., 2019). Cre recombinase activity was induced through oral administration of Tamoxifen (Sigma-Aldrich, T5648) to pregnant dams at E12, with embryo collection occurring 48 hours post-treatment. *Phf6*$^{-/Y}$*/Nestin-CreERT2*$^{+}$ mice were analyzed alongside *Phf6*$^{loxP/Y}$*/Nestin-CreERT2*$^{-}$ control mice that also received tamoxifen treatment. Male mice were utilized throughout these experiments.

2.2 Genotyping

Mouse tissue samples (ear biopsies) were lysed in alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA, pH 12) and incubated at 95°C for 30 minutes. Samples were subsequently neutralized with an equivalent volume of neutralization buffer (40 mM Tris-HCl, pH 5.0).

For C99F-m and R342X genotyping, PCR reactions (25 µL total volume) contained: 2.5 µL 10× PCR buffer, 0.2 µL 25 mM dNTP, 6.5 µL betaine, 1 µL each of 10 µM forward and reverse/mutation primers, 0.2 µL Klentaq Thermostable DNA Polymerase (Jena Bioscience, #PCR-217L), 12.6 µL RNase-free H_2O, and 1 µL DNA template.

For $Phf6^{fl/fl}$ genotyping, PCR reactions (15 µL total) were prepared with: 7.5 µL 2× Green PCR Master Mix (ZmTech Scientific, #S2100G), 0.4 µL each of 10 µM forward and reverse primers, 5.7 µL RNase-free H_2O, and 1 µL DNA template.

Nestin-CreERT2 genotyping reactions (15.5 µL total) consisted of: 7.5 µL 2× Green PCR Master Mix (ZmTech Scientific, #S2100G), 1.5 µL each of 0.5 µM primers (oIMR1084, oIMR1085, oIMR7338, and oIMR7339), 0.975 µL 6.5% glycerol, and 1 µL DNA template.

PCR amplification was performed in a Bio-Rad T100 Thermal Cycler. For C99F-m, R342X, and $Phf6^{Loxp/Loxp}$, the cycling conditions were: initial denaturation at 95°C for 2 minutes; 34 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; followed by final extension at 72°C for 4 minutes.

The Nestin-CreERT2 samples underwent: 94°C for 2 minutes; 10 touchdown cycles of 94°C for 20 seconds, 65°C for 15 seconds (decreasing 0.5°C per cycle), and 68°C for 10 seconds; 28 cycles of 94°C for 15 seconds, 60°C for 15 seconds, and 72°C for 10 seconds; with final extension at 72°C for 2 minutes. The Nestin-Cre genotyping used: 94°C for 2 minutes; 35 cycles of 94°C for 20 seconds, 60°C for 20 seconds, and 72°C for 25 seconds; followed by 72°C for 2 minutes.

Amplified products were analyzed by electrophoresis on 3% agarose gels at 100 V for 40 minutes (C99F-m, R342X, Nestin-CreERT2, and Nestin-Cre) or 60 minutes (*Phf6*$^{fl/fl}$). Primers listed in Table 1.

Table 1. Genotyping PCR Primers

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

2.3 Tamoxifen Induction

For temporal control of Cre recombinase in $Phf6^{fl/fl}$/Nestin-CreERT2 mice, pregnant dams at gestational day E12 received a single 0.1 mL oral gavage of Tamoxifen (Sigma-Aldrich, T5648) at 20 mg/mL concentration using a 1 mL syringe fitted with a 22-gauge feeding needle (Instech Solomon, #FTP-22-25-5).

2.4 Embryonic neural stem cell culture

Embryonic neural stem cells (eNSCs) were obtained by culturing whole brains isolated from E14 mice (excluding cerebellum). Pregnant mice were euthanized, and embryos were harvested by dissecting the uterine horns. Embryos were transferred into cold 1× Hank's Balanced Salt Solution (HBSS). Brain tissues were dissected from embryos and immediately placed into a 15mL Falcon containing 1mL cold 1× HBSS. The tissue was allowed to settle, HBSS was replaced with 1 mL fresh HBSS for washing, then replaced again with 1 mL of stem cell medium (SCM) containing 1:1 DMEM-F12 (Wisent, 319-005-CL) (ThermoFisher, 31765035), 50 units/mL penicillin-streptomycin (Wisent, 450-201-EL), 1× B-27 supplement (Invitrogen, 17504044), 10 mg/mL Heparin (Stemcell Technologies, 07980), 100µg/mL EGF

(Cell Signaling, 5331SC), 25µg/mL bFGF (Abbiotec, 600182). The tissue was triturated 10 times with a P1000 pipette, then 10 times with a P200 pipette. The single cell was plated in 6 mL of SCM and incubated for 6–7 days, allowing neurospheres to grow.

2.5 Adult neural stem cell culture

Adult neural stem cells (aNSCs) were extracted from the brains of mice at 6-8 weeks. Mice were euthanized, the subventricular zone (SVZ) and subgranular zone (SGZ) were dissected and transferred to sterile microcentrifuge tubes. Papain working solution (PWS, 15µL of Papain suspension enzyme is added to 1 mL of 0.53 mM EDTA and 10µL L-Cysteine) was filter-sterilized using a 0.22 µm syringe filter into the Falcon. Dissected tissue was transferred into Falcon using a plastic pipette pre-wetted with PWS. Tissue was mechanically dissociated by pipetting until freely entering the tip, followed by 20 rounds of trituration using a P1000 pipette set to 800 µL. Samples were incubated at 37 °C for 10 minutes and homogenized again (20 cycles, P1000 set to 800 µL). DNase I (5 µL of 10 ng/µL) was added to each sample, mixed by gentle flicking, and incubated at 37 °C for 20 minutes. Samples were homogenized again (20 times), followed by a second DNase treatment using the same conditions, flicked gently, and incubated at 37 °C for 15 minutes. This was followed by two additional cycles of 15-minute incubations and 20 homogenization strokes. After the final incubation, 400 µL of trypsin inhibitor (1 mg/mL) was added to each sample to terminate enzymatic digestion. Five millilitres of base media containing 3:1 of DMEM-F12 and 250 µL of 1% penstrep were added to each tube. For each sample, the cell suspension was filtered through a 40 µm cell strainer pre-wetted

with 8 mL of base media placed on a fresh 50 mL Falcon tube; the 8 mL was discarded before slowly pouring the sample through the strainer and centrifuged at 1000 rpm for 10 minutes. The supernatant was carefully aspirated, and the cell pellet was resuspended in 1 mL of proliferation media containing 3:1 of DMEM-F12, 500 µL of 1% penstrep, 1mL 2% B27, and 20µL of 50µg/ mL mouse EGF. Cells were resuspended and transferred to a T75 flask containing 29mL of fresh proliferation media and incubated for 14 days, allowing neurospheres to grow.

2.6 Electroporation

Tcf4 was knocked down (KD) using short interfering RNA (siRNA) in eNSCs and aNSCs with ON TARGET-plus SMART pool mouse *Tcf4* siRNA (Santa Cruz, sc-61658) and ON TARGET-plus non-targeting pool (Santa Cruz, #sc-36869) at a concentration of 100 nM. A lentiviral vector for overexpressing the TCF4 transcription factor open reading frame (ORF), tagged with a unique 24-bp barcode to enable identification in pooled screens, was obtained from Addgene (plasmid #141560). siRNA/plasmid was nucleofected into NSCs (10^6 cells) and cultured in NSCs media at 37 °C in a humidified atmosphere of 5% CO_2. Electroporation was delivered using the BTX™ Gemini X2 Electroporation System set at 250 volts, resistance 1050 Ω, capacitance 250 uF, and an electrode gap of 2 mm.

2.7 Extreme Limiting Dilution Assay (ELDA)

Extreme limiting dilution assays (ELDA) were conducted to assess self-renewal capacity of NSCs. Single-cell NSCs suspensions were prepared using Accumax. Single cells were counted, and 100µl of the diluted single-cell suspensions were plated in a 96-well plate, with 12 wells plated for dilutions of 25, 12, 6, 3, and 1 cell(s) per well. Plates were incubated at 37°C with 5% CO_2 for 7 days, after which wells were scored for the presence or absence of neurospheres. ELDA data was analyzed through http://bioinf.wehi.edu.au/software/elda/, which calculates stem cell frequency (SCF) and provides statistical estimates for the minimum number of cells required to initiate sphere formation under each condition.

2.8 Limiting Dilution Assay (LDA)

Neurospheres were enzymatically dissociated into single-cell suspensions, then serially diluted in complete NSC medium. Diluted single-cell suspensions (100 µL per well) were seeded into 96-well ultra-low attachment plates at limiting dilutions of 200, 100, 50, 25, 12, and 6 cell(s) per well, with 3 replicate wells per condition. Plates were incubated at 37°C with 5% CO_2 for 7 days, after wells were scored for the number of neurospheres present.

2.9 Quantitative real-time PCR

RNA was isolated from cells and 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 min, followed by incubation at 42 °C for 15 min, and finally at 85 °C for 5 min to inactivate the reaction. See primers listed in Table 2.

Table 2. RT-qPCR Primers

Primers	**Sequence 5'➔3'**
TCF4-F	GATGGGACTCCCTATGACCAC
TCF4-R	GAAAGGGTTCCTTTATTGCCC
PHF6-F	TGAAATATGAGCTGGTCAATCAC
PHF6-R	TACAGTATTTTGGGGAAGCTGG

2.10 Immunoblotting

Protein lysates were prepared from brain tissue using 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. A total of 30 µg of protein was loaded for each condition. PVDF membranes were activated in methanol for 5 min and then blocked in 5% BSA in TBST. Membranes were probed with anti-PHF6 (NOVUS, NB100-68262, 1:1000), anti-TCF4 (ThermoFisher, PA5-75987, 1:1000), anti-SOX2 (Abcam, ab97959, 1:250), anti-NESTIN (Santa Cruz, sc-23927, 1:100 or R&D Systems, MAB2736, 1:500), anti-beta-Actin (Sigma-Aldrich, a5316, 1:2000), alpha-Tubulin (Abcam, 9074, 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 h at room temperature. Proteins were visualized using ECL (Bio-Rad), and images were captured with a Chemidoc imaging system (Bio-Rad).

2.11 Chromatin immunoprecipitation (ChIP)

Cell pellets were collected and washed with PBS supplemented with protease inhibitors (Thermo Fisher Scientific, #A32959) before fixation. Cross-linking was performed using 1% formaldehyde in PBS for 10 minutes, followed by quenching with 0.125 M glycine in PBS for 5 minutes at room temperature. Washing, fixation, and quenching were done in 15 mL tubes while rotating at RT. After quenching, cells were washed twice with PBS containing protease inhibitors. Cell pellets were collected by spinning at 150 × *g* for 5 min at 4 °C. Next, 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 tip sonication on ice at 25% amplitude for 2 minutes using 10 second sonication followed by 20 second pauses. Sonication was optimized to generate DNA fragments ranging from approximately 300–800bp, assessed by agarose gel electrophoresis. Cell lysates were centrifuged at 12,000 × g for 15 minutes at 4°C, and the resulting supernatant was diluted 1:1 with 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). Antibody-protein-DNA complexes were collected, washed, and then eluted. Reverse cross-linking was performed as described in Soleimani et al., 2013 (Soleimani et al.,

2013). Immunoprecipitated DNA was quantified by qPCR, and binding enrichment was calculated as a percentage relative to input DNA. Primers were designed to target genomic regions corresponding to ChIP-seq peaks listed in Table 3.

