The X-linked Intellectual Disability Protein PHF6 Associates with the PAF1 Complex and Regulates Neuronal Migration in the Mammalian Brain

Citation

Permanent link

Terms of Use

Share Your Story

The X-linked Intellectual disability Protein PHF6 Associates with the PAF1 Complex
and Regulates Neuronal Migration in the Mammalian Brain

Abstract

Intellectual disability is a prevalent developmental disorder for which no effective treatments are available. Mutations of the X-linked protein PHF6 cause the Börjeson–Forssman–Lehmann syndrome (BFLS) that is characterized by intellectual disability and epilepsy. However, the biological role of PHF6 relevant to BFLS pathogenesis has remained unknown. Here, I present my dissertation research demonstrating that knockdown of PHF6 profoundly impairs neuronal migration in the mouse cerebral cortex *in vivo*, leading to the formation of white matter heterotopias that harbor aberrant patterns of neuronal activity. Importantly, BFLS patient specific mutation of PHF6 blocks its ability to promote neuronal migration. I also elucidate the mechanism by which PHF6 drives neuronal migration in the cerebral cortex. PHF6 physically associates with the PAF1 transcription elongation complex, and inhibition of PAF1 phenocopies the PHF6 knockdown-induced migration phenotype *in vivo*. I further identify Neuroglycan C (NGC), a susceptibility gene for schizophrenia, as a critical downstream target of PHF6 and the PAF1 complex, and I demonstrate that NGC mediates PHF6-dependent neuronal migration. These findings define PHF6, the PAF1 transcription elongation complex, and NGC as components of a novel cell-intrinsic transcriptional pathway that orchestrates neuronal migration in the brain, with important implications for the pathogenesis of intellectual disability and potentially other neuropsychiatric disorders.

Table of Contents

Acknowledgements

I would like to thank my thesis adviser, Dr. Azad Bonni, for his support and mentorship throughout my graduate career. He supported the project all the way from the beginning, and allowed me to explore new directions. With his help and support I was able to carry out interesting projects. In addition, his mentorship, both scientific and personal, helped me grow into a mature scientist. As an insightful scientist, he always asks good questions that will lead to new directions or surprising findings in the project. As a mentor, he is always available and approachable for questions and concerns. His passion and dedication for his work are great stimulating forces that push me to perform my best. No less importantly, he assembled a team of great postdocs and graduate students, from whom I learnt tremendously. I truly appreciate his support and guidance throughout my graduate school career.

I would like to thank my colleagues in the lab who have helped me along the way, including Tomoko Yamada, Yoshiho Ikeuchi, Gilbert Gallardo, Arezu Jahani-Asl, Luis de la Torre, Yue Yang, Parizad M. Bilimoria, Mai Anh Huynh, Nadia Litterman, Sidharth Puram, Luis Mejía, Julius Anckar, Pamela Valnegri, Ju Huang, Celine Vuong, Zengqiang Yuan and Jessica Barowski. I've spent an unforgettable 6 years with everyone and I truly enjoyed the time here. I also learned tremendously from all members of the lab and I really appreciate their help.

I would like to thank my dissertation advisory committee members Xi He, John Flanagan, and Andrew Lassar for sharing with me their advice on my science and my career. Their

knowledge and enthusiasm really helped me go through the difficult times of my graduate career.

I would like to thank Bjorn Olsen, my director in the graduate program, for taking me into the program, and more importantly, always supporting me and backing me up whenever I get into trouble. I truly enjoyed meeting and talking to Bjorn and take him as one of my closest friends here in Harvard.

Finally, I would like to thank my family, including my parents Baolin Zhang and Lihong Liu, my past grandfather Huangzhen Liu, my uncle Li'An Liu and aunt Dongmei Zhang, my cousins Xinyu Liu and Xinyang Liu, for always caring about me and cheering for me. I could not have done that without you!

Chapter 1

Introduction

Intellectual disability is a prevalent disorder

Intellectual disability (ID) is a very prevalent disorder, affecting 1 to 3 percent of the general population (Bhasin et al., 2006; Larson et al., 2001). It is defined as a condition marked by an intelligence quotient (IQ) of <70, and characterized by impaired intellectual functioning and adaptive skills. The severity of intellectual disability is further defined as mild (IQ: 50–70), moderate (IQ: 35–49), severe (IQ: 20–34), and profound (IQ: <20) intellectual disability. Unfortunately there is currently no treatment for intellectual disability, and because ID patients usually need special education program and sometimes assistance in carrying out daily activities, it poses a huge social economic burden for the society, with an estimated lifetime cost of $1,014,000 per person (2004).

It was noted from a very early point that intellectual disability is more prevalent among males than females (Penrose, 1938). Epidemiological studies repeatedly showed a sex bias, with 30–50% excess of males over females, and led to the assumption that much of the excess of male MR may be due to X-linked genes (Fishburn et al., 1983; Herbst and Miller, 1980). Indeed, both human genetics and functional studies have revealed that the X chromosome harbors many genes essential for brain development and homeostasis, and since males have only 1 copy of the X chromosome, they suffer from an increased susceptibility to intellectual disability.

X-Linked intellectual disability (XLID) accounts for roughly 5%–10% of intellectual disability in males, and mutations of 102 genes have been described that lead to XLID (Lubs et al., 2012). These are further divided into syndromic and non-syndromic XLIDs. Syndromic ID describes patients with intellectual disability together with somatic,

metabolic, or neuromuscular manifestations, whereas non-syndromic ID patients typically have intellectual disability alone or with inconsistent abnormalities.

Recent human genetics studies have contributed significantly to our understanding of cognitive disorders. Genetic mappings and genome wide association studies have identified numerous genes, when mutated, cause cognitive dysfunction. For example, the identification of the FMR1 (Fragile X Mental Retardation) gene clearly elucidated the transmission of the disease within Fragile X families (Verkerk et al., 1991). Studying the functions of these genes and how they regulate brain development and homeostasis, on the other hand, have led to new understanding of the underlying pathophysiology of cognitive disorders. For example, studies of FMR1 has revealed important roles in the regulation of protein translation in neurobiology, and in particular, synapse biology (Wang et al., 2012). Hopefully, a detailed understanding of the function of MR genes will lead to novel therapeutic treatments for cognitive disorders.

The Börjeson-Forssman-Lehmann syndrome (BFLS) was initially characterized by Mats Börjeson et al in 1962 (Borjeson et al., 1962). They described 3 men who suffered from severe intellectual disability, hypogonadism, obesity, abnormal faces with fatty swelling of the soft tissues and large ears. Two of the 3 patients suffered from epilepsy. Notably, none of the females in the family had the syndrome, and only moderate mental deficiency was noted in three women, with one woman showing certain physical abnormalities in the probands. Based on the inheritance pattern and severity of cognitive impairment within the family, they hypothesized that a recessive mutation of an X-linked gene likely causes the disease.

Over the years, more cases of BFLS patients have been identified, although BFLS remains a rare form of syndromic intellectual disability. In general, patients are born with normal pregnancy and delivery. Many infants exhibit generalized hypotonia and poor feeding. Developmental delay appears very early during postnatal life, usually evident after the first birthday. Their ears are enlarged and genitalia are small. In contrast to the earliest cases, most BFLS patients exhibit mild to modest intellectual disability and epilepsy occurs in a substantial but not major fraction of patients. Female heterozygotes may have learning problems and show part of the facial features including shortened toes, and thickened and fleshy ears. Nevertheless, most female carriers are spared or show very mild symptoms in BFLS (Gecz et al., 2006; Robinson et al., 1983).

Molecular genetics studies have gone a long way toward identifying the underlying genetic cause of BFLS (Baumstark et al., 2003; Lower et al., 2002). Through gene mapping approaches, Lower et all identified a number of mutations in the PHD finger protein 6 (PHF6) gene in 9 families of BFLS, including a mutation that occurs in two independent families. Follow up studies examining additional BFLS families have added to the number of mutations along PHF6 that are specifically associated with BFLS (Baumstark et al., 2003; Berland et al., 2011; Carter et al., 2009; Turner et al., 2004). Multiple missense and truncation mutations of PHF6 were identified in different patients, strongly arguing that mutation of PHF6 is likely the cause of BFLS.

PHF6 belongs to the PHD (plant homeodomain) zinc finger protein family, a group of proteins that have been implicated in a variety of biological processes. PHF6 is a 365 amino acid protein that contains two imperfect PHD zinc finger domains. PHD zinc fingers are structurally conserved modules commonly found in proteins that interact with

chromatin and mediate protein-protein interactions during gene transcription. The chromatin is the combination of DNA and proteins that make up most of the contents of the nucleus in a cell. DNA molecules wrap around histone proteins, which help package the DNA molecule into the small compartment of the nucleus. In addition, recent studies implicate that modification of histones are important regulators of gene transcription. For example, acetylation of histone is generally coupled to a relaxed chromatin structure and enhanced transcriptional activity. Methylation of histones, however, plays a more diverse role in transcriptional regulation. For example, tri-methylation of histone H3 Lysine 4 (H3K4) residue results in transcriptional activation, whereas tri-methylation of H3K9 marks transcriptional repression. In sum, the modification status of histones is a critical regulator of gene transcription.

PHD finger proteins regulate transcription in part through recognizing specific histone modifications using the PHD domain. The PHD domain contains the Cys_4-His-Cys_3 motif that adopts a globular fold and coordinates two Zn^{2+} ions. Not surprisingly, different PHD fingers recognize different modifications of the histone. For example, the double PHD fingers of DPF3b preferentially recognize acetylated lysine 14 at histone H3 through forming a hydrophobic pocket, while the PHD finger of nucleosome remodeling factor (NURF) directly associates with H3K4me3 tails (Wysocka et al., 2006; Zeng et al., 2010). Therefore, PHD finger containing proteins are generally thought to regulate transcription by recognizing specific chromatin marks, and subsequently either enzymatically modifying histone tails, or recruiting other protein complexes.

Although very little is known about PHF6 function and the pathology of BFLS, two lines of evidence suggest a neurodevelopmental origin of BFLS. First, symptoms of

5

cognitive dysfunction in BFLS patients are evident from a very early age, suggesting that abnormalities in the early brain development might underlie the mental disabilities and other neuroendocrine dysfunctions. Secondly, *in situ* RNA hybridization analysis showed that PHF6 is highly expressed in the developing central nervous system (CNS), while expression declines along the course of development (Voss et al., 2007). Therefore, I hypothesized that PHF6 might play an essential role during early development of the CNS, and mutations of PHF6 leads to abnormal development of the brain, which results in cognitive dysfunction.

Development of the cerebral cortex

The development of the cerebral cortex is a very complicated process involving proliferation of progenitors, generation of neurons, migration of neurons into their destined location, development and refinement of synaptic connections. Disruption of any of these processes would be detrimental to cognitive functions.

Neuronal migration refers to the directed movement of newly born neurons to their final location. The proper migration and positioning of neurons is critical to the correct wiring of the nervous system. In the brain, neurons are generated along the proliferative neuroepithelium along the neural tube termed the ventricular zone. Excitatory pyramidal neurons are generated from the embryonic pallium while GABAergic inhibitory neurons originate from the subpallium. There exist two types of migration: radial migration and tangential migration (Marin and Rubenstein, 2003; Nadarajah et al., 2001). In the cerebral cortex, pyramidal neurons migrate into the cortical plate through radial migration and inhibitory neurons adopt a tangential manner of migration initially before switching to radial migration into the cerebral cortex. Here I

6

will mainly focus on the migration of excitatory pyramidal neurons due to the relevance to the topic.

Migration of excitatory pyramidal neurons

Excitatory neurons in the cerebral cortex are generated through asymmetric divisions of progenitors that line the ventricle surface along the developing pallium. After neuronal lineage commitment, neurons initially undergo bipolar-to-unipolar transition and migrate toward the differentiating cell zones. Although not much is known about the mechanisms of movement during this process, time lapse imaging studies showed that twisting of the pial process exerts a mechanical pulling force onto the soma, towards the subventricular zone (Miyata, 2007; Miyata and Ogawa, 2007). During this stage, reverse movement toward the ventricle surface has been observed although its functional significance remains unclear.

Upon reaching the subventricular zone, the neurons start to adopt a multipolar morphology. These multipolar cells do not exhibit fixed polarity and instead, extend and retract thin processes in various directions dynamically. Time-lapse imaging studies suggest that speed of somal movement at this stage is very slow and therefore, it does not contribute significantly to overall movement of the cell (Hatanaka and Yamauchi, 2013). However, it has been speculated that this is the stage when the neurons sense the environmental cues that direct radial migration. Also the tangential movement of neurons at this stage is critical for intermingling and dispersion of neurons across cortical columns, which might be essential for establishing proper connections of the radial units (O'Rourke et al., 1992).

Migration along the radial glial fiber starts when the neuron switches from multipolar to bipolar morphology, after which nucleokinesis becomes the predominant manner of movement. Nucleokinesis is a manner of movement where neurons migrate discontinuously, guided by a leading process of constant length (Nadarajah et al., 2001). This is a tightly regulated step and a lot of factors are critical for the transit of multipolar-to-bipolar morphology. Here I will elaborate on a few factors that are critical for nucleokinesis mediated neuronal migration.

First, the protein reelin, secreted by the Cajal-Retzius neurons from the pre-plate, is critical for multiple events during neuronal migration. Reelin acts on multiple levels that cooperate in promoting migration. Reelin signals through VLDLR and ApoER2 receptors, leading to phosphorylation of the cytoplasmic adaptor Dab1 in migrating neurons. Dab1 is required for detachment of the migrating neuron from the radial glial fiber, and loss of Dab1 leads to termination of migration beneath preplate zone (Sanada et al., 2004). Reelin stabilizes the leading process through acting on the phosphorylation status of cofilin and therefore the actin cytoskeleton (Chai et al., 2009). In addition, Reelin also regulates adhesion of neurons to radial glia through the integrin pathway (Dulabon et al., 2000; Sanada et al., 2004). Finally, Reelin controls transcription by inhibiting the degradation of Notch intracellular domain (Hashimoto-Torii et al., 2008).

From a cell biological perspective, subcellular organelles play important roles in the cytoskeleton reorganizations that are critical for neuronal migration. For example, the centrosome orients toward the pia surface before the multipolar-to-bipolar transition. Disruption of centrosomal function through inactivation of Centrin 2 or depletion of centrosomal proteins block this transition and further migration (de Anda et al., 2010). In

8

addition, extensive remodeling of the cytoskeleton network is critical to the

morphological and behavioral changes during migration (Heng et al., 2009). Protrusion of

the leading process occurs concomitantly with actin polymerization, a rather dynamic

process that requires both assembly and disassembly on the actin filaments (Pollard and

Borisy, 2003; Theriot and Mitchison, 1991). Polymerization of actin bundles also

generates protrusive force that enables extension of the membrane (Condeelis, 1993). On

the other hand, the microtubule network serves as the major scaffold of the cell and the

tracks on which proteins and mRNA are transported. Neucleokinesis is critically

dependent on the microtubule network because movement of the soma occurs through a

cage of microtubules that surround the nucleus. The leading process exerts a pulling force

onto the microtubule cage which then pulls the nucleus forward (Lambert de Rouvroit

and Goffinet, 2001; Solecki et al., 2004). The microtubule network directly contributes to

leading process stabilization, not only through structural support but also through proper

transport of vesicles along microtubules. Disruption of microtubule-based transport

machinery including the dynein complex and microtubule associated proteins (MAPs)

block migration (Shim et al., 2008; Tsai et al., 2007). Fourthly, adhesion of migrating

neurons to radial glia provides the scaffold on which the cell moves. The neuron-radial

glia interaction through integrin signaling is critical for establishing adhesion of the

leading process to the extracellular matrix (ECM) in the marginal zone (Sekine et al.,

2012). Moreover, gap junctions between the migrating neuron and radial glia participate

in stabilization of the leading process and somal translocation (Elias et al., 2007). Fifth,

dynamic membrane trafficking is also necessary for proper glia-directed migration.

Inhibition of endocytic pathways block migration through abnormal accumulation of N-cadherin, which is involved in neuron-glia contact (Kawauchi et al., 2006).

The application of molecular biology techniques in developmental neurobiology has greatly advanced our knowledge of molecular mechanisms of brain development. The use of transgenic mice, including knockouts and knockins, and the development of *in utero* electroporation approach, has enabled efficient genetic manipulation in the developing brain. As a result, key molecular mechanisms critical for brain development have been identified. The *in utero* electroporation, a method of delivering DNA plasmids into the embryonic brain, has emerged as a useful tool for studies of molecular functions during brain development. In addition to its rapid turnaround and wide range of functional readouts, a huge advantage of this approach is that transfected cells represent only a very small percent of the whole brain. In other words, transfected cells are grown in an otherwise normal environment. Therefore, compared to the traditional knockout approach, *in utero* electroporation is a powerful tool where one can examine the cell-autonomous effect of genetic manipulation on transfected cells.

Transcriptional regulation of neuronal migration

Although previous studies have mostly focused on factors regulating the cytoskeleton and cell-cell interaction, it is becoming clear that transcriptional regulation is also a key component of regulation of neuronal migration. In particular, several transcription factors have been shown to play a key role in directing radial migration within the developing cerebral cortex.

The bHLH transcription factor Ngn2 and the POU-domain transcription factors Brn1 and Brn2 are good examples of transcriptional regulators that contribute to cortical neuron migration. Ngn2 is thought to promote migration of cortical neurons in part by inducing the formation of a polarized leading edge and regulating the expression of Dcx, RhoGAPs, RhoA, the Rho GTPase Rnd2, and the Cdk5 activator p35 (Chen et al., 2009; Ge et al., 2006; Heng et al., 2008). Brn1 and Brn2 control cortical radial migration by directly stimulating the expression of Dab1 and the Cdk5 activators p35 and p39, which engage in reelin-dependent signaling and nucleokinesis, respectively (McEvilly et al., 2002; Sugitani et al., 2002). Therefore, these studies suggest that Brn1 and Brn2 regulate migration by controlling the expression of multiple downstream effectors of reelin signaling.

Epigenetic regulation of transcription is a recent topic that received a lot of attention. It is based on the finding that histone and DNA molecules are covalently modified at multiple locus, and more importantly, the modifications can lead to conformational changes of chromatin, rendering it either compact or loose (Strahl and Allis, 2000). The conformational changes further influence the accessibility of DNA molecules wrapped by the histones, therefore regulating transcription. In addition, the histone or DNA modifications are able to recruit effector proteins that mediate the downstream effects (Lauberth et al., 2013). Epigenetic proteins regulate transcription through either directly or indirectly modulating histones or DNA molecules(Greer and Shi, 2012). Given the established role of transcription factors in neuronal developoment, it would be interesting to examine the role of epigenetic proteins in neuronal development.

Chapter 2

PHF6 plays an essential role in radial neuronal migration in the cerebral cortex *in vivo*

Introduction

Intellectual disability is a devastating cognitive disorder where no treatment is available. Therefore, there is a huge need to understand the underlying pathophysiology of intellectual disability.

Various abnormalities of early brain development are usually associated with intellectual disability, and in a lot of cases, are key contributing factors of the disease symptoms. For example, abnormalities associated with progenitor cell proliferation can lead to microcephaly or macrocephaly (Mahmood et al., 2011). Alterations of neuronal migration result in accumulation of neurons in ectopic locations in the cerebral cortex (Liu, 1007). In addition, defective axonal, dendritic and synaptic development impairs neuronal connectivity, which in turn impair brain function on a circuit level (Chechlacz and Gleeson, 2003). Therefore, studying the role of intellectual disability genes during early brain development should provide important information regarding the pathogenesis of the disease.

Human genetic studies have firmly established PHF6 as the causative gene for the Börjeson-Forssman-Lehmann syndrome. Multiple mutations, including missense, nonsense and insertion/deletion mutations, have been identified in independent families of BFLS. However, the functions of PHF6 relevant to BFLS remain unknown. Since previous *in situ* hybridization studies suggest a high level of PHF6 expression during embryonic brain development, I reasoned that PHF6 might play a critical role in the development of the cerebral cortex (Voss et al., 2007).

In this chapter, I carried out functional studies of PHF6 in the developing cerebral cortex. Using RNAi-mediated gene silencing approach, I uncovered a critical role of PHF6 in regulating neuronal migration in the cerebral cortex. Knockdown of PHF6 leads to massive neuronal migration arrest, and patient mutations of PHF6 block its ability to

promote neuronal migration. Therefore, I uncovered a pathophysiologically relevant function of PHF6 in BFLS.

Results

       To interrogate PHF6 function in the mammalian brain, I first characterized the expression of PHF6 in the developing cerebral cortex. I found that PHF6 was highly expressed during early phases of cortical development (Figure 2-1). In primary rat cortical neurons, PHF6 levels peaked between day 1 *in vitro* (DIV1) to DIV3, a period when neurons are actively polarizing and extending neurites (Figure 2-1A). In the developing mouse brain, PHF6 protein was highly expressed throughout the period of corticogenesis, declined in the first postnatal week, and became barely detectable in the adult cerebral cortex (Figure 2-1B). PHF6 was found to be broadly expressed in the ventricular and subventricular zones (VZ/SVZ), intermediate zone (IZ), and cortical plate (CP) in embryonic day 17 (E17) mouse cortex (Figure 2-1C). The temporal profile of PHF6 expression raised the possibility that PHF6 might play a role in cortical development.

Figure 2-1

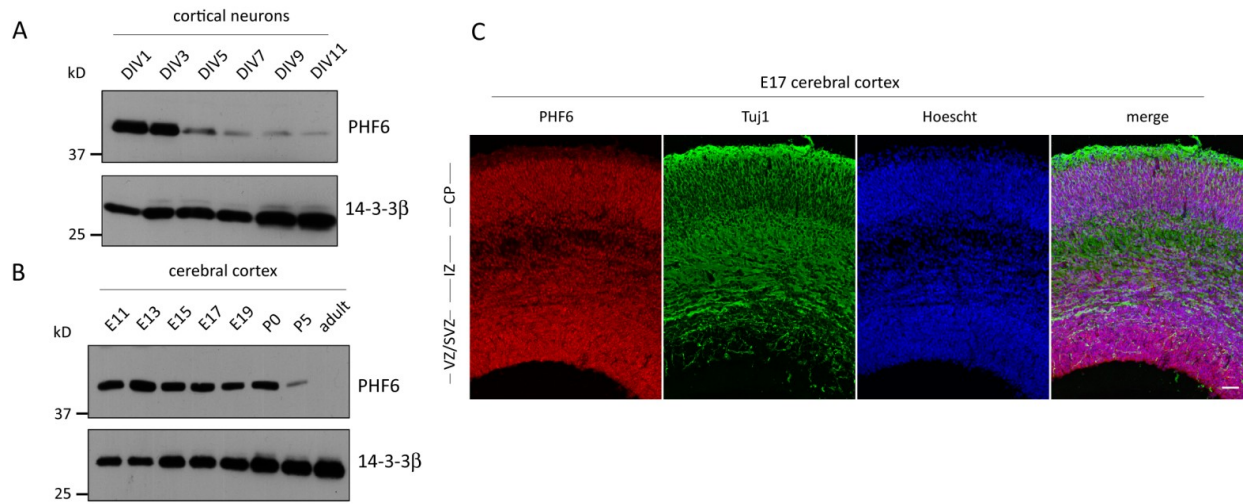Figure 2-1. PHF6 is highly expressed during early development of the cerebral cortex. **(A-B)** Lysates of primary rat cortical neurons (A) or mouse cerebral cortex (B) were immunoblotted with the PHF6 and 14-3-3β antibodies. **(C)** Immunohistochemical analyses of the cerebral cortex in E17 mouse embryos with the PHF6 and neuron-specific β- tubulin type III (Tuj1) antibodies. Scale bar: 50μm in (C).

To determine PHF6 function in cortical development, I used a plasmid-based method of RNA interference (RNAi) to acutely knockdown PHF6 in the developing cerebral cortex (Gaudilliere et al., 2002). I designed three short hairpin RNAs (shRNAs) targeting distinct regions of PHF6 mRNA, and confirmed that expression of these three shRNAs induced the knockdown of exogenous PHF6 protein in Human Embryonic Kidney 293 cells (293T cells) and endogenous PHF6 in primary mouse cortical neurons (Figures 2-2A, 2-2B, 2-2D, and 2-2F). In addition, I confirmed that introducing these shRNAs *in vivo* during development of the cerebral cortex similarly induced efficient knockdown of PHF6 *in vivo* (Figures 2-2C and 2-2E).

