探討 KBP 在神經發育的功能以及導致 Goldberg-Shprintzen 症候群所扮演的角色

Functions of KBP in Neural Development and its Role in Causing Goldberg-Shprintzen Syndrome

研究生：張馨云 (Hsin-Yun Chang)

指導教授：蔡金吾 博士 (Jin-Wu Tsai, Ph.D.)

國立陽明大學

腦科學研究所

碩 士 論 文

Institute of Brain Science

National Yang-Ming University

Master Thesis

中華民國一0七年八月

August, 2018

Contents 目錄

Contents 目錄---------- i

Acknowledgments ---------- iv

Abstract ---------- viii

摘要---------- x

Chapter 1: Introduction

1.1 Early development of the cerebral cortex---------- 1

1.2 Migration and maturation of cortical projection neurons---------- 2

1.3 Malformations of cortical development---------- 3

1.4 Goldberg-Shprintzen syndrome (GOSHS)---------- 4

1.5 Molecular functions of kinesin binding protein (KBP)---------- 5

1.6 Roles of KBP in neural development---------- 6

1.7 Significance---------- 7

Chapter 2: Materials and Methods

2.1 Constructs---------- 7

2.2 Electrophoresis and Western Blotting---------- 9

2.3 *In utero* electroporation (IUE)---------- 10

2.4 Immunofluorescence staining---------- 11

2.5 TUNEL assay---------- 12

2.6 Image analysis---------- 12
2.7 Statistical analysis---------- 13

Chapter 3: Results

3.1 KBP knockdown affects neuronal migration but not neural differentiation process---------- 13
3.2 KBP knockdown delays neuronal migration until P6 while arrested cells have become mature neurons---------- 15
3.3 KBP knockdown impairs cortical neurons morphology in both dendritic arborization and axon projection---------- 16
3.4 In vitro quantification of the neuritic complexity---------- 17
3.5 KBP knockdown induces cell apoptosis---------- 17

Chapter 4: Discussion---------- 18

4.1 Potential mechanisms of KBP in neuronal migration---------- 20
4.2 Proposed mechanisms of KBP in dendrite morphogenesis---------- 22
4.3 Proposed roles of KBP in axon outgrowth and extension---------- 23
4.4 Potential mechanisms responsible for apoptosis found in KBP knockdown brains---------- 24

Chapter 5: Reference---------- 25

Figure 1---------- 31

Figure 2---------- 32
Figure 3---------- 35
Figure 4---------- 38
Figure 5---------- 40
Figure 6---------- 42
Figure 7---------- 44

本研究榮獲財團法人罕見疾病基金會

「第十九屆罕見疾病博碩士論文獎助學金」獎助

謹於此特別致謝

Acknowledgments

碩班一轉眼就過了，卻是目前求學生涯最充實的一段。慶幸進入一個很棒的實驗室，開啟我進行科學研究的大門，並順利在此完成碩班的研究。這裡沒有澆熄我的熱忱，反倒澆灌了更多研究的熱情和勇氣。除了慶幸，更是感謝！

能遇上蔡金吾老師是我最大的幸運！大一時系上的物理課改由金吾老師教學，課堂被教學的熱情感動，也因而接觸到老師研究的神經發育領域。非常感謝當時老師同意我到實驗室見習，給予很棒的環境，在自然氛圍下引發對科學研究的興趣。謝謝老師在大學時協助申請科技部計畫，還支持我申請五年一貫繼續到腦科所就讀。真正進入碩士班後，我獲得了很棒的題目，很快的開始。雖然過程中很多不如意，甚至喪志灰心，但老師總是教我們正向思考，提醒我們用不同的角度看待問題，永遠鼓勵我們當下的研究成果。謝謝老師的器重，即使經常覺得自己不夠厲害，成果和預期有落差，但我時刻提醒自己，老師說：「我們要更有自信，要相信自己絕對有能力可以在未來不管什麼樣的環境跟別人競爭。」

除了老師，研究過程最重要的就是學長姐和同學的教學與陪伴，我們的「金金LAB」就像個大家庭，研究生的生活很沉悶，幸好有家人一般的大家，才能順利熬過。首先要謝謝大學時期帶我的佩雯學姊，他雖然是超級忙的行政兼研究助理，卻仍願意花時間教我們什麼都不懂的大學生做實驗。很謝謝她！不僅是教我們實驗操作，更仔細地協助我完成報告，對於之後進入碩士班有很大的幫助。再來要感謝主要帶我的嘉偉學長－「黃老師」。從「黃老師」身上我學到如何從生活中看到不同研究的題材，並思考應用在自己研究的可能性。黃老師也教會我動物實驗的技術，讓我之後可以獨力完成自己的題目。除此之外，他也常熱心地跟我分

享申請獎學金和出國留學相關的資訊，真的非常謝謝他。接著要感謝很多實驗室的前輩，他們總是願意在百忙之中抽空幫我解決問題。芳馨學姊有很棒的分生實驗知識，一開始教會我許多不同的分生實驗技術，之後碰到問題時也願意理解並花時間教我如何解決。另外學姊也會關心我們平常的生活，經常會有意外的小驚喜，真的很溫馨。嘉隆學長－「隆哥」，雖然表面上看起來有點難親近，但後來發現找他幫忙，絕對是用心幫到最後。可能因為題目跟隆哥比較接近，很常找他討論研究方向，他總不厭煩聽我的想法，也會適時給我一些建議，真的很謝謝！好險隆哥今年初才離開，否則我們就遇不到傳承老師技術的元老了。家祥學長是我們的偶像。學長不只學識淵博、做實驗厲害，還非常謙虛而且總是不厭煩的幫我們解決問題：記得有一次學長在我報告完後，主動找我討論，真的很感動。怡陵學姐也總默默的關心我，仔細聽我實驗遇到的困難並給我一些建議。在研究題目進行到最後的階段，很感謝有學姊幫助我完成神經細胞培養，非常認真地幫我想辦法證明細胞凋亡的假說。雖然不善於溝通但能感受到她的關心，真的很感謝！原本和我們一起進入碩一，現在卻變成博班學長的皓元。原本跟我們一樣什麼都不懂，現在已經能獨當一面，動物實驗非常熟練且傑出。遇到問題時會第一個問他，可能因為我們算是同屆，請他幫忙比較不會有壓力，而他也總義不容辭。很謝謝他不管是碩班時還是博班後，總陪我們一起經歷各種關卡。

接著是同屆一起努力打拚的夥伴們。竣弘是聰明王也是遊戲王，我總是非常佩服他做實驗思考周全可以同時完成好多事，也不會被一時的失敗給打倒，會一直想解決方法。有一陣子我 cloning 一直卡關，他陪我一起想解決方法，也讓我從他身上學到做實驗的態度。原本跟蕾立比較少有接觸的機會，一起出國後多了很多相處的時間，也發現他是個很細心，很為別人著想的人。很謝謝他聽我講很多心事，而且總能給我一些鼓勵。他也當了畢業負責人，為班上默默做了很多事，在大家忙著寫論文時，常看到他還要花心思擔憂這些公共事務，真的覺得非常感謝。以寧是個各方面都非常周到的人，很善良，不管什麼事總先為大家著想，也很謹

慎，是個很棒的學習對象。雖然沒有和大家坐在同一間休息室，但總能感受到他的溫暖，期望我們都可以順利申請上好學校!以後我去德國找你玩你也來美國找我玩~最後是晚半年加入我們的劉臻。可能因為坐在我旁邊，我們的個性又都很大剌剌、不拘小節，所以變成很好的朋友。雖然我們還有另一個很頭痛的共通點，我們實驗好像常常不是那麼順利，總會碰到離奇的事情，但我相信我們那麼努力過一定會有很棒的結果。很謝謝你總是聽我抱怨、包容我的雷，也給我很多歡笑，期待美國行，我們一起征服美西。

除了實驗室的夥伴，生活中還好有很多永遠支持我的朋友和家人。首先要感謝我的爸爸媽媽，給我很好的成長環境，也支持我的每一個選擇，雖然我總是隔很久才回家，常常你們問我:我再從事什麼研究，我也很難解釋給你們聽，但你們絕對是我最大的支柱! 再來要感謝我的好室友 YT，謝謝你這兩年忍耐包容我的情緒，每次在實驗室遇到什麼挫敗，我總會在宿舍發洩，還好你總是聽我說個不停並給我很多鼓勵和建議，也願意說走就走，讓我可以跳脫實驗室，轉換心情，重新出發。總之真的很謝謝念碩班時能遇到這麼好的室友，未來能成為一輩子的朋友真是我的幸運。最後最後要感謝大學時期最好的朋友培雯，我們在生活、課業上總是非常有默契，常常不用說你就能知道我在想什麼。你不只像陪我聊心事的好姊妹，更像家人一樣全心的關心我，跟你相處總是可以感受到快樂和無限的正能量，生活中有你真是幸福，謝謝你。

Abstract

Background and Aims: Kinesin-binding protein (KBP; KIF1BP; KIAA1279) functions as a regulator of a subset of kinesins, many of which play important roles in neural development. Previous studies have shown that KBP is expressed in nearly all tissue with cytoplasmic localization. Autosomal recessive mutations in KIAA1279 cause a rare neurological disorder, Goldberg-Shprintzen syndrome (GOSHS), characterized by microcephaly, polymicrogyria, intellectual disability, axonal neuropathy, thin corpus callosum and peripheral neuropathy. Most KIAA1279 mutations found in GOSHS patients are homozygous nonsense mutations that result in KBP loss-of-function. However, the mechanism of KBP dysfunction in causing these defects remains not fully understood. Our aim is to identify the function of KBP in cortical development and its relationship to GOSHS.

Hypothesis: We hypothesized that KBP knockdown may cause neuronal migration defect.

Materials and Methods: We used *in utero* electroporation (IUE) to express KBP shRNA with green fluorescent protein (GFP) in neural progenitor cells in embryonic day (E) 14 mice and collected brain slices at different developmental stages. By immunostaining of neuronal lineage markers, we identified the fate of cells of our interest.

Results: We found that KBP knockdown did not affect neural differentiation process. However, four days after IUE, KBP knockdown led to many cells located in the

intermediate zone (IZ) where neural progenitors undergo multipolar-bipolar transition before they start radial migration. Moreover, at postnatal day (P) 6, about one thirds of the cells, which have become mature neurons, remained ectopically in the white matter (WM). For those cells that have reached Layer II/III of the cortex, dendrite outgrowth and axon projection was impaired. Besides, we also found KBP knockdown induces apoptosis during the postnatal period.

Conclusions: Our findings indicate that loss of KBP function leads to defects in neuronal migration, morphogenesis, maturation and survival, which may be responsible for brain phenotypes observed in GOSHS.