Table 3. ChIP-qPCR Primers

Primers	**Sequence 5'→3'**
TCF4-F	CCGAGCCAGGTGCATAATCT
TCF4-R	CTCACATGAGTGTTCCCCCAA
EphA4-F	GGAGGGGGAGAGAGACAGAC
EphA4-R	TTTTTCTGTCCGAGGTGAGG
ZFP735-F	TGGTCCATCCTTTTGACACA
ZFP735-R	ACTTTGCCCCTTCGAATTTT

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 *TCF4* luciferase reporter genes, primers were designed based on the PHF6 ChIP-seq peak to amplify an approximately 1kb genomic region encompassing the TCF4 promoter. The plasmid and the annealed primer pair were digested using KpnI (NEB, #R3142) and HindIII (NEB, #R3104) then ligating them with T4 DNA ligase (NEB, #M0202L). The constructs were confirmed by DNA sequencing. eNSCs were electroporated with the TCF4-pGL4.23 or the empty pGL4.23. Luciferase assays were performed 48h after electroporation with the Dual-Luciferase Reporter Assay system (Promega, #E1910) with Synergy H1 BioTek

(luminance mode). In all experiments, cells were electroporated with a Renilla firefly reporter control, and the firefly luminescence signal was normalized to the Renilla luminescence signal. Primers listed in Table 4.

Table 4. Luciferase Primers

Primers	**Sequence 5'→3'**
TCF4-F	GCCATCGGTACCAAACCGAGCCAGGTGCA
TCF4-R	GCCATCAAGCTTCCCCTCAGATCGCCAGTT

2.13 Immunofluorescence staining of tissue

Mouse brains were fixed in 4% paraformaldehyde (PFA) for 24 hours, followed by 48 hours of 30% sucrose fixation, brains were frozen in OCT on dry ice. 8 μm frozen sections were cut using a cryostat (HM525 MX). Brain sections were washed three times in PBS for 5 minutes each on a shaker, followed by permeabilization in 0.1% Tween-20 detergent (PBST) for 20 min at room temperature. Sections were then washed three times in PBS for 5 min each on a shaker. The tissue was outlined using a DAKO pen before blocking. Samples were then blocked in 20% horse serum, 0.1% Triton X-100, and 0.1% Tween-20 diluted in PBS for 30 min at room temperature. After blocking, sections were subjected to the following primary antibody for TCF4 immunofluorescence (overnight at 4 °C): anti-TCF4 (1:100, ThermoFisher, PA5-75987), and DAPI (1:1000, ThermoFisher, D1306). Secondary antibodies (1:500); Anti-rabbit IgG, Alexa Fluor® 594 Conjugate (Cell Signaling, 8889S), and DAPI (1:1000 of 1 μg/ml) (ThermoFisher,

D1306) were applied for 60 minutes at room temperature in a humid chamber. Slides were mounted with ProLong Gold Antifade Mountant (ThermoFisher, P36934) with a coverslip. Images were obtained with Zeiss Axio Observer 7 microscope.

2.14 Leveraging published RNA sequencing datasets

Single cell RNA-seq data from the developing mouse cerebral cortex were obtained from (Di Bella et al., 2021). Log-normalized counts, cell type annotations and UMAP coordinates were retrieved from the original publication and used to generate UMAP plots. For the correlation analyses, MAGIC (v.2.0.3) (van Dijk et al., 2018) was applied to obtain imputed gene-expression values, and Pearson correlation coefficients were computed using the imputed matrix. For human bulk RNA-seq profiles, normalized RPKM (Reads per Kilobase Million) values were retrieved from the Allen Brain Atlas BrainSpan dataset (BrainSpan (Atlas of the Developing Human Brain Secondary BrainSpan, 2011) and filtered to include only samples from the ventral frontal cortex (VFC). Average RPKM values were calculated for each developmental time point. All analyses and plots were generated using R (v.4.3.1).

2.15 Statistical analysis

All experiments were performed with three independent biological replicates ($n \geq 3$). Statistical analysis was performed with software Prism 8. Two-tailed unpaired Student t-tests were used to compare two conditions. One-way ANOVA Tukey's Honest Significant Difference

test was used to analyze multiple groups. Data are shown as mean with standard error of mean (mean ± SEM). *p*-values of 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 ***. Methods of statistical analysis and *p*-values employed are reported in the corresponding figure legends. 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.

3. Results

3.1 Gene expression analyses of PHF6 and TCF4 during cortical neurogenesis

Previous analysis of PHF6 genome-wide occupancy using ChIP-seq established that PHF6 directly binds genomic DNA in the embryonic mouse brain. Building on these findings, we further investigated the mechanism of BFLS by defining how PHF6 regulates NSC fate through its downstream transcriptional effectors. Integration of PHF6 ChIP-seq and transcriptomic analyses identified *Tcf4* as a candidate downstream target, with significant PHF6 occupancy at the *Tcf4* regulatory region ($p = 7.32 \times 10^{-5}$) (Rasool et al., 2024). Given the well-established role of TCF4 in embryonic neurogenesis and its association with neurodevelopmental disorders, we assessed whether *Tcf4* is a potential target of PHF6-dependent regulation of cortical development. To begin with, we characterized the relationship between *Phf6* and *Tcf4* expression during neurodevelopment, via querying a publicly available single-cell RNA-seq atlas of the developing mouse cortex (Di Bella et al., 2021). Our analysis revealed that both genes were broadly expressed across neurodevelopmental stages, spanning embryonic day 10 (E10) to postnatal day 4 (P4) (**Fig. 6A-C**). Pearson correlation analysis of imputed gene expression values demonstrated a positive association between *Phf6* and *Tcf4* across multiple cortical cell populations, including apical progenitors and cycling glial cells (**Fig. 6D, E**). To determine whether *Phf6* and *Tcf4* exhibit coordinated temporal expression across developmental time, we also examined normalized expression across progenitor populations. Both genes showed sustained expression across embryonic stages, with enrichment in apical progenitors, intermediate progenitors, and cycling glial cells (**Fig. 6F, G**). Overall, *Phf6* and *Tcf4* exhibited positive correlation in expression across multiple lineages, spanning from early neurogenesis

through late embryonic stages. This raised the question of whether these two transcription factors, known to be associated with different human disease, operate on the same pathway to regulate neurogenic processes.

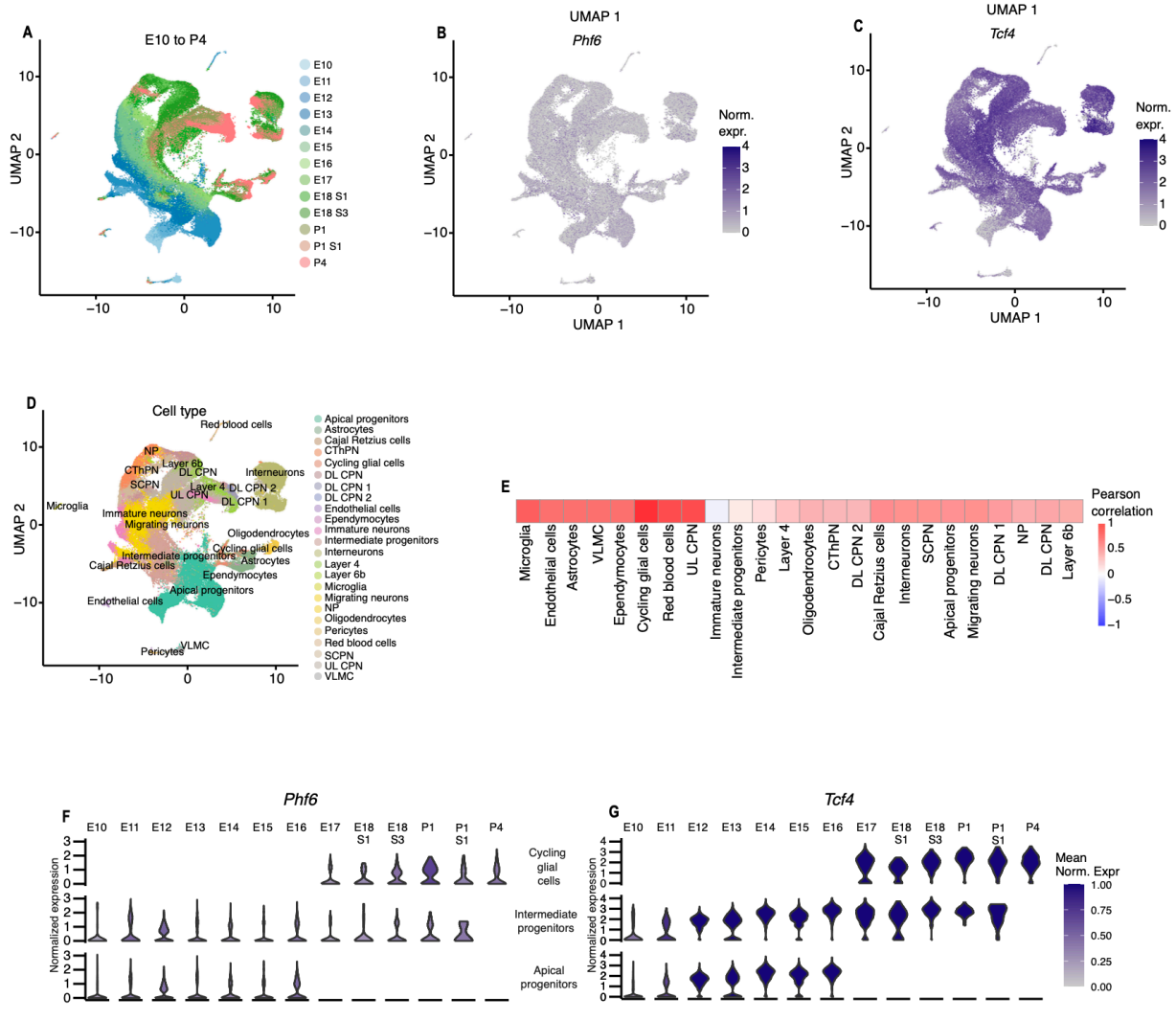
A
E10 to P4
UMAP 2
E10
E11
E12
E13
E14
E15
E16
E17
E18 S1
E18 S3
P1
P1 S1
P4
B
UMAP 1
Phf6
Norm. expr.
C
UMAP 1
Tcf4
Norm. expr.
D
Cell type
Red blood cells
NP
Layer 6b
CThPN
DL CPN
Interneurons
SCPN
Layer 4
DL CPN 2
UL CPN
DL CPN 1
Microglia
Immature neurons
Migrating neurons
Oligodendrocytes
Cycling glial cells
Intermediate progenitors
Astrocytes
Cajal Retzius cells
Ependymocytes
Apical progenitors
Endothelial cells
VLMC
Pericytes
Apical progenitors
Astrocytes
Cajal Retzius cells
CThPN
DL CPN
DL CPN 1
DL CPN 2
Endothelial cells
Ependymocytes
Immature neurons
Intermediate progenitors
Interneurons
Layer 4
Layer 6b
Microglia
Migrating neurons
NP
Oligodendrocytes
Pericytes
Red blood cells
SCPN
UL CPN
VLMC
E
Pearson correlation
Microglia
Endothelial cells
Astrocytes
VLMC
Ependymocytes
Cycling glial cells
Red blood cells
UL CPN
Immature neurons
Intermediate progenitors
Pericytes
Layer 4
Oligodendrocytes
CThPN
DL CPN 2
Cajal Retzius cells
Interneurons
SCPN
Apical progenitors
Migrating neurons
DL CPN 1
NP
DL CPN
Layer 6b
F
Phf6
Normalized expression
Cycling glial cells
Intermediate progenitors
Apical progenitors
G
Tcf4
Mean Norm. Expr

Figure 6: Cell type-specific co-expression analysis of *Phf6* and *Tcf4* in mouse cerebral cortex. Low-dimensional representation of single cells from the mouse cerebral cortex, derived from UMAP embedding of single-cell RNA-seq data are shown. (A) animal age, or expression level of (B) *Phf6* and (C) *Tcf4*. (D) cell-typed annotations. (E) Heatmap displaying Pearson correlation coefficients between *Phf6* and *Tcf4* across various cell types. (F-G) Violin plots showing the expression of *Phf6* (F) and *Tcf4* (G) in the developing mouse cerebral cortex. Violin color represents the mean normalized expression level for each cell type at each developmental stage. Correlation values were computed using imputed gene expression profiles obtained after applying MAGIC (van Dijk et al., 2018). Single cell RNA-seq data was obtained from Di Bella et al, 2021.