Figure 2-2
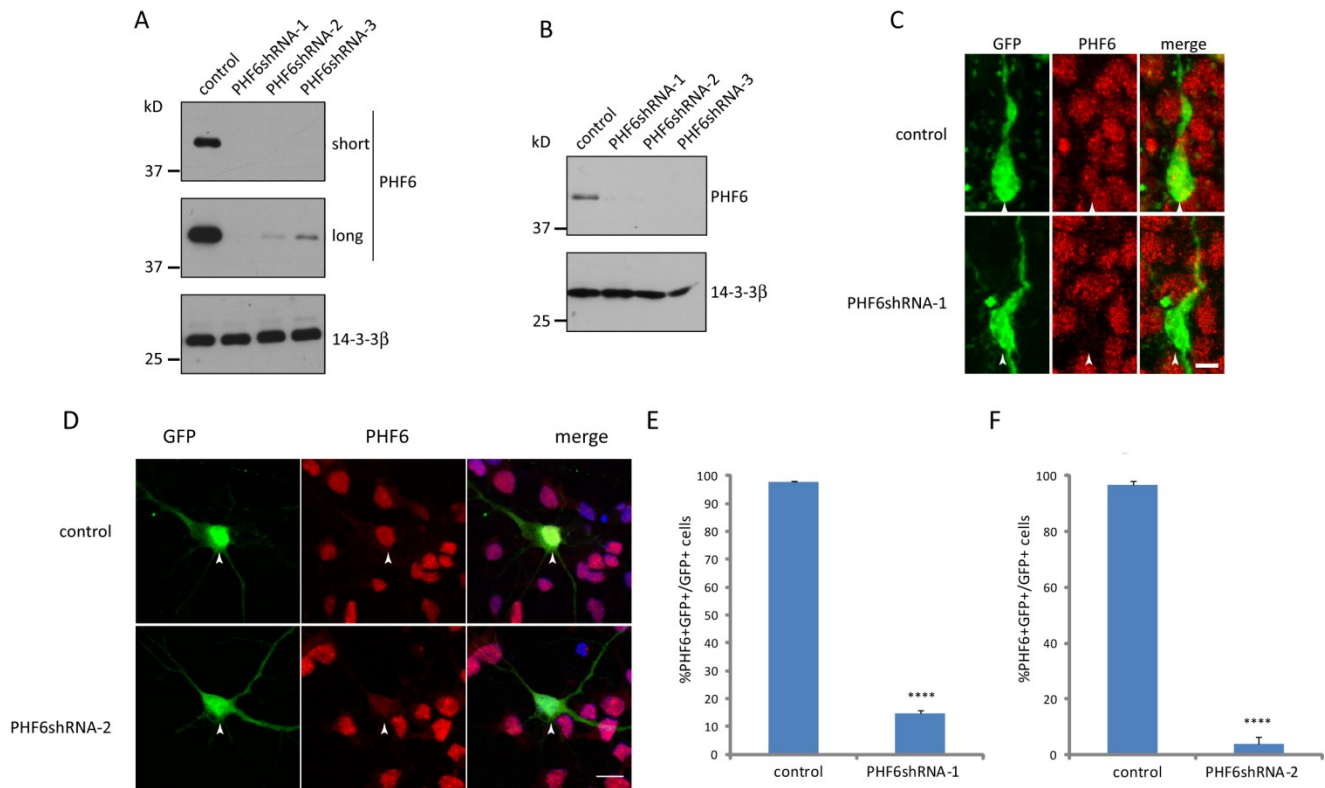
Figure 2-2. Validation of shRNA-mediated knockdown of PHF6. (**A**) Lysates of 293T cells transfected with an expression plasmid encoding FLAG-PHF6 together with an RNAi plasmid encoding PHF6 shRNAs or control scrambled shRNAs were immunoblotted with the FLAG and 14-3-3β antibodies. Two exposures (short and long) are shown for PHF6. (**B**) Lysates of primary mouse cortical neurons infected with lentivirus expressing PHF6 shRNAs or control scrambled shRNAs were immunoblotted with the PHF6 and 14-3-3β antibodies. (**C**) E14 mouse embryos electroporated with an RNAi plasmid encoding PHF6 shRNAs or control scrambled shRNAs together with a GFP expression plasmid were allowed to develop until E19. The cerebral cortex from the E19 embryos was subjected to immunohistochemical analyses with the PHF6 and GFP antibodies. PHF6 RNAi induced robust downregulation of PHF6 in cortical neurons *in vivo* (arrow heads). (**D**) Primary rat cortical neurons were transfected DIV0 with an RNAi plasmid encoding PHF6 shRNAs or control scrambled shRNAs together with GFP expression plasmid,

18

Figure 2-2. continued

and were subjected to immunocytochemistry analyses with the PHF6 and GFP antibodies at DIV3. PHF6 RNAi substantially reduced PHF6 immunoreactivity in cortical neurons. (**E**) Quantification of the percentage of GFP positive neurons as treated in (C) that were positive for PHF6 immunoreactivity is presented as mean + SEM. PHF6 RNAi significantly reduced the percentage of GFP positive neurons that were PHF6 positive. (**F**) Quantification of the percentage of GFP positive neurons as treated in (D) that were positive for PHF6 immunoreactivity is presented as mean + SEM. PHF6 RNAi significantly reduced the percentage of GFP positive neurons that were PHF6 positive in the cerebral cortex *in vivo*. Scale bar: 3μm in (C), 10μm in (D).

I next employed an *in utero* electroporation method to induce the knockdown of PHF6 in the developing mouse cerebral cortex *in vivo* (Figure 2-3). The PHF6 RNAi plasmids were electroporated together with a plasmid encoding GFP in the developing cortex in mice at E14, when superficial layer neurons are generated. Embryos were allowed to develop *in utero* until E19, and brains were harvested and subjected to immunohistochemical analyses. Upon characterizing the consequences of PHF6 knockdown on the development of the cerebral cortex, I found a striking migration phenotype. Neurons in control animals differentiated and migrated properly to the superficial layers of the cortical plate (Kawauchi et al., 2006). By contrast, cortical neurons in PHF6 knockdown animals failed to migrate to their proper location in the upper cortical plate (Figures 2-4A and 2-4B). Rather, PHF6 knockdown cortical neurons accumulated in the intermediate zone or lower cortical plate. Quantification of the percentage of neurons in different regions revealed that PHF6 RNAi reduced the percentage of neurons reaching the upper cortical plate by 2- to 3-fold, and increased the number of neurons in the intermediate zone by 3- to 5-fold. Notably, the extent of the migration defect in the PHF6 knockdown animals correlated with the degree of PHF6 knockdown (Figure 2-2A). In control analyses, I confirmed that the migration defect in PHF6 knockdown animals was not secondary to impaired neuronal differentiation (Figure 2-5A, 2-5F, 2-5G, 2-5H), altered progenitor cell proliferation (Figure 2-5B, 2-5C), effects on general cell health (Figures 2-5D, 2-5E) or changes of radial glial fiber structure (Figure 2-5I). Taken together, these results suggest that PHF6 knockdown profoundly and specifically impairs the radial migration of neurons in the developing cerebral cortex.

Figure 2-3
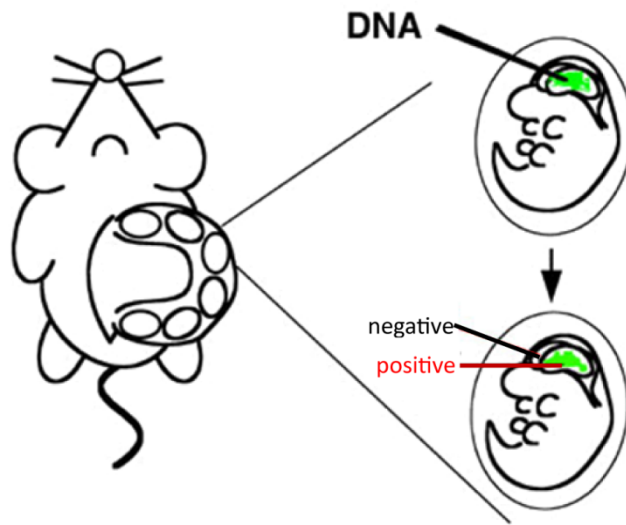
Figure 2-3. A diagram of *in utero* electroporation. DNA (green) is injected into the lateral ventricles of E14 mouse embryos, and electroporation is performed to transfect the DNA into the developing cerebral cortex.

Figure 2-4

A

B
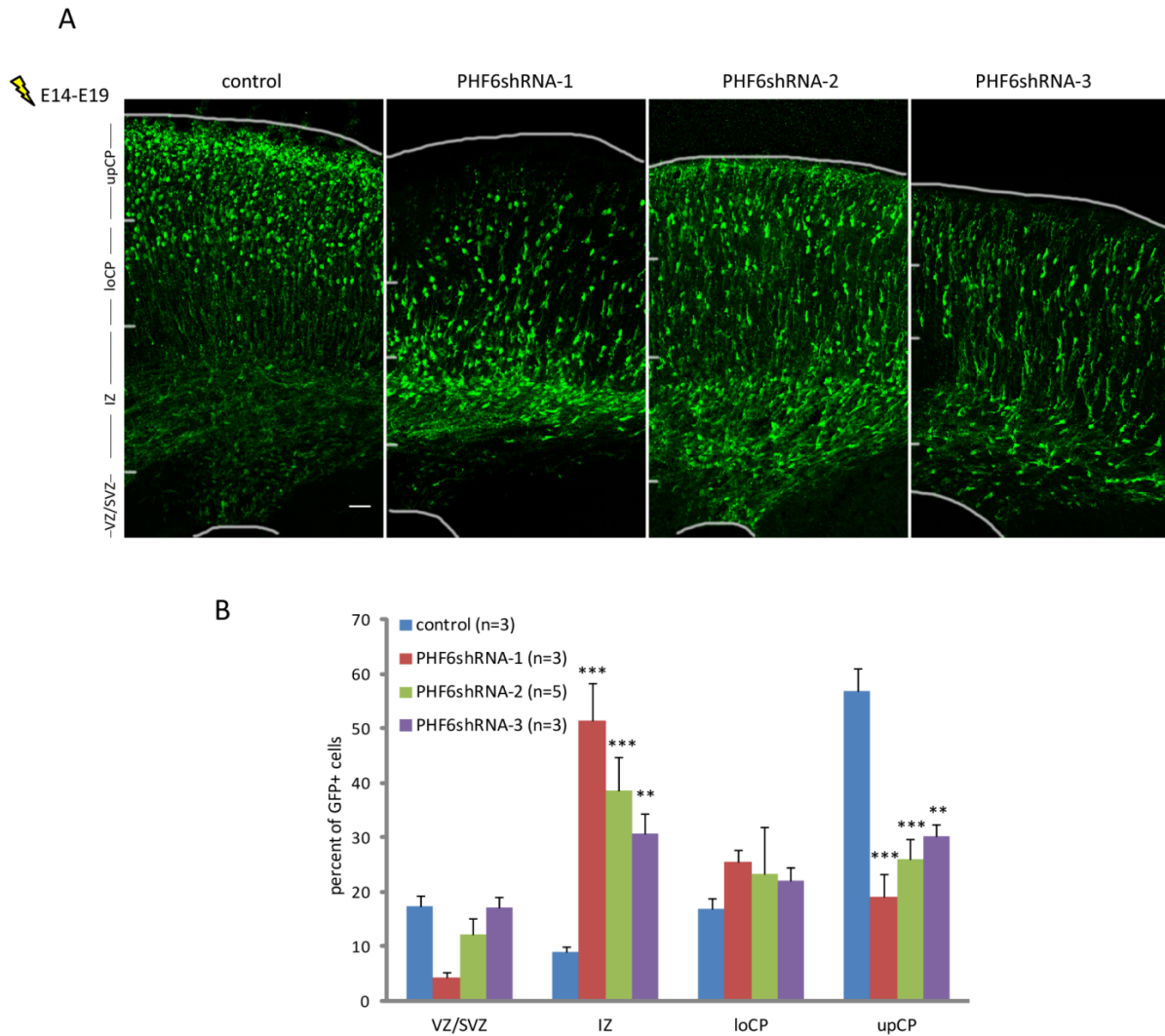
Figure 2-4. PHF6 knockdown impairs neuronal migration in the cerebral cortex. (**A**) E14 mouse embryos were subjected to *in utero* electroporation and the cerebral cortex of E19 embryos was analyzed by immunohistochemistry using the GFP antibody. PHF6 knockdown in the cerebral cortex *in vivo* profoundly impaired the radial migration of cortical neurons. upCP, upper cortical plate; loCP, lower cortical plate; IZ, intermediate zone; VZ/SVZ, ventricular zone/sub-ventricular zone. (**B**) Quantification of the percentage of GFP positive neurons in distinct regions of the cerebral cortex in E19 mouse embryos treated as in (A) is presented as mean + SEM. PHF6 RNAi

22

Figure 2-4. continued

significantly reduced the percentage of neurons that reached the upper cortical plate, and concomitantly increased the percentage of neurons stalled in the intermediate zone. Scale bars: 50μm in (A).

Figure 2-5

A

B

C

D

E

Figure 2-5 (Continued)

F

Figure 2-5 (Continued)

G

	GFP	Cux1	Merge

E14-E19

control

PHF6shRNA-1

PHF6shRNA-2
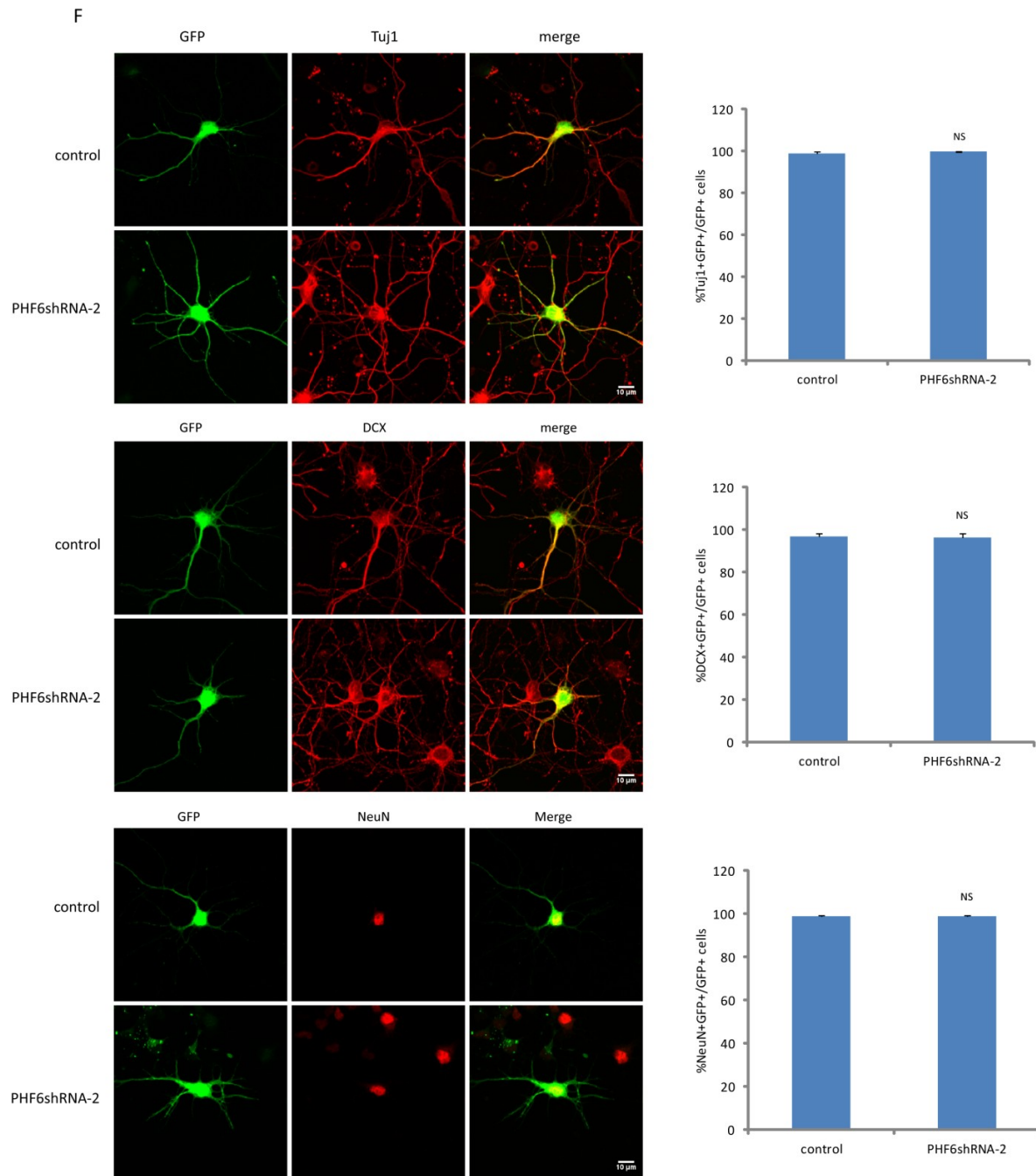
Figure 2-5 (Continued)

H

Figure 2-5 (Continued)

Figure 2-5. PHF6 knockdown induced migration phenotype is not due to abnormal neuronal differentiation, alterations of proliferation or cell death. (**A**) E14 mouse embryos were electroporated and allowed to develop until E19. The cerebral cortex from the E19 embryos was
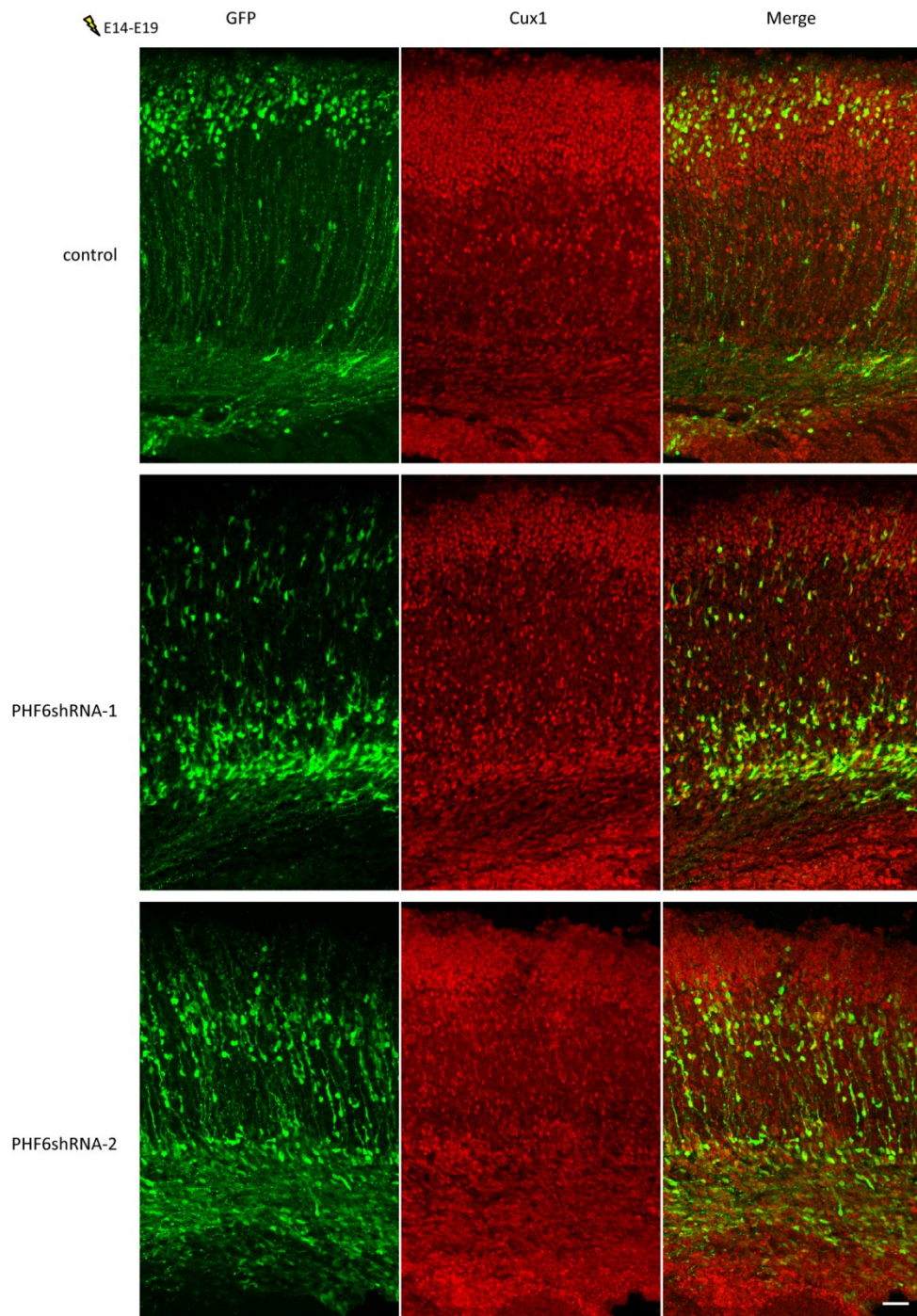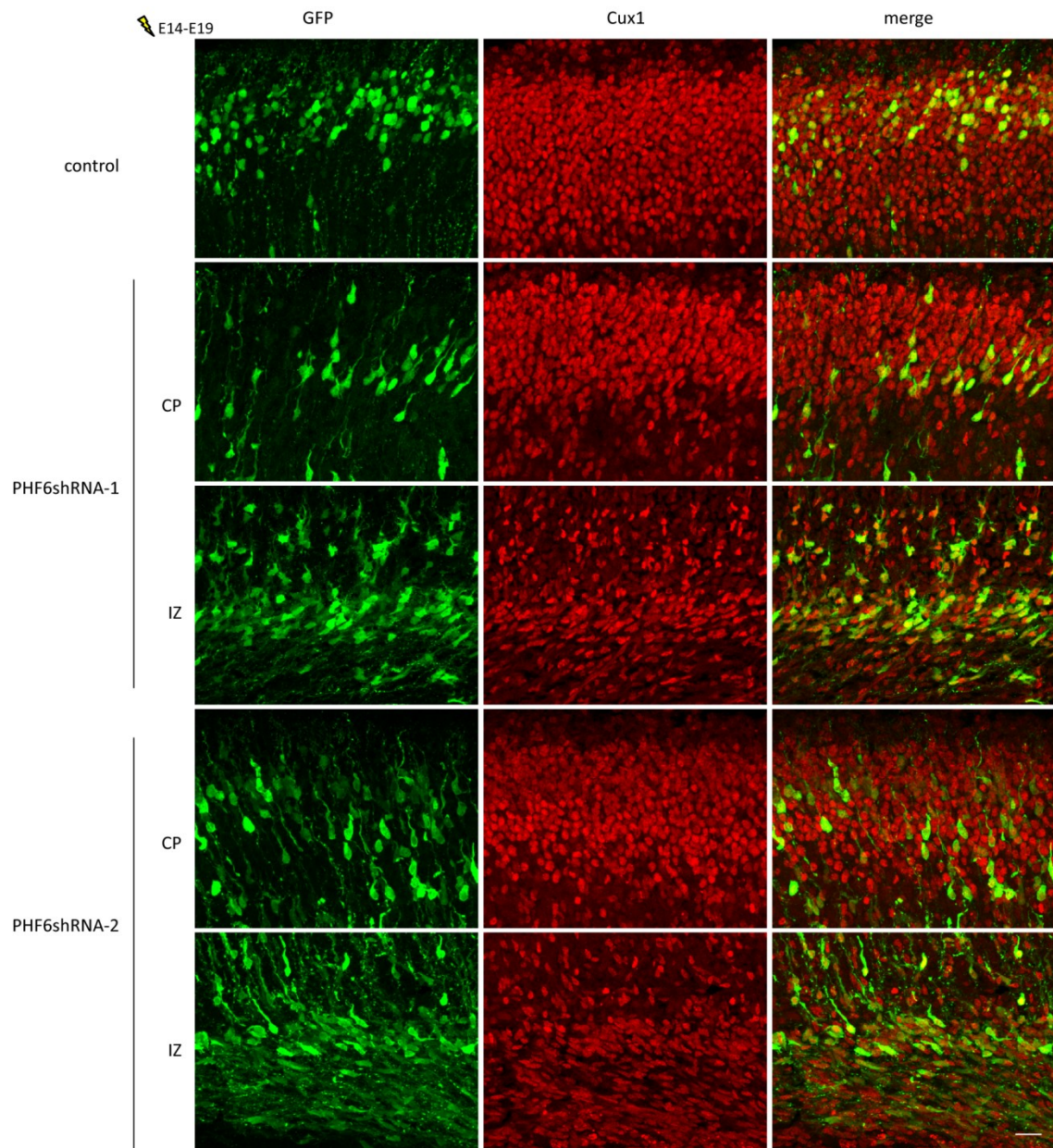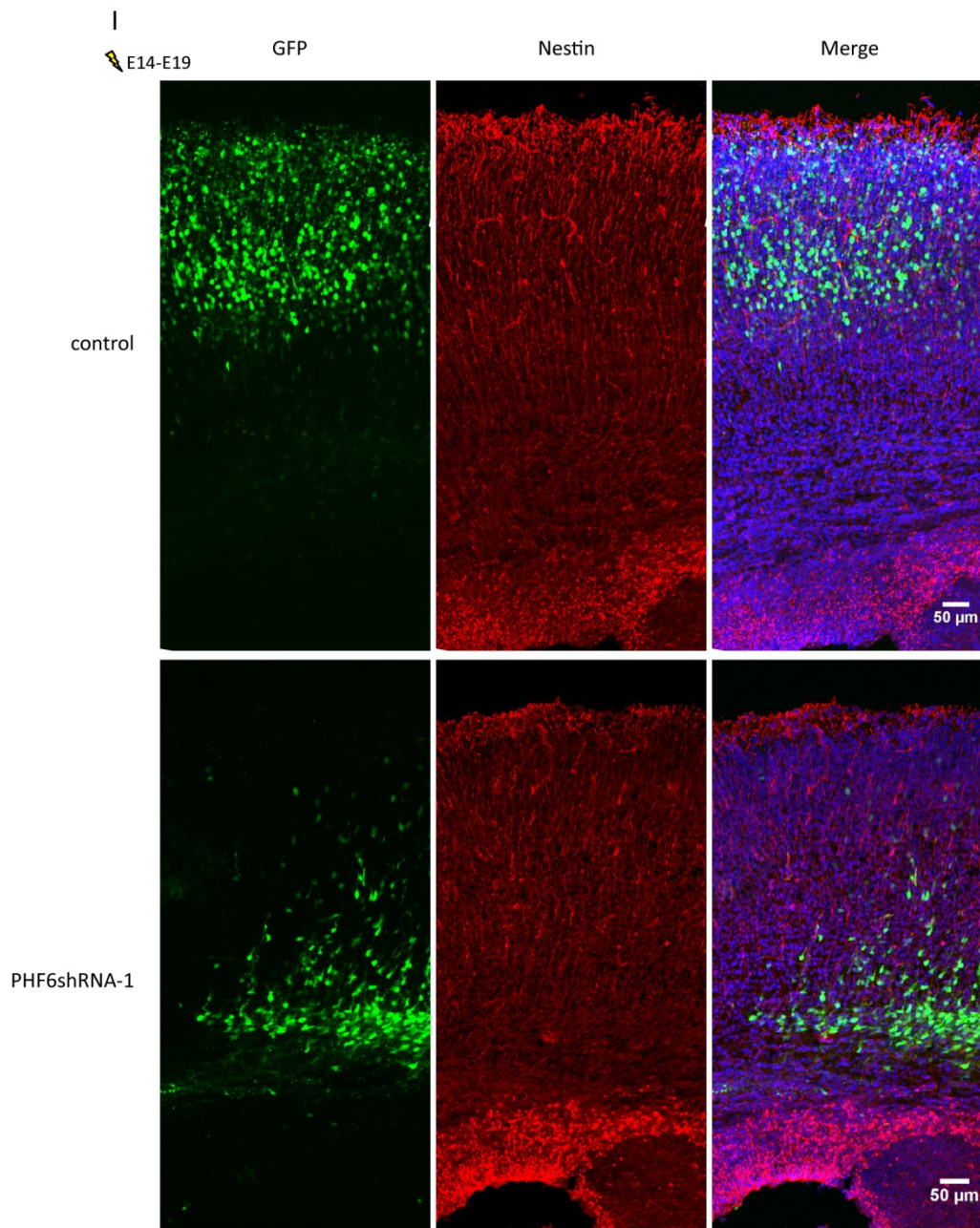
Figure 2-5. continued

subjected to immunohistochemical analyses with the GFP and the neuron-specific β-tubulin type
III (Tuj1) antibodies. Cells transfected with PHF6 shRNAs were positive for the neuron-specific
marker (arrowheads). (**B**) E14 mouse embryos were electroporated and pregnant dams were
pulsed with BrdU (100g/g body weight) at E17 for 2hr before harvest. Quantification of the
percentage of GFP positive cells that were BrdU positive in VZ/SVZ of E17 mouse embryos is
presented as mean + SEM. PHF6 knockdown did not significantly affect incorporation of BrdU in
progenitor cells. (**C**) E14 embryos were electroporated as in (A) and harvested at E17. The
cerebral cortex from the E17 embryos were subjected to immunohistochemical analysis with the
GFP and phospho-histone H3 (pH3) antibodies. Quantification of the percentage of GFP positive
cells that were pH3 positive in E17 embryos is presented as mean + SEM. PHF6 knockdown did
not significantly affect the percent of pH3 positive cells among the progenitor cells. (**D**) E14
mouse embryos were electroporated as in (A) and allowed to develop until E19. The cerebral
cortex from the E19 embryos was subjected to immunohistochemical analyses with the GFP and
cleaved caspase 3 antibodies. For PHF6 shRNAs, cells in the intermediate zone are shown. (**E**)
Quantification of the percentage of GFP positive cells that were cleaved caspase 3 negative or
positive in E19 embryos treated as in (D) is presented as mean + SEM. (**F**) Primary rat cortical
neurons were transfected DIV0 with an RNAi plasmid encoding PHF6 shRNAs or control
scrambled shRNAs together with GFP expression plasmid, and were subjected to
immunocytochemistry analyses with the GFP and Tuj1/DCX/NeuN antibodies at DIV3. The
percentage of GFP positive cells that are positive for the indicated markers is quantified on the
right. PHF6 knockdown does not affect the differentiation status of cortical neurons. (**G-H**) E14
mouse embryos were electroporated as in (A) and allowed to develop until E19. The cerebral
cortex from the E19 embryos was subjected to immunohistochemical analyses with the GFP and
Cux1 antibodies. PHF6 knockdown neurons are positive for Cux1, suggesting that they are

29

Figure 2-5. continued

properly differentiated to occupy the superficial layers of the cerebral cortex. (**I**) E14 mouse embryos were electroporated as in (A) and allowed to develop until E19.PHF6 knockdown does not significantly affect the radial glial scaffold. Scales: 5μm in (A), 20μm in (D), 10μm in (F), 50μm in (G) and (I), 30μm in (H).