摘要

背景與目標：驅動蛋白結合蛋白 （KBP; KIAA1279)具有調控特定驅動蛋白的功能，而已知驅動蛋白在神經發育中扮演重要角色。KBP 產生同合子無義突變可能導致嚴重的神經疾病－Goldberg-Shprintzen 症候群 （GOSHS)。這種疾病患者主要有小頭畸形、多小腦回、智能障礙和軸突病變等病徵。然而，KBP 突變造成疾病的分子機制尚未清楚了解。我的研究目標是探討 KBP 在大腦皮質發育中所扮演的角色以及導致 GOSHS 疾病的可能原因。

假設：我們推測抑制 KBP 表現量會造成大腦皮質神經細胞遷徙異常。

材料與方法：我們利用子宮內電穿孔技術將 KBP 的小髮夾 RNA 以及帶有綠色螢光蛋白的質體同時表現於小鼠胚胎的神經幹細胞中，並收集不同發育時期的大腦組織切片進行神經標記物免疫染色。

結果：當 KBP 的表現量被抑制時，皮質發育中的神經細胞遷徙受到延遲，但神經幹細胞分化的能力並未受到影響。出生後六天雖然仍有三分之一已分化完成的神經細胞異常分布在白質，大約一半的神經細胞已遷徙至皮質第二、三層。然而這些錐體神經細胞的樹突和軸突皆無法正常發育及生成。我們推測神經細胞遷徙受到延遲的可能原因是神精細胞往皮質遷徙之前，從多極變成雙極性的型態轉換過程受度阻礙。此外，我們也發現 KBP 缺失會促進細胞凋亡。

結論：當 KBP 無法正常表先功能時，會影響神經細胞的遷徙、型態、成熟過程及存亡。我們的發現可幫助釐清造成 Goldberg-Shprintzen 症候群大腦病變的原因。

Chapter 1: Introduction

1.1 Early development of the cerebral cortex

Neural development of the vertebrates starts with neurulation from the ectoderm at early embryonic stage and encompasses a series of complex processes to form the most highly organized organ (Jiang and Nardelli, 2016). During this process, the neural plate folds into the neural tube, which eventually forms the central nervous system. The anterior part of the neural tube then enlarges to form the forebrain, midbrain and hindbrain in the early developmental stage. The forebrain then separates into the diencephalon and the telencephalon, which develops into the cerebrum. Cerebral cortex in mammals differentiated from the dorsal telencephalon finally develops into a six-layered architecture responsible for highly cognitive functions (Kawauchi et al., 2013).

In the early brain development, neuroepithelial cells (NECs), which expand between the ventricular surface and the pial lamina of the neural tube, form a layer of pseudostratified epithelium. NECs then turn into radial glial cells (RGCs), which gradually extend the basal process to keep contact with the pial surface as the cerebral cortex thickens (Florio and Huttner, 2014; Sun and Hevner, 2014). These cells keep self- renewing by symmetric division to expand the pool of progenitors (Hansen et al., 2010; Jiang and Nardelli, 2016). Later on, RGCs also undergo asymmetric divisions to produce neurons, intermediate progenitors (IPCs) or glia cells. During proliferation, NECs and RGCs undergo a special cell cycle-dependent nuclear oscillation termed interkinetic nuclear migration (INM). In this process, the nuclei stay at the basal side during G1 and S phases, move to the ventricular surface for mitosis, and then return to

basal surface during G2 phase (Kosodo, 2012; Taverna and Huttner, 2010).

Postmitotic neurons directly from RGCs then start a series morphogenesis and migration program while IPCs divide a few more times in the subventricular zone (SVZ) before neuronal migration (Campbell and Gotz, 2002; Florio and Huttner, 2014; Kriegstein et al., 2006; Kriegstein and Noctor, 2004).

1.2 Migration and maturation of cortical projection neurons

Neuronal migration is a complex process encompassed by a variety of intracellular and extracellular events of changes in cell shape, polarity, motility and maturity (Jiang and Nardelli, 2016; Kawauchi et al., 2013; Kon et al., 2017). During the development of the neocortex, pyramidal projection neurons, which comprise 80-90% of the cortical neurons, go through a dynamic radial migration pathway. The immature neurons generated from RGCs first appear as multipolar cells, which extend and retract processes in different directions. At this stage, these cells also generate an axon that extends within the lower IZ, which becomes the future WM. The cells then extend a pia-directed leading process, exhibiting a bipolar morphology, and migrate along radial glia fibers of RGCs toward the CP. These neurons then translocate to the final position and gradually mature into pyramidal projection neurons (Arikkath, 2012; Cooper, 2014; Hatanaka et al., 2004).

The maturation process of projection neurons starts with the outgrowth of dendrites and axons, which represent information inputs and outputs of neurons, respectively. Morphology of mature pyramidal neuron is characterized by long apical and short basal dendrites from the soma with multiple branches. The dendritic arborization determines

the field of information reception. The postsynaptic complex develops along the dendrites in the form of dendritic spines, followed by neuronal connections and neural circuit development. Meanwhile, the long axon projects to other brain regions during neural maturation, guided by the growth cone to sense the guidance cues for pathfinding and target recognition. Finally, experience- and environment-dependent synapse pruning and stabilization provide a plastic neural network to support highly orchestrated functions of the brain (Arikkath, 2012; Jiang and Nardelli, 2016; Wu et al., 1999).

1.3 Malformations of cortical development

Disruption in key steps of the neural development may result in malformations of cortical development (MCD) (Jiang and Nardelli, 2016; Lu et al., 2018; Pang et al., 2008). MCD represents a major cause of developmental disorders, which include diverse clinical presentations. It can be classified by defects at different stages in the cortical development. For example, disorders resulted from the abnormalities of the cell proliferation may cause microcephaly, macrocephaly or cortical dysplasia; disorders of neuronal migration and cortical layer organization may result in lissencephaly, subcortical heterotopia, periventricular heterotopia and polymicrogyria (Spalice et al., 2009). However, cortical development is an interlinked dynamic process, and thus some MCDs with multiple dysfunctions were difficult to classify.

So far, more than 100 genes have been identified to be associated with MCDs (Andrade, 2009; Barkovich et al., 2012; Kato, 2015). These genes play roles in different biological pathways including cell-cycle regulation, cell-fate specification, cytoskeleton function, cell adhesion, metabolism, and apoptosis. For example, LIS1 and DCX both playing

roles in microtubule-based behavior are main causing genes of lissencephaly (Vallee and Tsai, 2006); Tubulin- associated genes, TUBA1A, TUBB2B, TUBB3 and TUBB5 are related to many cortical disorders such as polymicrogyria, microcephaly, subcortical heterotopia and lissencephaly ; centrosome and cell cycle-related genes, MCPH1, ASPM and CEP152 are microcephaly-related genes (Gilmore and Walsh, 2013); many genes involved in mTOR pathway related to cell metabolism are causing genes of focal cortical dysplasia and other MCD disorders (Lim et al., 2015).

1.4 Goldberg-Shprintzen syndrome (GOSHS)

Goldberg-Shprintzen syndrome (OMIM 609460) (GOSHS) is a rare congenital anomaly syndrome associated with Hirschsprung's disease, which is characterized by multiple dysfunctions in both central nervous system (CNS) and peripheral nervous system (PNS) (Hirst et al., 2017). It is an autosomal recessive disorder first described by Goldberg and Shprintzenin in 1981 (Goldberg and Shprintzen, 1981). In 2005, Brooks et al. identified *KIAA1279* located on chromosome 10q21.1. as the disease-causing gene by homozygosity mapping in a large consanguineous family (Brooks et al., 2005). So far, seven different *KIAA1279* mutations have been reported in GOSHS patients and most of them are homozygous nonsense mutations (Salehpour et al., 2017).

Classical characteristics of GOSHS are intellectual disability, microcephaly, dysmorphic facial, and Hirschsprung disease. However, different mutations on *KIAA1279* may also show distinct phenotypes (Drevillon et al., 2013; Salehpour et al., 2017). For example, a case reported in 2013 implicated a GOSHS patient with polymicrogyria and thin corpus callosum (Valence et al., 2013). Therefore, this syndrome revealed diverse dysfunctions.

1.5 Molecular functions of kinesin binding protein (KBP)

KIAA1279 gene produces the Kinesin binding protein (KBP) containing 621 amino acids with predicted molecular weight of 71 kDa. KBP has two tetratricopeptide repeats (TPRs) protein-functional domains that mediate protein-protein interactions (Blatch and Lassle, 1999; Drevillon et al., 2013). KBP express in various human tissue. KBP is also named KIF1-binding protein (KIF1BP), since it was first discovered to interact with kinesin-like proteins KIF1C by yeast two-hybrid assay and was found to be associated with other kinesin-3 family members (Wozniak et al., 2005).

KBP shows diverse subcellular localization in different cell types and conditions. In NIH3T3 cells, KBP mainly localized to mitochondria; however, it is neither a mitochondrial receptor nor adapter protein (Wozniak et al., 2005). In mouse neuroblastoma cell line, N1E-115, and zebrafish, KBP was shown to interact with SCG10, a neuron-specific microtubule destabilizing protein (Alves et al., 2010). It is also demonstrated that in human fibroblasts, KBP interacts with microtubule and actin cytoskeleton and may function as an actin microtubule cross-link protein (Drevillon et al., 2013).

Recently, Josta T. Kevenaar et al. used pull-down assays combined with mass spectrometry to identify additional KBP-binding partners. They indicated that KBP binds to motor domain of specific subset of kinesin family proteins such as KIF1A, KIF1B, KIF1C, KIF3A, KIF13B, KIF14, KIF15 and KIF18A (Kevenaar et al., 2016). They also indicated that KBP inhibits kinesin motility by preventing microtubule binding and modulates neuronal cargo transport and microtubule dynamics (Kevenaar

et al., 2016). These studies provided insights into the molecular functions of KBP in regulating kinesins and the cytoskeleton.

1.6 Roles of KBP in neural development

In spite of the widespread expression of KBP, primary defects of GOSHS is about the development of neurons and neural crest-derived tissues (Hirst et al., 2017). Previous studies have using cell lines, zebrafish and mouse model to identify essential roles of KBP in neural development.

For neurite outgrowth, knockdown of KBP decreased neurite length in PC12 cells and SH-SY5Y (human neuroblastoma) cells. Overexpression of KBP increased neurite length in SH-SY5Y cells.(Alves et al., 2010; Drevillon et al., 2013; Kevenaar et al., 2016). For axon extension, overexpression of KBP reduced axon length in cultured mouse hippocampal neurons and KBP mutants disrupted axonal microtubules and thus defected axon outgrowth and maintenance in zebrafishes (Lyons et al., 2008) Kevenaar et al., 2016.

In addition, KBP, as a kinesin regulator, is important to assure correct function of kinesins, which play a fundamental role for neural development by controlling cargo transport and microtubule organization. Therefore, misregulation of kinesins as a result of KBP dysfunction may lead to abnormal neural function (Kevenaar et al., 2016).

Last year, a research team in Australia created KBP knockout mice by CRISPR-Cas9 editing to investigate defects of KBP loss in the peripheral and central nervous system. Mice lacking KBP underwent perinatal death and exhibited smaller brain, olfactory

bulbs and anterior commissures, as well as defects in vegal and sympathetic innervation of the gut (Hirst et al., 2017). This study focused on in vivo role of KBP in axon extension. Although study of KBP mutant zebrafish did not show Hirschsprung disease-like phenotype as GOSHS, KBP $^{-/-}$ mice showed delay in the colonization of the gut, which is related to Hirschsprung disease. (Hirst et al., 2017; Lyons et al., 2008).

1.7 Significance

So far, studies including molecular mechanism and neural development had identified essential functions of KBP by cell lines, zebrafish and KBP knockout mice. However most of the models were not precise enough to present roles of KBP in cortical development. By using a cutting-edged technology, *in utero* electroporation, we directly investigate KBP functions in mouse cerebral cortex and demonstrate that KBP is required for neuronal migration, maturation, morphogenesis and cell survive. Moreover, our results bring us to look more deep insight to the cellular mechanisms of KBP-dependent cortical dysfunctions in GOSHS.

Chapter 2: Materials and Methods

2.1 Constructs

Knockdown of KBP by IUE was achieved by using PGPU6/GFP/Neo vectors (BIOTOOLS CO., Ltd.) encoding shKBP target sequence (GCTCAAGTCTACCAGCACATG), or shCtrl (TTCTCCGAACGTGTCACGT) as negative control. For shRNA virus construction, shKBP target sequence was cloned into

PLKO TRC011 vector by following Academia Sinica RNAi core annealing method. The US2-GFP construct used to co-electroporate with shKBP is a generous gift from Dr. Jenn-Ya Yu, National Yang-Ming University.

For KBP expression in the cell lines, Mouse 2510003E04RIK ORF mammalian expression plasmid, C-GFPSpark tag (Sino Biological Inc.; Catalog Number: MG51968-ACG; RefSeq: NM_02817.2) was used.