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3.2 TCF4 expression is attenuated upon deletion of *Phf6* in Nestin-expressing cells

To begin with, we set out to characterize how TCF4 expression is altered in eNSCs lacking PHF6. We induced genetic deletion of *Phf6* via breeding *Phf6*$^{loxP/loxP}$ with Nestin-CreERT2^{+} mice, and administered tamoxifen at E12 to delete *Phf6* exons 4 and 5 in Nestin-expressing cells embryonically, followed by analysis of brain sections at E14 (**Fig. 7A**). Immunohistochemical analysis of the embryonic brain sections revealed consistent expression of TCF4 in the subventricular zone (SVZ) of Phf6$^{loxP/Y}$/Nestin-CreERT2^{-} control (**Fig. 7B**), consistent with its role in late embryonic and early postnatal neurogenesis (Burette et al., 2024; Mesman et al., 2020). In contrast, TCF4 expression was markedly reduced upon deletion of *Phf6*, as revealed by analysis of brain sections from the Phf6$^{-/Y}$/Nestin-CreERT2^{+} mice (**Fig. 7B**). To validate our observation, we next examined the mRNA and protein expression levels of TCF4 in brain tissue. RT-qPCR analysis revealed a significant reduction in *Tcf4* mRNA expression at E14 in Phf6$^{-/Y}$/Nestin-CreERT2^{+} mice compared to the Phf6$^{loxP/Y}$/Nestin-CreERT2^{-} control (**Fig. 7C**). Consistent with these data, immunoblotting of E14 cortical lysates of Phf6$^{-/Y}$/Nestin-CreERT2^{+} model revealed a marked reduction in TCF4 protein levels (**Fig. 7D, E**). Together, our data revealed that PHF6 deletion in Nestin-expressing cells results in a significant reduction in TCF4 expression.

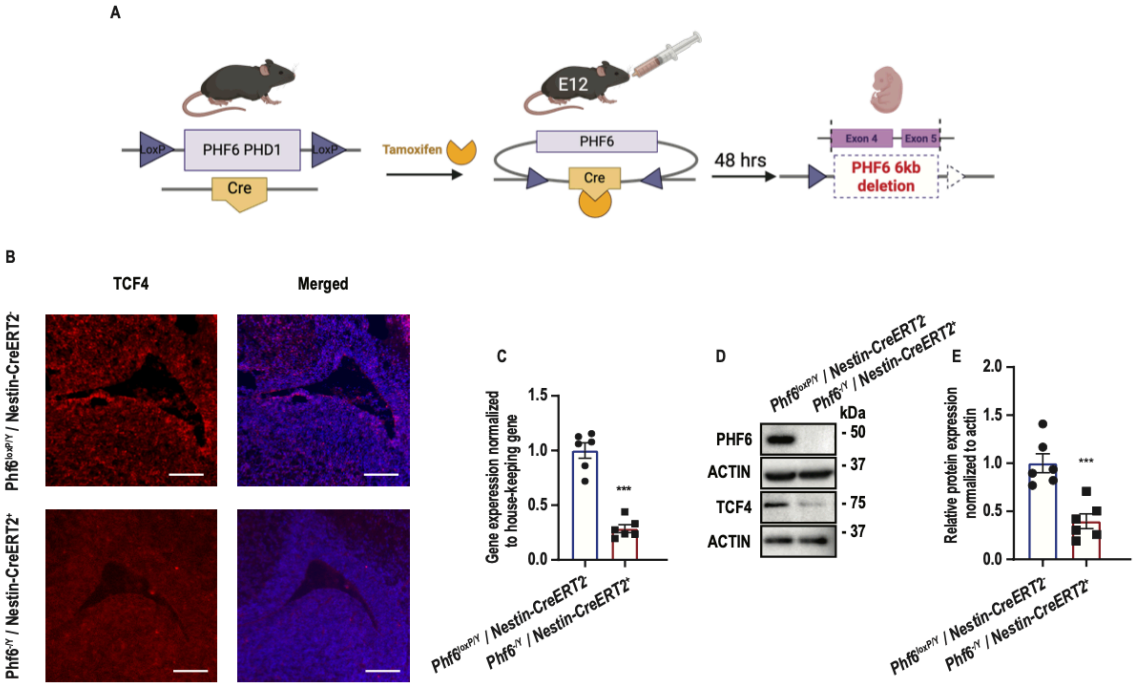
A
LoxP
PHF6 PHD1
LoxP
Cre
Tamoxifen
E12
PHF6
Cre
48 hrs
Exon 4
Exon 5
PHF6 6kb deletion
B
TCF4
Merged
Phf6loxP/Y / Nestin-CreERT2-
Phf6-/Y / Nestin-CreERT2+
C
Gene expression normalized to house-keeping gene
1.5
1.0
0.5
0.0
***
Phf6loxP/Y / Nestin-CreERT2-
Phf6-/Y / Nestin-CreERT2+
D
Phf6loxP/Y / Nestin-CreERT2-
Phf6-/Y / Nestin-CreERT2+
kDa
PHF6
- 50
ACTIN
- 37
TCF4
- 75
ACTIN
- 37
E
Relative protein expression normalized to actin
2.0
1.5
1.0
0.5
0.0
***
Phf6loxP/Y / Nestin-CreERT2-
Phf6-/Y / Nestin-CreERT2+

Figure 7: TCF4 expression is impaired in PHF6 cKO mice. (A) A floxed allele of PHF6, with LoxP sites flanking the PHD1 domain, was used to generate a conditional knockout mouse model. Upon tamoxifen administration at E14, Cre recombinase driven by the Nestin-CreERT2 promoter is activated and translocates to the nucleus, where it mediates site-specific recombination at the LoxP sites. This results in a 6 kb genomic deletion of the PHF6 gene at exons 4 and 5. Figure made with BioRender. (B) Immunofluorescence (IF) staining of coronal sections from E14 for $Phf6^{-/Y}$/Nestin-CreERT2^{+} and control $Phf6^{loxP/Y}$/Nestin-CreERT2^{-} male mice using a TCF4 antibody (red). (C-E) mRNA and protein of $Phf6^{-/Y}$/Nestin-CreERT2^{+} and control $Phf6^{loxP/Y}$/Nestin-CreERT2^{-} mice ~E14 were subjected to RT-qPCR and immunoblotting analysis (n=3). Statistical comparisons were performed using an unpaired two-tailed Student's t-test. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n represents an independent biological sample.

3.3 TCF4 expression is reduced in BFLS mouse models

Next, to address whether PHF6 regulation of TCF4 expression is impaired in the disease context, we set out to assess the TCF4 expression in two BFLS mouse models harboring *PHF6* patient mutations, R342X and C99F-m. The R342X mutation is the most recurrent BFLS patient-mutation occurring at exon 10 (C.1024 C > T), impairing the ePHD2 domain, whereby PHF6 is proposed to function as a truncated protein(Ahmed et al., 2021; Chao et al., 2010; Crawford et al., 2006; Gécz et al., 2006; Lower et al., 2004; Lower et al., 2002). Another BFLS patient point mutation (m) in *Phf6* is wherein cysteine-99 is replaced with phenylalanine (C99F), caused by a nucleotide change at position 296 (G>T), impairing the function of the PHD1 domain (**Fig. 8A**). We subjected brain sections from R342X and C99F-m to immunostaining analysis. Similar to results obtained in the PHF6 cKO model, we observed a reduction in TCF4 expression at E14 in both R342X and C99F-m mutant models, with the most pronounced effects in the brain of R342X mice (**Fig. 8B**). RT-qPCR analysis revealed a significant decrease in *Tcf4* mRNA in both R342X and C99F-m brain tissue compared to control mice (**Fig. 8C, F**). Moreover, immunoblotting analysis showed that both R342X and C99F-m mutants displayed reduced TCF4 protein expression compared to corresponding controls (**Fig. 8D-E, G-H**). Together, our data using three different mouse models reveals that genetic deletion of *Phf6* or its loss-of-function mutations impairs the expression of TCF4, raising the question of whether TCF4 is a downstream effector of PHF6 in the regulation of neurogenesis.