To determine whether the PHF6 RNAi-induced migration phenotype is due to specific knockdown of PHF6 rather than off-target effects of RNAi, I performed a rescue experiment. I used an expression plasmid encoding rat PHF6 (PHF6-Res), which contains two mismatches with shRNA-2 targeting mouse PHF6. Whereas expression of mouse PHF6 (PHF6-WT) was effectively downregulated by PHF6 RNAi, expression of PHF6-Res was refractory to PHF6 RNAi (Figure 2-6A). I next determined the effect of PHF6-Res expression on neuronal migration in the background of PHF6 knockdown in the cerebral cortex *in vivo*. I found that expression of PHF6-Res in PHF6 knockdown animals largely restored the normal migration pattern of cortical neurons (Figures 2-6C and 2-6D). These data indicate that the PHF6 RNAi-induced migration phenotype is the result of specific knockdown of PHF6.

I next asked whether the migration phenotype induced by PHF6 knockdown is relevant to intellectual disability. The rescue experiment allowed in structure-function analyses the assessment of the effect of BFLS patient specific mutations on PHF6 function in neuronal migration. I focused on the patient mutation C99F, because cysteine 99 is a highly conserved and critical residue in PHF6's first PHD finger domain (Lower et al., 2002) (Figure 2-6B). Strikingly, I found that the C99F mutation blocked the ability of PHF6-Res to drive neuronal migration in the cerebral cortex *in vivo* in the background of PHF6 RNAi (Figures 2-6C and 2-6D). Similarly, deletion of 86 amino acids at the C-terminus of PHF6, a mutation identified in a female patient of BFLS, also blocked its ability to promote neuronal migration (Berland et al., 2011). These data suggest that PHF6 function in neuronal migration is perturbed by BFLS patient mutations, and thus impaired neuronal migration might underlie the pathogenesis of BFLS.

Figure 2-6
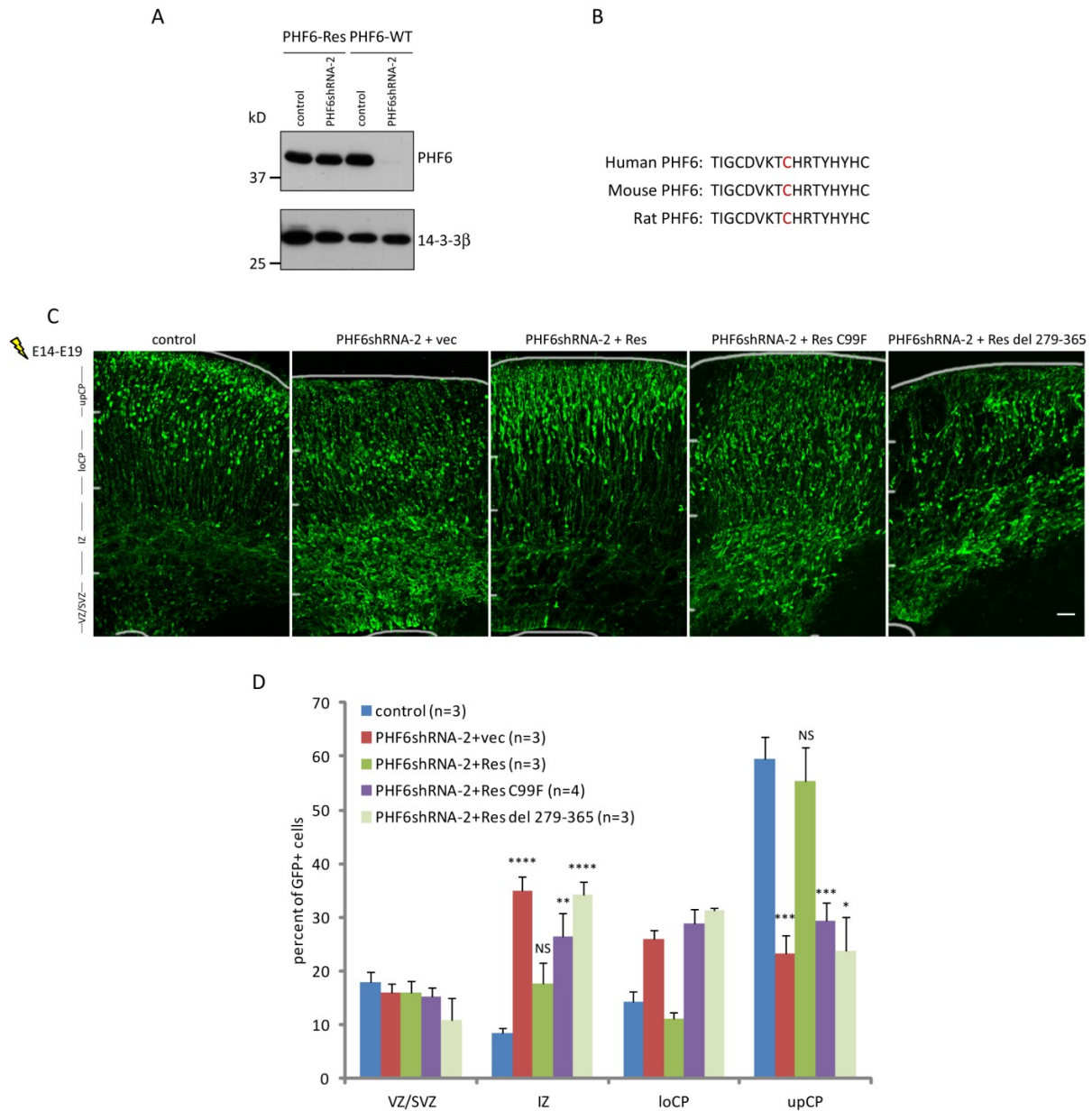
Figure 2-6 PHF6 knockdown induced migration arrest is specifically due to loss of PHF6, and is pathophysiologically relevant. (**A**) Lysates of 293T cells transfected with an expression plasmid encoding FLAG-rat PHF6 (PHF6-Res) or FLAG-mouse PHF6 (PHF6-WT) together with the PHF6 RNAi or control RNAi plasmid were immunoblotted with the FLAG and 14-3-3β antibodies. Expression of PHF6-Res is refractory to PHF6 RNAi. (**B**) Protein sequences of human,

32

Figure 2-6. continued

mouse and rat PHF6 are aligned and cysteine 99 residue is labeled red. This cysteine residue is highly conserved across the three species. (**C**) E14 embryos were electroporated and analyzed as in Figure 2-3A with the PHF6 RNAi or control RNAi plasmid together with an expression plasmid encoding wild type or the C99F mutant, or deletion 279-365 PHF6-Res protein or the corresponding control vector. vec, control vector; Res, PHF6-Res. (**D**) Quantification of the percentage of GFP positive neurons in distinct regions of the cerebral cortex in E19 mouse embryos treated as in (G) is presented as mean + SEM. Expression of PHF6-Res significantly reduced the percentage of neurons stalled in the intermediate zone and significantly increased the percentage of neurons that reached the upper cortical plate in the background of PHF6 RNAi in the cerebral cortex *in vivo*. The C99F mutation and the deletion 279-365 mutation blocked the ability of PHF6-Res to drive neuronal migration. Scale bars: 50μm in (C).

Having established a critical role of PHF6 in neuronal migration, I next asked how PHF6 functions in neurons at the cellular level. Radial neuronal migration in the cerebral cortex results from major cell morphological rearrangements, including the transition from multipolar to bipolar neuronal morphology in the intermediate zone and extension of the leading process toward the pia (Ayala et al., 2007; Elias et al., 2007; Kerjan and Gleeson, 2007; Kriegstein and Noctor, 2004; Nadarajah et al., 2001). Knockdown of PHF6 substantially increased the number of multipolar neurons and concomitantly reduced the number of bipolar neurons in the intermediate zone in the cerebral cortex in E17 embryos (Figures 2-7A, 2-7B, 2-7C). The multipolar neurons displayed a few short, highly branched processes with multiple orientations. Notably, the remaining bipolar neurons in the PHF6 knockdown embryos harbored a thick leading process with numerous filopodia-like protrusions (Figures 2-7C and 2-7D). I also characterized the morphology of the leading process in migrating neurons in the lower cortical plate in E19 embryos. PHF6 knockdown dramatically increased the number of neurons that lacked the leading process or that had a short, poorly developed, or aberrantly-branched leading process (Figures 2-7E and 2-7F). Collectively, these data suggest that PHF6 plays a critical role in the multipolar-to-bipolar transition and the morphogenesis of the leading process in migrating neurons.

Figure 2-7
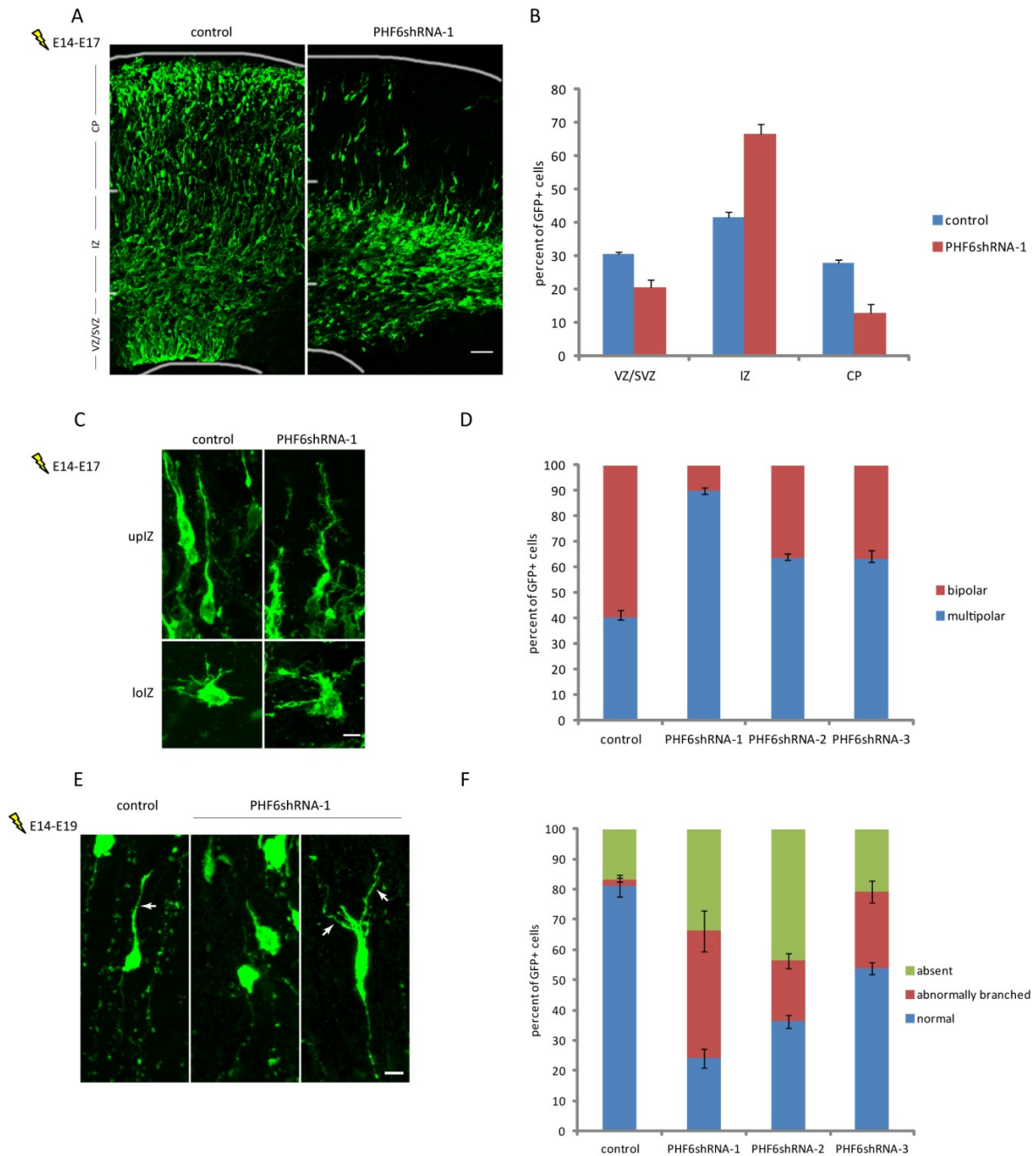
Figure 2-7. PHF6 is important for the morphological changes during neuronal migration. (A) E14 embryos were electroporated and allowed to develop until E17. The cerebral cortex from the E17 embryos was subjected to immunohistochemistry with the GFP antibody. CP, cortical plate. (B) Quantification of the percentage of GFP positive neurons in distinct regions of the cerebral cortex

35

Figure 2-7. continued

in E17 mouse embryos as treated in (A) is presented as mean + SEM. PHF6 knockdown in the cerebral cortex *in vivo* significantly reduced the percentage of neurons that reached the cortical plate and concomitantly increased the percentage of neurons stalled in the intermediate zone. (C) E14 embryos were electroporated and analyzed as in (A). Representative images for neurons in the lower or upper intermediate zone are shown. loIZ, lower intermediate zone; upIZ, upper intermediate zone. (D) Quantification of the percentage of GFP positive neurons in the intermediate zone exhibiting bipolar or multipolar morphology in E17 mouse embryos treated as in (C) is presented as mean + SEM. PHF6 knockdown in the cerebral cortex *in vivo* significantly increased the percentage of multipolar cells in the IZ. (E) E14 embryos were electroporated as in (A) and allowed to develop until E19. The cerebral cortex from the E19 embryos was subjected to immunohistochemistry with the GFP antibody. Representative images of migrating neurons in the lower cortical plate are shown. PHF6 knockdown in the cerebral cortex *in vivo* leads to loss or aberrant branching of the leading process, which is indicated by arrows. (F) Quantification of the percentage of GFP positive cells exhibiting different leading process morphologies in E19 embryos treated as in (E) is presented as mean + SEM. PHF6 knockdown significantly reduced the percentage of cells bearing a normal leading process. Scale bar: 50μm in (A), 5μm in (C), 10μm in (E).

Discussion

In this chapter, I identified a critical role of PHF6 in the regulation of neuronal migration. Loss of PHF6 during cortical development, induced by RNAi-mediated gene silencing, leads to profound impairment of neuronal migration. Moreover, this is likely to be pathologically relevant because PHF6 patient mutants fail to promote neuronal migration. Taken together, my results suggest that abnormal neuronal migration represents a key component of the pathophysiology of BFLS.

The finding that PHF6 is critical to neuronal migration is somewhat unexpected. Although prior to our studies nothing was known concerning the cellular function of PHF6, it was speculated to regulate cell growth and proliferation. This is based on its genetic linkage to acute myeloid leukemia, where mutations of PHF6 were identified in the leukemia cells (Van Vlierberghe et al., 2010). In the literature, both macrocephaly and microcephaly have been documented in BFLS patients, and there is no consensus on whether changes in brain size are a typical presentation of BFLS patients (Gecz et al., 2006). Therefore it is unclear whether alterations of brain size are a pathological feature of BFLS.

In our system, it is unclear whether PHF6 is critical for progenitor cell proliferation in the cerebral cortex. Our data regarding the effect of PHF6 knockdown on proliferation is equivocal and therefore I cannot reach a conclusion. However, this also argues that the migration arrest is unlikely due to alterations of progenitor cell proliferation. Therefore, our data support a specific function of PHF6 in the regulation of neuronal migration.

The pathology of BFLS is very poorly documented. Examination of one of the probands described in the earliest families of BFLS disease revealed cerebral malformation with clear though not profound abnormalities mainly due to disturbed

migration (Brun et al., 1974). In particular, the cerebral cortex showed widespread cortical dysplasia with large areas of indistinct or absent lamination, a critical feature of neuronal migration disorders. Therefore, the pathological findings, together with our data regarding the patient mutants, suggest that our findings are of critical relevance to the pathogenesis of BFLS syndrome.

Methods

Animals

      Timed pregnant CD-1 mice were purchased from Charles River Laboratories. All animal experiments were conducted under the institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC).

Plasmids

      The PHF6 plasmids were generated by PCR using mouse or rat cortical neuron cDNA. The shRNA plasmid targeting sequences are: control: TACGCGCATAAGATTAGGGTA; PHF6shRNA-1 (mouse): GTTCAGCTCACAACAACATCA; PHF6shRNA-1 (rat): GTGCAGCTCACAACAACATCA; PHF6shRNA-2 (mouse): AAGTAGCTCCTACCGAGATAG; PHF6shRNA-2 (rat): AAGCAGTTCCTACCGAGATAG; PHF6shRNA-3: AACTGTACTTCAGGAGATTAA; Different shRNA targeting sequences were designed due to differences between mouse and rat coding sequences.

In utero electroporation

      In utero electroporations were performed as described (Ge et al., 2010). Briefly, venus plasmid (final concentration 0.5ug/ul) was co-injected with the RNAi plasmid (final concentration 2ug/ul) into the lateral ventricle of E14 mouse embryos within the uterine sac, and electroporation was performed (35V for 50ms, with 950ms intervals, 6 pulses). The uterine sac was then returned to the abdominal cavity and the abdominal wall was sutured. E19 embryos were harvested and fixed in 4% paraformaldehyde for 1hr. P6 brains were fixed by intracardiac perfusion using 4% paraformaldehyde followed by post-fixation in 4% paraformaldehyde for 2hr. After fixation, brains were subjected to

cryoprotection in 30% sucrose in PBS overnight. Afterwards, brains were embedded in OCT and flash-frozen, and 20 m coronal sections were prepared. At least 3 animals were analyzed for each condition.

Immunoblotting

Immunoblotting was performed as described (Lehtinen et al., 2006). The PHF6 (Novus Biologicals), 14-3-3β(Santa Cruz) antibodies were used.

Immunohistochemistry

Cortical sections were rehydrated in PBS for 30min, followed by incubation in blocking solution (10% goat serum, 3% BSA, 0.5% Triton X-100 in PBS) for 30min at room temperature. Subsequently, sections were incubated in primary antibodies diluted in blocking solution overnight at 4°C. The PHF6 (Novus Biologicals), Tuj1 (Covance), GFP (chicken, Abcam) and Cux1 (Santa Cruz) antibodies were used. Sections were then washed three times with PBS and incubated with appropriate secondary antibodies conjugated to Alexa 488 or Cy3 (1:1000,Jackson ImmunoResearch), followed by staining with the DNA dye bisbenzimide. Sections were mounted with fluoromount-G (SouthernBiotech) for fluorescence microscopy.

Immunocytochemistry

Primary cultured neurons were fixed in 4% paraformaldehyde for 5-20min followed by permeabilization using 0.4% Triton X-100 in PBS. Samples were then blocked in blocking solution containing 10% milk and 1% goat serum, followed by staining with primary and secondary antibodies. The PHF6 (Novus Biologicals), Tuj1 (Covance), GFP (chicken, Abcam), NeuN (Millipore) and Cux1 (Santa Cruz) antibodies were used.

Lentivirus

shRNA sequences were cloned into pLentiLox 3.7 vector and lentivirus production was performed as described (Rubinson et al., 2003).

Attributions

All experiments in this chapter were performed by Chi Zhang. The *in utero* electroporation was developed with the help of Yoshiho Ikeuchi.

Chapter 3

The PAF1 complex interacts with PHF6 and plays a critical role in radial migration of neurons in the cerebral cortex *in vivo*

Introduction

In the previous chapter, I described functional characterization of PHF6 during embryonic brain development. I identified a critical function of PHF6 in the regulation of neuronal migration, and uncovered an intimate link between this function and the pathophysiology of intellectual disability in BFLS.

In this chapter, I will explore the molecular mechanisms by which PHF6 functions. I hypothesized that PHF6 functions through interacting with other proteins, because it does not seem to bear any enzymatic activity by itself. To test this hypothesis, I adopted the unbiased immunoprecipitation followed by mass spectrometry (IP-MS) approach to search for interacting partners of PHF6. This approach is very powerful because it allows us to compare a specific IP-MS dataset with hundreds of IP-MS datasets that were performed in a similar manner, thereby effectively eliminating the background interacting proteins and identifying the specific interacting partners (Sowa et al., 2009).

Using the IP-MS approach, we identified the PAF1 transcription elongation complex as an interacting partner of PHF6. The PAF1 complex has been established as the RNA polymerase II associated complex that efficiently promotes transcriptional elongation. Originally identified in yeast, it has been shown to promote transcriptional elongation along the chromatin substrate in a variety of systems (Chen et al., 2009; Kim et al., 2010; Krogan et al., 2002; Marton and Desiderio, 2008; Rondon et al., 2004; Squazzo et al., 2002). It functions through a number of molecular mechanisms: 1) the PAF1 complex can directly stimulate RNA polII transcription along the chromatin, through physical association with the polymerase; 2) the PAF1 complex acts synergistically with other factors that promote transcriptional elongation, such as the transcription elongation factor SII; 3) the PAF1 complex promotes posttranslational modifications of the chromatin including H2B ubiquitination and H3K4/K79 methylation,

which was suggested to facilitate future rounds of transcription at the modified genomic loci (Kim et al., 2010).

Although the biochemical functions of the PAF1 complex has been studied quite extensively, its role in development and homeostasis remains very poorly characterized. Loss of Leo1, a critical component of the PAF1 complex, leads to defective development of neural crest-derived cells in zebrafish (Nguyen et al., 2010), whereas mutation of cdc73, a tumor suppressor gene, is directly involved in predisposition to Hyperparathyroidism-Jaw tumor syndrome (HPT-JT) and in development of some sporadic parathyroid tumors (Carpten et al., 2002). However, the biological functions of the PAF1 complex during brain development remain unstudied. I established a critical role of the PAF1 complex during neuronal migration in the mammalian cerebral cortex, thereby providing the first insight into the role of PAF1 complex and transcriptional elongation in brain development. In addition, the identification of the PAF1 complex as a novel interacting complex for PHF6 provides important insights to the functions of PHF6 and underlying mechanisms of BFLS pathophysiology.

Results

Having established a critical role of PHF6 in the regulation of neuronal migration, I next determined the mechanism by which PHF6 functions. I first characterized the subcellular localization of PHF6. I found that PHF6 protein was localized in the nucleoplasm in primary cortical neurons, consistent with the possibility that PHF6 might regulate transcription (Figure 3-1A) (Lower et al., 2002). This immunopositive signal is likely to be specific because knockdown of PHF6 abrogates the signal (Figure 2-2 D and F). Similarly, ectopically expressed PHF6 localized to the nucleoplasm in 293T cells (Figure 3-1B). Since PHF6 is not known to harbor enzymatic activity, I reasoned that PHF6 might associate with other factors that in turn regulate transcription.

Figure 3-1

A

B
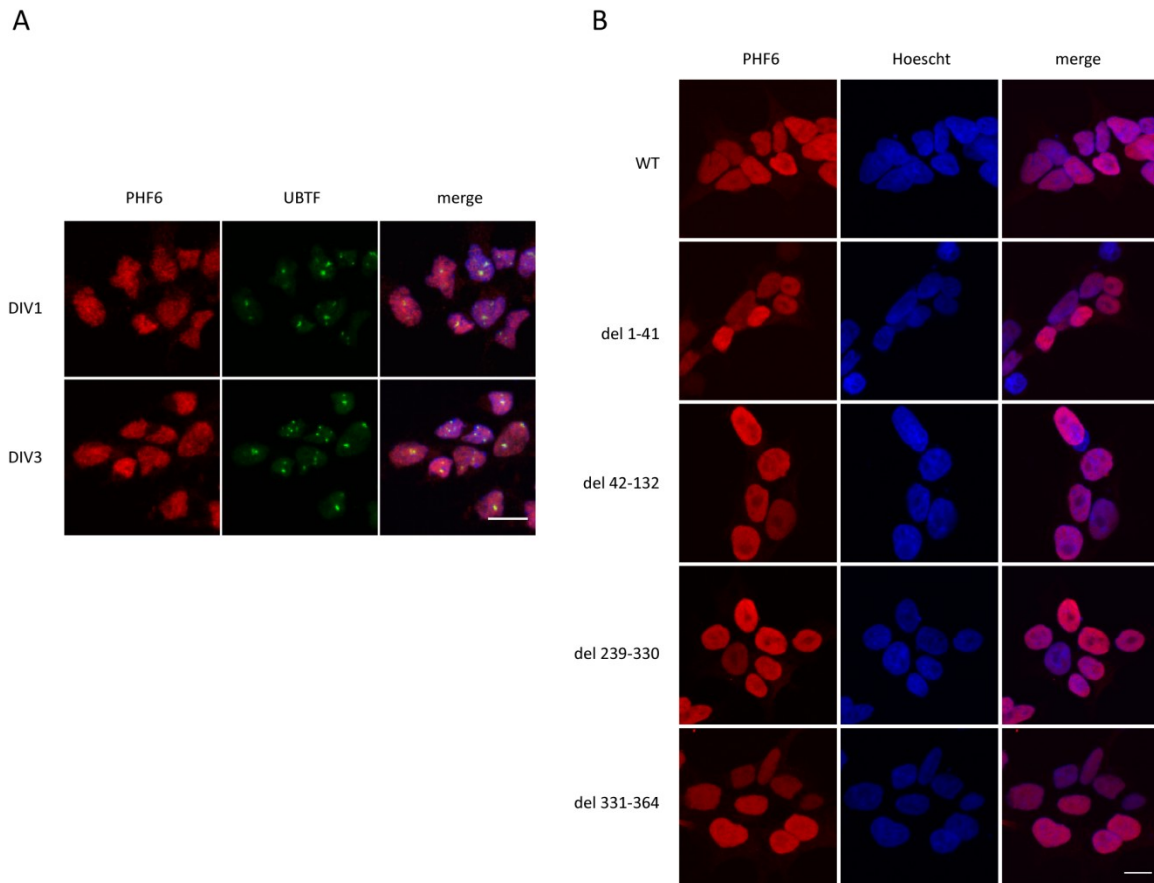
Figure 3-1. PHF6 is localized in the nucleus. (**A**) Immunocytochemical analyses of primary rat cortical neurons at DIV1 or DIV3 with the PHF6 and UBTF antibodies, the latter to label the nucleolus. PHF6 is localized in the nucleoplasm in neurons. DNA was stained with bisbenzimide in the right panel (in blue). (**B**) 293T cells stabling expressing HA-tagged PHF6 wildtype (WT) or different deletion mutants are subjected to immunocytochemical analyses using the HA antibody. PHF6 is localized in the nucleoplasm. Scale bar: 10μm in (A) and (B).