For expressing KBP in the animal model, KBP-cDNA (NM_02817.2) was digested from Mouse 2510003E04RIK ORF mammalian expression plasmid and inserted into the PCIG2-IRES-GFP vector by using Xhol1 and EcoR1 restriction enzymes. HA was tagged at the N-terminus. PCIG2-IRES-GFP construct containing CAG promoter is a generous gift from Dr. Olivier Ayrault, Institute of Curie.

For knockdown efficiency test, HEK293 cells derived from human embryonic kidney cells were used because of their propensity for transfection. We cultured HEK293 cells in 6cm dishes with $1x10^6$ seeding densities, and then co-transfected KBP-cDNA and shKBP plasmids by a ratio of 1:9 into cells after 8 hours of passaging cells, and cells were harvested 48 hours after transfection. Transfection was carried out using the Lipofectamine 3000 reagent (Invitrogen).

For analysis of neuronal morphology and in vitro RNAi assay, cultured cortical neurons were isolated from E14.5 ICR mice. Cultured plates were coating with poly-D-lysine and incubated at 37℃ overnight. After washed two times in distilled deionized water (ddw), each cultured plates were incubated with HBSS and 5% Matrigel at 4℃ and then incubated at 37℃ overnight. Cortex and meninges dissection were performed in

the dishes containing HBSS and the dissected cortical tissues were placed in HBSS. Discarded the flow-through and dissociated tissues in papain solution (prepared papain vial: 5 ml EBSS to papain vial; o.5 ml EBSS to DNAse vial and add 0.25 ml to the prepared papain vial), and then incubated at 37℃ for 15 minutes. Collected the supernatant and centrifuged at 4℃, 1100g for 5 min. Discarded the flow-through and re-suspensed tissues in the re-suspension medium (3.6 ml cultured medium, 0.4ml albumin-ovomucoid mixture, 0.2ml DNAse solution). Cells were seeded on 3.5 cm plates with 1.25 x10^6 seeding densities, and exchanged cultured medium on the next day. Lentivirus carrying shKBP target sequence were produced by Academia Sinica RNAi core and were infected into neurons at DIV3 according to manufacturer's protocol. Cultured cortical neurons were harvested at DIV7 and DIV9.

Harvested cells were lysed in RIPA buffer (50 Mm Tris-HCl, pH 8.0, 150 Mm NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with 10% protease inhibitor and 10% phosphatase inhibitor (Sigma Aldrich).

2.2 Electrophoresis and Western Blotting

Protein concentration was determined by BCA protein assay kit. Protein samples (from HEK293 cells: 40μg/lane; primary cortical neurons: 40μg/lane; mouse brain lysate: 60μg/lane) were separated on 4-20% Gradient gel SDS-PAGE (Tools Biotechnology) and then transfer to polyvinylidene difluoride (PVDF) membrane (Millipore) with a transfer apparatus (Bio-red). PVDF membranes were incubated in 5% milk and 1% BSA in 0.1%TTBS buffer (0.1% TWEEN20 in TBS buffer). Membranes were then incubated in primary antibodies overnight at 4℃. Primary antibodies including anti-KBP antibody (Proteintech, Catalog Number: 25653-1-AP, 1:500), anti-GFP antibody

(Abcam, 1:5000), anti-HA antibody (Proteintech, 1:500), anti-Pro-Casepase3 antibody (1:1000), anti-Cleaved-Casepase3 antibody (1:500), anti-MAP2 antibody (GeneTex, 1:100) anti-tubulin alpha antibody (Proteintech, 1:5000) and anti-beta-actin antibody (Proteintech, 1:5000). The immunoblots were then washed in 0.1% TTBS buffer for 10 minutes three times and then immersed in the second antibody solution including goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP (Santa Cruz) for two hours. The immunoblotted proteins were visualized by an enhanced chemiluminescence ECL reagent (Santa Cruz) and detected by Luminescence Imaging system LAS-4000 (Fujifilm). At last, we will quantify the signal by Image-J.

2.3 *In utero* electroporation (IUE)

Pregnant ICR (BioLASCO) mouse at E14.5 were undergone surgery, and were anesthetized by Isoflurane. The abdominal area was shaved and cleaned with alcohol. And then we made an incision around 3cm through the skin and abdominal muscle to expose the underlying viscera. The uterine horns will be carefully externalized. 1 μl solution containing DNA plasmid (1-5μg/μl) with Fast green (2.5 mg/ml, Sigma) were injected into lateral ventricle of the brain and electroporated by forceps electrodes (5 mm in diameter; Harvard Apparatus). Forceps electrodes provided five electric pulses separated by 450 ms in a voltage of 40V. The uterine horns were put back into the abdominal cavity and the incision closed.

The embryos were harvested four days after electroporation or at postnatal day six and 12 for further experiments. Embryos and infant mice were transcranial perfused by PBS and fixed by 4% PFA. Brains were collected immediately and immersed in 4% PFA for 12 hours as post-fixation and then removed into PBS.

2.4 Immunofluorescence staining

For brain slice staining, fixed brains were embedded in 4% low-melting-agarose (Amresco, USA) dissolved in PBS and sectioned coronally on a Vibratome (Leica) for 100 μm in each slices and storaged in the PBS with 0.05% sodium azide (Sigma) at 4℃. Brain slices were incubated in the blocking buffer (10% normal goat serum, 5% BSA and 0.2% Triton X-100 in PBS) for 1 hour shaking at room temperature, followed by placing in the primary antibodies over two nights at 4℃. After primary antibodies incubation, slices were washed in PBS ten minutes for three times and then incubated with the secondary antibodies for 2 hours at room temperature.

For cultured neurons staining, cells were fixed in 4% PFA in PBS and incubated at 37℃ for 15 minutes, and then placed in o.1% TritonX-100 diluted in PBS for permeabilization. After incubated in blocking buffer (5% normal goat serum, 5% BSA and 0.1% Triton X-100 in PBS) for 30 minutes at room temperature, cells were placed in the primary antibodies for two hours at 4℃ followed by washed in PBS and incubated with the secondary antibody for one hours at room temperature.The primary antibodies used for immunostaining were: rabbit anti-Pax6 (BioLegend, 1:500), rabbit anti-ki67 (Millipore, 1:600), rabbit anti-NeuN (Millipore, 1:500), rabbit anti-cux1 (Santa Cruz, 1:200), rabbit anti-Tbr1 (1:500), anti-beta III Tubulin antibody (abcam, 1:1500), mouse anti-MAP2 antibody (GeneTex, 1:100). The goat anti-rabbit or anti-mouse secondary antibodies used for immunofluorescence staining were conjugated with Alexa flour 546 (1:500), and 647 (1:500) (Thermo Fisher Scientific). At last, slices were counterstained with 0.5μg/ml 4’,6- diamidino-2-phenylindole (DAPI) (Molecular Probes) for one hour and mounted by VECTASHIELD mounting media before sealing

the slides.

2.5 TUNEL assay

TUNEL staining was carried out using Cell Death Detection kit (Roche, TMR red, #12156792910). Cultured cortical neurons were wash in PBS once followed by fixing in 4% PFA dissolved in PBS and incubated at 37℃ for 15 minutes and then wash in PBS three times. Cell slides were then incubated with TUNEL solution followed the manufacturer's instructions for staining.

2.6 Image analysis

All images were collected with a four laser, point scanning confocal microscopy (Zeiss LSM 700), and analyzed with ZEN software (Zeiss). ImageJ software was used to quantified cells distribution in the cortex.

NeuromanticV1_6_3 (Yale. edu) software was used to trace cell bodies and apical dendrites extending from the cortical layer II/III pyramidal neurons expressing GFP (P6). Tracing images were converted by NEURON 7.5 documentation and processed by CorelDRAW X3 software.

For analysis of neuronal morphology, cultured cortical neurons at DIV7 and DIV9 expressing GFP were randomly selected and analyzed semi-automatically with a customized Matlab program. Circles of increasing radii (25μm, 50μm, 75μm) are manually centered over the cell soma by sholl analysis.

2.7 Statistical analysis

Statistics were presented as mean ± SEM. Student's t-test was used to compare difference between two experimental conditions. All statistical tests were two sided. $P < 0.05$ was considered statistically significant.

Chapter 3: Results

3.1 KBP knockdown affects neuronal migration but not neural differentiation process

To explore possible involvement of KBP in cortical development, we first examined whether KBP is expressed in the developing brain. We collected mouse cortices and extracted protein lysates at different embryonic stages between E14 and E18, during which time neurogenesis and neuronal migration are most active. By western blot analysis for cortical protein lysates, we confirmed that KBP is expressed in the developing mouse cortex at all stages examined between E14 and E18 (Fig. 1A).

To identify the function of KBP in cortical development, we next investigated the effects of KBP loss of function by RNA interference (RNAi) of KBP in the developing brain. Therefore, we constructed 4 short hairpin RNA (shRNA) constructs targeting KBP coding region to knockdown KBP expression (referred as shKBP#1-4). To screen the best shRNA sequence for KBP knockdown, cultured HEK293 cells were co-transfected with shKBP or a negative control construct (shCtrl) along with a GFP-tagged KBP (GFP-KBP). Forty-eight hours after transfection, cell lysates were

collected and analyzed by western blotting with anti-GFP antibody. Among all 4 constructs, shKBP#4 showed the best knockdown efficiency at ~70% (Fig. 1B); thus we used this construct (referred as shKBP hereafter) for following experiments.

Since most of our experiments would be done in the mouse brain, we further verified knockdown efficiency of shKBP in cultured cortical neurons. Due to the low efficiency of liposome-based transfection in neurons, we inserted the shRNA sequence into a lentiviral vector and packaged the construct into lentiviral particles. Cultured cortical neurons from E14.5 ICR mice were infected with these viruses and lysed after 4 days for western blot analysis by KBP antibody. Consistent with previous results in HEK293 cells, we observed a similar knockdown effect at ~60% efficiency (Fig. 1C).

We then examined the effects of KBP dysfunction on the developmental processes of the cortex by IUE of KBP shRNA into neural progenitor cells. Mouse embryonic brains at E14.5 were co-electroporated with shKBP or shCtrl along with US2-GFP construct, which expresses GFP to label transfected cells (Fig. 2A, B). Four days after IUE, brains were collected, sliced, and stained with the cortical layer marker Tbr1, which is localized to cortical layers V-VI. We found that the majority of GFP+ shCtrl-transfected cells were found to localize above layer V-VI marked by Tbr1 (77.8 ± 4.3 %, n = 4 animals), indicating that these cells had migrated and reached the upper CP. In contrast, most of the GFP+ shKBP-transfected cells were located in the IZ (68.0 ± 3.9 %, n = 8 animals) beneath the Tbr1+ layers and only ~20% of the cells had reached the CP (20.1 ± 4.0 %). This result demonstrated that KBP knockdown altered neural progenitor cell distribution, which can result from defects in neural progenitor cell proliferation, differentiation, and/or migration.

To further determine which developmental stage that KBP affects to cause abnormal distribution of neural progenitor cells, we examined the identity of cells located in the IZ. By immunostaining of markers for neural progenitor cells Pax6 and early differentiated neurons Tuj1, we found that most of GFP+ shKBP-transfected cells were Tuj1 positive and Pax6 negative (Fig. 2C, D), indicating that KBP-knockdown cells abnormally located in the IZ have started to differentiate into neurons as in controls. This result showed that the altered cell distribution in the E18.5 cerebral cortex by KBP knockdown may not result from defects in neural progenitor cell differentiation.

3.2 KBP knockdown delays neuronal migration until P6 while arrested cells have become mature neurons

Cerebral cortex is divided into VZ, SVZ, IZ and CP during the early cortical development, which takes place in the embryonic stage. While in the late cortical development which occur during the postnatal period, VZ, SVZ and IZ are replaced by WM and the CP is divided into six layers.