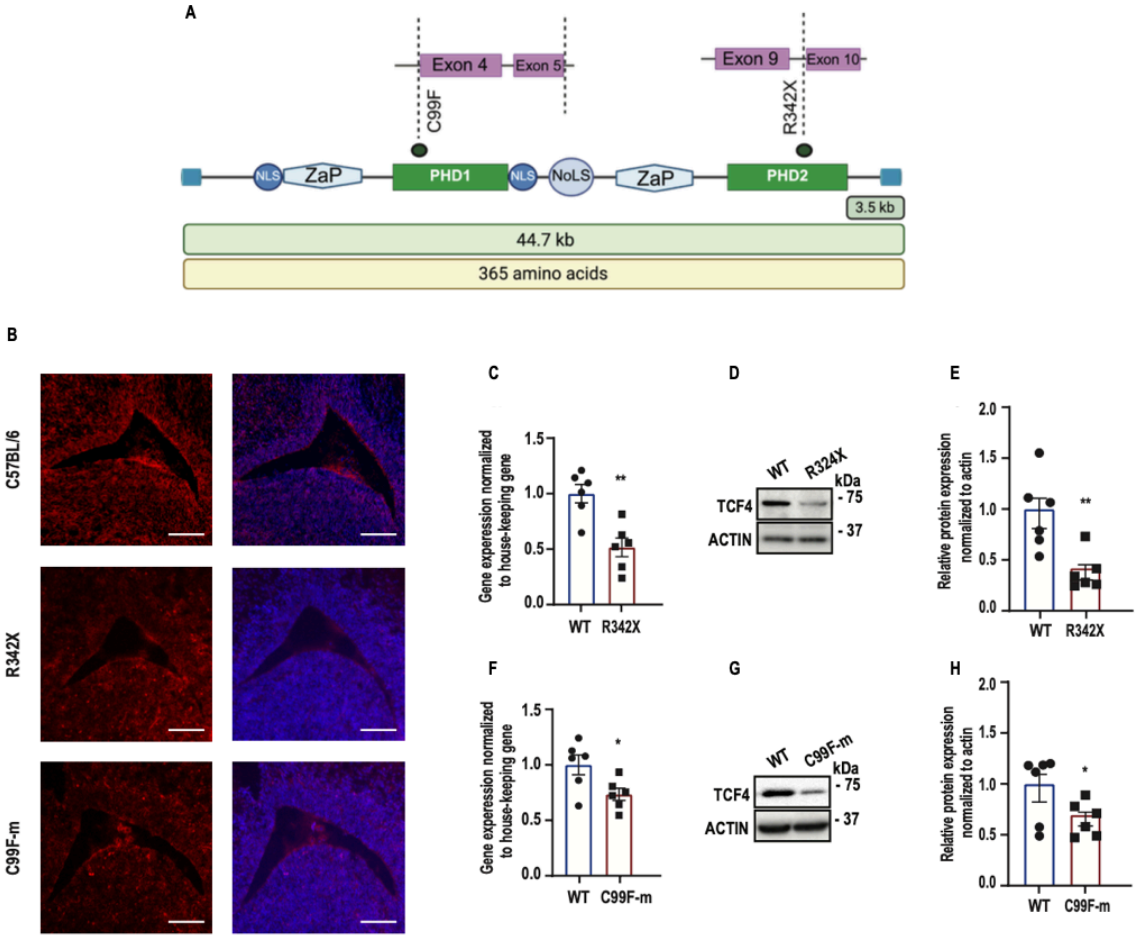
A
Exon 4
Exon 5
Exon 9
Exon 10
C99F
R342X
NLS
ZaP
PHD1
NLS
NoLS
ZaP
PHD2
3.5 kb
44.7 kb
365 amino acids
B
C57BL/6
R342X
C99F-m
C
Gene expression normalized to house-keeping gene
WT
R342X
D
WT
R324X
kDa
TCF4
ACTIN
- 75
- 37
E
Relative protein expression normalized to actin
WT
R342X
F
Gene expression normalized to house-keeping gene
WT
C99F-m
G
WT
C99F-m
kDa
TCF4
ACTIN
- 75
- 37
H
Relative protein expression normalized to actin
WT
C99F-m

Figure 8: TCF4 expression is reduced in BFLS mice. (A) Illustration of the full *Phf6* gene**.** The two recurrent BFLS mutations are shown: C99F and R342X (truncation at exon 10). Made with BioRender. (B) Immunofluorescence (IF) staining of coronal sections from E14 for R342X, C99F-m, and littermate C57BL/6 wild-type (WT) males as controls using a TCF4 antibody (red). (C-H) mRNA and protein of R342X, C99F-m, and wild-type control mice ~E14 were subjected to RT-qPCR and immunoblotting analysis (n=3). Statistical comparisons were performed using an unpaired two-tailed Student's t-test. Data information: Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. n represents an independent biological sample.

3.4 TCF4 is a direct transcriptional target of PHF6

Having established the significant deregulation of TCF4 expression in a PHF6-dependent manner in BFLS and Phf6$^{-/Y}$/Nestin-CreERT2^{+} mice, we next asked if TCF4 is a direct transcriptional target of PHF6. We performed ChIP-qPCR in embryonic cortical tissue, utilizing an antibody to endogenous PHF6 for chromatin precipitation. A non-specific IgG antibody was used in parallel as a negative control to assess the background signal. Following immunoprecipitation, we performed qPCR using primers designed against the *Tcf4* peak summit at the promoter, in which PHF6 binding was predicted from prior ChIP-seq analysis (Rasool et al., 2024). As controls, we included EphA4, a known PHF6 target, as a positive control, and Zfp735, a genomic locus with no predicted PHF6 occupancy, as a negative control (Rasool et al., 2024). In cortices from wild-type mice, PHF6 was significantly enriched at *Tcf4* loci (**Fig. 9A**), supporting robust PHF6 binding to the *Tcf4* promoter. To assess whether this regulatory interaction is disrupted in BFLS models, we performed ChIP-qPCR on E14 cortical tissues from Phf6$^{-/Y}$/Nestin-CreERT2^{+}, R342X, and C99F-m mice. In Phf6$^{-/Y}$/Nestin-CreERT2^{+} mice, PHF6 binding at the *Tcf4* promoter was markedly reduced compared to control littermates (**Fig. 9B**). Similarly, PHF6 occupancy at *Tcf4* promoters significantly decreased in R342X (**Fig. 9C**) and C99F-m (**Fig. 9D**) cortices, consistent with the loss of DNA-binding ability in these mutant mice. Our findings demonstrate that PHF6 directly binds to the *Tcf4* promoter *in vivo* and this interaction is consistently disrupted across different mouse models upon PHF6 loss-of-function.

To assess whether the direct binding of PHF6 to *Tcf4* promoter induces *Tcf4* expression, we designed a dual-luciferase reporter assay. PHF6 sequence within the peak identified by ChIP-seq[36] were cloned into the pGL4.23 luciferase reporter vector. We performed a dual-luciferase

reporter assay in eNSCs from Phf6$^{-/Y}$/Nestin-CreERT2^{+} and BFLS mutant (R342X & C99Fm). eNSCs were electroporated with a firefly luciferase reporter plasmid containing the *Tcf4* promoter or the control pGL4.23-basic vector, and a Renilla luciferase expression plasmid. Luciferase activity was evaluated 48 hours post-electroporation. Our results demonstrated that PHF6 regulation of *Tcf4* transcriptional activity was significantly impaired in the Phf6$^{-/Y}$/Nestin-CreERT2^{+} compared to Phf6$^{loxP/Y}$/Nestin-CreERT2^{-} control (**Fig. 9E**). Similarly, eNSCs from the R342X exhibited a significant reduction (**Fig. 9F**), while eNSCs from the C99Fm also showed a decrease trend in luciferase activity (**Fig. 9G**), supporting that PHF6 directly upregulates *Tcf4* transcription, and PHF6 genetic deletion or truncation significantly attenuates *Tcf4* expression.

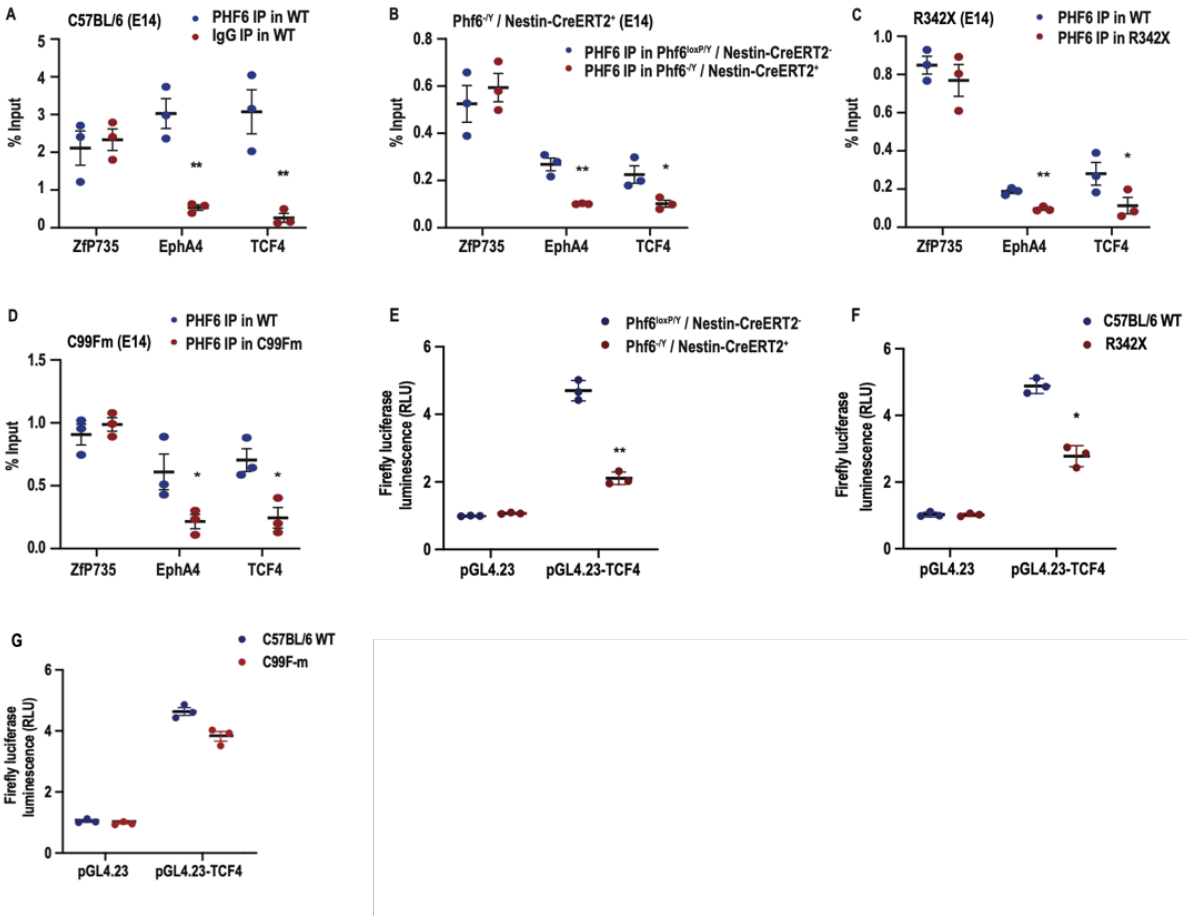
A
C57BL/6 (E14)
PHF6 IP in WT
IgG IP in WT
% Input
ZfP735
EphA4
TCF4
B
Phf6-/Y / Nestin-CreERT2+ (E14)
PHF6 IP in Phf6loxP/Y / Nestin-CreERT2-
PHF6 IP in Phf6-/Y / Nestin-CreERT2+
C
R342X (E14)
PHF6 IP in WT
PHF6 IP in R342X
D
C99Fm (E14)
PHF6 IP in WT
PHF6 IP in C99Fm
E
Phf6loxP/Y / Nestin-CreERT2-
Phf6-/Y / Nestin-CreERT2+
Firefly luciferase luminescence (RLU)
pGL4.23
pGL4.23-TCF4
F
C57BL/6 WT
R342X
G
C57BL/6 WT
C99F-m

Figure 9: TCF4 is a direct transcriptional target of PHF6. (A-D) Cerebral cortical tissues were isolated from Phf6$^{-/Y}$ / Nestin-CreERT2^{+} (cKO), Phf6$^{loxP/Y}$ / Nestin-CreERT2^{-} (control); R342X, and C99F-m and their littermate C57BL/6 as controls at E14. Samples were subjected to ChIP-PCR using a PHF6 antibody. Zfp735 loci were used as a negative control, and EphA4 as a positive control for the PCR. (E-G) Dual luciferase reporter assay was performed in eNSC 48 h following electroporation with a firefly luciferase reporter plasmid containing the TCF4 promoter or the control pGL4.23-basic vector. Statistical comparisons were performed using an unpaired two-tailed Student's t-test. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Representative data of n=3 independent replicates are shown in panels. n represents an independent biological sample.