To identify proteins that physically associate with PHF6, I used an approach of immunoprecipitation followed by mass spectrometry (IP/MS). I used a rigorous proteomics method that compares a specific IP/MS dataset against a large set of unrelated parallel IP/MS datasets, thus distinguishing high-confidence candidate interacting proteins (HCIPs) from background proteins (Behrends et al., 2010; Litterman et al., 2011; Sowa et al., 2009). I infected 293T cells with a lentivirus encoding HA-PHF6, and immunopurified PHF6 from cells stably expressing HA-PHF6. The purified PHF6 immunoprecipitate was subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS) analyses. The Comparative Proteomics Analysis Software Suite (Comp*PASS*) platform was then used to identify HCIPs from greater than 500 proteins identified by LC-MS/MS (Sowa et al., 2009). Remarkably, all four core components of the PAF1 transcription elongation complex, PAF1, LEO1, CDC73 and CTR9, were found as robust HCIPs of PHF6 (Figure 3-2A).

I validated the interaction of HA-PHF6 and the endogenous PAF1 complex in co-immunoprecipitation analyses in 293T cells (Figure 3-2B). Importantly, I also found that endogenous PHF6 associated with all four components of the endogenous PAF1 complex in the cerebral cortex in mouse embryos at E17, an age that coincides temporally with migration of neurons to the superficial layers (Figure 3-2C). These data suggest that the PAF1 complex might represent a physiological interacting partner of PHF6.

Figure 3-2

A

tsc	symbol	dscore	ndscore	wdscore	nwdscore	zscore
5	FKBP3	174.41	3.3	174.41	2.58	8.67
5	PAF1	174.41	3.3	174.41	2.58	8.67
17	PHF6	160.8	3.04	160.8	2.38	33.84
4	UBTF	156	2.95	156	2.31	8.78
3	CDC73	135.1	2.56	135.1	2	8.71
11	CKAP5	129.35	2.45	457.89	6.78	8.68
2	CTR9	110.31	2.09	110.31	1.63	8.57
2	CCDC86	110.31	2.09	110.31	1.63	8.57
2	RCL1	110.31	2.09	110.31	1.63	8.57
2	LEO1	110.31	2.09	110.31	1.63	8.57
9	MAP4	78	1.48	78	1.15	6.84
4	DIMT1L	78	1.48	82.68	1.22	8.38
1	GTF2B	78	1.48	78	1.15	9
1	METAP2	78	1.48	78	1.15	9
1	C11orf31	78	1.48	78	1.15	9
1	METAP1	78	1.48	78	1.15	9
1	FEN1	78	1.48	78	1.15	9
1	ANP32B	78	1.48	78	1.15	9
1	FAM69A	78	1.48	78	1.15	9
1	MRPL20;LOC642393	78	1.48	78	1.15	9
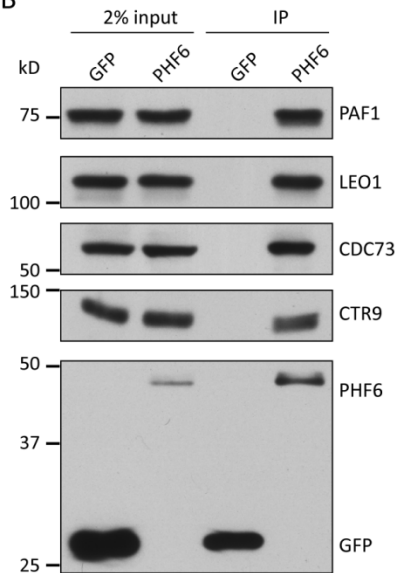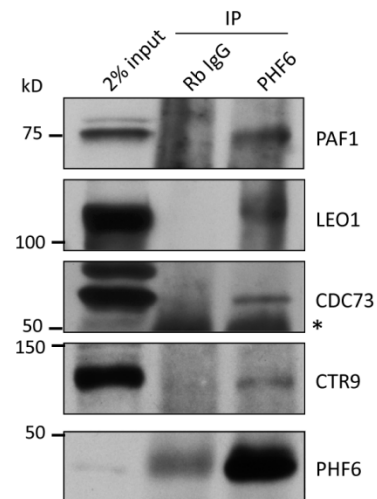
Figure 3-2. PHF6 interacts with the PAF1 transcription elongation complex. (A) Lysates of 293T cells stably expressing N-terminally tagged HA-PHF6 were immunoprecipitated with the HA beads and subjected to proteomic analyses using LC-MS/MS. Peptide spectra were analyzed using the CompPASS software program to interrogate datasets against parallel IP/MS databases and assign scoring metrics. High-confidence candidate interacting proteins (HCIPs) of PHF6 are

49

Figure 3-2. continued

shown. Rows in pink denote components of the PAF1 complex. (B) Lysates of 293T cells expressing HA-PHF6 or HA-GFP were subjected to immunoprecipitation with the HA beads followed by immunoblotting with the HA, PAF1, LEO1, CDC73 and CTR9 antibodies. Input was also immunoblotted with the antibodies. (C) Lysates of E17 mouse cerebral cortex were immunoprecipitated with the PHF6 antibody or rabbit IgG followed by immunoblotting with the PAF1, LEO1, CDC73, CTR9 and PHF6 antibodies. Asterisk denotes a non-specific band. Rabbit IgG signal from the PHF6 immunoblot is non-specific immunoreactivity from the rabbit heavy chain.

The PAF1 transcription elongation complex was originally identified in yeast as an RNA polymerase II-associated complex, and plays a critical role in efficient transcriptional elongation along chromatin in yeast and mammalian cells (Marton and Desiderio, 2008; Rondon et al., 2004; Shi et al., 1996). The PAF1 complex has been implicated in tumorigenesis (Chaudhary et al., 2007; Lin et al., 2008; Newey et al., 2009). However, the role of the PAF1 complex in the brain has remained unexplored.

To assess the role of the PAF1 complex in the developing mammalian brain, I first characterized the expression profile of the four components of the PAF1 complex during neuronal development. Interestingly, all four components were highly expressed during early neuronal development in both primary cortical neurons and the cerebral cortex *in vivo* (Figure 3-3A).

I next asked whether the PHF6-PAF1 interaction is functionally relevant in neuronal migration. I reasoned that if PHF6 acts via the PAF1 complex to regulate neuronal migration, loss of PAF1 would be predicted to disrupt neuronal migration. Consistent with this prediction, knockdown of PAF1 by two distinct shRNAs substantially impaired neuronal migration in the cerebral cortex *in vivo*, thus phenocopying the effect of PHF6 knockdown (Figures 3-3B, 3-3C, 3-3D and 3-3E). Collectively, these data suggest that PHF6 physically associates with the PAF1 complex and thereby drives neuronal migration.
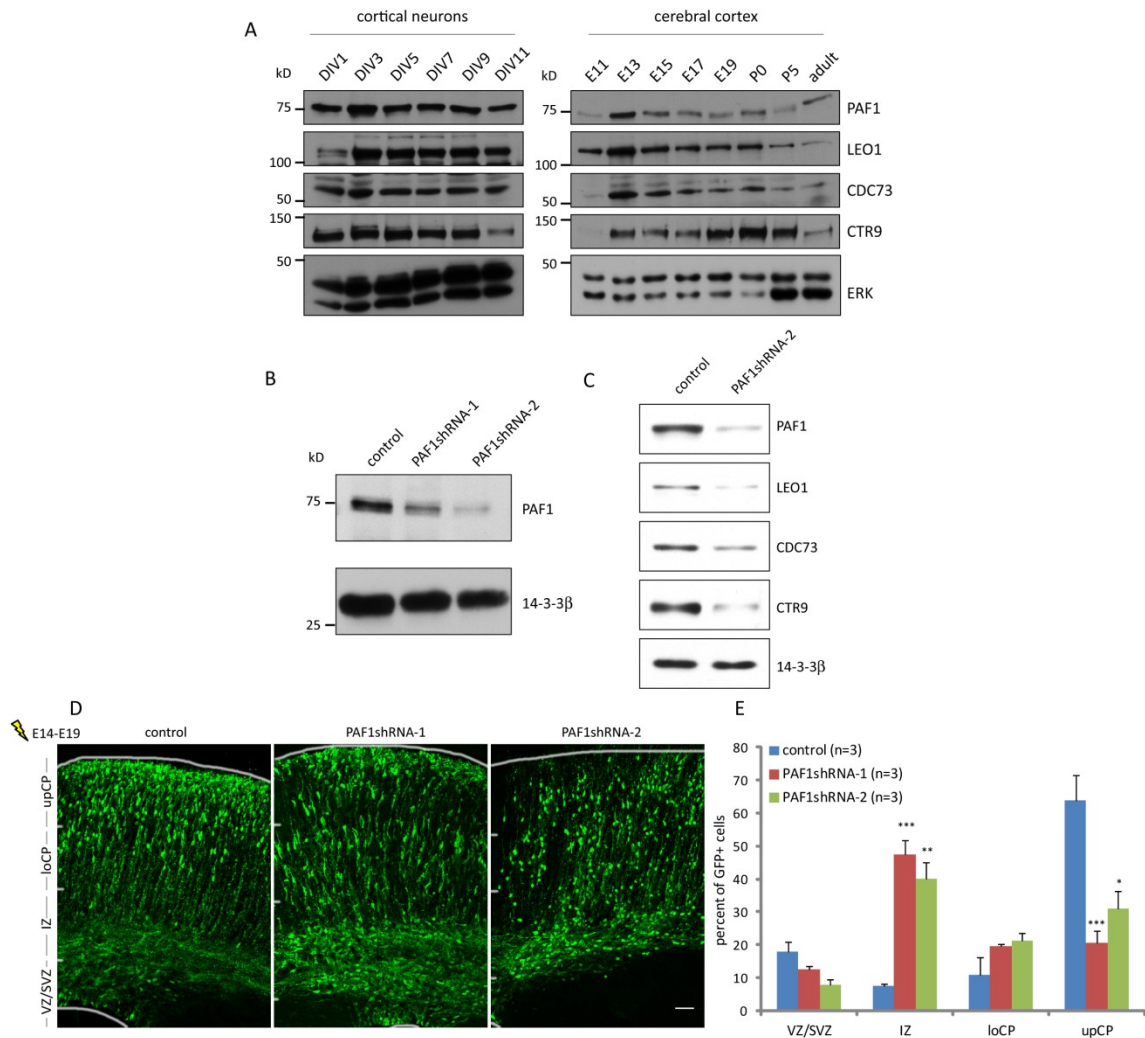
Figure 3-3. Downregulation of the PAF1 transcription elongation complex impairs neuronal migration in the cerebral cortex. (A) Lysates of primary rat cortical neurons or mouse cerebral cortex were immunoblotted with the PAF1, LEO1, CDC73, CTR9 and ERK antibodies. (B) Lysates of mouse cortical neurons infected with lentivirus expressing PAF1 shRNAs or control scrambled shRNAs were immunoblotted with the PAF1 and 14-3-3β antibodies. (C) Lysates of N2A cells infected with lentivirus expressing PAF1 shRNAs or control scrambled shRNAs were immunoblotted with the PAF1, LEO1, CDC73, CTR9 and 14-3-3β antibodies. (D) E14 mouse embryos were electroporated with an RNAi plasmid encoding PAF1 shRNAs or control scrambled shRNAs together with the GFP expression plasmid and allowed to develop until E19.

Figure 3-3. continued

The cerebral cortex from the E19 embryos was subjected to immunohistochemical analyses with the GFP antibody. PAF1 knockdown in the cerebral cortex *in vivo* profoundly impaired the radial migration of cortical neurons. (E) Quantification of the percentage of GFP positive neurons in distinct regions of the cerebral cortex in E19 mouse embryos treated as in (D) is presented as mean + SEM. PAF1 knockdown in the cerebral cortex *in vivo* significantly reduced the percentage of neurons that reached the upper cortical plate and concomitantly significantly increased the percentage of neurons stalled in the intermediate zone. Scale bar: 50µm in (D).

Discussion

In contract with previous studies, I find that PHF6 is mostly localized in the nucleoplasm, instead of the nucleolus, in immature neurons *in vitro* and *in vivo*, although in more mature neurons, there is some enrichment of PHF6 in the nucleolus. Several possibilities would explain this discrepancy: 1) certain studies showed the nucleolar localization of overexpressed PHF6. Indeed, when I overexpressed PHF6 by transfecting GFP-tagged PHF6 into 293T cells or primary cultured cortical neurons, I observed a strong nucleolar localization of GFP-PHF6. However, I think this reflects an abnormal localization of PHF6 due to an excessive expression level. This is further supported by the observation that when I expressed HA-PHF6 using lentivirus, which is thought to express exogenous proteins at a lower (and probably more physiological) level, it is localized diffusely in the nucleoplasm; 2) PHF6 likely localizes to different compartments in the nucleus in different cell types, and probably during different developmental stages. Since knockdown of PHF6 largely abrogates the immunopositive signal in cortical neurons, I believe the nucleoplasmic localization is specific for PHF6.

The identification of the PAF1 transcription elongation complex as an interacting partner of PHF6 implies a potential role of PHF6 in regulating transcription. PHF6 has long been speculated to play a role in transcription because it harbors two imperfect PHD finger domains. A lot of PHD finger domains recognize specific histone modifications and mediate downstream effects either through enzymatic function or through protein-protein interactions. However, our preliminary studies failed to identify any histone marks that PHF6 recognizes. Therefore it remains unanswered whether PHF6 regulates transcription through interacting with histone marks, and thus it would be interesting to perform more detailed biochemical analysis to determine the molecular mechanisms of PHF6 function.

The study of the PAF1 transcription elongation complex has mostly focused on its biochemical function in regulating transcription elongation. Previous *in vitro* and *in vivo* studies have established a role of the PAF1 complex in positively promoting transcription elongation along a chromatin template (Kim et al., 2010; Marton and Desiderio, 2008; Mueller et al., 2004; Squazzo et al., 2002). Yet the biological and cellular functions of the PAF1 complex remain largely unexplored. Our results reveal an essential role of the PAF1 complex in the regulation of neuronal migration, and potentially, the pathogenesis of X-linked intellectual disability.

Methods

Plasmids

The shRNA plasmid targeting sequences are: control: TACGCGCATAAGATTAGGGTA; PAF1shRNA-1 (mouse): TATGACTATAAGATTGCTCGG; PAF1shRNA-1 (rat): TATGACTACAAGATTGCTCGA; PAF1shRNA-2 (mouse): TTGGTAGTGATGCTGATTCAG; PAF1shRNA-2 (rat): TCGGTAGTGATGCTGATTCAG. Different shRNA targeting sequences were designed due to differences between mouse and rat coding sequences.

Immunoprecipitation/mass spectrometry

To identify specific PHF6 interactors, 293T cells stably expressing N-terminally HA-tagged PHF6 were generated using lentivirus followed by antibiotic selection. Four confluent 15cm dishes of stable PHF6-expressing cells were lysed in 0.5% Nonidet P40 and subjected to immunoprecipitation using anti-HA resin (clone HA-7, Sigma). Immunoprecipitated PHF6 was eluted using HA peptide (Anaspec), and the eluted proteins were precipitated with trichloroacetic acid and digested with trypsin. The resulting tryptic peptides were desalted over C18 resin, and then loaded onto an LTQ linear ion trap mass spectrometer (Thermo Finnigan) for LC-MS/MS analyses. MS/MS spectra were searched using SEQUEST against a target-decoy database of tryptic peptides, and protein assignments were further deconvolved using the CompPASS software platform (Behrends et al., 2010; Litterman et al., 2011; Sowa et al., 2009). Using CompPASS, the uniqueness, abundance, and reproducibility of each protein assignment was compared across parallel sets of MS data generated from multiple

56

unrelated immunoprecipitations to distinguish high-confidence protein interactors (HCIPs) from non-specific interacting proteins. HCIPs have WDN-scores >1.0.

Immunoblotting

Immunoblotting was performed as described (Lehtinen et al., 2006). The PAF1 (Bethyl laboratories), LEO1 (Bethyl laboratories), CDC73 (Bethyl laboratories), and CTR9 (Bethyl laboratories) antibodies were used.

Attributions

The IP-MS was performed by Luis Mejia in our lab, with assistance of Eric Bennett from the Harper lab (Department of Cell Biology, Harvard Medical School).

The remaining experiments described in this chapter were carried out by Chi Zhang.

Chapter 4

The Neuroglycan C (NGC) gene is a key downstream target of PHF6 and PAF1 in the control of neuronal migration *in vivo*

Introduction

In the previous chapters, I uncovered a critical function of the PHF6-PAF1 interacting complex in the regulation of neuronal migration. Knockdown of PAF1 phenocopies PHF6 knockdown, suggesting that they act in a similar fashion to promote neuronal migration.

The fact that PAF1 is positively involved in transcription elongation suggests that PHF6 might exert its function through regulation of transcription. Therefore an important remaining question is what are the downstream transcriptional events of PHF6 and PAF1.

In this chapter, I characterize the downstream events of PHF6 and the PAF1 complex. I identify Neuroglycan C (NGC) as a critical downstream target of PHF6 and PAF1 complex, and NGC mediates their effect on neuronal migration. Interestingly, NGC was identified as a potential susceptibility gene for schizophrenia. Therefore, my results suggest a potentially common basis of the underlying pathophysiology for BFLS and schizophrenia.

Results

The finding that PHF6 interacts with the PAF1 transcription elongation complex and thereby promotes neuronal migration in the cerebral cortex led me to determine whether PHF6 exerts its function via regulation of gene expression. Because the PAF1 complex promotes transcription, I reasoned that PHF6 might stimulate the expression of genes that mediate neuronal migration. I therefore performed microarray analyses of control and PHF6 knockdown primary cortical neurons. A large number of genes were downregulated in cortical neurons upon PHF6 knockdown (Table 4-1), consistent with the conclusion that PHF6 regulates gene expression in neurons. Among the downregulated genes, several genes were found to encode proteins with potential roles in neuronal migration, including 14-3-3γ, Dync1i2, Ephb1, Abl1, Apbb2, Srgap3, Stmn1 and Neuroglycan C (NGC). I validated the downregulation of selected genes in PHF6 knockdown cortical neurons using two distinct PHF6 shRNAs. Among these genes, NGC was the most consistently and robustly downregulated gene in PHF6 knockdown neurons (Figure 4-1A and C). NGC was also robustly downregulated by PAF1 knockdown in primary cortical neurons, suggesting that NGC is coordinately regulated by PHF6 and the PAF1 transcription elongation complex (Figure 4-1B and D).

Table 4-1

GeneSymbol	PHF6shRNA-2/control exp 1	PHF6shRNA-2/control exp 2	average fold change
PHF6	0.276676514	0.276959361	0.276817937
Etnk2	0.46403582	0.350641693	0.407338757
Nt5dc3	0.394824313	0.452586666	0.423705489
Sec11c	0.350106195	0.498601502	0.424353848
Nipsnap3b	0.438226028	0.445362986	0.441794507
Etnk2	0.446746234	0.45082538	0.448785807
Ccdc6	0.347082097	0.556912574	0.451997336
Stk32b	0.374674105	0.542877097	0.458775601
Dnm3	0.458681043	0.48233965	0.470510346
Nsg1	0.424637957	0.527390856	0.476014407
Far2	0.477756399	0.505250314	0.491503356
Hivep1	0.462376047	0.529773192	0.496074619
Stox2	0.549052324	0.466949003	0.508000663
Fut8	0.374087813	0.652309916	0.513198865
Elavl3	0.506635479	0.544333789	0.525484634
Stk32b	0.453116697	0.607346304	0.530231501
Arl8a	0.498962249	0.567781514	0.533371881
Ywhag	0.493128928	0.598219182	0.545674055
Scarb2	0.511171167	0.588823816	0.549997492
Tnfrsf21	0.485934628	0.617280998	0.551607813
Cdc2l6	0.566046095	0.537480059	0.551763077
Dync1i2	0.495952709	0.612885116	0.554418912
Cspg5	0.526441186	0.584902071	0.555671628
Fam20b	0.524515771	0.587403884	0.555959827
Tmem48	0.511078028	0.607882888	0.559480458
Pphln1	0.558160765	0.563633568	0.560897167
Stox2	0.550064663	0.580055428	0.565060045
Scn3a	0.592323264	0.548558522	0.570440893
Adamts3	0.540822185	0.600188088	0.570505137
RGD1562079	0.5017112	0.642546901	0.57212905
Clta	0.582157506	0.566213785	0.574185645
Rgs4	0.57171304	0.576965331	0.574339186
Abl1	0.56554631	0.586426996	0.575986653
Commd3	0.498192189	0.65389329	0.576042739
Ephb1	0.501533863	0.651108706	0.576321285
RGD1310110	0.699595927	0.457742169	0.578669048
Klhl7	0.529433998	0.630842738	0.580138368
Scn3a	0.55936536	0.611552315	0.585458837
Rbpj	0.525521099	0.648866498	0.587193798
Fem1b	0.57411303	0.601175789	0.58764441
Smarca5	0.552482718	0.624803046	0.588642882
Pcdh20	0.630354069	0.548802729	0.589578399
Lrrc8b	0.637061888	0.545015906	0.591038897
Ranbp6	0.585230554	0.596911294	0.591070924
Socs2	0.686071159	0.496565114	0.591318137
Apbb2	0.605289896	0.587962958	0.596626427
Adamts3	0.540339524	0.655156505	0.597748014
Lrrc61	0.536061034	0.660324728	0.598192881
Arl8b	0.530767534	0.66775279	0.599260162
Utp15	0.474879299	0.723646462	0.599262881

Table 4-1. continued

Table 4-1. Microarray analyses of primary rat cortical neurons infected with lentivirus expressing PHF6 shRNAs or control scrambled shRNAs. Selected downregulated genes upon PHF6knockdown in cortical neurons are listed with fold change in their expression level.
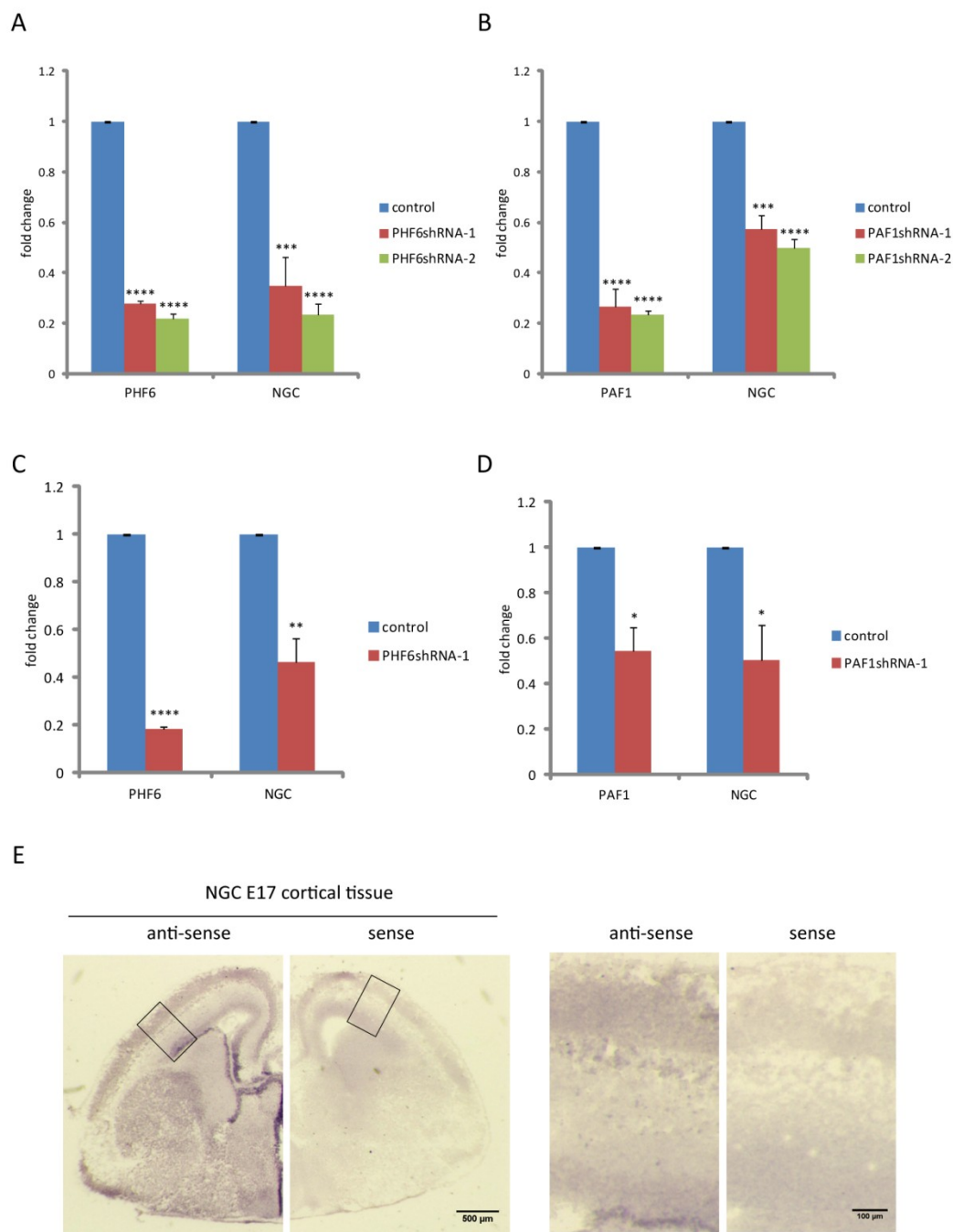
Figure 4-1

Figure 4-1. Neuroglycan C (NGC) is a downstream target of PHF6 and PAF1 complex. (**A**) Quantitative RT-PCR analyses of primary rat cortical neurons infected with lentivirus expressing PHF6 shRNAs or control scrambled shRNAs. Levels of PHF6 and NGC were normalized to GAPDH levels (n=3). (**B**) Quantitative RT-PCR analyses of primary rat cortical neurons infected

Figure 4-1. continued

with lentivirus expressing PAF1 shRNAs or control scrambled shRNAs. Levels of PAF1 and NGC were normalized to GAPDH levels (n=3). (**C**) Quantitative RT-PCR analyses of primary mouse cortical neurons infected with lentivirus expressing PHF6 shRNAs or control scrambled shRNAs. Levels of PHF6 and NGC were normalized to GAPDH levels (n=3). (**D**) Quantitative RT-PCR analyses of primary mouse cortical neurons infected with lentivirus expressing PAF1 shRNAs or control scrambled shRNAs. Levels of PAF1 and NGC were normalized to GAPDH levels (n=3). (**E**) *In situ* hybridization was performed on sections of E17 mouse cerebral cortex using probes for NGC. Sense probe was used as a negative control for the *in situ* hybridization. NGC is highly expressed in the VZ/SVZ and CP. Zoom in images of boxed regions are shown on the right. Scale bars in (E), 500µm on the left, 100µm on the right.