To investigate whether cells located in the IZ at E18.5 in the KBP knockdown brains would catch up the normal migrating process in the late cortical development stage, we collected P6 brain slices after electroporation at E14.5. In control brains electroporated with shCtrl, the majority of electroporated cells were found in layer II/III labeled by Cux1 (92.4 ± 3.2 %; n = 4 animals). In KBP knockdown group, about one third (34.3 ± 4.9%) of GFP+ cells remained ectopically in the WM although half of GFP+ cells (47.7 ± 5.6 %) had reached cortical layer II/III (Fig. 3A, B). Interestingly, some (18.1 ± 0.7 %) cells appearing as migrating neurons below layer II/III in the KBP knockdown brains extended long leading processes (Fig. 3A), suggesting abnormalities in neuronal

migration.

To characterize the cell identity of these ectopically located cells in the WM, we stained the brain slices with differentiated post-mitotic neuronal cells marker NeuN (Fig. 3C). In the control brain, the majority of the cells had reached the cortex and were all NeuN+ (NeuN+ cells/ total GFP+ cells in the CP: 100 ± 0%; *n*=3). Surprisingly, most of the cells located in the WM of KBP knockdown brains were also NeuN+ (NeuN+ cells / total GFP+ cells in the WM: 88.5 ± 2.9%; *n*=3. Fig. 3D).

3.3 KBP knockdown impairs cortical neurons morphology in both dendritic arborization and axon projection

Even though nearly half of KBP knockdown neurons had migrated toward cortical layer II/III as in normal brain, we observed abnormalities in neurite morphology of these neurons. To analyze the neurite structures of these neurons, we reconstructed 3D images of GFP+ neurons from confocal image stacks by manual tracing and showed each processes extending from the cell soma (Fig. 4A, B). Prominent apical dendrite and its branches were easily seen for comparison. We found that, while normal layer II/III pyramidal neurons exhibited a well-developed dendritic “tree” with multiple branches, KBP knockdown neurons contained few branches extending from the apical dendrite.

In addition to hypoplastic dendrites, we also examined the change of callosal axon projection originated from the layer II/III pyramidal neurons. Callosal axon bundles are the main composition of the corpus callosum, which connects two hemispheres of the cortex by axon projection from one side of the cortex to the contralateral side. Callosal axon projections in the mouse develop during the prenatal and early postnatal periods;

therefore, we collected brains at P6 and P12 after *in utero* electroporation of GFP to label the axonal projections at E14.5 (Fig 4C). In the control coronal brain sections, we observed axons projected down toward the WM, crossed the midline and extended through the contralateral cortex at P6. Axonal tracts extended to the contralateral cortex even further at P12. On the contrary, in the KBP knockdown brain sections, we found that axons only projected down to the WM and failed to reach the midline at P6. Moreover, the impaired callosal axon projections was even more severe at P12 showing scarce axonal projections in the WM.

3.4 In vitro quantification of the neuritic complexity

To further illustrate the altered neuron morphology by KBP knockdown, we cultured cortical neurons from E14.5 ICR mouse, the same developmental stage of the IUE experiments. We then introduced lentivirus carrying shCtrl or shKBP as well as GFP into cultured neurons at DIV3. The cells were then fixed at DIV7 or DIV9 (Fig. 5A) and immunostained with neuron-specific marker MAP2. GFP+ cells co-localized with MAP2 were identified as cortical neurons for further analysis. We then took images from single neurons selected randomly from each slides and quantified neuritic complexity by sholl analysis. Neurite intersections with circles of 25, 50, and 75 μm radii were plotted for analyzing each selected neuron (Fig. 5B). The analysis showed reduced numbers of intersections from proximal to distal of dendrites in the KBP knockdown group both at DIV7 and DIV9. These results revealed KBP may be required for dendritic branches formation (Fig. 5C).

3.5 KBP knockdown induces cell apoptosis

When collecting brain sections at P12, we also found an apparent decrease in the number of total GFP+ cells in the KBP knockdown group (Fig. 6A). This observation led us to speculating that loss of KBP may lead to cells apoptosis since the initial number of GFP+ cells did not appear to be affected. Therefore, we used western blot analysis and TUNEL assay to confirm and quantify the apoptosis effect in the cultured cortical neurons. Cortical neurons were isolated from E14.5 days and introduced with shKBP or shCtrl lentiviruses at DIV3 for 24h. Protein levels of Caspase-3, which is cleaved during apoptosis, were detected at DIV7 and DIV9. Western blot analysis showed that relative levels of cleaved Caspase-3 to procapase-3 at DIV7 and DIV9 both increase in the KBP knockdown group. Furthermore, the ratio becomes larger as the growing days of neurons increase (Fig 6B, C). Moreover, we did the TUNEL assay staining in DIV9 cells and found significant increase of TUNEL positive cells in the KBP knockdown group (Fig. 6D, E). This result revealed that loss of KBP leads to progress cell death as the development progresses.

Chapter 4: Discussion

In our study, we have provided sufficient evidences to show that loss of KBP leads to multiple dysfunction in the cerebral cortical development prior to synaptogenesis including delay of neuronal radial migration, disruption of neurons morphological maturation, and induction of programmed cell death (Fig. 7). All these results we found in the mouse model show high correlations to clinical phenotypes in GOSHS.

First, we had seen migration defect in KBP knockdown brains 4 days after *in utero*

electroporation until P6, this phenotype can relate to polymicrogyria (PMG) in GOSHS patients. PMG characterized by numerous small, unfolding gyri and disorganized lamination is a significant pathology found in GOSHS patients (Kato, 2015; Squier and Jansen, 2014; Valence et al., 2013). Although it's hard to represent the abnormal gyri formation in the mouse model, migration defects can indirectly reveal the PMG manifestation. Since studies had established that main causes of PMG are impaired migration of neuroblasts in the early development stage and disrupted post-migrational maturation in the late development stage. A study of *TUBB2B* mutation resulting in PMG shows migration defects after *in utero* knockdown of TUBB2B expression in rat which is similar to our phenotype when we knockdown KBP in mouse (Jaglin et al., 2009). Moreover, our study shows mature neurons arrested in the WM of P6 KBP knockdown mouse brain, which is also similar to pathology found in PMG patient (Valence et al., 2013). By magnetic resonance imaging, the paper revealed the presence of radial columnar heterotopic neurons in the WM of PMG patients.

Secondly, to see the altered of pyramidal neurons dendritic outgrowth, it can reflect GOSHS patients with intellectual disability. Dendrites, as integrators of synaptic information in neurons play crucial roles in brain cognitive function. Dendritic arborization involving dendrite extension, addition, elongation, retraction and pruning is a critical process to determines the synaptic input field (Arikkath, 2012). Therefore, aberrant dendrite morphogenesis such as reduce of dendritic branching we found in KBP knockdown neurons may cause human diseases related to mental retardation.

Besides the altered of neuronal information input, axon representing information output is also impaired in our KBP study and this result can somehow explain the thin corpus callosum formation in GOSHS patients (Valence et al., 2013). The corpus callosum is

the major forebrain commissure which connecting right and left cerebral cortex. The corpus callosum is associated with cognitive abilities and many medical conditions such as seizures and schizencephaly. Some researches indicates that defects in midline crossing of callosal axons by guidepost cells is one of primary cause of corpus callosum agenesis (Gelot et al., 1998; Magnani et al., 2014); however, our results revealing axon extension defects in KBP knockdown brain may result in abnormal formation of corpus callosum through another mechanism.

Finally, as cell apoptosis we found in the comparatively late developmental stage, it may be responsible for the microcephaly phenotype in GOSHS. Microcephaly is a condition in which brain size is abnormal, and according to different pathogenic mechanisms of causing this disorder, it can be classified into primary and secondary two types (Gilmore and Walsh, 2013). For primary microcephaly, it presents at birth (congenital microcephaly) due to dysregulation of centrosomes or DNA-repair defects in causing abnormal proliferation of redial glia cells or cell fate changes in early cortical development. On the other hand, secondary microcephaly happens after birth (postnatal microcephaly) is associated with increased apoptosis (Nakayama et al., 2015). Accordingly, our results of cell apoptosis in KBP knockdown neurons may result from neuronal apoptosis rather than decreased proliferation of radial glia cells while we need to further confirm change in proliferation rate by Ki67 cell cycle marker staining with E16.5 (2 days after IUE) brain sections.

4.1 Potential mechanisms of KBP in neuronal migration

Our current results demonstrate that KBP knockdown lead to neuronal migration delay. This primary observation makes us think of possible molecular mechanisms of KBP in

neuronal migration that KBP may interact with kinesin motor proteins, KIF1A and KIF3A, which our group and others have identified to regulate neuronal migration previously (Baffet et al., 2015; Chen et al., 2018; Hu et al., 2013; Tsai et al., 2010). KIF1A was identified as basally direction regulator of INM, and KIF3A was also found to play a role in INM regulated by Gli2 and Cyclin D1 system.

However, according to cells distribution pattern in KBP knockdown brains, most of cells delayed in the IZ but not VZ and SVZ at E18.5. Therefore, we assert that KBP may not associated with KIF1A and KIF3A in the INM but plays a role in reorientation of immature projection neurons to transform from multipolar into bipolar form and exits the IZ for further radial migration.

Many researches have explored the molecules and mechanisms that regulate multipolar migration in the IZ before postmitotic neurons turning into bipolar form and exit the IZ (Cooper, 2014). Multipolar migration can be classified into three more precise stages, including initial multipolar migration of newborn neurons, axon formation, and leading process stabilization. These stages are possibly regulated by intrinsic and extrinsic signal while more evidences need to be provided. So far protein kinase, GTPases and cytoskeletal proteins are implicated in these signaling pathways and the following is one of the essential pathway. p27, a well-known cyclin-dependent kinase (CDK), is stabilized by Cdk5, another CDK, to increase the amount of activated cofilin, by inhibiting RhoA, a small GTPase (Kawauchi et al., 2006). Since cofilin is an actin-severing protein, this pathway is shown to be required for actin cytoskeleton reorganization to promote IZ exit in the process of neuronal migration. Despite of protein kinase and GTPases, cytoskeleton-related protein Kinesin6 is also found to regulate multipolar-bipolar transition by maintaining microtubules and concentrating

actin in the base of the leading process. (Falnikar et al., 2013; Lin et al., 2012). Furthermore, a neuron-specific protein SCG10, functioned as microtubules stabilizer when phosphorylating by Jnk1, c-Jun N-terminal kinase, inhibits microtubule reorganization for neuronal transition from multipolar to bipolar form (Grenningloh et al., 2004; Westerlund et al., 2011).

Looking back on our study, which pathway might be KBP involved in? First possibility is that KBP, as a kinesin binding protein, interacts with Kinesin6 to regulate leading process formation during the late multipolar migration. Although Kinesin6 was not detected in the previous research of screening a lot amount of KIF proteins as KBP-binding partners. (Kevenaar et al., 2016). In addition, since KBP has been found to interact with KIF1B to regulate SCG10 for axon extension (Drerup et al., 2016), it is proposed that KBP interacts with SCG10 to modulate multipolar-bipolar transition by regulating microtubule function. Alternatively, KBP is identified to interact with F-actin (Drevillon et al., 2013), which enhances the possibility that it function as cofilin activator for regulating neuronal migration process.

4.2 Proposed mechanisms of KBP in dendrite morphogenesis

In the recent decade, more intrinsic and extrinsic signaling pathways have been found to involved in dendritic arborization, while still many unknown molecular mechanisms need to be explored. The dendritic arborization process involves dendrite extension, retraction stabilization and pruning, which is regulated by multiple cellular molecules such as transcription factor, cell adhesion molecules, calcium signaling proteins and regulator of the actin cytoskeleton (Arikkath, 2012; Emoto, 2012). Previous studies indicated that KBP functioned as a cytoskeleton regulator (Kevenaar et al., 2016), and

thus we will further discuss mechanisms of KBP regulating dendrite morphogenesis through this pathway.