3.5 TCF4 restricts eNSC self-renewal and rescues PHF6-deficient-eNSCs phenotype

Having shown that PHF6 directly binds *Tcf4* promoter to upregulate its expression, led us next to the question of whether genetic KD of *Tcf4* functionally phenocopies PHF6-loss-of-function defects in the regulation of NSCs. We employed an siRNA-mediated approach in primary E14 WT eNSCs and confirmed efficient KD of *Tcf4* (**Fig. 10A**). To evaluate eNSC self-renewal, we conducted ELDA to assess the self-renewal capacity of eNSCs. Our results revealed a significant increase in eNSC self-renewal upon knockdown of *Tcf4* (**Fig. 10B, C**). In parallel, we also conducted LDA, which demonstrated an increase in the number of neurosphere formation in *Tcf4* KD compared to non-targeting control (**Fig. 10D**). Consistent with these data, sphere size analysis revealed a significant increase in diameter in *Tcf4* KD cultures (133.1 μm) compared to controls (61.1 μm) (**Fig. 10E**), further supporting a role of TCF4 in restricting eNSC expansion. Next, we subjected the *Tcf4* KD and control eNSCs to RT-qPCR analysis to evaluate changes in the mRNA expression of stem cell markers, including *Sox2* and *Nestin*. We found that the KD of *Tcf4* led to a significant increase in the mRNA levels of both genes (**Fig. 10F**). To determine whether these changes in gene expression were reflected at the protein level, we conducted immunoblotting analysis, and detected elevated protein levels of SOX2 and NESTIN in *Tcf4* KD cells compared to non-targeting control siRNA (**Fig. 10G, H**). Collectively, these findings demonstrate that *Tcf4* KD enhances eNSC self-renewal and stemness, mirroring the effects of PHF6 loss.

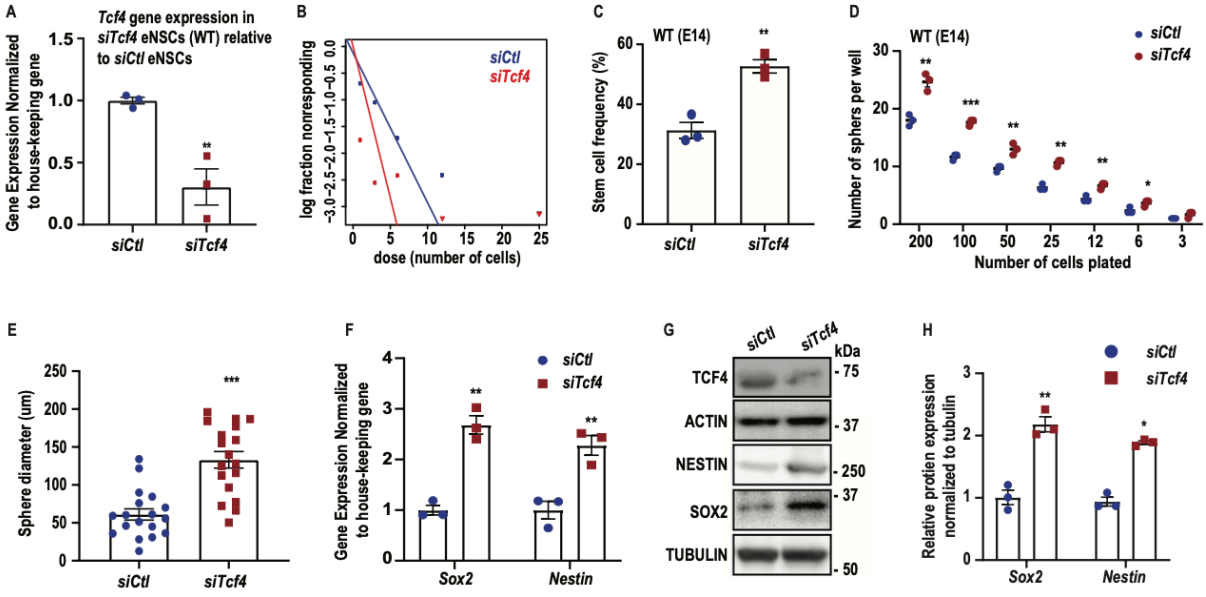
A
Tcf4 gene expression in siTcf4 eNSCs (WT) relative to siCtl eNSCs
Gene Expression Normalized to house-keeping gene
1.5
1.0
0.5
0.0
**
siCtl
siTcf4
B
log fraction nonresponding
−3.0 −2.5 −2.0 −1.5 −1.0 −0.5 0.0
siCtl
siTcf4
0 5 10 15 20 25
dose (number of cells)
C
WT (E14)
Stem cell frequency (%)
60
40
20
0
**
siCtl
siTcf4
D
WT (E14)
siCtl
siTcf4
Number of spheres per well
30
20
10
0
**
***
**
**
**
*
200 100 50 25 12 6 3
Number of cells plated
E
Sphere diameter (um)
250
200
150
100
50
0
***
siCtl
siTcf4
F
Gene Expression Normalized to house-keeping gene
4
3
2
1
0
siCtl
siTcf4
**
**
Sox2
Nestin
G
siCtl
siTcf4
kDa
TCF4
ACTIN
NESTIN
SOX2
TUBULIN
75
37
250
37
50
H
Relative protien expression normalized to tubulin
3
2
1
0
siCtl
siTcf4
**
*
Sox2
Nestin

Figure 10: Knockdown of *Tcf4* impairs NSCs in the embryonic brain. eNSCs were isolated and cultured from WT mice at E14, and Tcf4 KD was induced using an siRNA approach (A). (B-D) Samples were analyzed using ELDA and LDA 7 days post-plating. (E) sphere diameter, (F) RT-qPCR analysis using Nestin and Sox2 primers. (G, H) Immunoblotting using NESTIN and SOX2 antibodies was also performed 7-days post-plating. Statistical analyses were performed using unpaired two-tailed Student's t-test or one-way ANOVA Tukey's Honest Significant Difference test, where appropriate. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Representative plots of n=3 independent replicates.

3.6 TCF4 Rescues the Effects of PHF6 Deficient-Phenotype in eNSCs

We next asked whether forced expression of TCF4 rescues the NSC defects observed in PHF6-deficient cultures. We conducted electroporation of TCF4 or an empty vector control in eNSC obtained from Phf6$^{-/Y}$/Nestin-CreERT2^{+}, and subjected the cultures to a series of functional and molecular assays to assess self-renewal and stemness. ELDA revealed that forced expression of TCF4 significantly suppressed aberrant self-renewal in Phf6$^{-/Y}$/Nestin-CreERT2^{+} eNSCs (**Fig. 11A**). Consistently, LDA showed a decrease in neurosphere-forming frequency in TCF4-expressing cells compared to controls (**Fig. 11B**). In parallel, we examined the mRNA expression of *Sox2* and *Nestin*, revealing a significant attenuation at transcript levels for both markers in TCF4-expressing cells relative to control (**Fig. 11C**). This was further supported by immunoblotting, which confirmed reduced protein levels of SOX2 and NESTIN (**Fig. 11D**), indicating that TCF4 forced expression suppresses the expression of stemness markers.

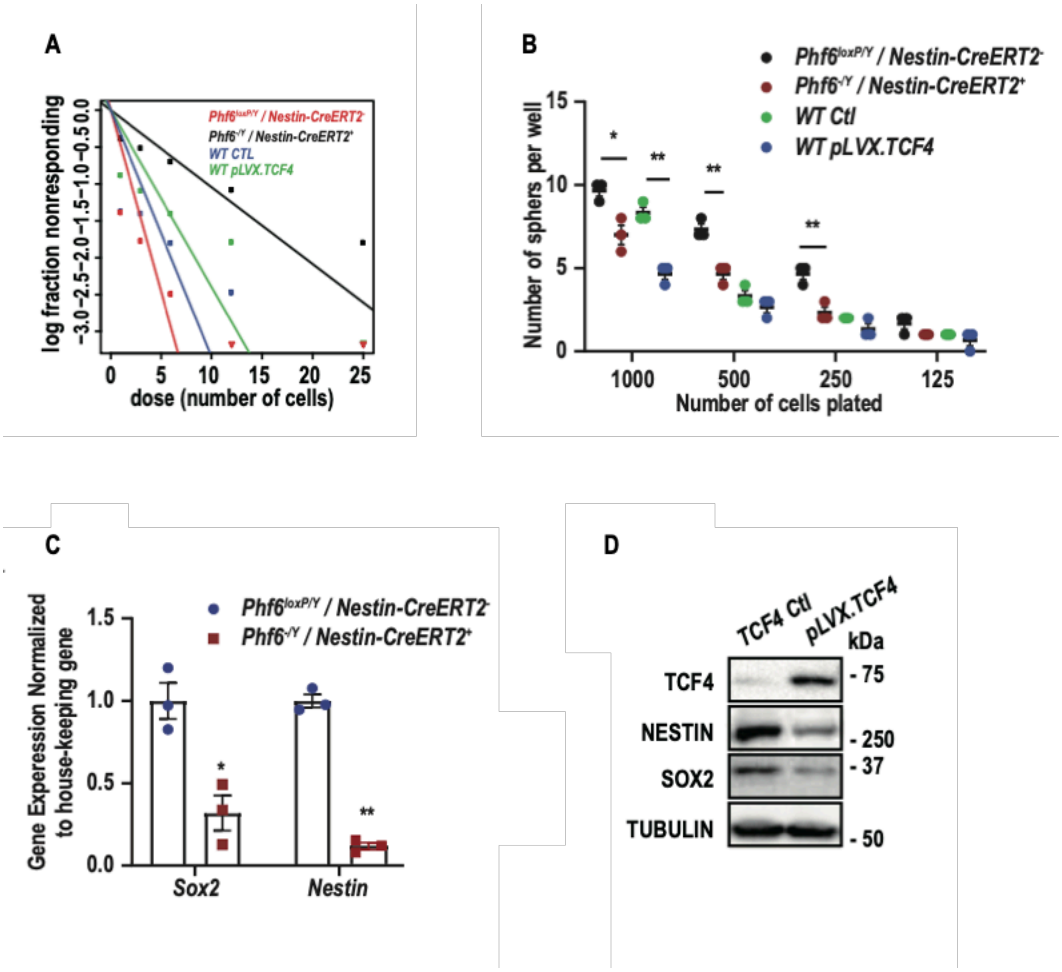
A
log fraction nonresponding
dose (number of cells)
Phf6loxP/Y / Nestin-CreERT2-
Phf6-/Y / Nestin-CreERT2+
WT CTL
WT pLVX.TCF4
B
Number of sphers per well
Number of cells plated
Phf6loxP/Y / Nestin-CreERT2-
Phf6-/Y / Nestin-CreERT2+
WT Ctl
WT pLVX.TCF4
C
Gene Expression Normalized to house-keeping gene
Sox2
Nestin
D
TCF4 Ctl
pLVX.TCF4
kDa
TCF4
NESTIN
SOX2
TUBULIN
- 75
- 250
- 37
- 50

Figure 11: Forced expression of TCF4 rescues eNSC defects in cKO PHF6. Phf6 cKO and WT eNSCs cultured at E14 were electroporated with pLVX.TCF4 and samples were subjected to (A) ELDA, (B) LDA, (C) RT-qPCR, and (D) immunoblotting analysis with SOX2 and NESTIN. Statistical analyses were performed using unpaired two-tailed Student's t-test or one-way ANOVA Tukey's Honest Significant Difference test, where appropriate. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Representative plots of n=3 independent replicates.