The NGC gene encodes a transmembrane chondroitin sulfate glycoprotein that is expressed predominantly in the brain (Brandt et al., 2007; Kinugasa et al., 2004; Nakanishi et al., 2006), and is a member of the neuregulin family of proteins, which is implicated in neuronal migration (Anton et al., 1997; Kinugasa et al., 2004; Rio et al., 1997). However, NGC function in the brain has remained unknown. Interestingly, the NGC gene was recently identified as a potential susceptibility locus in schizophrenia, a neuropsychiatric disorder in which impaired neuronal migration is thought to play a pathogenic role (Impagnatiello et al., 1998; Lee et al., 2011; So et al., 2010; Tomita et al., 2011). Together, these observations raised the possibility that NGC might represent a physiologically relevant downstream target of the PHF6-PAF1 pathway in the control of neuronal migration.

I assessed the role of NGC in neuronal migration. *In utero* knockdown of NGC in mouse embryos using two distinct shRNAs profoundly impaired neuronal migration in the cerebral cortex *in vivo* (Figures 4-2A, B and C). The extent of the migration defect correlated with the knockdown efficiency of NGC knockdown. Thus, NGC knockdown phenocopied the PHF6 knockdown phenotype. I next asked whether NGC mediates the ability of PHF6 to drive neuronal migration. Remarkably, in epistasis analyses, expression of exogenous NGC in PHF6 knockdown animals largely restored the normal migration pattern in the cerebral cortex *in vivo*, suggesting that NGC represents a critical target of PHF6 in the control of neuronal migration (Figures 4-2D and E). Taken together, our data suggest that NGC mediates the ability of PHF6 to promote neuronal migration in the developing brain *in vivo*.
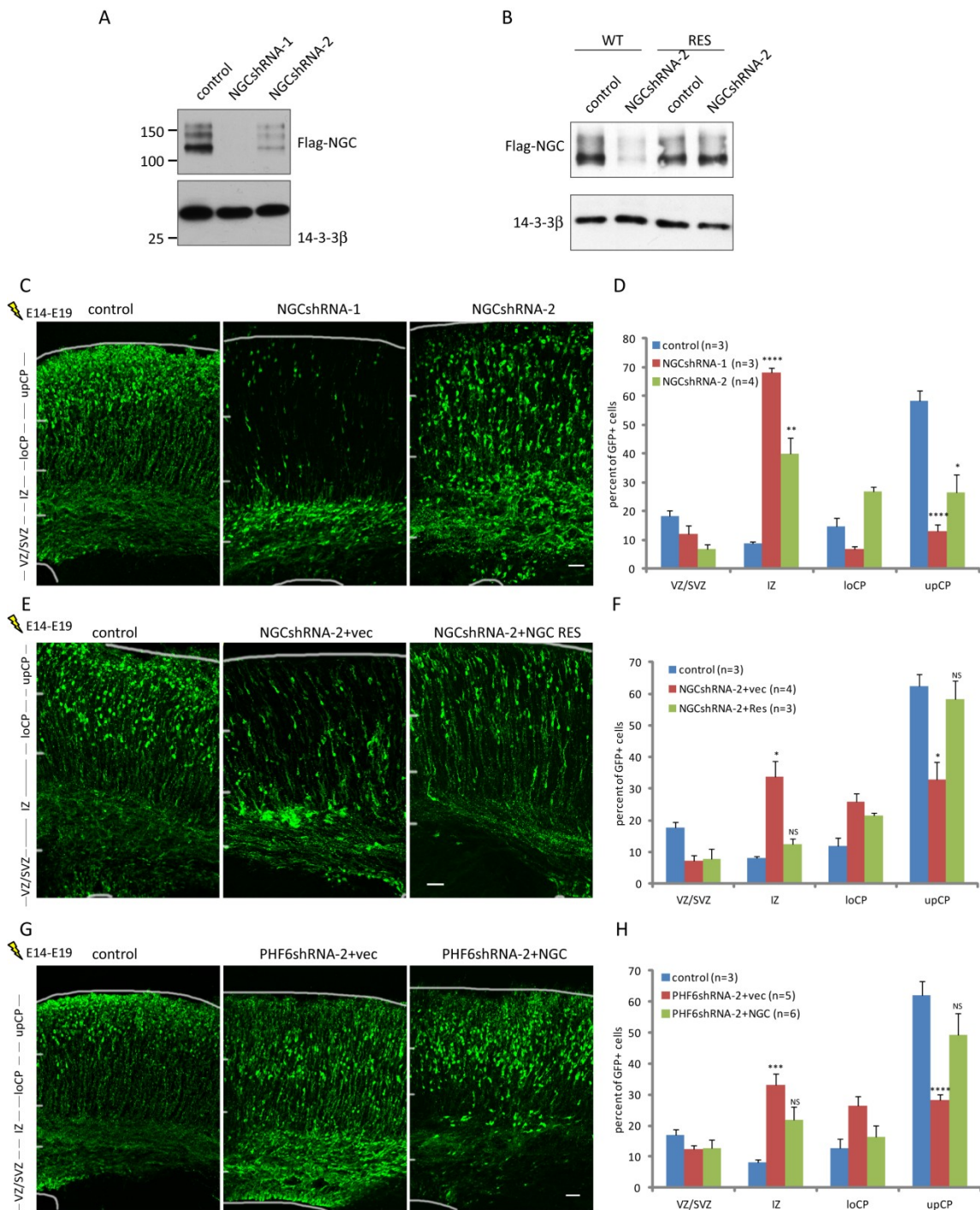
Figure 4-2

Figure 4-2. NGC is a key downstream target of PHF6 in the control of neuronal migration. (**A**) Lysates of 293T cells transfected with an expression vector encoding FLAG-NGC together with an RNAi plasmid encoding NGC shRNAs or control scrambled shRNA were immunoblotted with the FLAG and 14-3-3β antibodies. (**B**) Lysates of 293T cells transfected with an expression

Figure 4-2. continued

vector encoding FLAG-NGC wildtype (WT) or rescue (RES) together with an RNAi plasmid encoding NGC shRNAs or control scrambled shRNA were immunoblotted with the FLAG and 14-3-3β antibodies. (**C**) E14 mouse embryos electroporated with an RNAi plasmid encoding NGC shRNAs or control scrambled shRNAs were allowed to develop until E19 and subjected to immunohistochemistry with the GFP antibody. NGC knockdown in the cerebral cortex *in vivo* profoundly impaired the radial migration of cortical neurons. (**D**) Quantification of the percentage of GFP positive neurons in distinct regions of the cerebral cortex in E19 mouse embryos treated as in (C) is presented as mean + SEM. Knockdown of NGC in the cerebral cortex *in vivo* significantly reduced the percentage of neurons that reached the upper cortical plate and concomitantly increased the percentage of neurons stalled in the intermediate zone. (**E**) E14 mouse embryos electroporated with the NGC RNAi or control RNAi plasmid together with an expression plasmid enconding NGC-Res or its corresponding control vector were allowed to develop until E19 and subjected to immunohistochemistry with the GFP antibody. Expression of NGC-Res suppressed the NGC knockdown-induced positioning defect in the cerebral cortex *in vivo*. (**F**) Quantification of the percentage of GFP positive neurons in distinct regions of the cerebral cortex in E19 mouse embryos treated as in (E) is presented as mean + SEM. Expression of NGC-Res significantly increased the percentage of neurons reaching the upper cortical plate in the background of NGC RNAi in the cerebral cortex *in vivo* (ANOVA, p<0.01). (**G**) E14 mouse embryos electroporated with the PHF6 RNAi or control RNAi plasmid together with an expression plasmid enconding NGC or its corresponding control vector were allowed to develop until E19 and subjected to immunohistochemistry with the GFP antibody. Expression of NGC suppressed the PHF6 knockdown-induced positioning defect in the cerebral cortex *in vivo*. (**H**) Quantification of the percentage of GFP positive neurons in distinct regions of the cerebral cortex in E19 mouse embryos treated as in (G) is presented as mean + SEM. Expression of NGC significantly increased the percentage of neurons reaching the upper cortical plate in the

Figure 4-2. continued

background of PHF6 RNAi in the cerebral cortex *in vivo* (ANOVA, p<0.01). Scale bars: 50µm in

(C), (E) and (G).

Discussion

The identification of Neuroglycan C (NGC) as a target gene of PHF6 and PAF1 that mediates PHF6-dependent migration has implications for the biology of both NGC and PHF6. PHF6 knockdown induces robust downregulation of NGC, and NGC knockdown phenocopies PHF6 knockdown-induced migration arrest. In addition, expression of NGC largely compensates PHF6 knockdown-induced migration phenotype. Therefore NGC is a critical target of PHF6 in the pathogenesis of BFLS. In addition, since NGC has been implicated as a potential locus for schizophrenia (So et al., 2010), our findings raise the possibility that mutations of PHF6 may contribute to the pathogenesis of neuropsychiatric disorders beyond intellectual disability. Conversely, it will be interesting to determine whether deregulation of NGC might play a role in intellectual disability and epilepsy.

How does NGC regulate neuronal migration? Given that NGC is a transmembrane molecule with a large ectodomain, NGC might act as a receptor or ligand that mediates cell-cell interaction. Indeed, a similar mechanism of action has been reported for neuregulins in the cerebellar granule neurons, where neuronally expressed neuregulin acts on ErbB receptors on the radial glial fiber, and blocking the activation of ErbB4 on the radial glia impairs neuronal migration (Rio et al., 1997). Therefore I hypothesize that NGC probably mediates interaction between neurons and radial glia. In the future it will be interesting to identify the proteins that interact with NGC, with a particular interest on the ones with extracellular domains, which might mediate this neuron-glia interaction.

In addition to identifying the molecular mechanisms of BFLS pathophysiology, our results suggest that stimulating NGC expression or activity might confer a potential therapeutic benefit for BFLS. Because NGC is a transmembrane protein and a member of the neuregulin family (Kinugasa et al., 2004), NGC might represent an attractive target

for drug development. Thus, in addition to providing insights into the molecular pathogenesis of intellectual disability and potentially neuropsychiatric diseases, our study may provide novel molecular targets for future therapies for these devastating disorders.

Methods

RT-PCR

 RT-PCR was performed as described (Yuan et al., 2008). Primers used for RT-PCR are: PHF6 F: GCGCAAATGTGGCTTTTGTA, PHF6 R: TGCCTCTTTTAATTTCCTTTTGGA. PAF1 F: CATGACCTCCTGACTGAGCC, PAF1 R: GCTTCTCATTGGAGATGCCA. NGC F: ACACTCCCTGTGTCTCCTCC, NGC R: CAGGTCAGGTTATTCTGGAGACAG. GAPDH F: TGCTGGTGCTGAGTATGTCG, GAPDH R: GCATGTCAGATCCACAACGG.

Plasmids

 The NGC expression plasmids were generated by PCR using mouse or rat cortical neuron cDNA. NGCshRNA-1: TGGCTCACTATGATAACATTG; NGCshRNA-2: TCCATGATAAGCCTAGTGTAG.

In situ hybridization

 In situ hybridization was performed using coronal cortical sections of 30-μm thickness, prepared from E17 mouse embryos. The full length cDNA clone (NM_001166273, National Center for Biotechnology Information) was used for synthesis of digoxigenin (DIG)-labeled antisense and sense probes. Hybridization was carried out as described (Trimarchi et al., 2007).

Attributions

The *in situ* hybridization was performed by Pamela Valnegri in our lab, with assistance of Sui Wang from the Cepko lab (Department of Genetics, Harvard Medical School).

The remaining experiments described in this chapter were carried out by Chi Zhang.

Chapter 5

PHF6 knockdown triggers the formation of white matter heterotopias with aberrant neuronal activity

Introduction

In the previous chapters, I examined the molecular and cellular functions of PHF6 during embryonic brain development. I identified the PHF6-PAF1-NGC transcriptional pathway that mediates its role in the regulation of neuronal migration. In this chapter, I extend our analysis to examine the broader question of how mutations of PHF6 would lead to cognitive dysfunction.

Abnormal neuronal migration can impair cognitive functions in a number of ways. Most commonly, abnormal neuronal migration can present itself as ectopically located clusters of neurons below, within, or above the brain. For example, in females affected by the double cortex syndrome, ectopically located neurons form abnormal areas that appear as band-like clusters of cells underneath the gray tissue of the cerebral cortex (subcortical), creating the appearance of a double cortex (Gleeson et al., 1998). In the case of Cobblestone lissencephaly, overmigration of the neuroblasts and glial cells beyond the external glial limitations into the subarachnoid space leads to a nodular brain surface (Abdel Razek et al., 2009). Another possible scenario is that neuronal migration is not completely arrested but delayed. In this situation, there is unlikely to be dramatic changes in the gross brain structure. However, since the establishment of synaptic connection is a highly coordinated process that requires a number of spatiotemporal cues, including both cell-autonomous and non cell-autonomous signals, a delay of neuronal migration might lead to changes in the environmental signals or responsiveness of the target cell, and thereby impairing the brain connectivity. Finally, it is becoming clear that the brain develops in a concerted manner where different cell types interact with each other, and abnormalities of one cell type can, in certain cases, lead to abnormalities of other cell types. For example, genetic manipulation of excitatory neurons, which induce the formation of heterotopic clusters of these neurons beneath the cortical plate, is able to recruit inhibitory neurons into the cluster, therefore resulting in further changes of brain

connectivity (Lodato et al., 2011). Therefore, it would be important to understand how loss-of-PHF6 induced migration defect leads to cognitive impairment in BFLS patients.

I addressed this question by first asking whether PHF6 induced neuronal migration arrest is permanent or temporary. Intriguingly, PHF6 knockdown resulted in formation of subcortical heterotopia in postnatal brains, suggesting that loss of PHF6 leads to permanent changes in the brain structure. Interestingly, electrophysiological recordings revealed remarkable hyperexcitability of heterotopic neurons, which is potentially relevant to the elevated seizure susceptibility observed in BFLS patients. Therefore, our results provided a potential explanation for the cognitive dysfunction induced by PHF6 mutation.

Results

Having elucidated a mechanism by which PHF6 orchestrates neuronal migration in the developing cerebral cortex *in vivo*, I next turned my attention to the broader question of how loss of PHF6 might contribute to disease pathogenesis in BFLS. I first asked whether PHF6 knockdown simply delays neuronal migration during development or whether consequences of impaired migration persist beyond the formation of the cerebral cortex. To address this question, I electroporated E14 mouse embryos and allowed embryos to develop past birth until postnatal day 6 (P6). In these analyses, almost all transfected neurons in control animals were found in layers II-IV and expressed Cux1, a marker of superficial layer neurons (Nieto et al., 2004). Strikingly, neurons in PHF6 knockdown animals failed to reach the superficial layers in mouse pups at P6. Rather, they formed subcortical heterotopias in the white matter and were also found ectopically in layers V-VI. Quantification revealed that 98% of Cux1 positive, transfected cortical neurons reached layers II-IV in control animals, whereas only 32% of Cux1 positive, transfected neurons reached the superficial layers in PHF6 knockdown animals (Figures 5-1A and 5-1B). The failure of PHF6 knockdown neurons to reach layers II-IV in P6 animals was not due to impaired neuronal differentiation, as the heterotopic neurons expressed Cux1 but not Ctip2, a marker for layer V neurons (Figure 5-1C, 5-1D and 5-1E). These data suggest that loss of PHF6 triggers the formation of heterotopia in the cerebral cortex *in vivo*.

Figure 5-1
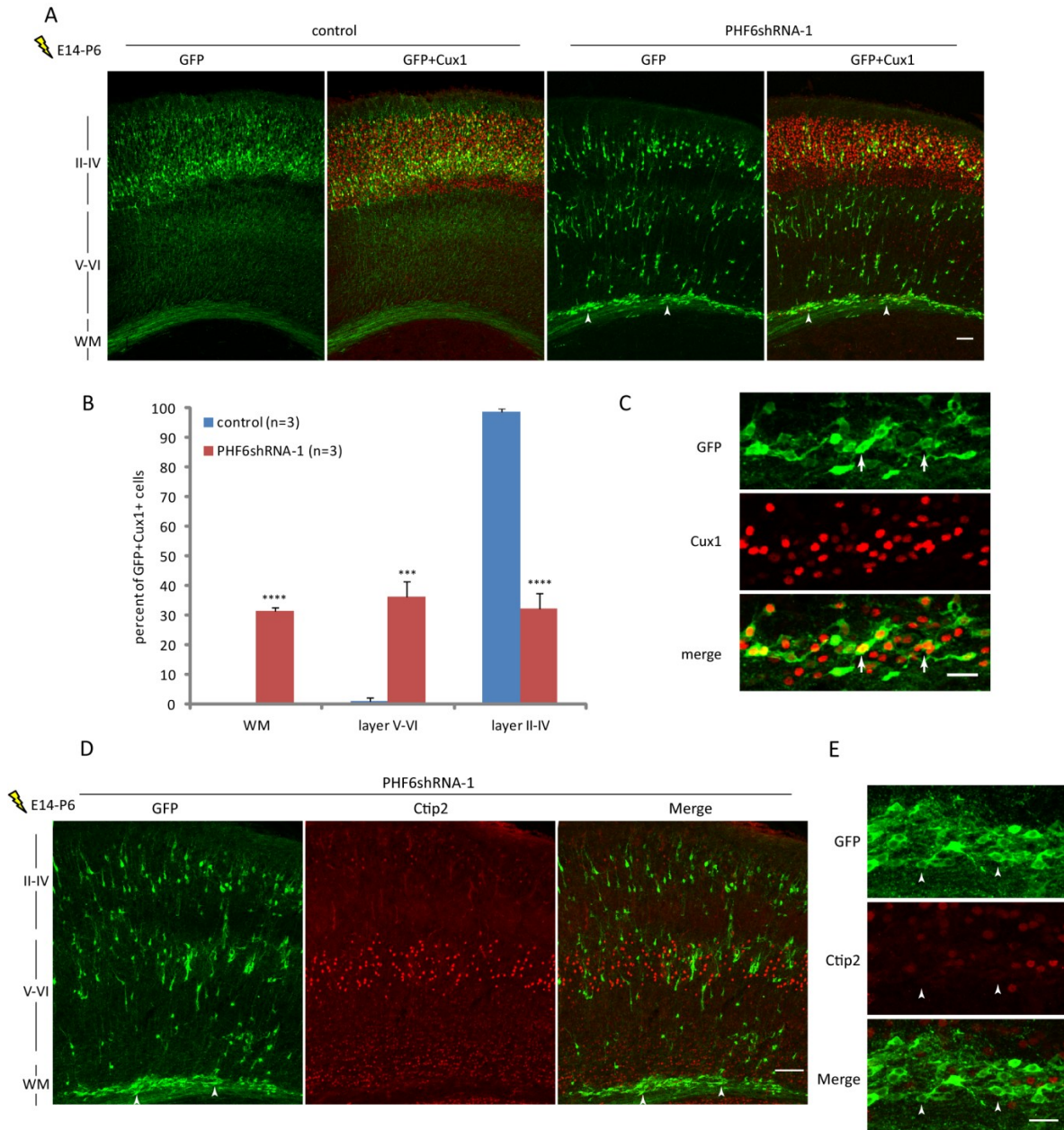
Figure 5-1. PHF6 knockdown leads to heterotopia formation in the postnatal brains. (**A**) E14 mouse embryos were electroporated with the RNAi plasmid encoding PHF6 shRNAs or control scrambled shRNAs together with the GFP expression plasmid and allowed to develop until P6. The cerebral cortex from the P6 mouse pups was subjected to immunohistochemical analyses with the GFP and Cux1 antibodies. Arrowheads denote heterotopic neurons in PHF6 knockdown

Figure 5-1. continued

animals. WM, white matter. (**B**) Quantification of the percentage of GFP positive, Cux1 positive neurons in distinct regions of the cerebral cortex in P6 mouse pups treated as in (A) is presented as mean + SEM. PHF6 knockdown significantly increased the percentage of GFP positive, Cux1 positive neurons in the white matter *in vivo*. (**C**) Representative image of heterotopic neurons in the white matter in PHF6 knockdown animals. GFP positive neurons are positive for Cux1 (arrows). (**D**) E14 mouse embryos were treated the same way as in (A) and the cerebral cortex of P6 animals were subjected to immunohistochemistry with the GFP and Ctip2 antibodies. (**E**) Representative image of heterotopic neurons in the white matter in PHF6 knockdown animals. GFP positive neurons are negative for Ctip2 (arrowheads). Scale bars: 100μm in (A) and (D), 30μm in (C) and (E).

We next examined the electrophysiological properties of transfected neurons in the electroporated animals. Whole-cell patch clamp recordings were performed using acute cortical slices prepared from P10 control or PHF6 knockdown animals. Under current clamp configuration, we observed an aberrant pattern of activity in 70% of heterotopic neurons (9 out of 13 cells recorded), but not in neurons that reached layers II-IV, in PHF6 knockdown animals (Figure 5-2A). The membrane potential of heterotopic neurons oscillated, leading to frequent firing of action potentials (Figure 5-2A). In other analyses, spontaneous excitatory postsynaptic currents (sEPSCs) were observed in layer II-IV neurons from control or PHF6 knockdown animals but not in heterotopic neurons from PHF6 knockdown animals, suggesting that the membrane potential of heterotopic neurons oscillated spontaneously in the absence of synaptic inputs (Figures 5-2C, 5-2D and 5-2E). The membrane potential oscillation in heterotopic neurons was blocked by nimodipine, which inhibits L-type voltage gated calcium channels, suggesting that calcium currents might underlie the spontaneous depolarization (Figure 5-2B). Interestingly, knockdown of NGC leads to formation of heterotopia that harbors a similar pattern of neuronal activity (Figure 5-2F). Together, these data suggest that inhibition of the PHF6 pathway triggers the formation of heterotopias in which neurons are hyperexcitable.
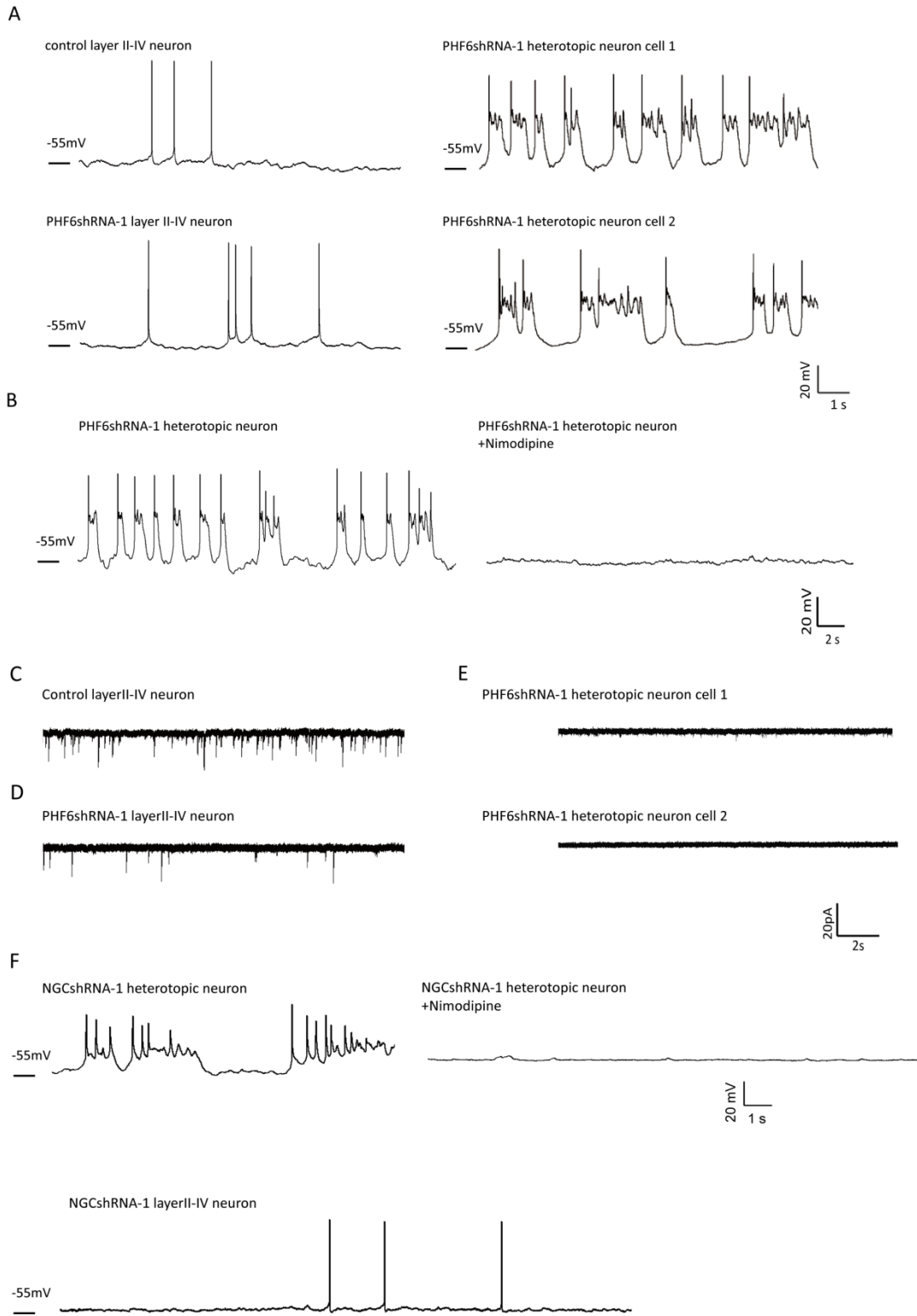
Figure 5-2

A

control layer II-IV neuron

-55mV

PHF6shRNA-1 heterotopic neuron cell 1

-55mV

PHF6shRNA-1 layer II-IV neuron

-55mV

PHF6shRNA-1 heterotopic neuron cell 2

-55mV

20 mV

1 s

B

PHF6shRNA-1 heterotopic neuron

-55mV

PHF6shRNA-1 heterotopic neuron
+Nimodipine

20 mV

2 s

C

Control layerII-IV neuron

E

PHF6shRNA-1 heterotopic neuron cell 1

D

PHF6shRNA-1 layerII-IV neuron

PHF6shRNA-1 heterotopic neuron cell 2

20pA

2s

F

NGCshRNA-1 heterotopic neuron

-55mV

NGCshRNA-1 heterotopic neuron
+Nimodipine

20 mV

1 s

NGCshRNA-1 layerII-IV neuron

-55mV

Figure 5-2. PHF6 knockdown induces ectopic neuronal activity in the heterotopic neurons. (**A**)

Figure 5-2. continued

E14 mouse embryos were electroporated with the RNAi plasmid encoding PHF6 shRNAs or control scrambled shRNAs together with the GFP expression plasmid and allowed to develop until P10. Representative images for whole-cell recordings in current clamp of GFP positive neuron in cortical slices from P10 PHF6 knockdown or control mouse pups are shown. Heterotopic neurons in P10 PHF6 knockdown animals exhibit aberrant neuronal activity. (**B**) Representative images for whole-cell recordings in current clamp of GFP positive heterotopic neurons in cortical slices from P10 PHF6 knockdown mouse pups are shown. Abnormal firing patterns of heterotopic neurons are blocked by bath application of 10μM nimodipine. Left and right panels are recordings from the same neuron before and after adding the inhibitor. (**C-E**) Representative images for whole-cell patch clamp recordings of spontaneous EPSCs under voltage clamp configuration of GFP positive neurons in cortical slices from P10 control (C) or PHF6 knockdown (D and E) mouse pups are shown. Neurons were held at their resting membrane potentials during sEPSC recordings. Heterotopic neurons do not exhibit synaptic current input. The two heterotopic neurons are the same neurons shown in (A). (**F**) E14 mouse embryos were electroporated with the RNAi plasmid encoding NGC shRNAs with the GFP expression plasmid and analyzed as in (A). Heterotopic neurons in P10 NGC knockdown animals exhibit aberrant neuronal activity.