Cytoskeletons forms the primary dendrite structure; therefore, regulators of cytoskeleton play a crucial role in dendritic arborization. So far, small GTP binding proteins, Rac, Rho, and Cdc42 are found to mediate neurites extension and retraction by cooperating with cadherin—catenin cell adhesion complex (Elia et al., 2006; Konietzny et al., 2017). While it is known that δ-catenin, a component of the cadherin-catenin cell adhesion complex, regulates dendrites morphology through its upstream activator, erbin, it is probably not the only one regulating upstream of this pathway (Arikkath et al., 2008). Besides, there must be a variety of other unknown factors to control the actin cytoskeleton to further regulate dendritic arborization. KBP has high potential to be one of the factor involved in this pathway, since our results showed severely abnormal dendrites extension with few branches in postnatal mouse cerebral cortex.

4.3 Proposed roles of KBP in axon outgrowth and extension

Axon outgrowth involves protrusion, engorgement, and consolidation three stages which requires function of F-actin and microtubules to promote structural changes and locomotion (Dent et al., 2011). Therefore, regulating dynamic of these cytoskeletons and transporting vital components is necessary for axon outgrowth, extension and maintenance. One essential mechanism of regulating microtubule dynamic is through microtubule destabilizing proteins, SCG10, and this mechanism had been linked to KBP function by Catherine M. Drerup et al. recently. They used zebrafish to demonstrate that KBP interacts with KIF1B motor for transporting SCG10 to growth

cones and completing axon extension (Drerup et al., 2016).

However, KBP may also be involved in other molecular pathways to regulate axonal outgrowth. For example, interaction of KBP with F-actin may also regulate axon outgrowth. Accordingly, actin dynamics mainly play role in guidance instead of elongation in the axonal outgrowth (Dent et al., 2011). Our data shows that although KBP knockdown impaired axon projecting to the contralateral side, axon still can extend to the WM in P6 mouse brains. Thus, these views enhance the probability that KBP regulates axon outgrowth through F-actin pathway.

4.4 Potential mechanisms responsible for apoptosis found in KBP knockdown brains

Apoptosis is a form of programmed cell death that is important for tissue homeostasis. In nervous system, neuronal apoptosis occurs extensively not only during the pathology but during the development to establish correct population of neural progenitor cells, post-mitotic neurons and glial cells in the limited brain size. Neuronal apoptosis during development may induce by several ways such as axotomy, aberrant of cell-cycle re-entry, loss of connected neurons, oxidants, and microglial-mediated inflammation (Fricker et al., 2018; Kristiansen and Ham, 2014).

According to our KBP knockdown study, neuronal cell death occurs followed by dendritic and axonal outgrowth defects which disrupt neuronal function of contacting to targeting neurons both in input and output ways. Therefore, loss of connected neurons may be the mechanism of KBP-mediated neuronal apoptosis.

Previous study of KIF1A motor protein in mouse model showed that KIF1A mutant leads to neuronal degeneration and decrease in both densities of synaptic vesicles and synapses. Since removing afferent input of synaptic transmission can inhibit neuronal survival during the development stage, increased neuronal cell death might be caused by defects in synaptic function. In addition, using cultured hippocampal neurons, Kevenaar et al found that KBP regulates trafficking of axonal cargo Rab3, presynaptic regulator, by controlling KIF1A activity (Kevenaar et al., 2016). Overall, KBP may regulate neuronal apoptosis through interacting with KIF1A to transport vital molecules to synapses which is responsible for neuronal connection and survive.

Chapter 5: References

1. Alves, M.M., Burzynski, G., Delalande, J.M., Osinga, J., van der Goot, A., Dolga, A.M., de Graaff, E., Brooks, A.S., Metzger, M., Eisel, U.L*., et al.* (2010). KBP interacts with SCG10, linking Goldberg-Shprintzen syndrome to microtubule dynamics and neuronal differentiation. Human molecular genetics *19*, 3642-3651.
2. Andrade, D.M. (2009). Genetic basis in epilepsies caused by malformations of cortical development and in those with structurally normal brain. Human genetics *126*, 173-193.
3. Arikkath, J. (2012). Molecular mechanisms of dendrite morphogenesis. Frontiers in cellular neuroscience *6*.
4. Arikkath, J., Israely, I., Tao, Y., Mei, L., Liu, X., and Reichardt, L.F. (2008). Erbin controls dendritic morphogenesis by regulating localization of delta-catenin. The Journal of neuroscience *28*, 7047-7056.
5. Baffet, A.D., Hu, D.J., and Vallee, R.B. (2015). Cdk1 Activates Pre-mitotic Nuclear Envelope dynein recruitment and apical nuclear migration in neural stem cells. Developmental cell *33*, 703-716.
6. Barkovich, A.J., Guerrini, R., Kuzniecky, R.I., Jackson, G.D., and Dobyns, W.B. (2012). A developmental and genetic classification for malformations of cortical

development: update 2012. Brain : a journal of neurology *135*, 1348-1369.

7. Blatch, G.L., and Lassle, M. (1999). The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. BioEssays : news and reviews in molecular, cellular and developmental biology *21*, 932-939.
8. Brooks, A.S., Bertoli-Avella, A.M., Burzynski, G.M., Breedveld, G.J., Osinga, J., Boven, L.G., Hurst, J.A., Mancini, G.M., Lequin, M.H., de Coo, R.F.*, et al.* (2005). Homozygous nonsense mutations in KIAA1279 are associated with malformations of the central and enteric nervous systems. American journal of human genetics *77*, 120-126.
9. Campbell, K., and Gotz, M. (2002). Radial glia: multi-purpose cells for vertebrate brain development. Trends in neurosciences *25*, 235-238.
10. Chen, J.L., Chang, C.H., and Tsai, J.W. (2018). Gli2 Rescues Delays in Brain Development Induced by Kif3a Dysfunction. Cerebral cortex.
11. Cooper, J.A. (2014). Molecules and mechanisms that regulate multipolar migration in the intermediate zone. Frontiers in cellular neuroscience *8*, 386.
12. Dent, E.W., Gupton, S.L., and Gertler, F.B. (2011). The growth cone cytoskeleton in axon outgrowth and guidance. Cold Spring Harbor perspectives in biology *3*.
13. Drerup, C.M., Lusk, S., and Nechiporuk, A. (2016). Kif1B Interacts with KBP to Promote Axon Elongation by Localizing a Microtubule Regulator to Growth Cones. The Journal of neuroscience : the official journal of the Society for Neuroscience *36*, 7014-7026.
14. Drevillon, L., Megarbane, A., Demeer, B., Matar, C., Benit, P., Briand-Suleau, A., Bodereau, V., Ghoumid, J., Nasser, M., Decrouy, X.*, et al.* (2013). KBP-cytoskeleton interactions underlie developmental anomalies in Goldberg-Shprintzen syndrome. Human molecular genetics *22*, 2387-2399.
15. Elia, L.P., Yamamoto, M., Zang, K., and Reichardt, L.F. (2006). p120 catenin regulates dendritic spine and synapse development through Rho-family GTPases and cadherins. Neuron *51*, 43-56.
16. Emoto, K. (2012). Signaling mechanisms that coordinate the development and maintenance of dendritic fields. Current opinion in neurobiology *22*, 805-811.
17. Falnikar, A., Tole, S., Liu, M., Liu, J.S., and Baas, P.W. (2013). Polarity in migrating neurons is related to a mechanism analogous to cytokinesis. Current biology : CB *23*, 1215-1220.

18. Florio, M., and Huttner, W.B. (2014). Neural progenitors, neurogenesis and the evolution of the neocortex. Development (Cambridge, England) *141*, 2182-2194.
19. Fricker, M., Tolkovsky, A.M., Borutaite, V., Coleman, M., and Brown, G.C. (2018). Neuronal Cell Death. Physiological reviews *98*, 813-880.
20. Gelot, A., Lewin, F., Moraine, C., and Pompidou, A. (1998). [Agenesis of the corpus callosum. Neuropathologic study and physiopathologic hypotheses]. Neuro-Chirurgie *44*, 74-84.
21. Gilmore, E.C., and Walsh, C.A. (2013). Genetic causes of microcephaly and lessons for neuronal development. Wiley interdisciplinary reviews Developmental biology *2*, 461-478.
22. Goldberg, R.B., and Shprintzen, R.J. (1981). Hirschsprung megacolon and cleft palate in two sibs. Journal of craniofacial genetics and developmental biology *1*, 185-189.
23. Grenningloh, G., Soehrman, S., Bondallaz, P., Ruchti, E., and Cadas, H. (2004). Role of the microtubule destabilizing proteins SCG10 and stathmin in neuronal growth. Journal of neurobiology *58*, 60-69.
24. Hansen, D.V., Lui, J.H., Parker, P.R., and Kriegstein, A.R. (2010). Neurogenic radial glia in the outer subventricular zone of human neocortex. Nature *464*, 554-561.
25. Hatanaka, Y., Hisanaga, S., Heizmann, C.W., and Murakami, F. (2004). Distinct migratory behavior of early- and late-born neurons derived from the cortical ventricular zone. The Journal of comparative neurology *479*, 1-14.
26. Hirst, C.S., Stamp, L.A., Bergner, A.J., Hao, M.M., Tran, M.X., Morgan, J.M., Dutschmann, M., Allen, A.M., Paxinos, G., Furlong, T.M.*, et al.* (2017). Kif1bp loss in mice leads to defects in the peripheral and central nervous system and perinatal death. Scientific reports *7*, 16676.
27. Hu, D.J., Baffet, A.D., Nayak, T., Akhmanova, A., Doye, V., and Vallee, R.B. (2013). Dynein recruitment to nuclear pores activates apical nuclear migration and mitotic entry in brain progenitor cells. Cell *154*, 1300-1313.
28. Jaglin, X.H., Poirier, K., Saillour, Y., Buhler, E., Tian, G., Bahi-Buisson, N., Fallet-Bianco, C., Phan-Dinh-Tuy, F., Kong, X.P., Bomont, P.*, et al.* (2009). Mutations in the beta-tubulin gene TUBB2B result in asymmetrical polymicrogyria. Nature genetics *41*, 746-752.
29. Jiang, X., and Nardelli, J. (2016). Cellular and molecular introduction to brain

development. Neurobiology of disease *92*, 3-17.

30. Kato, M. (2015). Genotype-phenotype correlation in neuronal migration disorders and cortical dysplasias. Frontiers in neuroscience *9*, 181.
31. Kawauchi, T., Chihama, K., Nabeshima, Y., and Hoshino, M. (2006). Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration. Nature cell biology *8*, 17-26.
32. Kawauchi, T., Shikanai, M., and Kosodo, Y. (2013). Extra-cell cycle regulatory functions of cyclin-dependent kinases (CDK) and CDK inhibitor proteins contribute to brain development and neurological disorders. Genes to cells : devoted to molecular & cellular mechanisms *18*, 176-194.
33. Kevenaar, J.T., Bianchi, S., van Spronsen, M., Olieric, N., Lipka, J., Frias, C.P., Mikhaylova, M., Harterink, M., Keijzer, N., Wulf, P.S.*, et al.* (2016). Kinesin-Binding Protein Controls Microtubule Dynamics and Cargo Trafficking by Regulating Kinesin Motor Activity. Current biology : CB *26*, 849-861.
34. Kon, E., Cossard, A., and Jossin, Y. (2017). Neuronal Polarity in the Embryonic Mammalian Cerebral Cortex. Frontiers in cellular neuroscience *11*, 163.
35. Konietzny, A., Bar, J., and Mikhaylova, M. (2017). Dendritic Actin Cytoskeleton: Structure, Functions, and Regulations. Frontiers in cellular neuroscience *11*, 147.
36. Kosodo, Y. (2012). Interkinetic nuclear migration: beyond a hallmark of neurogenesis. Cellular and molecular life sciences : CMLS *69*, 2727-2738.
37. Kriegstein, A., Noctor, S., and Martinez-Cerdeno, V. (2006). Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. Nature reviews Neuroscience *7*, 883-890.
38. Kriegstein, A.R., and Noctor, S.C. (2004). Patterns of neuronal migration in the embryonic cortex. Trends in neurosciences *27*, 392-399.
39. Kristiansen, M., and Ham, J. (2014). Programmed cell death during neuronal development: the sympathetic neuron model. Cell death and differentiation *21*, 1025-1035.
40. Lim, J.S., Kim, W.I., Kang, H.C., Kim, S.H., Park, A.H., Park, E.K., Cho, Y.W., Kim, S., Kim, H.M., Kim, J.A.*, et al.* (2015). Brain somatic mutations in MTOR cause focal cortical dysplasia type II leading to intractable epilepsy. Nature medicine *21*, 395-400.
41. Lin, S., Liu, M., Mozgova, O.I., Yu, W., and Baas, P.W. (2012). Mitotic motors

coregulate microtubule patterns in axons and dendrites. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 14033-14049.