3.7 TCF4 reverses the BFLS-derived eNSCs phenotype

Next, we assessed whether TCF4 rescues PHF6-deficient phenotypes in the R342X model. We induced the expression of TCF4 in eNSCs derived from R342X mutant mice. TCF4 forced expression led to a significant reduction in neurosphere formation, as assessed by ELDA and LDA (**Fig. 12A, B**). Consistently, expression levels of the stemness markers SOX2 and NESTIN were significantly attenuated at both mRNA and protein levels upon induction of TCF4 (**Fig. 12C, D**). Similarly, in examining the effect of TCF4 in eNSCs derived from C99F-m mutant mice, enforced expression of TCF4 significantly reduced self-renewal and neurosphere formation, as assessed by ELDA and LDA, respectively (**Fig. 12E, F**). In addition, TCF4 forced expression resulted in a marked downregulation of SOX2 and NESTIN at both the transcript and protein levels (**Fig. 12G, H**). Together, these findings demonstrate that TCF4 can mitigate the aberrant self-renewal phenotype caused by PHF6 deficiency or loss of function mutations.

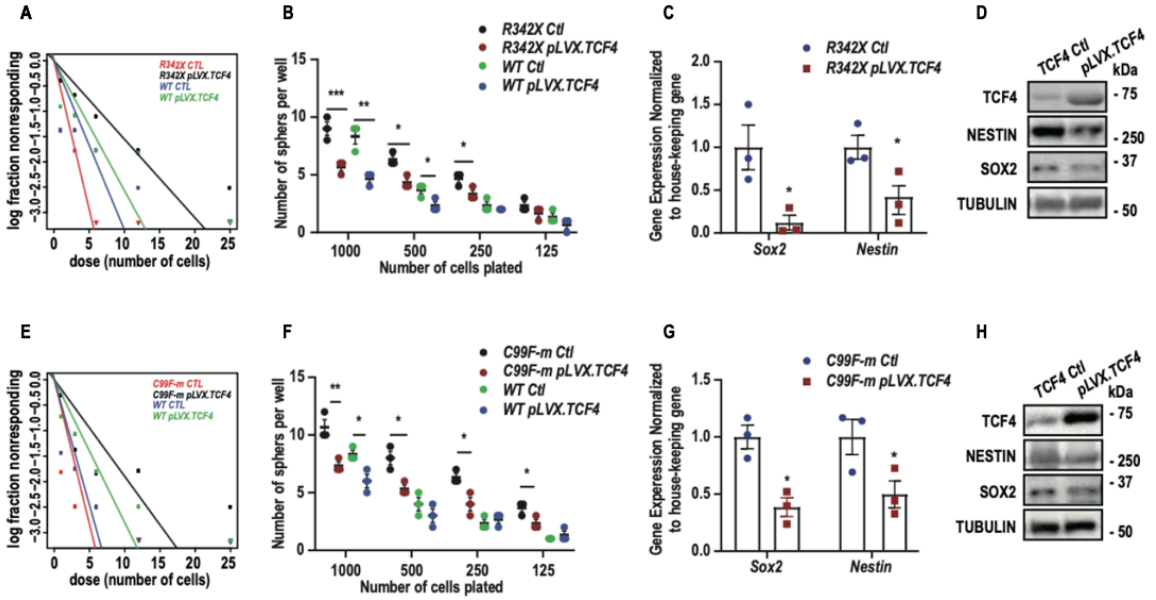
A
log fraction nonresponding
dose (number of cells)
R342X CTL
R342X pLVX.TCF4
WT CTL
WT pLVX.TCF4
B
Number of spheres per well
Number of cells plated
R342X Ctl
R342X pLVX.TCF4
WT Ctl
WT pLVX.TCF4
C
Gene Expression Normalized to house-keeping gene
Sox2
Nestin
R342X Ctl
R342X pLVX.TCF4
D
TCF4 Ctl
pLVX.TCF4
kDa
TCF4
NESTIN
SOX2
TUBULIN
- 75
- 250
- 37
- 50
E
log fraction nonresponding
dose (number of cells)
C99F-m CTL
C99F-m pLVX.TCF4
WT CTL
WT pLVX.TCF4
F
Number of spheres per well
Number of cells plated
C99F-m Ctl
C99F-m pLVX.TCF4
WT Ctl
WT pLVX.TCF4
G
Gene Expression Normalized to house-keeping gene
Sox2
Nestin
C99F-m Ctl
C99F-m pLVX.TCF4
H
TCF4 Ctl
pLVX.TCF4
kDa
TCF4
NESTIN
SOX2
TUBULIN
- 75
- 250
- 37
- 50

Figure 12: Forced expression of TCF4 rescues BFLS-derived eNSC defects. R342X, C99F-m, and WT eNSCs cultured at E14 were electroporated with pLVX.TCF4 and samples were subjected to (A, E) ELDA, (B, F) LDA, (C, G) RT-qPCR, and (D, H) immunoblotting analysis with SOX2 and NESTIN. Statistical analyses were performed using unpaired two-tailed Student's t-test or one-way ANOVA Tukey's Honest Significant Difference test, where appropriate. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Representative plots of n=3 independent replicates.

3.8 TCF4 regulates stemness in adult BFLS-derived NSCs

Given the established role of TCF4 in NSC regulation[44] and its high expression in adult neurogenic niches (Matthias Jung et al., 2018; Shariq et al., 2021), we next investigated whether its expression is altered in adult BFLS mouse models. RT-qPCR analysis of adult NSCs (aNSCs) derived from R342X and C99F-m revealed that *Tcf4* transcript levels were significantly reduced in both models compared to corresponding wild-type controls (**Fig. 13A**), with the R342X line showing a more pronounced reduction. To examine the functional consequences of *Tcf4* deficiency, we performed siRNA-mediated KD studies in aNSCs derived from wild-type mice (**Fig. 13B**). ELDA revealed that *Tcf4* KD significantly increased self-renewal and stem cell frequency (**Fig. 13C, D**). Similarly, LDA showed an increased number of neurospheres across multiple dilutions in *Tcf4*-deficient aNSCs (**Fig. 13E**), reinforcing the conclusion that *Tcf4* loss promotes aNSC expansion. To uncover the molecular basis of this phenotype, we analyzed *Sox2* and *Nestin* expression using RT-qPCR and immunoblotting. Both mRNA and protein levels of these stemness markers were elevated in *Tcf4* KD aNSCs compared to controls (**Fig. 13F, G**), supporting that TCF4 negatively regulates stemness expansion. Overall, these findings indicate that TCF4 downregulation in aNSCs contributes to increased self-renewal, highlighting a key role for TCF4 in regulation of aNSCs behavior. Building on our observation that *Tcf4* expression is reduced in PHF6 mutant aNSCs, we next asked whether TCF4 forced expression could reverse the phenotype of aNSCs from R342X and C99F-m mice. aNSCs electroporated with TCF4 or empty vector control were subjected to ELDA, LDA, and analysis of stem cell markers. Similar to our results obtained from eNSCs, forced expression of TCF4 resulted in a marked decrease in neurosphere-forming frequency (**Fig. 13H, I**), and a marked reduction in the expression of SOX2

and NESTIN (**Fig. 13J, K**). Similarly, TCF4 rescued aNSCs defects derived from C99F-m mice (**Fig. 13L-O**). These data demonstrate that *Tcf4* KD significantly increases NSC self-renewal, suggesting that loss of TCF4 may restrict NSC commitment.

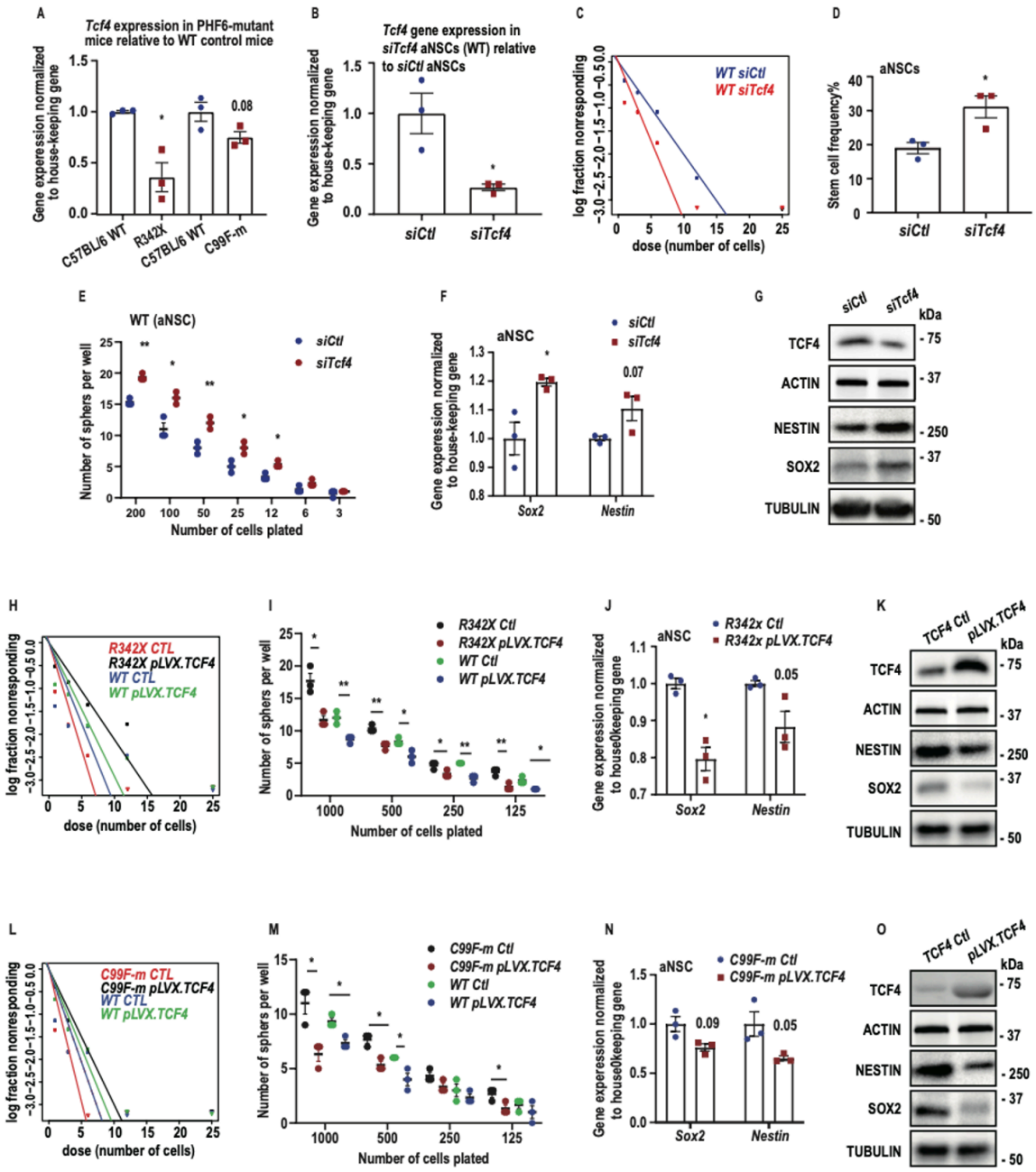
A
Tcf4 expression in PHF6-mutant mice relative to WT control mice
Gene experession normalized to house-keeping gene
C57BL/6 WT
R342X
C57BL/6 WT
C99F-m
0.08
B
Tcf4 gene expression in siTcf4 aNSCs (WT) relative to siCtl aNSCs
siCtl
siTcf4
C
WT siCtl
WT siTcf4
log fraction nonresponding
dose (number of cells)
D
aNSCs
Stem cell frequency%
siCtl
siTcf4
E
WT (aNSC)
siCtl
siTcf4
Number of spheres per well
Number of cells plated
F
aNSC
siCtl
siTcf4
0.07
Gene experession normalized to house-keeping gene
Sox2
Nestin
G
siCtl
siTcf4
kDa
TCF4
ACTIN
NESTIN
SOX2
TUBULIN
75
37
250
37
50
H
R342X CTL
R342X pLVX.TCF4
WT CTL
WT pLVX.TCF4
log fraction nonresponding
dose (number of cells)
I
R342X Ctl
R342X pLVX.TCF4
WT Ctl
WT pLVX.TCF4
Number of spheres per well
Number of cells plated
J
aNSC
R342x Ctl
R342x pLVX.TCF4
0.05
Gene experession normalized to house0keeping gene
Sox2
Nestin
K
TCF4 Ctl
pLVX.TCF4
kDa
TCF4
ACTIN
NESTIN
SOX2
TUBULIN
75
37
250
37
50
L
C99F-m CTL
C99F-m pLVX.TCF4
WT CTL
WT pLVX.TCF4
log fraction nonresponding
dose (number of cells)
M
C99F-m Ctl
C99F-m pLVX.TCF4
WT Ctl
WT pLVX.TCF4
Number of spheres per well
Number of cells plated
N
aNSC
C99F-m Ctl
C99F-m pLVX.TCF4
0.09
0.05
Gene experession normalized to house0keeping gene
Sox2
Nestin
O
TCF4 Ctl
pLVX.TCF4
kDa
TCF4
ACTIN
NESTIN
SOX2
TUBULIN
75
37
250
37
50