Discussion

The finding that loss of PHF6 induced migration arrest leads to heterotopia formation has important implications in the pathogenesis of BFLS. Clinically, heterotopia formation is highly associated with epileptic activity of the brain, which has been documented in certain BFLS patients (Robinson et al., 1983). Additionally, the heterotopic neurons are hyperexcitable, even without any synaptic current input. This raises the possibility that hyperexcitability of heterotopic neurons will increase the overall excitability of the brain and therefore, render the entire brain more susceptible to epileptic stimulus.

Heterotopia formation has been postulated to contribute to elevated seizure susceptibility via a number of means. One the one hand, heterotopia may by itself serve as the loci that initiates the seizure activity. Clinical evidence using depth electrode recordings or stereoencephalography (SEEG) recordings in patients support the idea that heterotopic loci plays a role in the generation of the epileptic activity, although it remains controversial whether the heterotopia is the initiator or propagator of seizure activity (Aghakhani et al., 2005; Kothare et al., 1998). Work with animal models also suggests a critical role of heterotopia in epileptogenesis (Ackman et al., 2009; Manent et al., 2009). Animals with genetically induced or chemically induced heterotopia show spontaneous epilepsy or reduced threshold for seizure induction. Notably, heterotopic neurons are often found to connect to diverse regions of the brain, and occasionally even outside the brain. For example, retrograde labeling experiments show connection of heterotopic neurons to the spinal cord and thalamus in the *tish* (telencephalic internal structural heterotopia) model (Lee et al., 1997). Additionally, heterotopic neurons participate in the whole neuronal network, and exhibit network-driven bursts of activity (Smith et al., 1999). Therefore, it is likely that heterotopic neurons, induced by loss of PHF6, are able

to transmit their ectopic activity pattern to the various parts of the brain through their synaptic connections.

Is heterotopia formation and associated susceptibility relevant to BFLS? It is unfortunate that detailed pathological studies of the BFLS brains are missing or very incomplete. Over the course of this study I obtained an MRI scan of a female BFLS patient whose genetic studies suggested a deletion of PHF6. However, we were unable to identify heterotopia within her brain, at least on a macroscopic level as MRI offers. Although this remains inconclusive, it is possible that heterotopia formation represents a more acute outcome of neuronal migration arrest, presumably due to the more acute effect of RNAi-mediated knockdown, whereas in patient brains, point mutation or truncation of PHF6 leads to partial loss-of-function, resulting in a milder migration arrest. In addition, compared to other disorders of neuronal migration such as the double cortex syndrome, the degree of intellectual disability is in general milder in BFLS: DCX mutation in males leads to prenatal lethality, and only female patients survive and exhibit intellectual disability, whereas BFLS predominantly affects males and female carries are mostly normal or affected very mildly. Therefore, the degree of brain malformation is likely lower in BFLS than other disorders such as double cortex syndrome.

Nevertheless, an earlier post-mortem pathology study in a patient suffering from severe intellectual disability revealed a brain structure with clear signs of widespread neuronal migration defect, including a coarse gyri, cortical dysplasia with large areas of indistinct or absent lamination, presence of excessive numbers of neurons or solid heterotopic grey matter in the subcortical white matter (Brun et al., 1974). All of these findings are highly consistent with my findings in the embryonic mouse brain, suggesting a critical pathophysiological relevance of our study. Thus, it will be important for future studies to perform detailed brain imaging and examine evidence of heterotopia in BFLS brains.

Collectively, I have identified a novel transcriptional pathway whereby the X-linked intellectual disability protein PHF6 forms a complex with the PAF1 transcription elongation complex and thereby induces the expression of NGC, leading to the migration of cortical neurons in the cerebral cortex. Deregulation of this novel transcriptional pathway may play a critical role in the pathogenesis of intellectual disability and epilepsy in the X-linked disorder BFLS.

Methods

Electrophysiology

Acute cortical slices (350μm) were prepared from the cerebral cortex of P10-P12 mice by vibratome (Leica VT1000s). After one hour recovery at 37°C , slices were maintained in a storage chamber containing ASCF (124mM NaCl, 5mM KCl, 1.25mM NaH_2PO_4, 1.3mM $MgCl_2$, 2.6mM $CaCl_2$, 26mM $NaHCO_3$, and 15mM D-Glucose, pH 7.4, 310 mOsM) gassed with 95% O2/ 5% CO2 at room temperature. Whole-cell patch-clamp recordings were performed in cortical pyramidal neurons. Recording electrodes (3-5MΩ) were filled with intracellular solution containing: 110mM K gluconate, 20mM KCl, 5mM $MgCl_2$, 20mM HEPES, 0.2mM EGTA, 2mM MgATP, 0.3mM Na_3GTP, pH 7.3, 300 mOsm. Electrophysiological signals were acquired by Axon-700B multiclamp amplifier, digitized at 10 kHz by a Digidata 1440A D-A converter and Bessel filtered at 1kHz. For current clamp recording of GFP-positive heterotopic neurons from PHF6 knockdown animals, the membrane potential was held at -55mV in current clamp by injecting hyperpolarizing current (5 to 10pA).

Attributions

All electrophysiological recordings were performed by Ju Huang in the Bonni lab.

The remaining experiments described in this chapter were carried out by Chi Zhang.

Chapter 6

Perspectives

Using RNAi-mediated knockdown in *in vivo* mouse models, I identified abnormal neuronal migration as the critical pathophysiological basis for intellectual disability in BFLS. I linked this function of PHF6 to regulation of transcription through identifying the PAF1 transcription elongation complex as an interacting partner, and NGC as a key downstream target. This study revealed important aspects of developmental neurobiology, and has broad implications in many fields of biology. Here I highlight a few exciting new areas that this study has opened up.

Important role of transcriptional elongation in neuronal development

Traditionally, transcriptional elongation has long been viewed as a passive, unregulated stage where polymerase II passes through the DNA spontaneously. A lot of studies have focused on regulation of transcriptional initiation and thus, ignored the importance of elongation during the transcription cycle.

However, this view is being challenged by recent findings that elongation is a heavily regulated process and is intimately linked to the transcription cycle. Seminal findings using drosophila and human cells identified paused RNA polII at the promoter region, and more importantly, these polII are transcriptionally engaged and have formed a nascent RNA chain of ~25 nucleotides (Bentley and Groudine, 1986; Rougvie and Lis, 1988). This finding was corroborated by numerous studies using a variety of modern techniques, and the emerging view is that nascent transcriptional initiation is wide spread along the genome, and the transition from initiating to elongating phase of transcription represents a critical step of regulation (Guenther et al., 2007; Krumm et al., 1995; Muse et al., 2007; Nechaev and Adelman, 2011)

The PAF1 transcription elongation complex plays critical roles in promoting transcription elongation along the chromatin template. Work from laboratories of Robert G. Roeder, Danny Reinberg and Hiroshi Handa, among others, have firmly established that the PAF1 complex cooperates with other factors, including DSIF and the elongation factor SII/TFIIS, and promotes elongation of polII along the gene body (Chen et al., 2009; Kim et al., 2010; Zhu et al., 2005).

My study highlights the first biological function of the PAF1 complex in the nervous system, and suggests that regulation of transcriptional elongation is a critical step of control in neuronal migration. Therefore, it opened up a new area of exciting possibilities for future studies. Potential remaining questions include:

1. How does PAF1 regulate NGC levels?

In my study, we performed RT-PCR analysis and observed a reduction of NGC levels upon knockdown of PAF1. Since the PAF1 complex positively regulates transcriptional elongation, an immediate hypothesis is that PAF1 complex regulates NGC levels through positively promoting transcriptional elongation at the genomic loci.

I performed preliminary CHIP (chromatin IP) experiment to examine the distribution of RNA polII, PAF1 complex on the NGC genomic loci. Unfortunately, I was unable to detect any significant enrichment of these factors along the NGC loci, although it is important to point out that this assay is limited by the detection sensitivity of quantitative PCR. Also, it is well documented in the literature that it is difficult to detect significant enrichment of polII and other polII associated factors under basal levels of transcription (Chen et al., 2009). Therefore, even the preliminary result is negative, it

is possible that at basal level, PAF1 complex and polII is present at a low level that is beyond the detection limit.

Nevertheless, I successfully performed CHIP experiments to examine the status of post-translational modifications on histones in the NGC genomic loci. The underlying rationale is that PAF1 complex was shown to be critical for modifications of histones, such as H3K4 tri-methylation (H3K4me3) or ubiquitination of histone H2B (ubH2B) (Krogan et al., 2003; Wood et al., 2003). Therefore the hypothesis is that PHF6 may promote modifications of histones along the NGC genomic loci through interactions with the PAF1 compex. I was able to detect specific H3K4me3 and ubH2B signal along the NGC loci, yet knockdown of PHF6 did not change the modification status of H3K4me3 or ubH2B, even though in the same experiments PHF6 knockdown led to a decrease of NGC RNA levels. This data argues against the hypothesis that changes in the modification status of histones are the underlying causes for a reduction of NGC RNA levels. It remains to be determined whether PAF1 knockdown leads to a change of the modification status along the NGC loci.

2. What are the additional targets and functions of PAF1 in neuronal development?

Given the ubiquitous role of transcriptional elongation in gene regulation, it is very likely that the PAF1 complex is involved in the regulation of other biological processes in neuronal development and homeostasis through regulation of additional targets. Therefore it will be interesting to perform genome wide studies to identify

additional targets of the PAF1 complex in the nervous system, which hopefully will lead to new findings of PAF1 function in the brain.

What is the biochemical function of PHF6

In my study, I established a physical interaction of PHF6 and the PAF1 transcription elongation complex. However, an important question that follows is what is the biochemical function of PHF6, and how it exerts its function.

Given the interaction between PHF6 and the PAF1 complex, one immediate hypothesis is that PHF6 might be somehow related to the function of PAF1 complex. To test this hypothesis, I adopted an activity-induced transcription assay as a readout of PAF1 function. It is already known that EGF induced expression of the immediate early gene c-fos in Hela cells is dependent on the PAF1 complex and efficient transcriptional elongation. Therefore, by examining the temporal dynamics of c-fos induction upon PHF6 knockdown, I can determine whether PHF6 is required for PAF1 function in this paradigm.

My preliminary results seemed to suggest that PHF6 is not required for c-fos induction under this experimental paradigm (Figure 6-1). Knockdown of PHF6 did not significantly impair induction of c-fos as PAF1 knockdown did. It would be important to repeat this experiment in the future to validate the result.
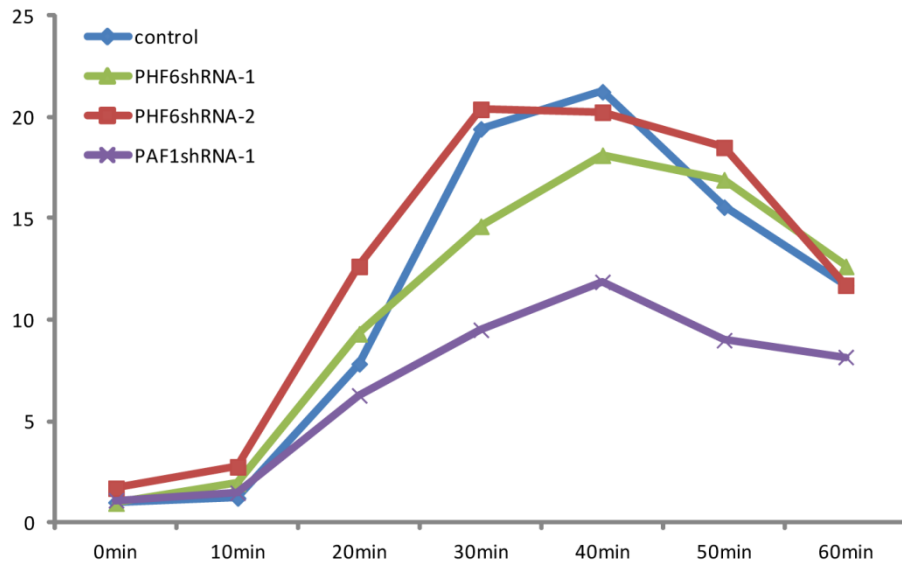
Figure 6-1

Figure 6-1. Preliminary data on the effect of PHF6 knockdown on EGF induced c-fos expression in Hela cells. PHF6 knockdown does not have a significant effect on c-fos induction whereas PAF1 knockdown blunts the induction of c-fos.

On the other hand, it is necessary to perform more detailed biochemical studies for PHF6. Important remaining questions include:

1. Does PHF6 interact with DNA or does it interact with histones, or even other molecules such as RNA?

2. What determines the specificity of regulation by PHF6?

The answer to these questions will add important insights to the understanding of how PHF6 functions in normal development.

Implications on cancer biology

Both PHF6 and the PAF1 complex were found to be involved in the development of leukemia. In particular, PHF6 mutations were identified in T-cell acute lymphoblastic leukemia (T-ALL), an aggressive form of hematological malignancy that has a high incidence in males (Van Vlierberghe et al., 2010). Somatic missense or truncating mutations of PHF6 were identified in T-ALL patients, suggesting that loss of PHF6 might play a critical role in the pathogenesis of T-ALL.

The PAF1 complex was found to interact with the MLL, translocations of which create potent oncogenic fusion proteins that account for the majority of infant acute leukemias. The PAF1 complex is required for MLL fusion protein mediated transcriptional activation and recruitment of MLL to target genes, therefore plays a critical role in the initiation of leukemia (Muntean et al., 2010).

The fact that both PHF6 and PAF1 complex are linked to the formation of leukemia raises the intriguing possibility that our study has important implications on cancer biology. Interesting questions to address include the followings:

1. Whether PHF6 and PAF1 complex interact in leukemia cells?

Although we validated the PHF6-PAF1 interaction in 293T cells, it is important to validate this in a relevant system, such as the Jurkat cells.

2. What are the physiological consequences upon loss of PHF6 in leukemia cells?

In view of the mutational loss of PHF6 in T-ALL, it would be important to perform functional studies of PHF6 in the formation of leukemia.

Functional consequence of ectopic neuronal activity on brain function

We have identified that PHF6 knockdown leads to heterotopia formation in the postnatal brain, which harbors ectopic neuronal activities. Therefore an important question remains as to what are the physiological consequences of having these heterotopic neurons in the otherwise normal brain.

Previous work from Alfonso Represa's group has shown that genetic knockdown rat models of human double cortex syndrome presented spontaneous seizure activity after adulthood (Lee et al., 1997; Manent et al., 2009). This raised the interesting possibility that PHF6 knockdown induced heterotopia formation might have similar effects on the overall brain activity.

To start testing this possibility, I performed some preliminary behavioral experiment where I injected the convulsant pentylenetetrazol (PTZ) to induce seizure activity in control or PHF6shRNA electroporated animals and examine the threshold for seizure. I did not observe any obvious differences in the threshold to PTZ-induced seizure. However, this is a rather crude experiment and might not allow detection of more minor differences in sensitivity to seizure. In the future, EEG recordings on the electroporated animals would be helpful in determining whether these animals suffer from spontaneous seizure activity.

Appendix

PHF6 is a potential substrate for the protein kinase Cdk5

<u>Introduction</u>

Having established a critical function of PHF6 in neuronal migration, I then considered potential regulators of PHF6 function. Protein phosphorylation is a common form of post-translational modification that impacts protein functions in a number of different ways, and is found to regulate multiple biological processes including neuronal migration. Therefore I considered the possibility that PHF6 might be subject to regulation through events such as phosphorylation.

A scan of the amino acid sequences of PHF6 revealed a few potential phosphorylation sites on PHF6, including potential sites for Abl kinase, PKC epsilon, ATM and Cdk kinases (http://scansite.mit.edu/). The potential phosphorylation sites for Cdk kinases are particularly interesting. This is because Cdk5, one of the Cdk family members, is highly implicated in neuronal morphogenesis, and in particular, regulation of neuronal migration. Knockout of Cdk5 leads to an inverted cerebral cortex where the brain loses the appropriate lamination pattern (Chae et al., 1997; Jin et al., 2010). A number of substrates have been identified that mediates its function in cell adhesion and neuronal migration, including PAK1, Tau, MAP1B and DCX ect (Dhavan and Tsai, 2001; Tanaka et al., 2004). Therefore, I think Cdk5 might be an interesting candidate kinase to examine that regulates PHF6 through phosphorylation.

I first examined the potential sites of phosphorylation on PHF6. Interestingly, out of the potential Cdk sites on the PHF6 protein, serine199 is highly conserved across the different species. Importantly, the +1 and +3 positions are also very conserved, and fits

really well for the consensus Cdk5 site (S/T)PX(K/H/R) (Figure S1). Therefore, I focused on our study on this site.

Human PHF6   SSYRDR<u>S</u>PHRSSPSDTR
Mouse PHF6   SSYRDR<u>S</u>PHRSSPNDTR
Rat PHF6   SSYRDR<u>S</u>PHRSSPNDTR
Xenopus PHF6   SSSRDR<u>S</u>PHRSSPSDTR
Chicken PHF6   SSYRDR<u>S</u>PHRSSPSDTK
Zebrafish PHF6 SSHRDR<u>S</u>PLRGSPGDSG

Figure S1. Alignment of PHF6 protein sequences cross different species around the consensus Cdk5 phosphorylation site. The serine residue is underlined, and the proline and arginine residues are highlighted in red to indicate that this is a conserved Cdk consensus site.

To examine the potential phosphorylation, I first performed *in vitro* kinase assay to test whether Cdk5 phosphorylates PHF6 *in vitro*. Incubation of recombinant PHF6 together with wildtype (WT) Cdk5 leads to robust phosphorylation of PHF6 assayed by incorporation of P^{32}. Importantly, incubation with kinase dead (KD) Cdk5 failed to phosphorylate PHF6 (Figure S2A), suggesting that the phosphorylation event is likely to be due to the presence of Cdk5 but not contaminating proteins in the immunoprecipitate.

I next asked whether the serine199 residue is the principle phosphorylation site on PHF6. To address this question, I mutated the serine residue to alanine, and subjected the recombinant protein to *in vitro* kinase assay. Interestingly, mutation of Ser199 strongly reduced the amount of phosphorylation on PHF6 by Cdk5 (Figure S2B), suggesting that this serine residue is the major site of phosphorylation by Cdk5.
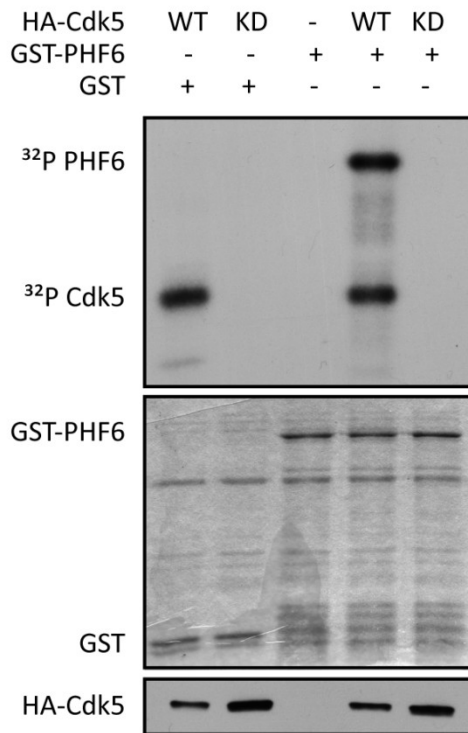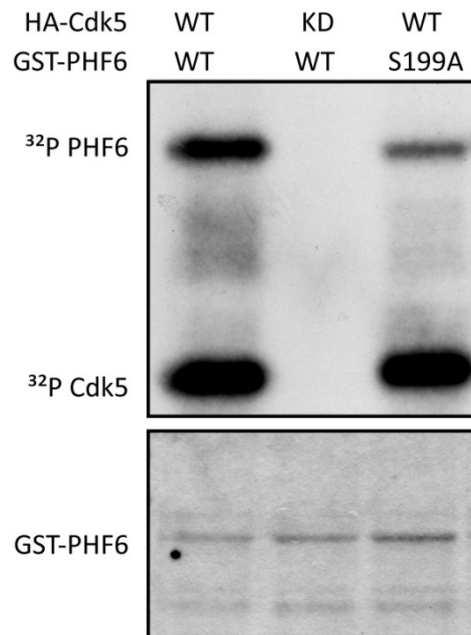
Figure S2. Cdk5 phosphorylates PHF6 *in vitro* on the Ser199 residue. (A) HA-Cdk5 immunoprecipitated from 293T cells are subjected to *in vitro* kinase assay with radioactive γ p^{32}-ATP and either GST or GST-PHF6 protein purified from E. coli. The upper panel shows radioactive signal, middle panel showing coomassie staining of the gel and the lower panel shows western blotting of HA tag. (B) *in vitro* kinase assay was performed as in (A) using either wildtype (WT) or S199A mutant GST-PHF6. Mutation of this serine residue significantly impairs the phosphorylation of PHF6 by Cdk5.

For future studies, it would be important to answer the following questions: 1) is PHF6 phosphorylated by Cdk5 in cells? 2) If PHF6 is indeed phosphorylated by Cdk5, what is the biological function of this phosphorylation? I have performed preliminary experiments examining the effect of the S199A mutant on neuronal migration. My preliminary data suggest that the S199A mutant is not as effective as the WT protein in promoting neuronal migration, although this result needs further validation. 3) Under what circumstances does Cdk5 phosphorylate PHF6? Since Cdk5 and PHF6 are both required for neuronal migration, one hypothesis would be that Cdk5 phosphorylation of PHF6 promotes its function of promoting neuronal migration.

PHF6 might control additional stages of neuronal maturation

In PHF6 knockdown animals, we examined the electrophysiological properties of GFP positive neurons that have reached layers II-IV, the proper location for that cohort of neurons. Interestingly, we observed a strong reduction of the frequency, but not much so for amplitude, for spontaneous excitatory postsynaptic current (sEPSC) (Figure S3A). Quantification revealed that frequency of sEPSC reduced more than 2 fold by PHF6 knockdown, whereas the amplitude of sEPSC in PHF6 knockdown neurons is only modestly lower than control neurons (Figure S3B, S3C, S3D and S3E).
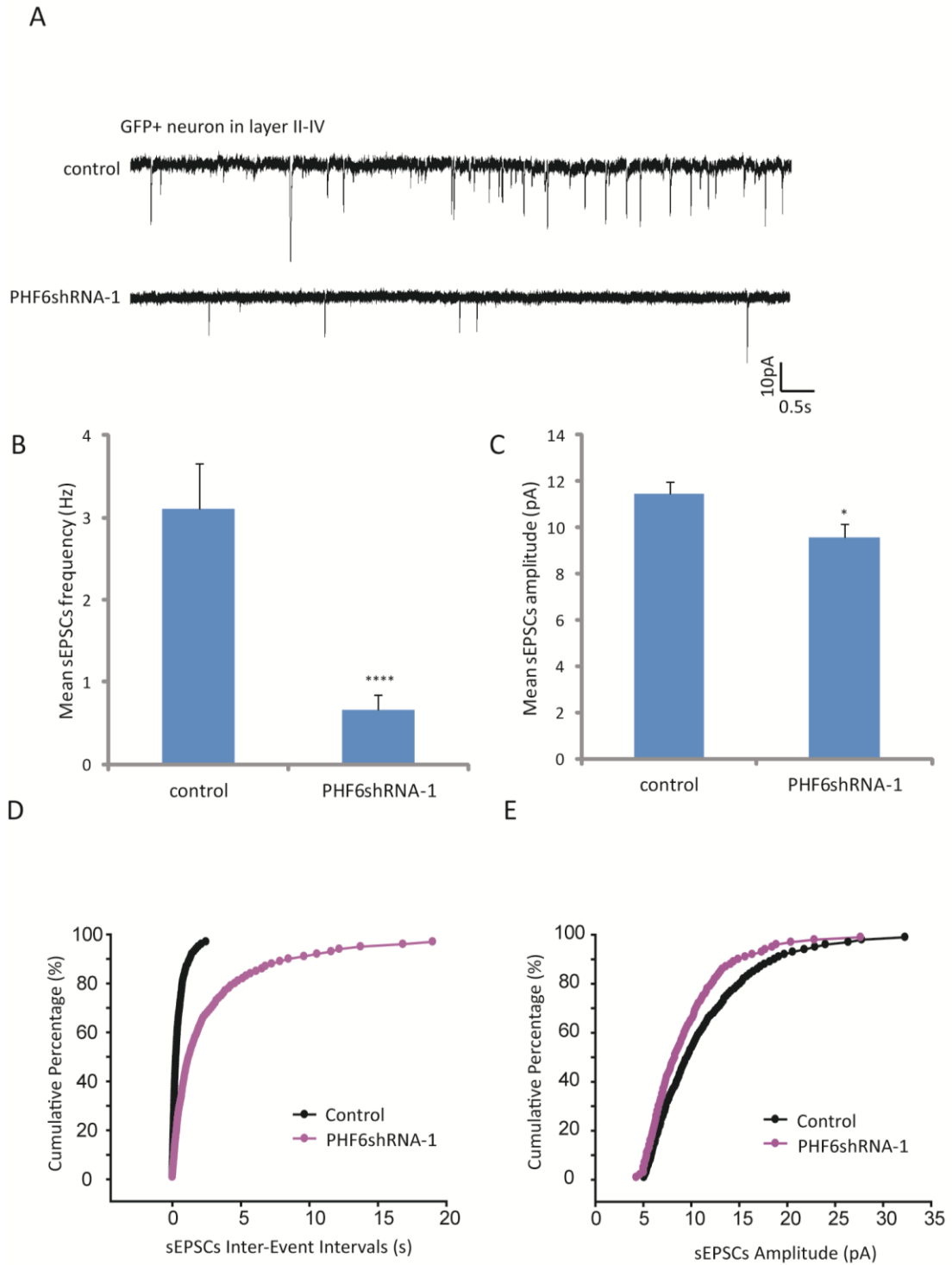
Figure S3. Knockdown of PHF6 reduces frequency of sEPSC in layer II-IV neurons. (A)

Representative traces of sEPSC recorded in GFP positive, layer II-IV neurons in P10

Figure S3. continued

electroporated animals. PHF6 RNAi reduces the frequency of sEPSC in layer II-IV neurons. (B-C)

Quantification of sEPSC frequency (B) and amplitude (C) of GFP positive layer II-IV neurons.