42. Lu, I.L., Chen, C., Tung, C.Y., Chen, H.H., Pan, J.P., Chang, C.H., Cheng, J.S., Chen, Y.A., Wang, C.H., Huang, C.W*., et al.* (2018). Identification of genes associated with cortical malformation using a transposon-mediated somatic mutagenesis screen in mice. Nature communications *9*, 2498.
43. Lyons, D.A., Naylor, S.G., Mercurio, S., Dominguez, C., and Talbot, W.S. (2008). KBP is essential for axonal structure, outgrowth and maintenance in zebrafish, providing insight into the cellular basis of Goldberg-Shprintzen syndrome. Development (Cambridge, England) *135*, 599-608.
44. Magnani, D., Hasenpusch-Theil, K., Benadiba, C., Yu, T., Basson, M.A., Price, D.J., Lebrand, C., and Theil, T. (2014). Gli3 controls corpus callosum formation by positioning midline guideposts during telencephalic patterning. Cerebral cortex (New York, NY : 1991) *24*, 186-198.
45. Nakayama, T., Al-Maawali, A., El-Quessny, M., Rajab, A., Khalil, S., Stoler, J.M., Tan, W.H., Nasir, R., Schmitz-Abe, K., Hill, R.S*., et al.* (2015). Mutations in PYCR2, Encoding Pyrroline-5-Carboxylate Reductase 2, Cause Microcephaly and Hypomyelination. American journal of human genetics *96*, 709-719.
46. Pang, T., Atefy, R., and Sheen, V. (2008). Malformations of cortical development. The neurologist *14*, 181-191.
47. Salehpour, S., Hashemi-Gorji, F., Soltani, Z., Ghafouri-Fard, S., and Miryounesi, M. (2017). Association of a Novel Nonsense Mutation in KIAA1279 with Goldberg-Shprintzen Syndrome. Iranian journal of child neurology *11*, 70-74.
48. Spalice, A., Parisi, P., Nicita, F., Pizzardi, G., Del Balzo, F., and Iannetti, P. (2009). Neuronal migration disorders: clinical, neuroradiologic and genetics aspects. Acta paediatrica (Oslo, Norway : 1992) *98*, 421-433.
49. Squier, W., and Jansen, A. (2014). Polymicrogyria: pathology, fetal origins and mechanisms. Acta neuropathologica communications *2*, 80.
50. Sun, T., and Hevner, R.F. (2014). Growth and folding of the mammalian cerebral cortex: from molecules to malformations. Nature reviews Neuroscience *15*, 217-232.
51. Taverna, E., and Huttner, W.B. (2010). Neural progenitor nuclei IN motion. Neuron

67, 906-914.

52. Tsai, J.W., Lian, W.N., Kemal, S., Kriegstein, A.R., and Vallee, R.B. (2010). Kinesin 3 and cytoplasmic dynein mediate interkinetic nuclear migration in neural stem cells. Nature neuroscience *13*, 1463-1471.
53. Valence, S., Poirier, K., Lebrun, N., Saillour, Y., Sonigo, P., Bessieres, B., Attie-Bitach, T., Benachi, A., Masson, C., Encha-Razavi, F*., et al.* (2013). Homozygous truncating mutation of the KBP gene, encoding a KIF1B-binding protein, in a familial case of fetal polymicrogyria. Neurogenetics *14*, 215-224.
54. Vallee, R.B., and Tsai, J.W. (2006). The cellular roles of the lissencephaly gene LIS1, and what they tell us about brain development. Genes & development *20*, 1384-1393.
55. Westerlund, N., Zdrojewska, J., Padzik, A., Komulainen, E., Bjorkblom, B., Rannikko, E., Tararuk, T., Garcia-Frigola, C., Sandholm, J., Nguyen, L*., et al.* (2011). Phosphorylation of SCG10/stathmin-2 determines multipolar stage exit and neuronal migration rate. Nature neuroscience *14*, 305-313.
56. Wozniak, M.J., Melzer, M., Dorner, C., Haring, H.U., and Lammers, R. (2005). The novel protein KBP regulates mitochondria localization by interaction with a kinesin-like protein. BMC cell biology *6*, 35.
57. Wu, G.Y., Zou, D.J., Rajan, I., and Cline, H. (1999). Dendritic dynamics in vivo change during neuronal maturation. The Journal of neuroscience : the official journal of the Society for Neuroscience *19*, 4472-4483.

Figure 1

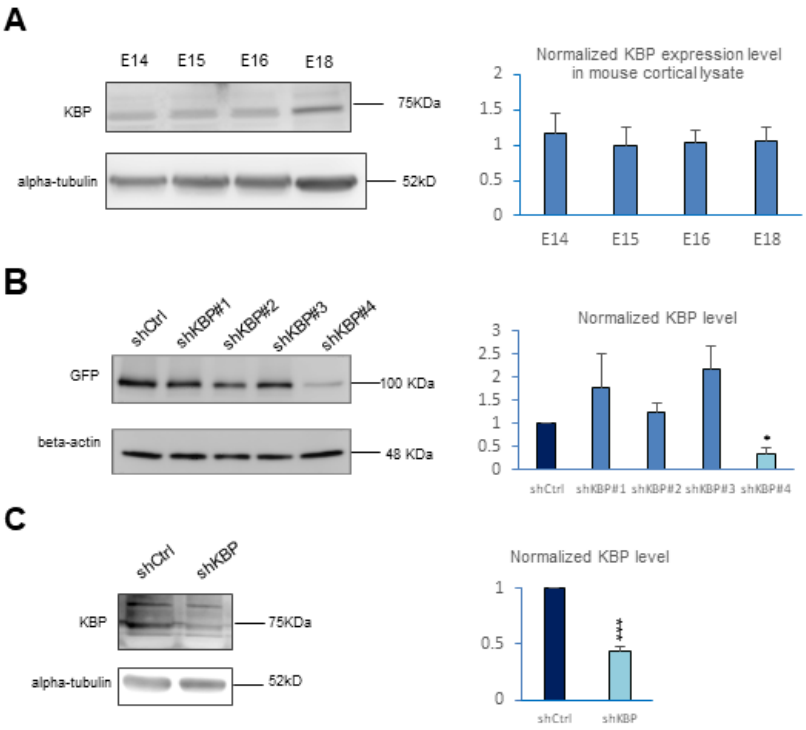
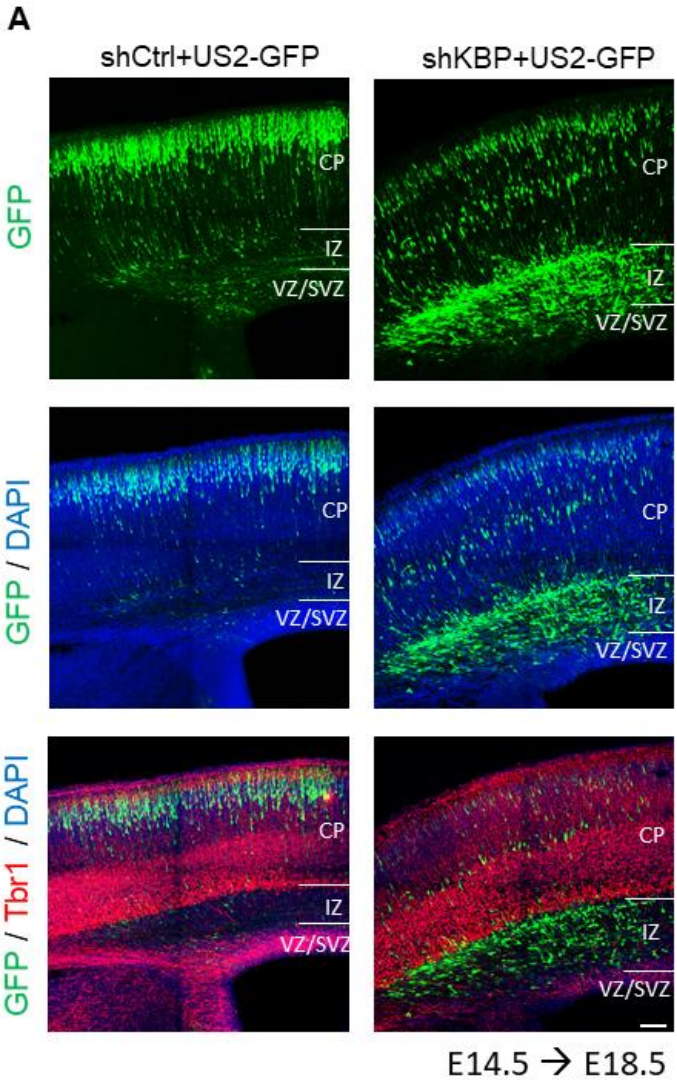


Figure 1. KBP is expressed in the developing brain and can be knockdown by shRNA. (A) Western blot for KBP expression in mouse cortical brain lysates at different embryonic days (E14, E15, E16, E18). The bar graph shows normalized KBP level. (B) KBP expression in HEK293 co-transfected with shRNA (shCtrl, shKBP#1-4) and KBP-cDNA fused with GFP for knockdown efficiency test. Western blot analysis shows KBP protein expression is significantly lower in shKBP#4 expressing cells. Beta-actin is used for loading control. (shCtrl: 1; shKBP:0.34 $\pm$14%, n=3), *: $p<0.05$, student's *t*-test. (C) KBP expression in cortical neurons introduced with lentivirus encoding shCtrl or shKBP. The results show similar knockdown effect. Alpha-tubulin is used for loading control. (shCtrl:1; shKBP: 0.44 $\pm$3%, n=4). ***: $p<0.001$, student's *t*-test.