Figure 13: TCF4 regulates stemness in adult BFLS-derived NSCs. (A) RT-qPCR was performed to investigate the expression level of Tcf4 in PHF6 mutant mouse models. (B) The aNSCs were isolated and cultured from WT mice and *Tcf4* KD was induced using a siRNA approach. (C-E) Samples were analyzed using ELDA and LDA. (F) RT-qPCR analysis using primers targeting Sox2 and Nestin, (G), and immunoblotting using NESTIN and SOX2 antibodies. R342X, C99F-m, and WT aNSCs cultured were electroporated with pLVX.TCF4 and samples were subjected to (H, L) ELDA, (I, M) LDA. (G, N) stem cell markers were examined using RT-qPCR using Sox2 and Nestin primers, and (K, O) immunoblotting analysis with SOX2 and NESTIN antibodies. Statistical analyses were performed using unpaired two-tailed Student's t-test or one-way ANOVA Tukey's Honest Significant Difference test, where appropriate. Data information: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.9 PHF6 and TCF4 correlation in human brain development

Our analysis on aNSC reveals that PHF6-dependent regulation of TCF4 has consequences extending beyond the embryonic stage, suggesting that such mutation-induced perturbations propagate across the lifespan and may contribute to persistent neurological dysfunction in the adult brain. To assess whether this relationship is conserved in humans, we analyzed bulk RNA-seq profiles from the human ventral frontal cortex across developmental time points (BrainSpan (Atlas of the Developing Human Brain Secondary BrainSpan, 2011). Consistent with the findings from the mouse dataset, *PHF6* and *TCF4* exhibited parallel expression trajectories throughout human cortical development (**Fig. 14A, B**). These findings suggest that the *PHF6-TCF4* signaling axis may operate as a developmentally conserved transcriptional mechanism in regulation of stem cell fate in human.

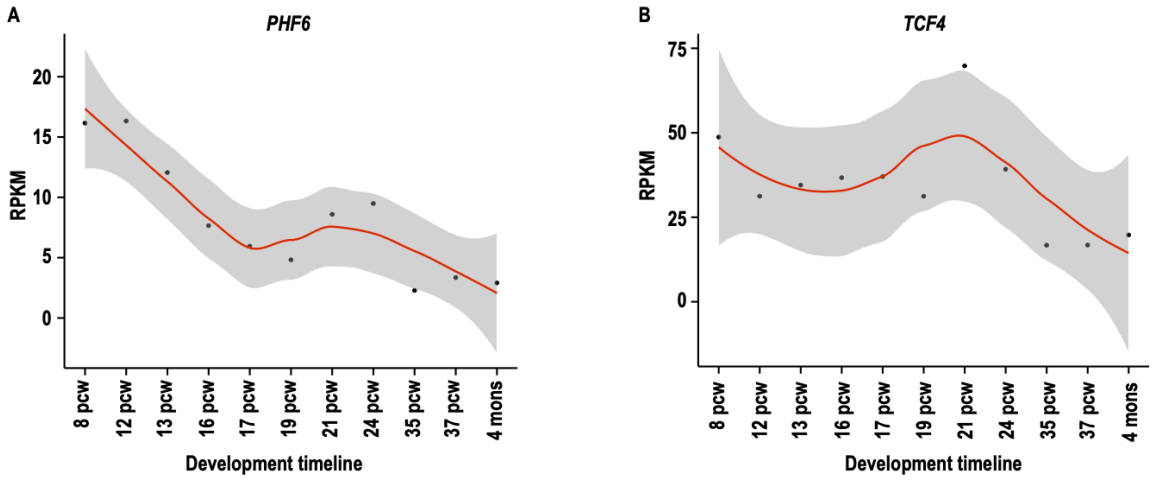
A
PHF6
RPKM
20
15
10
5
0
8 pcw
12 pcw
13 pcw
16 pcw
17 pcw
19 pcw
21 pcw
24 pcw
35 pcw
37 pcw
4 mons
Development timeline
B
TCF4
RPKM
75
50
25
0
8 pcw
12 pcw
13 pcw
16 pcw
17 pcw
19 pcw
21 pcw
24 pcw
35 pcw
37 pcw
4 mons
Development timeline

Figure 14: Analysis of PHF6 and TCF4 expression across development in human. (A-B) Analysis of PHF6 and TCF4 expression in the human cortex. Plots show average reads per kilobase per million mapped reads (RPKM) across human developmental time (post-conceptual weeks; pcw) for (A) PHF6 and (B) TCF4. Expression values were obtained from publicly available RNA-seq data generated from the human ventral frontal cortex (VFC) as part of the Allen Brain Atlas BrainSpan dataset (BrainSpan (Atlas of the Developing Human Brain Secondary BrainSpan, 2011).

4. Discussion

4.1 PHF6 directly regulates TCF4 transcription during corticogenesis

In this study, we identify a previously uncharacterized PHF6–*Tcf4* transcriptional mechanism that governs NSC fate. Using complementary BFLS and PHF6 mouse models, including R342X, C99F-m and a *Phf6* cKO, we demonstrate that PHF6 directly binds the *Tcf4* promoter to drive its expression in eNSCs, and that loss of PHF6 function consistently suppresses TCF4 levels across all models. *Tcf4* knockdown phenocopies PHF6 deficiency by enhancing NSC self-renewal and stemness marker expression, while forced TCF4 expression rescues these defects, establishing TCF4 as a functional effector downstream of PHF6. These findings provide the first evidence linking TCF4 dysregulation to BFLS pathogenesis.

PHF6 and TCF4 have each been independently studied in neurodevelopment, yet their functional relationship had not previously been explored. PHF6 operates through interactions with the PAF1 complex, UBF1, and NuRD complex to regulate neuronal migration and chromatin organization (Mittal et al., 2024; Soto-Feliciano et al., 2017; Todd & Picketts, 2012; Wang et al., 2013; Zhang et al., 2013), while TCF4 plays critical roles in neural progenitor proliferation, differentiation, and migration, exhibiting layer-specific expression in the developing neocortex and high expression in adult neurogenic niches (Braun et al., 2020; M. Jung et al., 2018; Mesman et al., 2020; Sepp et al., 2017). Although TCF4 has not been established as a direct epigenetic regulator, accumulating evidence suggests that it functions within an epigenetically regulated transcriptional network, with both its activity and expression being influenced by chromatin remodeling and DNA methylation mechanisms (Hennig et al.,

2017; van der Laan et al., 2024). Whether TCF4 itself directly functions as an epigenetic regulator remains to be explored. Despite their distinct molecular mechanisms, these two transcription factors appear to operate on the same signaling pathway in the regulation of stem cells. Our single-cell RNA-seq correlation analyses reveal positive co-expression of *Phf6* and *Tcf4* across progenitor populations throughout cortical development, consistent with their shared peak expression during mid-to-late embryogenesis. The data presented here establish TCF4 as a critical transcriptional effector in the PHF6-dependent program governing NSC fate.

4.2 The PHF6–TCF4 axis regulates eNSC fate

The second finding of this study is TCF4 functions as a downstream effector through PHF6 to regulate eNSC fate. Previous studies from our lab revealed that PHF6 deficiency promotes eNSC self-renewal while impairing neuronal differentiation. However, the molecular mediators responsible for these phenotypes had not been identified. The present study demonstrates that depletion of TCF4 phenocopies PHF6 deficiency, suggesting that disruption of TCF4 signaling contributes substantially to the developmental abnormalities in BFLS. KD of *Tcf4* significantly increased neurosphere forming frequency, stem cell frequency and neurosphere size, suggesting TCF4 loss enhances self-renewal. These functional changes were accompanied by elevated expression of the stemness markers SOX2 and NESTIN at both the transcript and protein levels, implying maintenance of the progenitor state. However, the possibility that TCF4 contributes to fate specification of NSCs into different neuronal lineages remains to be investigated in BFLS.

TCF4 is a class I bHLH E-protein that functions primarily through dimerization with proneural class II bHLH transcription factors, including ASCL1, NEUROG1/2, and NEUROD1, to regulate E-box-dependent transcriptional programs involved in progenitor differentiation and neuronal fate specification (Bertrand et al, 2002; Forrest et al., 2012; Savchenko & Skryabin, 2024). Previous studies have described TCF4 as a key transcription factor regulating neuronal differentiation during cortical development (Li et al., 2019; Mesman et al., 2020; Schoof et al., 2020). Our findings establish that TCF4 plays an essential role in regulating NSC self-renewal, and reduced TCF4 expression following PHF6 loss could impair proneural transcriptional activity, providing a potential mechanism linking the PHF6-TCF4 axis to the expansion of the neural progenitor pool and altered neuronal differentiation observed in PHF6 KO. Although the present study did not directly investigate TCF4 interactions with proneural bHLH factors or their downstream transcriptional targets, further studies could investigate whether these interactions contribute to the effects of PHF6–TCF4 regulation on progenitor maintenance and neuronal differentiation.