PHF6 knockdown significantly reduced the frequency but not amplitude of sEPSCs. (D-E)

Cumulative plot of sEPSC frequency (D) and amplitude (E) in GFP positive layer II-IV neurons.

To further validate this result, additional experiments, including testing an additional shRNA, will be needed.

The observation that knockdown of PHF6 leads to a reduction of sEPSC might be due to a few reasons. 1) PHF6 might have a function in neuronal development and maturation post-migration. Indeed, I observed that in P6 brains, knockdown of PHF6 by shRNA-2 in layer II-IV neurons lead to a reduction of dendritic complexity (Figure S4). Defects of post-synaptic development are also possible although I did not examine that possibility. 2) this reduction of spontaneous synaptic event is secondary to delayed neuronal migration. Establishment of neuronal connectivity is a spatiotemporally restricted process and a number of intrinsic and extrinsic cues come into play when synaptic connections are being established. Therefore it is possible that loss of PHF6 induced neuronal migration causes a delayed positioning of the neurons in the correct layer, therefore impairing the synaptic connections.

To test these possibilities, it would be interesting to use *in vitro* hippocampal neuron culture to ask whether PHF6 is required for dendritic complexity and post-synaptic development. Also this system would allow examination of synaptic connection without the confounding issue of neuronal migration. In addition, lentivirus injection into postnatal brains can also help us eliminate the confounding possibility that the reduction in sEPSC frequency is secondary to delayed neuronal migration, in an *in vivo* setting.
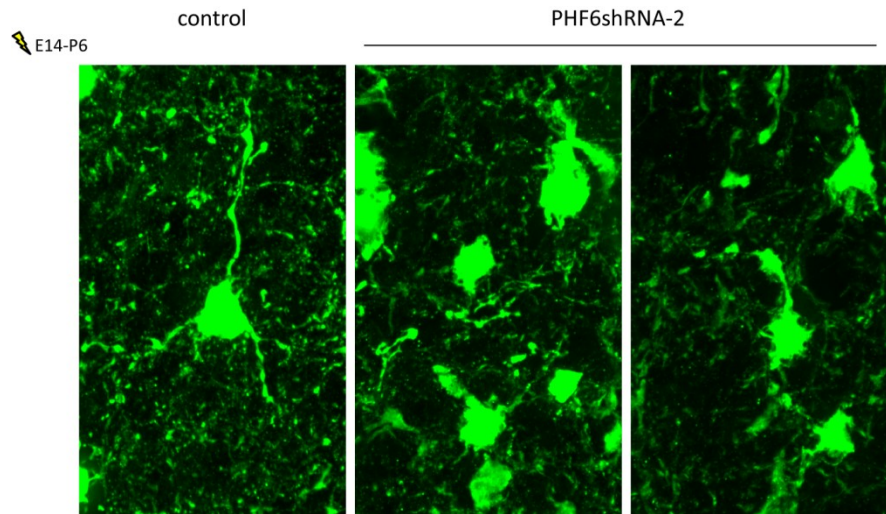
Figure S4. PHF6 knockdown reduces dendritic complexity in layer II-IV neurons. Animals were electroporated at E14 and brains were harvested at P6 followed by immunohistochemical analysis with the GFP antibody. Knockdown of PHF6 severely impaired the normal dendritic complexity of layer II-IV neurons.

I also explored other biological functions of PHF6. Especially, from the microarray, I observed that a number of molecules critical for establishing neuronal connectivity were downregulated by PHF6 knockdown. In particular, I was able to validate that EphB1 is likely to be an interesting downstream target of PHF6. Two shRNAs against PHF6 similarly lead to a downregulation of EphB1 (Figure S5), suggesting that it is likely a real downstream target for PHF6.

EphB1 is an interesting candidate for the study of neurobiology. It has been linked to multiple processes during neural development including axonal outgrowth, pathfinding, synaptic maturation and plasticity and neuronal apoptosis (Bilimoria et al., 2010; Depaepe et al., 2005; Gao et al., 1999). Therefore, it is possible that PHF6 also controls these biological processes through the regulation of EphB1 abundance, and thus, it remains interesting to test the role of PHF6 in these biological processes.
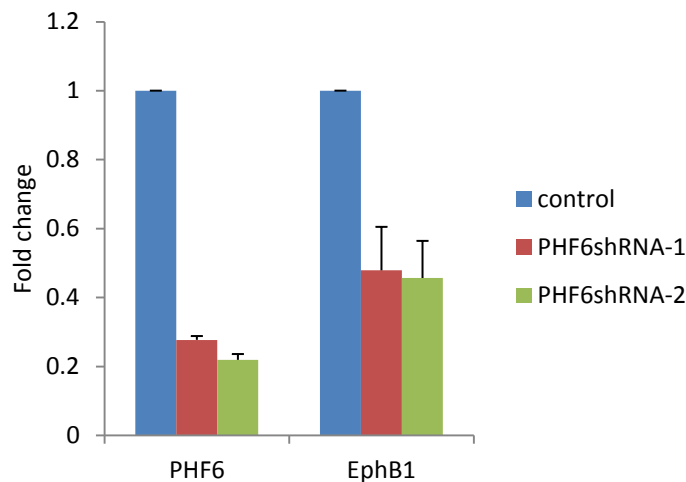

Figure S5. RT-PCR analysis of primary cultured rat cortical neurons infected with lentivirus expressing PHF6 shRNAs at DIV0 and harvested at DIV5. Levels of PHF6 and EphB1 were normalized by GAPDH. Knockdown of PHF6 by two independent shRNAs significantly reduced EphB1 levels.

FoxA family members are potential substrates for MST1, a protein kinase involved in apoptosis signaling

MST1 is a kinase that is implicated in regulation of apoptosis. Previously, our laboratory has identified that MST1 is critically involved in the process of oxidative stress induced neuronal cell death, and functions through phosphorylating the FOXO family members that in turn, promote transcription of downstream pro-apoptotic targets such as BIM (Lehtinen et al., 2006). To advance our understanding of the MST1 signaling pathway, I carried out both candidate studies and unbiased studies to identify novel substrates for MST1.

MST1 phosphorylates FOXO3 at Serine 207, a residue within the forkhead domain that is highly conserved in the forkhead family members. Therefore I searched for additional forkhead family members to see if any interesting candidates might be subject to regulation by MST1. In addition, the lab has established that the MST1-FOXO signaling pathway promotes longevity in C. elegans, and overexpression of the MST1 homolog cst-1 extends lifespan in a daf-16 dependent manner. Therefore, I searched for an additional downstream target that might link MST1 and longevity.

FoxA family members turn out to be very interesting candidates because recent studies show that it is essential for diet restriction induced longevity in C. elegans (Panowski et al., 2007). The potential MST1 phosphorylation site is highly conserved in FoxA family members, therefore they might be novel targets of MST1 (Figure S6).

```
Human FoxA1    QQRWQNSIRHSLSFND
Human FoxA2    QQRWQNSIRHSLSFND
Mouse FoxA1    QQRWQNSIRHSLSFND
Mouse FoxA2    QQRWQNSIRHSLSFND
Drosophila FoxA   QQRWQNSIRHSLSFND
C.elegans Pha-4   QQRWQNSIRHSLSFND
Human FoxO3    SAGWKNSIRHNLSLHS
```

Figure S6. Alignment of FoxA family members with FoxO3, highlighting the conservation of the potential MST1 phosphorylation site.

To test whether MST1 phosphorylates FoxA, I performed *in vitro* kinase assay using MST1 purified from 293T cells and GST-FoxA1/2 produced from E.coli. Interestingly, MST1 phoshorylates the forkhead domain of FoxA1/2 *in vitro* (Figure S7), suggesting that FoxA family members might be novel substrates of MST1. In addition, polyclonal antibodies raised against phospho-Ser205 reside on FoxA1 also confirmed that this residue is indeed phosphorylated by MST1 *in vitro* (Figure S8).

Additional questions remain regarding the phosphorylation event. For example, does the phosphorylation happen in cells? What is the physiological function and relevance of this modification?
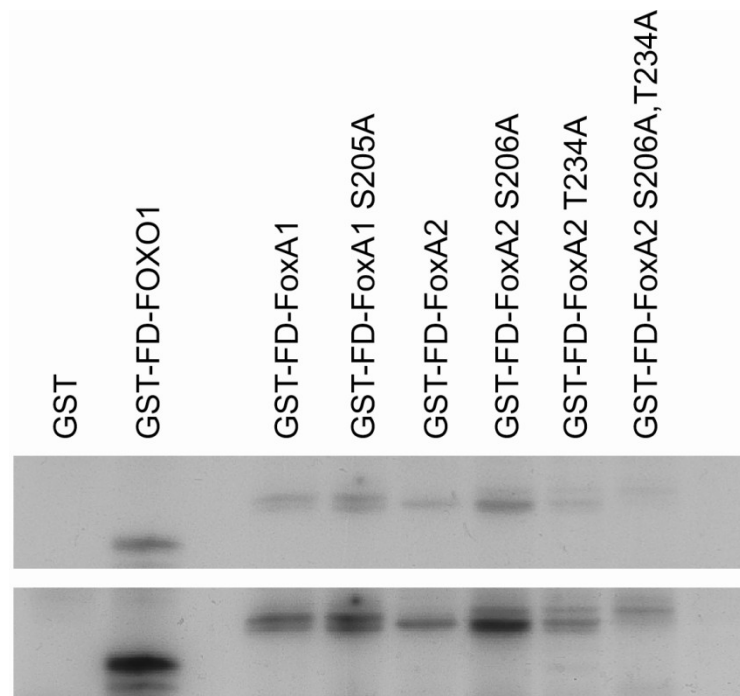

Figure S7. Cdk5 phosphorylates PHF6 *in vitro* on the Ser199 residue. Flag-MST1 immunoprecipitated from 293T cells are subjected to *in vitro* kinase assay with radioactive γ p^{32}-ATP and either GST or GST-FoxA1/2 or GST-FoxO1 purified from E. coli. The two different panels show two exposures of the film, with the lower panel showing a longer exposure.
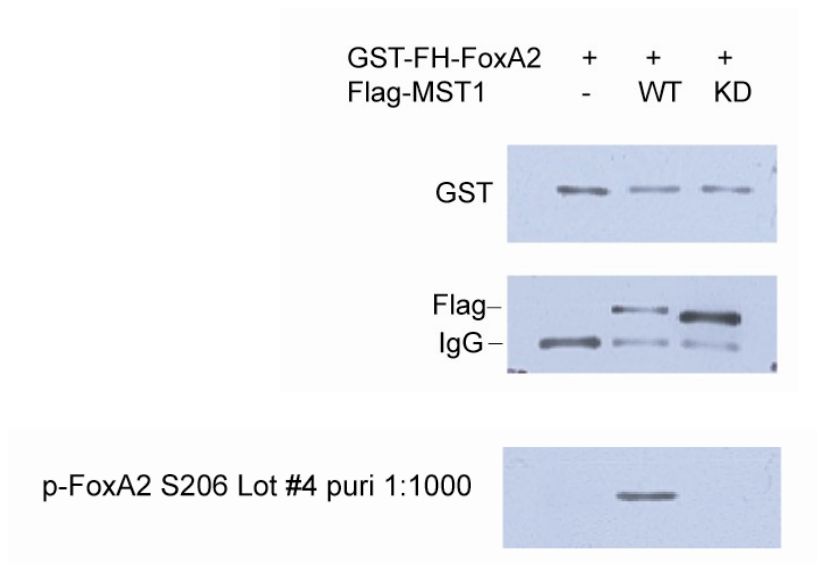
Figure S8. MST1 phosphorylates FoxA family members at the conserved Ser206 site. *In vitro* kinase assay was performed with Flag-MST1 and recombinant GST-Forkhead FoxA2 using cold ATP. The reaction is then subjected to immunoblotting using GST, Flag or an antibody directed at phosphorylated Ser206 site of FoxA2. Wildtype (WT) but not kinase dead (KD) MST1 phosphorylates FoxA2 at Ser206 site.

Unbiased screen to search for MST1 consensus phosphorylation site

In order to better understand MST1 functions, I sought to better characterize a consensus motif for MST1 kinase activity. I adopted an unbiased approach where the kinase of interest is applied to a library of peptides with fixed residues at particular locations (Hutti et al., 2004). This method takes advantage of a positional scanning peptide library consisting of 198 distinct peptide mixtures. In each mixture, one of the 20 naturally occurring proteogenic amino acids was fixed at each of 9 positions surrounding a central serine/threonine residue, with the remaining positions degenerate. In addition, peptides were generated that had either phosphothreonine or phosphotyrosine at each of the fixed positions. Through applying the kinase and radioactive ATP to the substrates and measuring the amount of radioactivity, I can generate information regarding the sequence preference of kinases on substrates.
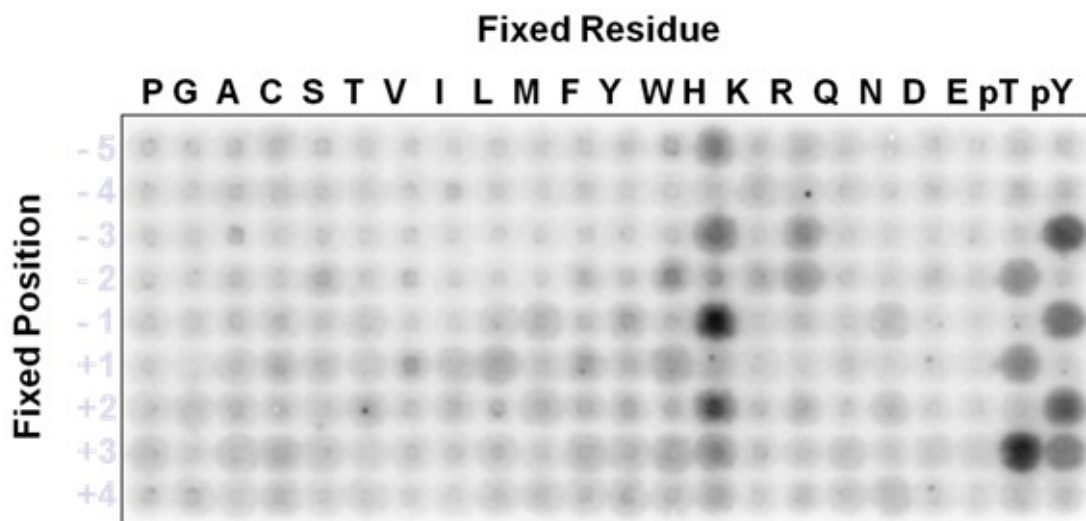

Figure S9. MST1 shows a preference on substrate selection where histidine residues occupy the -1 and -3 locations. Flag-MST1 immunoprecipitated from 293T cells and eluted using Flag peptide was subjected to *in vitro* kinase assay with radioactive γ p^{32}-ATP and the peptide library as

Figure S9. continued

described in Hutti et al. The stronger signal indicates a stronger preference on peptide sequence

by MST1 kinase.

Interestingly, I observed a pattern of strong MST1 preference on the peptides where histidine residues precede the serine/threonine residue, including the -1 and -3 sites preceding the ser/thr residue (Figure S9).

Future work will need to first validate the substrate preference by synthesizing a peptide with or without the histidine residues and testing whether mutating the histidine residue would significantly affect the substrate phosphorylation. After validation of this pattern, it would be interesting to scan potential substrates for this pattern to identify novel substrates that would like MST1 to interesting biological questions.

FoxA1 RNAi leads to altered Golgi and centrosomal location in cerebellar granule neurons

During the process of studying the MST1-FoxA link, I observed an interesting phenotype in cerebellar granule neurons where knockdown of FoxA1 leads to retraction of dendrites and concomitant enlargement of the proximal segment of the axon. A high portion of transfected neurons (~50%) exhibited this axon thickening phenotype, whereas none of the control transfected neurons showed this phenotype (Figure S10).
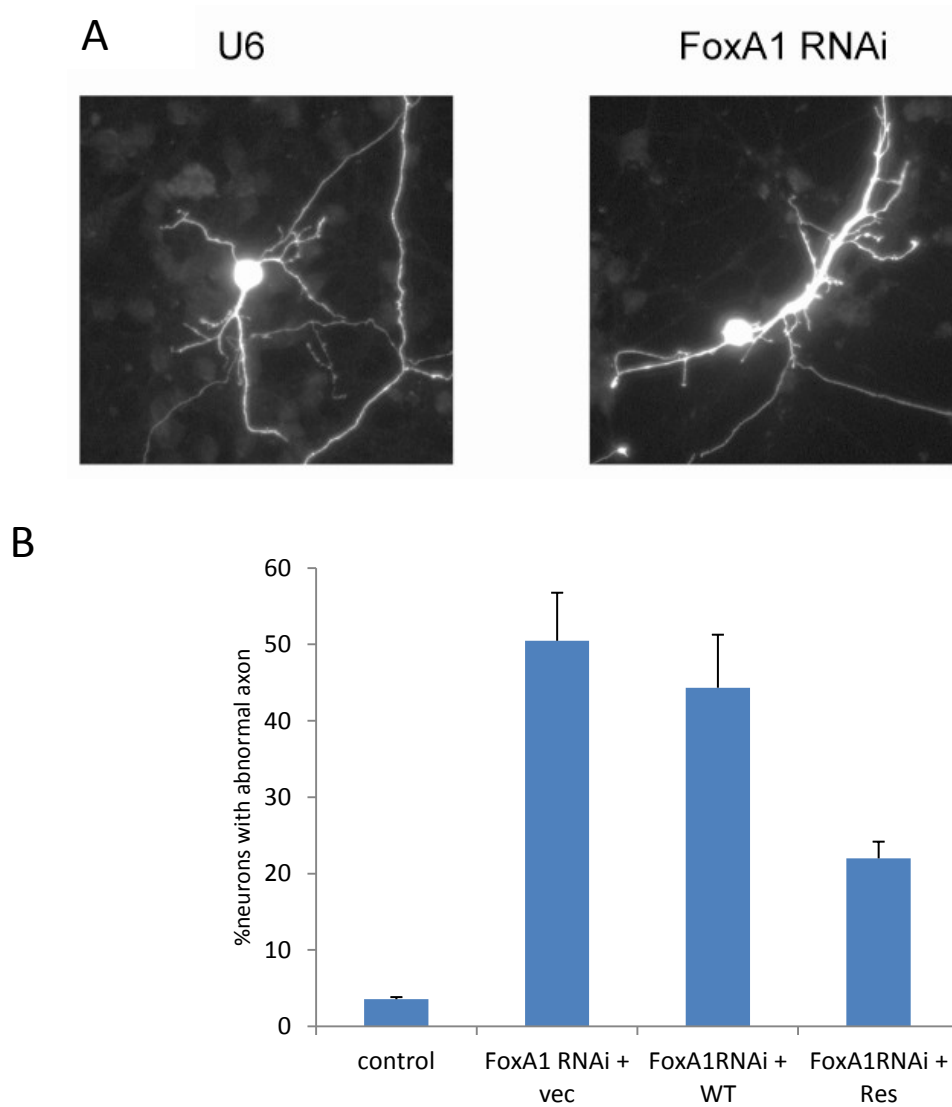

Figure S10

116

Figure S10. continued

Figure S10. FoxA1 RNAi leads to thickening of the proximal axon. (A) Representative image of the morphology of cerebellar granule neurons transfected with FoxA1 RNAi. Control (U6) cells have a thin axon extending out of the cell body, whereas FoxA1 RNAi leads to enlargement of the proximal axon with many protrusions. (B) Quantification of percent of neurons exhibiting the axonal phenotype induced by FoxA1 RNAi. Expression of RNAi resistant form of FoxA1 reduced the percent of neurons exhibiting this phenotype.

The specification of neuronal axon and dendrites is an important aspect of neural development, and a lot of studies have tried to identify the molecular and cellular mechanisms underlying axonal and dendritic specification. On a cellular level, differential orientation of subcellular organelles seems to play a role in this polarization process. For example, orientation of the centrosome and Golgi apparatus specifies the location of axon formation before it turns to the other side and establishes the leading process for migration (de Anda et al., 2010; Wiggin et al., 2005). Therefore I examined the localization of the centrosome and Golgi apparatus to examine whether location might underlie the abnormal formation of axons and dendrites.

Immunocytochemical analyses showed that under normal conditions, the centrosome is confined in the cell body, and the Golgi apparatus exhibits either an elongated or compact morphology in the soma, and occasionally with outposts in the dendritic arbors but never in the axon tracts. However, with FoxA1 RNAi, I observed a dramatic mislocalization of centrosome and the Golgi apparatus. The Golgi apparatus gets mislocalized into the axon, and interestingly, the location of the Golgi apparatus usually correlates with the site of enlargement along the axon (Figure S11A).
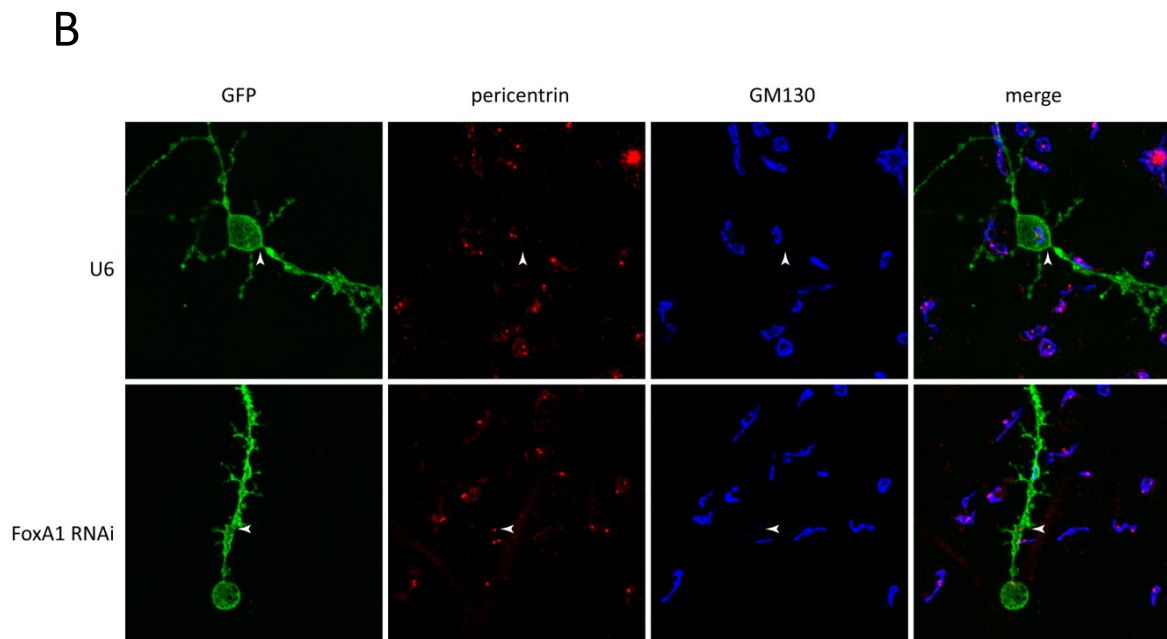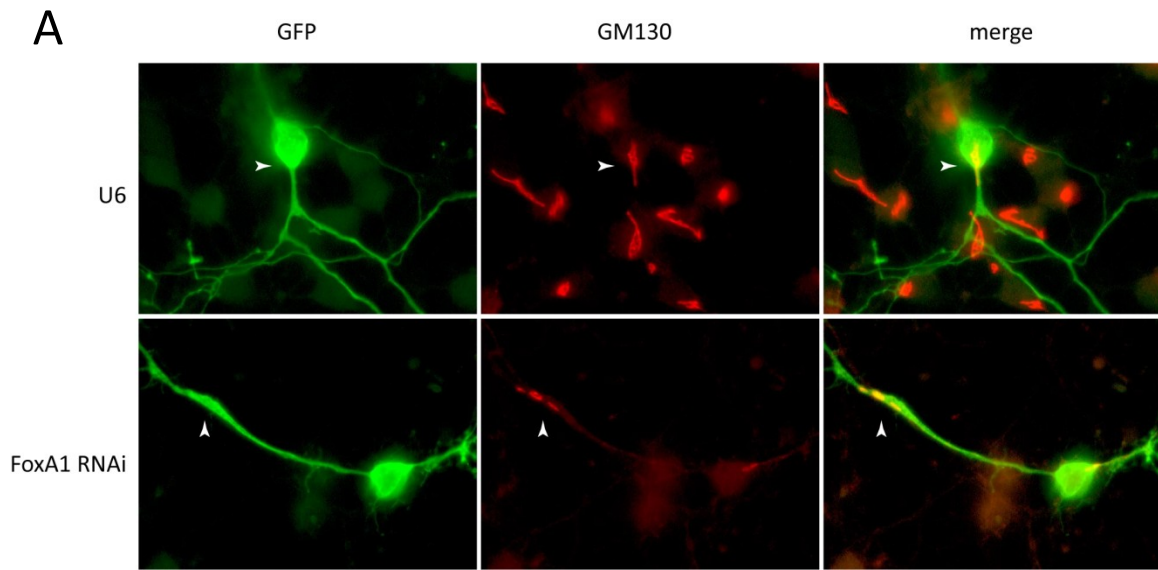
Figure S11. The Golgi apparatus and centrosome are mislocalized to the enlarged axons.