Figure 2


A
shCtrl+US2-GFP
shKBP+US2-GFP
GFP
GFP / DAPI
GFP / Tbr1 / DAPI
CP
IZ
VZ/SVZ
E14.5 → E18.5
B
VZ/SVZ
IZ
CP
Cell distribution
100%
80%
60%
40%
20%
0%
shCtrl
shKBP
***
**

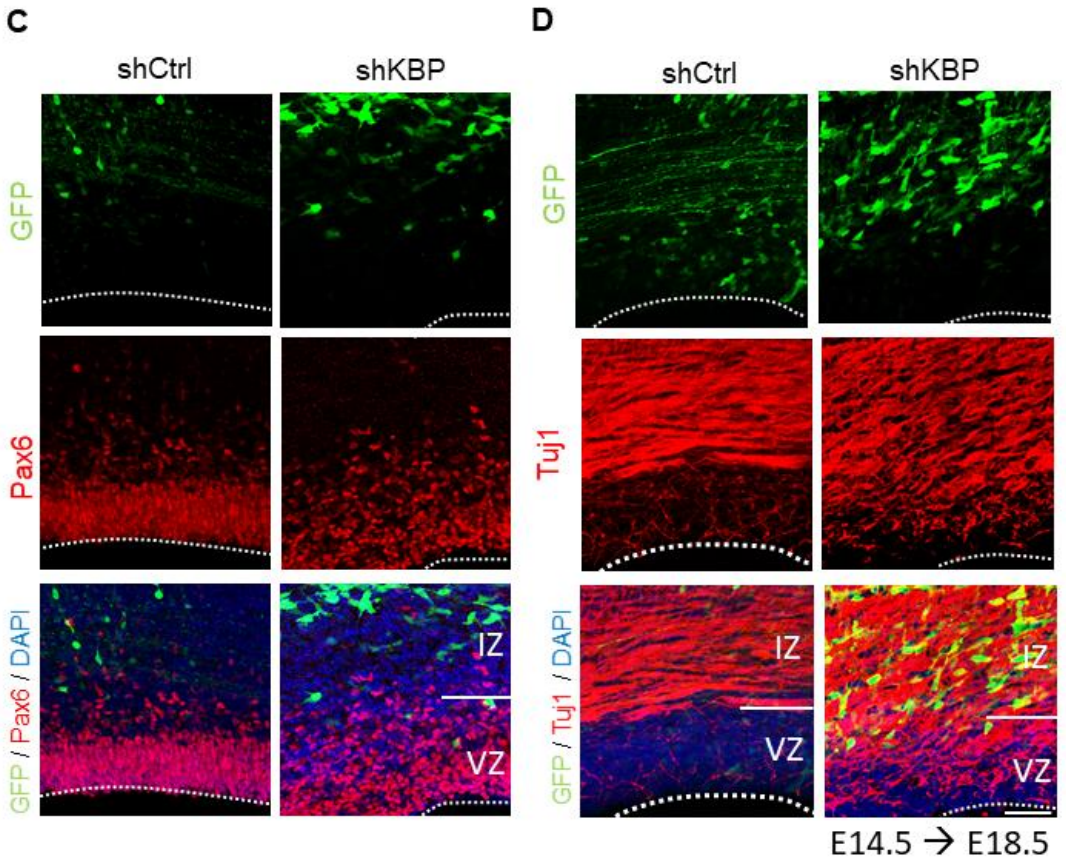
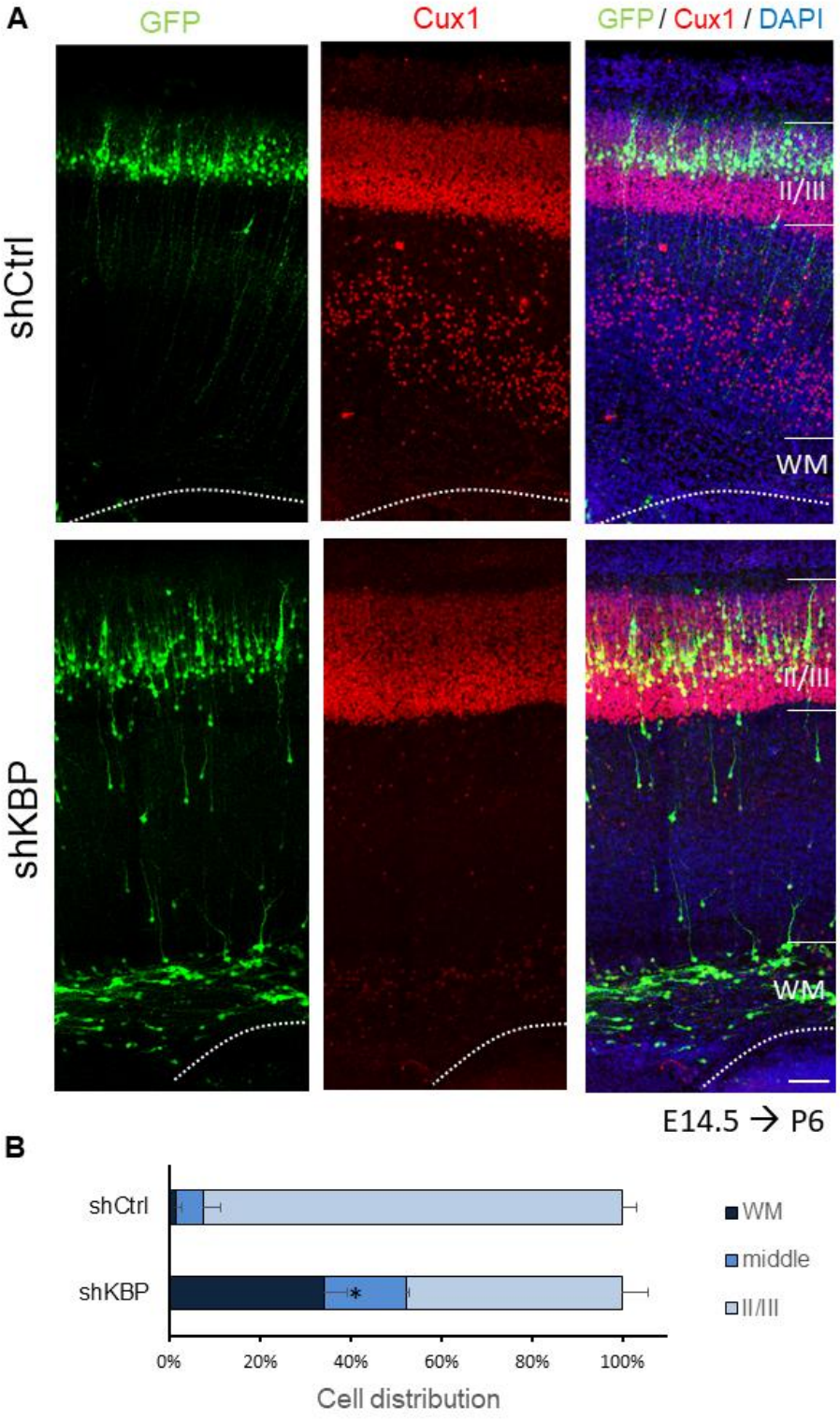


Figure 2. KBP knockdown arrested migrating cells in the intermediate zone (IZ) but did not affect neural differentiation process. (A) E18.5 brain sections introduced with shCtrl (left panel) or shKBP (right panel) examined 4 days after *in utero* electroporation. In the control brains, nearly 80% of GFP+ cells located in the CP, whereas nearly 70% of KBP knockdown cells were located in the IZ. Immunostaining with Tbr1 (red) labeled cortical layer V-VI. DAPI (blue) was used to stain nucleus. Scale bar=100μm. (B) Bar graph showed the percentage of GFP+ cells distributed in the VZ/SVZ, IZ, and CP. (shCtrl: n=4 animals; shKBP: n=8 animals) Error bars represent SEM. **: $p<0.01$, ***: $p<0.001$, student's *t*-test. (C) Immunostaining with neural progenitor cells marker Pax6 in E18.5 brain slices shows that most of GFP+ cells

in shKBP group did not colocalize to Pax6 signals (red) just as in control group. (D) Immunostaining for early differentiated neuron marker Tuj1 shows that GFP+ cells in shKBP group co-localize to Tuj1 signals (red). Scale bar= 50μm.

Figure 3


A
GFP
Cux1
GFP / Cux1 / DAPI
shCtrl
shKBP
II/III
WM
E14.5 → P6
B
shCtrl
shKBP
WM
middle
II/III
0%
20%
40%
60%
80%
100%
Cell distribution

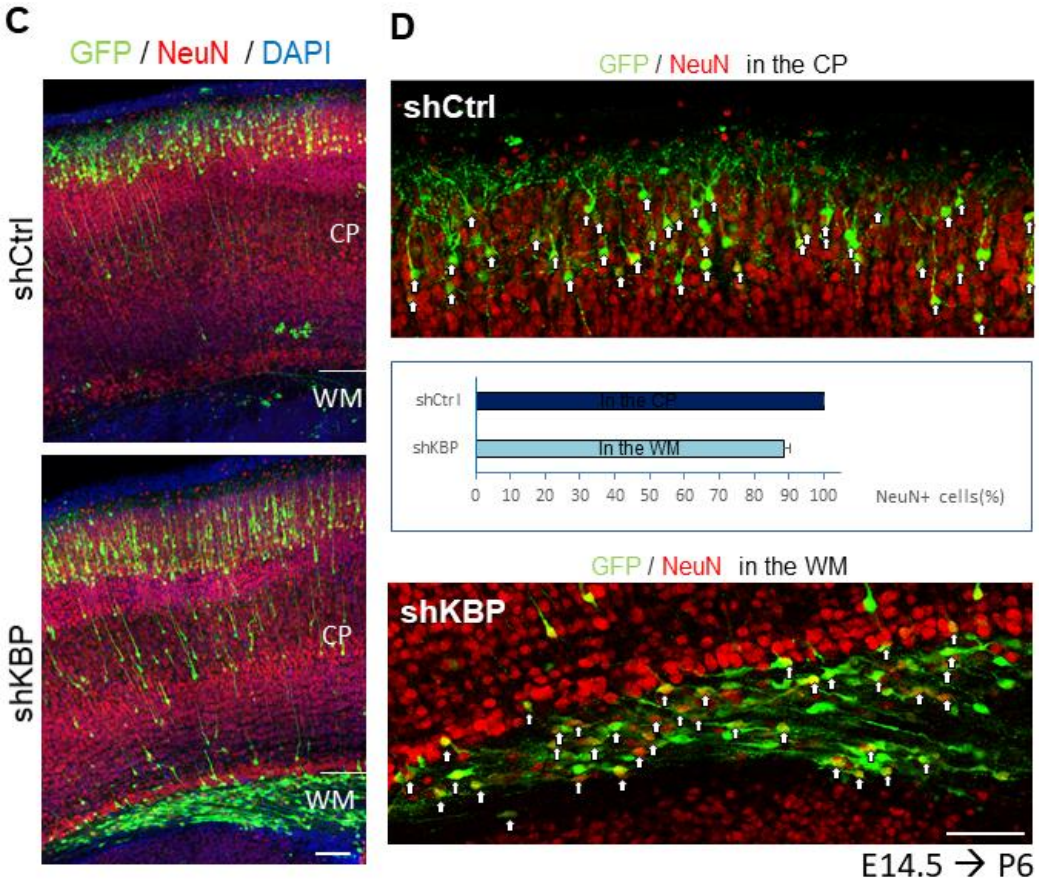


Figure 3. KBP knockdown delayed neuronal migration until P6 while arrested cells have become neurons. (A) Mouse brain sections from P6 electroporated with shCtrl or shKBP at E14.5 were immunostained with cortical layer II/III marker Cux1. While most of the GFP+ cells reached cortical layer II/III in the control brains, cells in the KBP knockdown brains had wide distribution in the whole cortex. (B) Bar graph showed the percentage of GFP+ cells distributed in the cortical layer II/III, WM and the cells migrating between the II/III and WM (middle). (n=4 animals in both conditions) Error bars represent SEM. *: $p<0.05$, ***. student's t-test. (C) Immunostaining with differentiated post-mitotic neuronal cells marker NeuN (red) in P6 brain slices. (D) Zooming in the CP of the control group, all GFP+ cells were co-localized to NeuN; Zooming in the WM of the KBP knockdown group, a majority of GFP+ cells were co-

localized to NeuN. Scale bar = 100. Bar graph showed the percentage of double GFP and NeuN positive cells in the CP and WM respectively in the control and KBP knockdown group.