4.3 PHF6–TCF4 Signaling Deficits Persist into Adulthood

While PHF6 is primarily expressed during embryonic stage, our findings suggest that PHF6-dependent regulation of TCF4 persists beyond embryonic development and remains important in aNSCs. Adult neurogenesis continues throughout life within the SGZ of the hippocampus and the SVZ. Proper regulation of stem cell quiescence, activation and differentiation within these niches is essential for maintaining brain homeostasis. Our study

demonstrates that TCF4 expression remains significantly reduced in aNSCs derived from both BFLS mouse models. Similar to our findings in eNSCs, TCF4 KD enhanced aNSC self-renewal and increased expression of SOX2 and NESTIN, whereas forced TCF4 expression rescued these abnormalities. These findings suggest that rather than functioning exclusively during embryonic stage, PHF6 appears to maintain stem cell homeostasis across multiple developmental stages. However, PHF6 expression declines substantially after birth, suggesting that additional transcriptional or epigenetic regulators may compensate for PHF6 in maintaining TCF4 expression within adult neurogenic niches. Studies of TCF4 indicated that its expression are cooperatively regulated by upstream TFs such as TCF3, and ZAC1 (Li et al., 2019; Schmidt-Edelkraut et al., 2014). These factors may contribute to sustaining TCF4 expression in the absence of PHF6 levels during adulthood, while the precise mechanisms remain unclear and require further investigation. Identifying these regulatory networks will provide important insight into how TCF4 expression is maintained throughout life and whether disruption of these pathways contributes to the persistent neurological deficits observed in BFLS. The persistence of PHF6-dependent TCF4 dysregulation in aNSCs further suggests that BFLS is not solely a disorder of embryonic cortical development, but may also involve long-term impairment of adult neurogenic niches. Such persistent alterations may contribute to the lifelong neurological manifestations observed in individuals with BFLS, including ID and other neurodevelopmental impairments.

4.4 Implications of the PHF6–TCF4 Axis in BFLS and Broader Neurodevelopmental Disorders

Although PHF6 mutations have been identified as the genetic cause of BFLS, the disorder mechanism has remained incompletely understood. Our identification of the PHF6–TCF4 regulatory axis provides an important conceptual understanding in how PHF6 dysfunction may contribute to the pathogenesis of BFLS. PHF6 dysfunction impairs the activation of TCF4, a transcription factor with established roles in neurogenesis. Therefore, rather than acting through isolated gene expression changes, PHF6 loss may disrupt a broader transcriptional network that governs the transition of NSCs from a proliferative state toward neuronal lineage commitment. These findings also raise the possibility that PHF6 dysfunction may contribute to TCF4 related disorders. Since PHF6 directly regulates TCF4 expression, variation in PHF6 function or its upstream regulatory pathways could potentially influence TCF4 activity, thereby modifying the severity of PTHS. Likewise, common genetic variants affecting TCF4 expression have been associated with schizophrenia and ASD, suggesting that disruption of the PHF6–TCF4 regulatory pathway may extend beyond rare monogenic disorders to broader neurodevelopmental and psychiatric conditions. Although this possibility remains speculative, it highlights the importance of investigating PHF6-dependent regulation of TCF4 across multiple disorders.

During cortical development, the balance between NSC self-renewal and differentiation must be tightly controlled to ensure the timely production of appropriate progenitor and neuronal populations. A shift toward excessive stemness may delay neuronal differentiation, alter the size or composition of progenitor pools and ultimately disrupt cortical organization. In this context,

reduced TCF4 expression downstream of PHF6 loss may represent a mechanism by which BFLS mutations impair neurogenesis. The phenotype is not simply a failure of one transcription factor, but rather a disruption in developmental timing, where progenitor cells may remain in a stem-like state when they should begin transitioning toward neuronal fate. Such alteration may result in long-lasting consequences for neuronal number, cortical layering, circuit formation, and cognitive function.

The PHF6–TCF4 axis also provides a potential elucidation in sharing neurological features between BFLS and other neurodevelopmental disorders. Clinically, both BFLS and PTHS present with overlapping features including ID, impaired language development, and abnormalities in social behaviour. Moreover, both disorders are associated with hypotonia, seizures, and structural brain abnormalities, suggesting disruption of common neurodevelopmental processes. More broadly, our findings support the idea that neurodevelopmental disorders often arise from disruption of shared transcriptional networks rather than single-gene defects acting in isolation. PHF6 may function as an upstream chromatin-associated regulator, while TCF4 acts as a downstream transcriptional effector that helps implement cell fate decisions. Disruption at either level could produce overlapping developmental consequences. This is particularly relevant for ID, where many causative genes encode transcription factors, chromatin regulators, or proteins involved in gene expression control. The PHF6–TCF4 pathway therefore provides a framework for understanding BFLS as part of a broader class of neurodevelopmental disorders caused by impaired transcriptional regulation of neural stem cell fate.

5. Future Directions: Investigating the PHF6–TCF4 Axis in an In Vivo Developmental Context

Although the present findings establish a critical role for TCF4 in mediating the NSC phenotypes associated with PHF6 deficiency, cortical development is governed by highly coordinated cellular interactions that cannot be fully recapitulated *in vitro*. While cell culture models provide valuable mechanistic insights into PHF6–TCF4 signaling, they lack the complex developmental environment of the embryonic brain, where cell–cell interactions, spatial patterning, and extracellular signaling collectively influence neurogenesis. Therefore, it is essential to determine whether restoring TCF4 expression in BFLS mouse models can rescue neurogenic defects within the embryonic brain. To address this, future studies can focus on in utero electroporation (IUE) to overexpress TCF4 in BFLS mouse at E14, followed by quantification of newborn neurons, committed progenitors, and neuronal subpopulations using markers to each progeny at E16 to E18. These experiments will assess whether ectopic expression of TCF4 can rescue changes in cell population dynamics induced by BFLS mutation. On top of that, conducting ChIP-seq and RNA-seq to establish the causal link between PHF6–TCF4 chromatin occupancy and gene expression will be important to assess. ChIP-seq for TCF4 will define its genome-wide binding sites, to test whether PHF6 loss alters TCF4 chromatin occupancy. In parallel, RNA-seq will be used to quantify transcriptional changes resulting from TCF4 deficiency and to determine how these changes overlap with the transcriptional defects observed following PHF6 loss. This analysis will allow us to distinguish genes that are directly regulated by TCF4 from broader secondary transcriptional changes associated with altered NSC state. Importantly, comparison with PHF6-dependent transcriptomic changes will help determine

whether TCF4 mediates a subset of the PHF6-regulated gene expression program. This integrated approach will clarify whether PHF6 regulates neurogenesis primarily through TCF4-dependent transcriptional networks, or whether PHF6 and TCF4 also control distinct but cooperating pathways during cortical development.

Finally, our findings raise the possibility that PHF6-dependent regulation of TCF4 may contribute more broadly to TCF4-associated neurodevelopmental phenotypes. Given that TCF4 is the causative gene of PTHS and has also been implicated in schizophrenia and ASD, these findings further support this model. Thus, alterations in PHF6 activity could potentially influence TCF4-associated phenotypes by modulating TCF4 expression and its downstream transcriptional programs. One approach would be to examine PHF6 expression in PTHS mouse models, to determine whether PHF6-dependent transcriptional regulation is altered in TCF4 haploinsufficiency. Conversely, PHF6 expression will be restored in TCF4-deficient models to determine whether it could partially rescue the neurogenic defects. Similar approaches could be extended to schizophrenia and autism-associated mouse models carrying TCF4 risk variants to investigate whether dysregulation of the PHF6–TCF4 axis represents a convergent molecular mechanism underlying these disorders. In parallel, comparative transcriptomic and epigenomic analyses across BFLS, PTHS, and TCF4-associated psychiatric disease models may identify shared downstream pathways that contribute to abnormal neurodevelopment. These studies would determine whether the PHF6–TCF4 signaling axis represents a common therapeutic target across multiple neurodevelopmental disorders rather than a mechanism unique to BFLS.

5. Conclusion

This study identifies TCF4 as a previously unrecognized downstream transcriptional target of PHF6 and establishes the PHF6–TCF4 axis as a key regulatory pathway controlling NSC cell fate during brain development. We demonstrated that PHF6 directly binds the *Tcf4* promoter to activate its transcription, whereas PHF6 deficiency or BFLS-associated mutations reduce TCF4 expression, leading to increased NSC self-renewal. Importantly, restoration of TCF4 expression rescues these cellular defects, demonstrating that TCF4 is a major functional mediator of PHF6 during neurogenesis. This work provides a mechanistic explanation for how PHF6 mutations disrupt cortical development and identifies TCF4 as a key downstream effector linking PHF6 dysfunction to abnormal neurogenesis and significantly advances our understanding of the molecular pathogenesis of BFLS. Future studies will determine whether restoration of TCF4 expression can rescue neurodevelopmental defects in vivo using BFLS mouse models. In parallel, integrated ChIP-seq and RNA-seq analyses will be essential to define the downstream transcriptional network regulated by the PHF6–TCF4 axis and identify the genes that mediate its effects on NSC cell fate. Together, our studies will further investigate the role of PHF6–TCF4 signaling during corticogenesis and may provide a foundation for developing targeted therapeutic strategies for BFLS and related neurodevelopmental disorders.

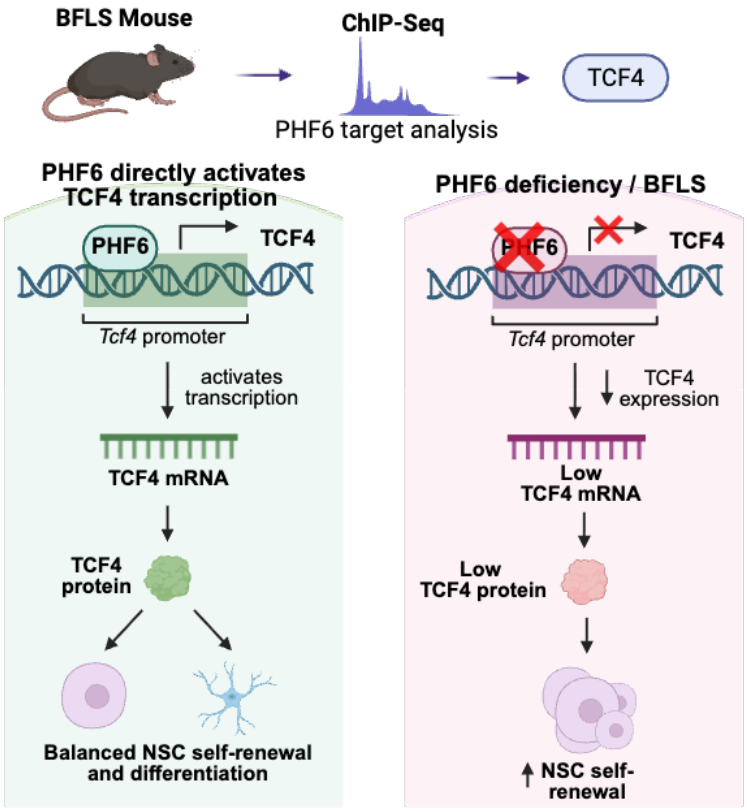

Figure 15. Summary of findings. This thesis revealed TCF4 as a direct downstream target of PHF6. PHF6/TCF4 pathway is impaired in the X-linked intellectual disability, BFLS. Figure created with BioRender.

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