Figure S11. continued

Figure S11. The Golgi apparatus and centrosome are mislocalized to the enlarged axons. Cerebellar granule neurons are transfected with control or FoxA1 RNAi at DIV2 and subjected to immunocytochemistry at DIV5 with antibodies against GFP and GM130 (A) or GM130 and pericentrin (B). In control cells the Golgi apparatus (arrowhead in A) localizes to the cell body whereas FoxA1 RNAi leads to an axonal localization of the Golgi, which correlates with the site of axonal enlargement. The centrosome (arrowhead in B) also gets mislocalized into the enlarged axon segment, suggesting that abnormal positioning of subcellular organelles underlies the ectopic thickening of axons.

The centrosome is known to initiate the reorganization and polarization of the Golgi apparatus. Therefore, I also examined the localization of centrosomes in the transfected cells. Interestingly, I observed that in a high percentage of cells transfected with FoxA1 RNAi, at least one centriole is abnormally located in the enlarged segment of the axon. Quantification revealed that up to 20% of the FoxA1 RNAi transfected neurons exhibit an axonal localization of at least one centriole, whereas only less than 5% of control neurons show this phenotype (Figure S11B).

I further performed live time imaging experiment to examine the cell biological basis for the thickened axon phenotype (Figure S12). Consistent with literature, control cells showed very dynamic remodeling of dendritic tree, whereas the axon remains a thin process extending out of the cell body that is relatively stable. However, neurons transfected with FoxA1 RNAi showed a collapse of dendritic tree and concomitant thickening of the axon. Although preliminary, the data seems to suggest fusion of dendrites to the initial segment of the axon, or at least mis-delivery of dendritic materials to the axon initial segment. This idea of mis-delivery of dendritic materials to the axon initial segment is consistent with the mislocalization of centrosome and Golgi apparatus in the proximal axon, since the polarized orientation of these organelles is known to direct cargo transport along microtubules (Trimarchi et al., 2007). Further, more detailed time lapse imaging is needed to confirm this and better understand the underlying mechanisms involved.

To summarize, I discovered a phenotype induced by FoxA1 RNAi in primary cultured cerebellar granule neurons where the proximal segment of the axon gets enlarged, and contains organelles such as the Golgi apparatus and centrosome. Future

work will need to address both on a cellular and molecular level how this happens and

what the physiological consequence of this phenotype is.

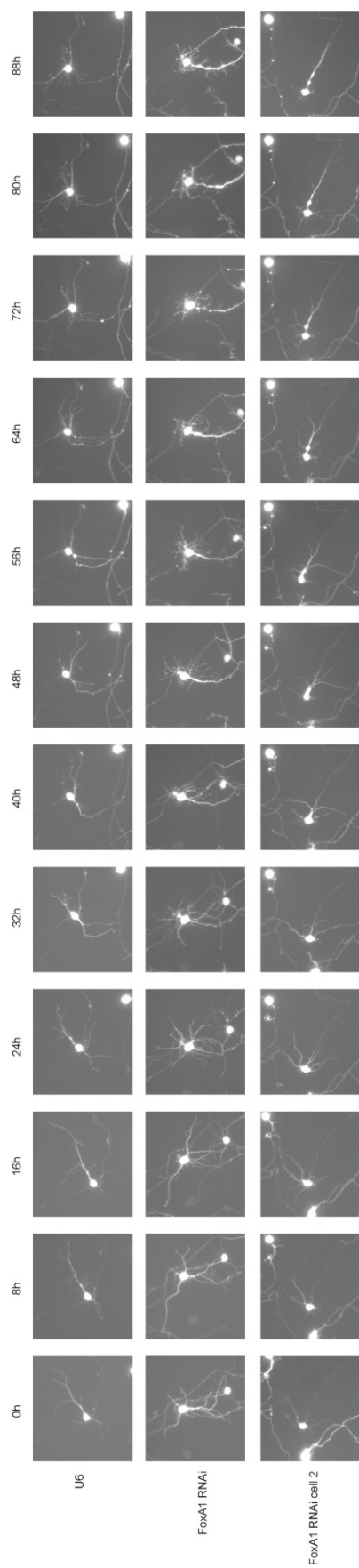Figure S12. Time lapse imaging of granule neurons transfected with GFP and control or FoxA1 RNAi. Granule neurons were transfected at DIV2 and imaged since DIV4 once every 8 hours. Whereas control cells showed very dynamic remodeling of dendritic tree, the axon remains a thin process extending out of the cell body. Cells transfected with FoxA1 RNAi show a collapse of dendritic tree and concomitant thickening of the axon.

References

(2004). Economic costs associated with mental retardation, cerebral palsy, hearing loss, and vision impairment--United States, 2003. MMWR Morb Mortal Wkly Rep *53*, 57-59.

Abdel Razek, A. A., Kandell, A. Y., Elsorogy, L. G., Elmongy, A., and Basett, A. A. (2009). Disorders of cortical formation: MR imaging features. AJNR Am J Neuroradiol *30*, 4-11.

Ackman, J. B., Aniksztejn, L., Crepel, V., Becq, H., Pellegrino, C., Cardoso, C., Ben-Ari, Y., and Represa, A. (2009). Abnormal network activity in a targeted genetic model of human double cortex. J Neurosci *29*, 313-327.

Aghakhani, Y., Kinay, D., Gotman, J., Soualmi, L., Andermann, F., Olivier, A., and Dubeau, F. (2005). The role of periventricular nodular heterotopia in epileptogenesis. Brain *128*, 641-651.

Baumstark, A., Lower, K. M., Sinkus, A., Andriuskeviciute, I., Jurkeniene, L., Gecz, J., and Just, W. (2003). Novel PHF6 mutation p.D333del causes Borjeson-Forssman-Lehmann syndrome. J Med Genet *40*, e50.

Behrends, C., Sowa, M. E., Gygi, S. P., and Harper, J. W. (2010). Network organization of the human autophagy system. Nature *466*, 68-76.

Bentley, D. L., and Groudine, M. (1986). A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. Nature *321*, 702-706.

Berland, S., Alme, K., Brendehaug, A., Houge, G., and Hovland, R. (2011). PHF6 Deletions May Cause Borjeson-Forssman-Lehmann Syndrome in Females. Mol Syndromol *1*, 294-300.

Bhasin, T. K., Brocksen, S., Avchen, R. N., and Van Naarden Braun, K. (2006). Prevalence of four developmental disabilities among children aged 8 years--Metropolitan Atlanta Developmental Disabilities Surveillance Program, 1996 and 2000. MMWR Surveill Summ *55*, 1-9.

Bilimoria, P. M., de la Torre-Ubieta, L., Ikeuchi, Y., Becker, E. B., Reiner, O., and Bonni, A. (2010). A JIP3-regulated GSK3beta/DCX signaling pathway restricts axon branching. J Neurosci *30*, 16766-16776.

Borjeson, M., Forssman, H., and Lehmann, O. (1962). An X-linked, recessively inherited syndrome characterized by grave mental deficiency, epilepsy, and endocrine disorder. Acta Med Scand *171*, 13-21.

Brun, A., Borjeson, M., and Forssman, H. (1974). An inherited syndrome with mental deficiency and endocrine disorder. A patho-anatomical study. J Ment Defic Res *18*, 317-325.

Carpten, J. D., Robbins, C. M., Villablanca, A., Forsberg, L., Presciuttini, S., Bailey-Wilson, J., Simonds, W. F., Gillanders, E. M., Kennedy, A. M., Chen, J. D.*, et al.* (2002). HRPT2, encoding parafibromin, is mutated in hyperparathyroidism-jaw tumor syndrome. Nat Genet *32*, 676-680.

Carter, M. T., Picketts, D. J., Hunter, A. G., and Graham, G. E. (2009). Further clinical delineation of the Borjeson-Forssman-Lehmann syndrome in patients with PHF6 mutations. Am J Med Genet A *149A*, 246-250.

Chae, T., Kwon, Y. T., Bronson, R., Dikkes, P., Li, E., and Tsai, L. H. (1997). Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. Neuron *18*, 29-42.

Chai, X., Forster, E., Zhao, S., Bock, H. H., and Frotscher, M. (2009). Reelin stabilizes the actin cytoskeleton of neuronal processes by inducing n-cofilin phosphorylation at serine3. J Neurosci *29*, 288-299.

Chechlacz, M., and Gleeson, J. G. (2003). Is mental retardation a defect of synapse structure and function? Pediatr Neurol *29*, 11-17.

Chen, Y., Yamaguchi, Y., Tsugeno, Y., Yamamoto, J., Yamada, T., Nakamura, M., Hisatake, K., and Handa, H. (2009). DSIF, the Paf1 complex, and Tat-SF1 have nonredundant, cooperative roles in RNA polymerase II elongation. Genes Dev *23*, 2765-2777.

Condeelis, J. (1993). Life at the leading edge: the formation of cell protrusions. Annu Rev Cell Biol *9*, 411-444.

de Anda, F. C., Meletis, K., Ge, X., Rei, D., and Tsai, L. H. (2010). Centrosome motility is essential for initial axon formation in the neocortex. J Neurosci *30*, 10391-10406.

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

Dhavan, R., and Tsai, L. H. (2001). A decade of CDK5. Nat Rev Mol Cell Biol *2*, 749-759.

Dulabon, L., Olson, E. C., Taglienti, M. G., Eisenhuth, S., McGrath, B., Walsh, C. A., Kreidberg, J. A., and Anton, E. S. (2000). Reelin binds alpha3beta1 integrin and inhibits neuronal migration. Neuron *27*, 33-44.

Elias, L. A., Wang, D. D., and Kriegstein, A. R. (2007). Gap junction adhesion is necessary for radial migration in the neocortex. Nature *448*, 901-907.

Fishburn, J., Turner, G., Daniel, A., and Brookwell, R. (1983). The diagnosis and frequency of X-linked conditions in a cohort of moderately retarded males with affected brothers. Am J Med Genet *14*, 713-724.

Gao, P. P., Yue, Y., Cerretti, D. P., Dreyfus, C., and Zhou, R. (1999). Ephrin-dependent growth and pruning of hippocampal axons. Proc Natl Acad Sci U S A *96*, 4073-4077.

Ge, W., He, F., Kim, K. J., Blanchi, B., Coskun, V., Nguyen, L., Wu, X., Zhao, J., Heng, J. I., Martinowich, K.*, et al.* (2006). Coupling of cell migration with neurogenesis by proneural bHLH factors. Proc Natl Acad Sci U S A *103*, 1319-1324.

Ge, X., Frank, C. L., Calderon de Anda, F., and Tsai, L. H. (2010). Hook3 interacts with PCM1 to regulate pericentriolar material assembly and the timing of neurogenesis. Neuron *65*, 191-203.

Gecz, J., Turner, G., Nelson, J., and Partington, M. (2006). The Borjeson-Forssman-Lehman syndrome (BFLS, MIM #301900). Eur J Hum Genet *14*, 1233-1237.

Gleeson, J. G., Allen, K. M., Fox, J. W., Lamperti, E. D., Berkovic, S., Scheffer, I., Cooper, E. C., Dobyns, W. B., Minnerath, S. R., Ross, M. E., and Walsh, C. A. (1998). Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. Cell *92*, 63-72.

Greer, E. L., and Shi, Y. (2012). Histone methylation: a dynamic mark in health, disease and inheritance. Nat Rev Genet *13*, 343-357.

Guenther, M. G., Levine, S. S., Boyer, L. A., Jaenisch, R., and Young, R. A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. Cell *130*, 77-88.

Hashimoto-Torii, K., Torii, M., Sarkisian, M. R., Bartley, C. M., Shen, J., Radtke, F., Gridley, T., Sestan, N., and Rakic, P. (2008). Interaction between Reelin and Notch signaling regulates neuronal migration in the cerebral cortex. Neuron *60*, 273-284.

Hatanaka, Y., and Yamauchi, K. (2013). Excitatory cortical neurons with multipolar shape establish neuronal polarity by forming a tangentially oriented axon in the intermediate zone. Cereb Cortex *23*, 105-113.

Heng, J. I., Chariot, A., and Nguyen, L. (2009). Molecular layers underlying cytoskeletal remodelling during cortical development. Trends Neurosci *33*, 38-47.

Heng, J. I., Nguyen, L., Castro, D. S., Zimmer, C., Wildner, H., Armant, O., Skowronska-Krawczyk, D., Bedogni, F., Matter, J. M., Hevner, R., and Guillemot, F. (2008). Neurogenin 2 controls cortical neuron migration through regulation of Rnd2. Nature *455*, 114-118.

Herbst, D. S., and Miller, J. R. (1980). Nonspecific X-linked mental retardation II: the frequency in British Columbia. Am J Med Genet *7*, 461-469.

Hutti, J. E., Jarrell, E. T., Chang, J. D., Abbott, D. W., Storz, P., Toker, A., Cantley, L. C., and Turk, B. E. (2004). A rapid method for determining protein kinase phosphorylation specificity. Nat Methods *1*, 27-29.

Jin, J., Suzuki, H., Hirai, S., Mikoshiba, K., and Ohshima, T. (2010). JNK phosphorylates Ser332 of doublecortin and regulates its function in neurite extension and neuronal migration. Dev Neurobiol *70*, 929-942.

Kawauchi, T., Chihama, K., Nabeshima, Y., and Hoshino, M. (2006). Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration. Nat Cell Biol *8*, 17-26.

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

Kinugasa, Y., Ishiguro, H., Tokita, Y., Oohira, A., Ohmoto, H., and Higashiyama, S. (2004). Neuroglycan C, a novel member of the neuregulin family. Biochem Biophys Res Commun *321*, 1045-1049.

Kothare, S. V., VanLandingham, K., Armon, C., Luther, J. S., Friedman, A., and Radtke, R. A. (1998). Seizure onset from periventricular nodular heterotopias: depth-electrode study. Neurology *51*, 1723-1727.

Krogan, N. J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M. A., Dean, K., Ryan, O. W., Golshani, A., Johnston, M.*, et al.* (2003). The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol Cell *11*, 721-729.

Krogan, N. J., Kim, M., Ahn, S. H., Zhong, G., Kobor, M. S., Cagney, G., Emili, A., Shilatifard, A., Buratowski, S., and Greenblatt, J. F. (2002). RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach. Mol Cell Biol *22*, 6979-6992.

Krumm, A., Hickey, L. B., and Groudine, M. (1995). Promoter-proximal pausing of RNA polymerase II defines a general rate-limiting step after transcription initiation. Genes Dev *9*, 559-572.

Lambert de Rouvroit, C., and Goffinet, A. M. (2001). Neuronal migration. Mech Dev *105*, 47-56.

Larson, S. A., Lakin, K. C., Anderson, L., Kwak, N., Lee, J. H., and Anderson, D. (2001). Prevalence of mental retardation and developmental disabilities: estimates from the 1994/1995 National Health Interview Survey Disability Supplements. Am J Ment Retard *106*, 231-252.

Lauberth, S. M., Nakayama, T., Wu, X., Ferris, A. L., Tang, Z., Hughes, S. H., and Roeder, R. G. (2013). H3K4me3 Interactions with TAF3 Regulate Preinitiation Complex Assembly and Selective Gene Activation. Cell *152*, 1021-1036.

Lee, K. S., Schottler, F., Collins, J. L., Lanzino, G., Couture, D., Rao, A., Hiramatsu, K., Goto, Y., Hong, S. C., Caner, H*., et al.* (1997). A genetic animal model of human neocortical heterotopia associated with seizures. J Neurosci *17*, 6236-6242.

Lehtinen, M. K., Yuan, Z., Boag, P. R., Yang, Y., Villen, J., Becker, E. B., DiBacco, S., de la Iglesia, N., Gygi, S., Blackwell, T. K., and Bonni, A. (2006). A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. Cell *125*, 987-1001.

Litterman, N., Ikeuchi, Y., Gallardo, G., O'Connell, B. C., Sowa, M. E., Gygi, S. P., Harper, J. W., and Bonni, A. (2011). An OBSL1-Cul7Fbxw8 ubiquitin ligase signaling mechanism regulates Golgi morphology and dendrite patterning. PLoS Biol *9*, e1001060.

Liu, J. S. (1007). Molecular genetics of neuronal migration disorders. Curr Neurol Neurosci Rep *11*, 171-178.

Lodato, S., Rouaux, C., Quast, K. B., Jantrachotechatchawan, C., Studer, M., Hensch, T. K., and Arlotta, P. (2011). Excitatory projection neuron subtypes control the distribution of local inhibitory interneurons in the cerebral cortex. Neuron *69*, 763-779.

Lower, K. M., Turner, G., Kerr, B. A., Mathews, K. D., Shaw, M. A., Gedeon, A. K., Schelley, S., Hoyme, H. E., White, S. M., Delatycki, M. B*., et al.* (2002). Mutations in PHF6 are associated with Borjeson-Forssman-Lehmann syndrome. Nat Genet *32*, 661-665.

Lubs, H. A., Stevenson, R. E., and Schwartz, C. E. (2012). Fragile X and X-linked intellectual disability: four decades of discovery. Am J Hum Genet *90*, 579-590.

Mahmood, S., Ahmad, W., and Hassan, M. J. (2011). Autosomal Recessive Primary Microcephaly (MCPH): clinical manifestations, genetic heterogeneity and mutation continuum. Orphanet J Rare Dis *6*, 39.

Manent, J. B., Wang, Y., Chang, Y., Paramasivam, M., and LoTurco, J. J. (2009). Dcx reexpression reduces subcortical band heterotopia and seizure threshold in an animal model of neuronal migration disorder. Nat Med *15*, 84-90.

Marin, O., and Rubenstein, J. L. (2003). Cell migration in the forebrain. Annu Rev Neurosci *26*, 441-483.

Marton, H. A., and Desiderio, S. (2008). The Paf1 complex promotes displacement of histones upon rapid induction of transcription by RNA polymerase II. BMC Mol Biol *9*, 4.

McEvilly, R. J., de Diaz, M. O., Schonemann, M. D., Hooshmand, F., and Rosenfeld, M. G. (2002). Transcriptional regulation of cortical neuron migration by POU domain factors. Science *295*, 1528-1532.

Mueller, C. L., Porter, S. E., Hoffman, M. G., and Jaehning, J. A. (2004). The Paf1 complex has functions independent of actively transcribing RNA polymerase II. Mol Cell *14*, 447-456.

Muntean, A. G., Tan, J., Sitwala, K., Huang, Y., Bronstein, J., Connelly, J. A., Basrur, V., Elenitoba-Johnson, K. S., and Hess, J. L. (2010). The PAF complex synergizes with MLL fusion proteins at HOX loci to promote leukemogenesis. Cancer Cell *17*, 609-621.

Muse, G. W., Gilchrist, D. A., Nechaev, S., Shah, R., Parker, J. S., Grissom, S. F., Zeitlinger, J., and Adelman, K. (2007). RNA polymerase is poised for activation across the genome. Nat Genet *39*, 1507-1511.

Nadarajah, B., Brunstrom, J. E., Grutzendler, J., Wong, R. O., and Pearlman, A. L. (2001). Two modes of radial migration in early development of the cerebral cortex. Nat Neurosci *4*, 143-150.

Nechaev, S., and Adelman, K. (2011). Pol II waiting in the starting gates: Regulating the transition from transcription initiation into productive elongation. Biochim Biophys Acta *1809*, 34-45.

Nguyen, C. T., Langenbacher, A., Hsieh, M., and Chen, J. N. (2010). The PAF1 complex component Leo1 is essential for cardiac and neural crest development in zebrafish. Dev Biol *341*, 167-175.

Nieto, M., Monuki, E. S., Tang, H., Imitola, J., Haubst, N., Khoury, S. J., Cunningham, J., Gotz, M., and Walsh, C. A. (2004). Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II-IV of the cerebral cortex. J Comp Neurol *479*, 168-180.

O'Rourke, N. A., Dailey, M. E., Smith, S. J., and McConnell, S. K. (1992). Diverse migratory pathways in the developing cerebral cortex. Science *258*, 299-302.

Panowski, S. H., Wolff, S., Aguilaniu, H., Durieux, J., and Dillin, A. (2007). PHA-4/Foxa mediates diet-restriction-induced longevity of C. elegans. Nature *447*, 550-555.

Penrose, L. (1938). A clinical and genetic study of 1280 cases of mental defect. London: HMSO *229*.

Pollard, T. D., and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell *112*, 453-465.

Rio, C., Rieff, H. I., Qi, P., Khurana, T. S., and Corfas, G. (1997). Neuregulin and erbB receptors play a critical role in neuronal migration. Neuron *19*, 39-50.

Robinson, L. K., Jones, K. L., Culler, F., Nyhan, W. L., Sakati, N., and Jones, K. L. (1983). The Borjeson-Forssman-Lehmann syndrome. Am J Med Genet *15*, 457-468.

Rondon, A. G., Gallardo, M., Garcia-Rubio, M., and Aguilera, A. (2004). Molecular evidence indicating that the yeast PAF complex is required for transcription elongation. EMBO Rep *5*, 47-53.

Rougvie, A. E., and Lis, J. T. (1988). The RNA polymerase II molecule at the 5' end of the uninduced hsp70 gene of D. melanogaster is transcriptionally engaged. Cell *54*, 795-804.

Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Zhang, M., Ihrig, M. M., McManus, M. T.*, et al.* (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet *33*, 401-406.

Sanada, K., Gupta, A., and Tsai, L. H. (2004). Disabled-1-regulated adhesion of migrating neurons to radial glial fiber contributes to neuronal positioning during early corticogenesis. Neuron *42*, 197-211.

Sekine, K., Kawauchi, T., Kubo, K., Honda, T., Herz, J., Hattori, M., Kinashi, T., and Nakajima, K. (2012). Reelin controls neuronal positioning by promoting cell-matrix adhesion via inside-out activation of integrin alpha5beta1. Neuron *76*, 353-369.

Shim, S. Y., Wang, J., Asada, N., Neumayer, G., Tran, H. C., Ishiguro, K., Sanada, K., Nakatani, Y., and Nguyen, M. D. (2008). Protein 600 is a microtubule/endoplasmic reticulum-associated protein in CNS neurons. J Neurosci *28*, 3604-3614.

Smith, B. N., Dudek, F. E., and Roper, S. N. (1999). Synaptic responses of neurons in heterotopic gray matter in an animal model of cortical dysgenesis. Dev Neurosci *21*, 365-373.

So, H. C., Fong, P. Y., Chen, R. Y., Hui, T. C., Ng, M. Y., Cherny, S. S., Mak, W. W., Cheung, E. F., Chan, R. C., Chen, E. Y.*, et al.* (2010). Identification of neuroglycan C and interacting partners as potential susceptibility genes for schizophrenia in a Southern Chinese population. Am J Med Genet B Neuropsychiatr Genet *153B*, 103-113.

Solecki, D. J., Model, L., Gaetz, J., Kapoor, T. M., and Hatten, M. E. (2004). Par6alpha signaling controls glial-guided neuronal migration. Nat Neurosci *7*, 1195-1203.

Sowa, M. E., Bennett, E. J., Gygi, S. P., and Harper, J. W. (2009). Defining the human deubiquitinating enzyme interaction landscape. Cell *138*, 389-403.

Squazzo, S. L., Costa, P. J., Lindstrom, D. L., Kumer, K. E., Simic, R., Jennings, J. L., Link, A. J., Arndt, K. M., and Hartzog, G. A. (2002). The Paf1 complex physically and functionally associates with transcription elongation factors in vivo. Embo J *21*, 1764-1774.

Strahl, B. D., and Allis, C. D. (2000). The language of covalent histone modifications. Nature *403*, 41-45.

Sugitani, Y., Nakai, S., Minowa, O., Nishi, M., Jishage, K., Kawano, H., Mori, K., Ogawa, M., and Noda, T. (2002). Brn-1 and Brn-2 share crucial roles in the production and positioning of mouse neocortical neurons. Genes Dev *16*, 1760-1765.

Tanaka, T., Serneo, F. F., Tseng, H. C., Kulkarni, A. B., Tsai, L. H., and Gleeson, J. G. (2004). Cdk5 phosphorylation of doublecortin ser297 regulates its effect on neuronal migration. Neuron *41*, 215-227.

Theriot, J. A., and Mitchison, T. J. (1991). Actin microfilament dynamics in locomoting cells. Nature *352*, 126-131.

Trimarchi, J. M., Stadler, M. B., Roska, B., Billings, N., Sun, B., Bartch, B., and Cepko, C. L. (2007). Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. J Comp Neurol *502*, 1047-1065.

Tsai, J. W., Bremner, K. H., and Vallee, R. B. (2007). Dual subcellular roles for LIS1 and dynein in radial neuronal migration in live brain tissue. Nat Neurosci *10*, 970-979.

Turner, G., Lower, K. M., White, S. M., Delatycki, M., Lampe, A. K., Wright, M., Smith, J. C., Kerr, B., Schelley, S., Hoyme, H. E*., et al.* (2004). The clinical picture of the Borjeson-Forssman-Lehmann syndrome in males and heterozygous females with PHF6 mutations. Clin Genet *65*, 226-232.

Van Vlierberghe, P., Palomero, T., Khiabanian, H., Van der Meulen, J., Castillo, M., Van Roy, N., De Moerloose, B., Philippe, J., Gonzalez-Garcia, S., Toribio, M. L*., et al.* (2010). PHF6 mutations in T-cell acute lymphoblastic leukemia. Nat Genet *42*, 338-342.

Verkerk, A. J., Pieretti, M., Sutcliffe, J. S., Fu, Y. H., Kuhl, D. P., Pizzuti, A., Reiner, O., Richards, S., Victoria, M. F., Zhang, F. P., and et al. (1991). Identification of a gene

(FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell *65*, 905-914.

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

Wang, T., Bray, S. M., and Warren, S. T. (2012). New perspectives on the biology of fragile X syndrome. Curr Opin Genet Dev *22*, 256-263.

Wiggin, G. R., Fawcett, J. P., and Pawson, T. (2005). Polarity proteins in axon specification and synaptogenesis. Dev Cell *8*, 803-816.

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

Wysocka, J., Swigut, T., Xiao, H., Milne, T. A., Kwon, S. Y., Landry, J., Kauer, M., Tackett, A. J., Chait, B. T., Badenhorst, P*., et al.* (2006). A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. Nature *442*, 86-90.

Yuan, Z., Becker, E. B., Merlo, P., Yamada, T., DiBacco, S., Konishi, Y., Schaefer, E. M., and Bonni, A. (2008). Activation of FOXO1 by Cdk1 in cycling cells and postmitotic neurons. Science *319*, 1665-1668.

Zeng, L., Zhang, Q., Li, S., Plotnikov, A. N., Walsh, M. J., and Zhou, M. M. (2010). Mechanism and regulation of acetylated histone binding by the tandem PHD finger of DPF3b. Nature *466*, 258-262.

Zhu, B., Mandal, S. S., Pham, A. D., Zheng, Y., Erdjument-Bromage, H., Batra, S. K., Tempst, P., and Reinberg, D. (2005). The human PAF complex coordinates transcription with events downstream of RNA synthesis. Genes Dev *19*, 1668-1673.