Figure 4

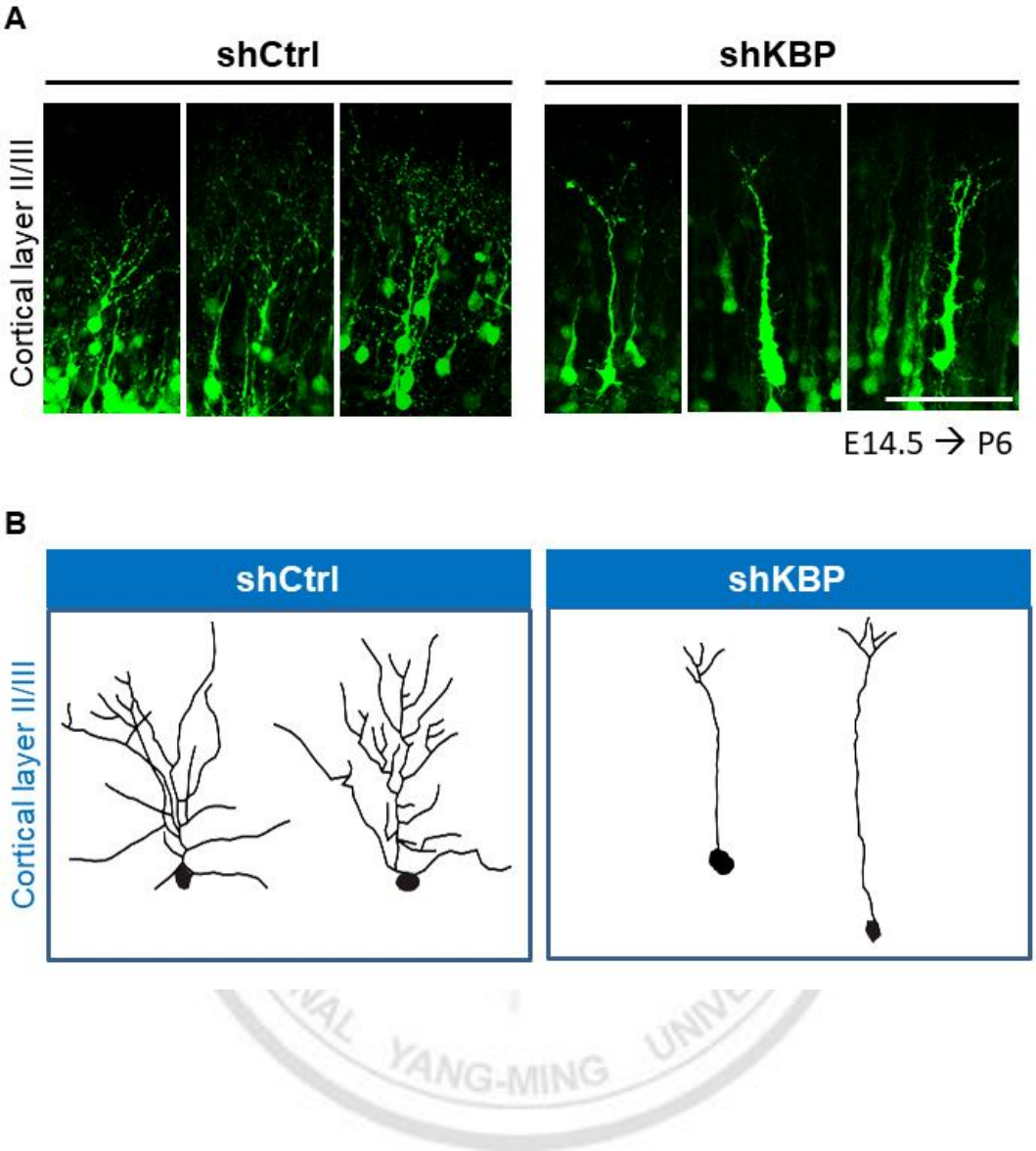
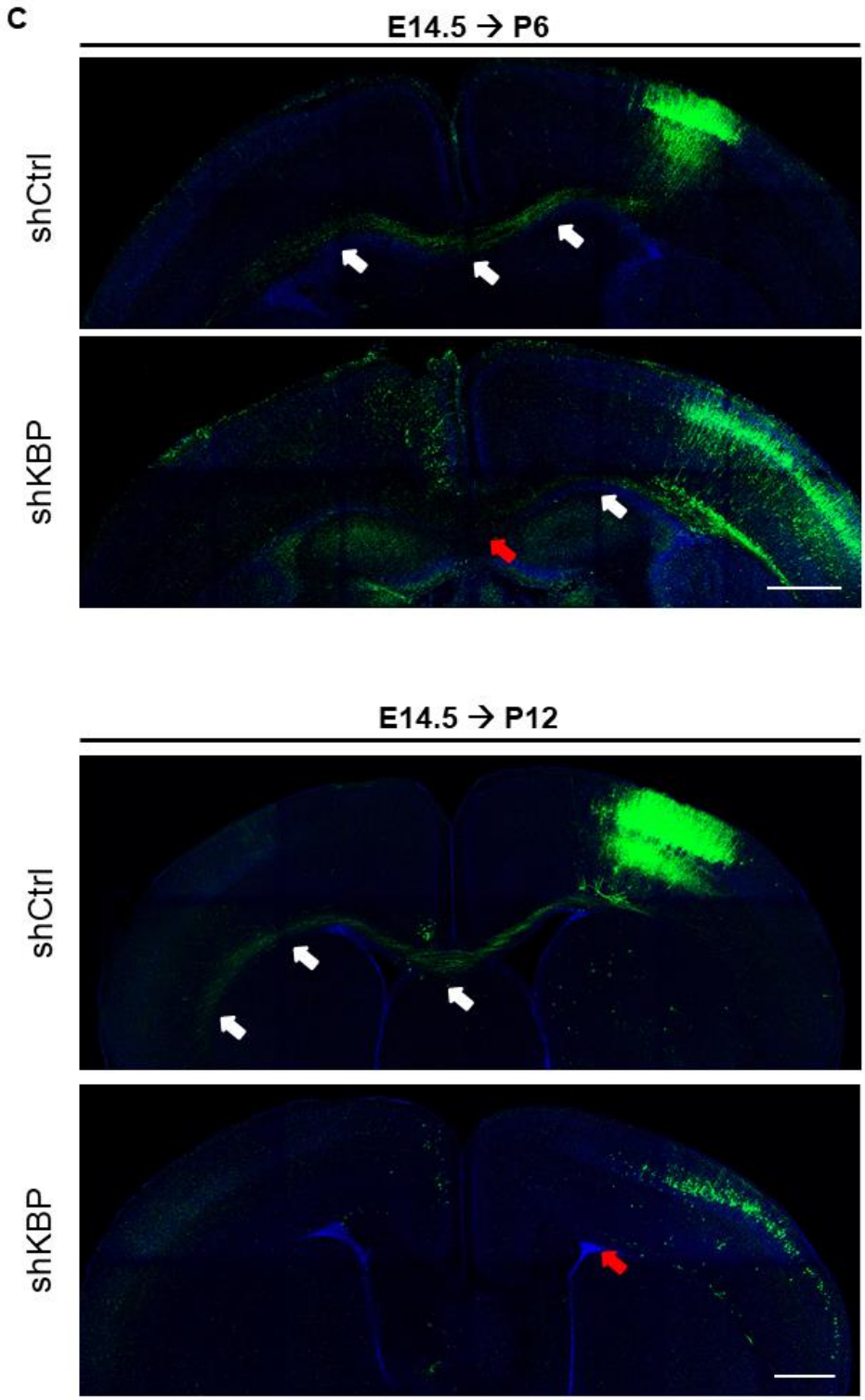


Figure 4. KBP is required for cortical neurons morphology in both dendritic arborization and axon projection in vivo. (A) Zooming in pyramidal neurons (GFP+) in cortical layer II and III of P6 brain sections. Confocal images showed KBP knockdown impairs neuronal morphological maturation for dendritic branches. Scale bar= 100μm. (B) Neuromantic-based reconstructions of layer II/III pyramidal neurons shows less dendritic branches in shKBP group.



(C) Callosal axon projection is impaired by KBP knockdown in P6 and P12 brain sections. White arrows indicate axon-projecting fibers in the transfected hemisphere, midline and contralateral cortex. Red arrows indicate axon-projecting terminate in the KBP knockdown group. Scale bar =1mm.

Figure 5

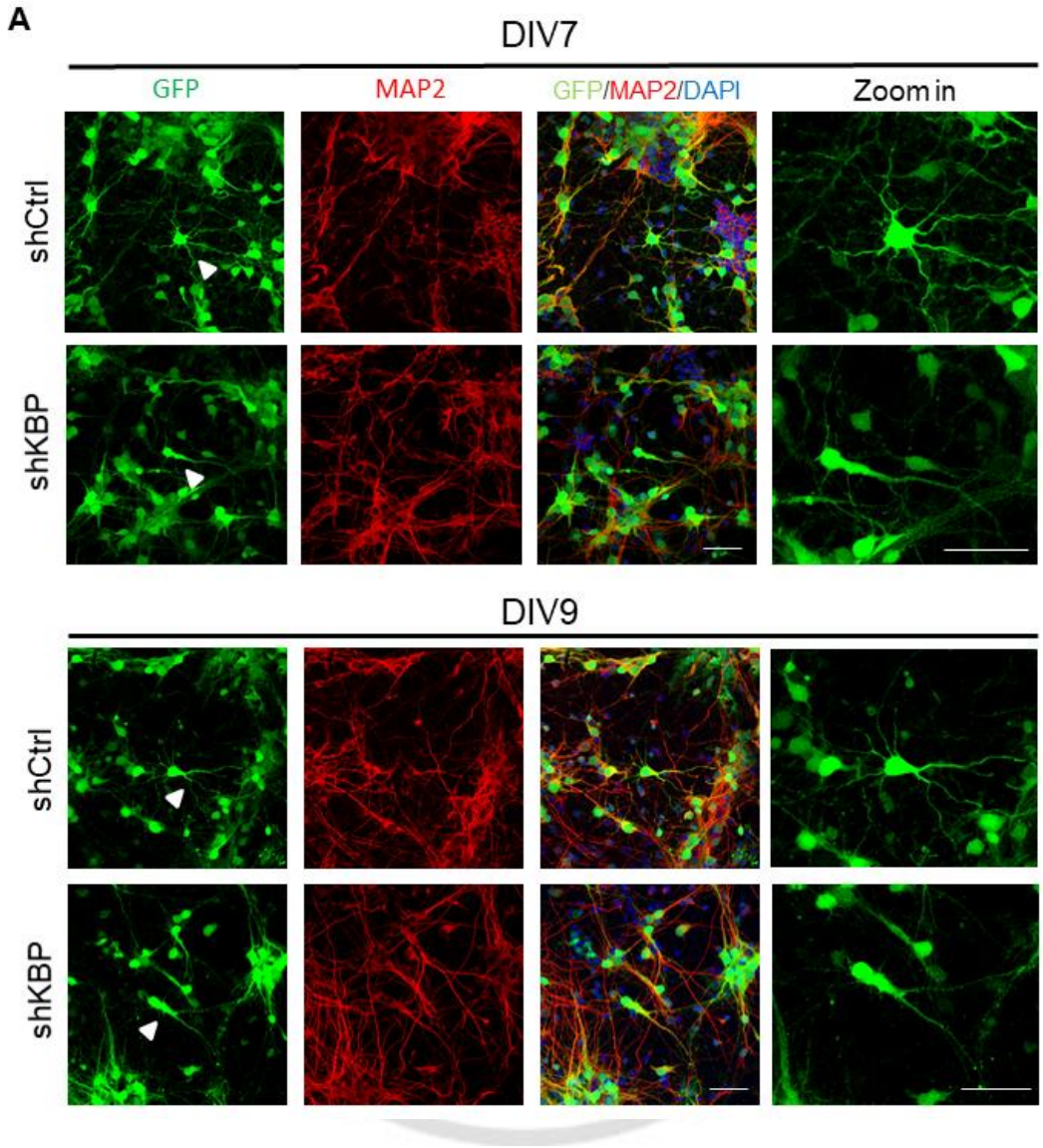
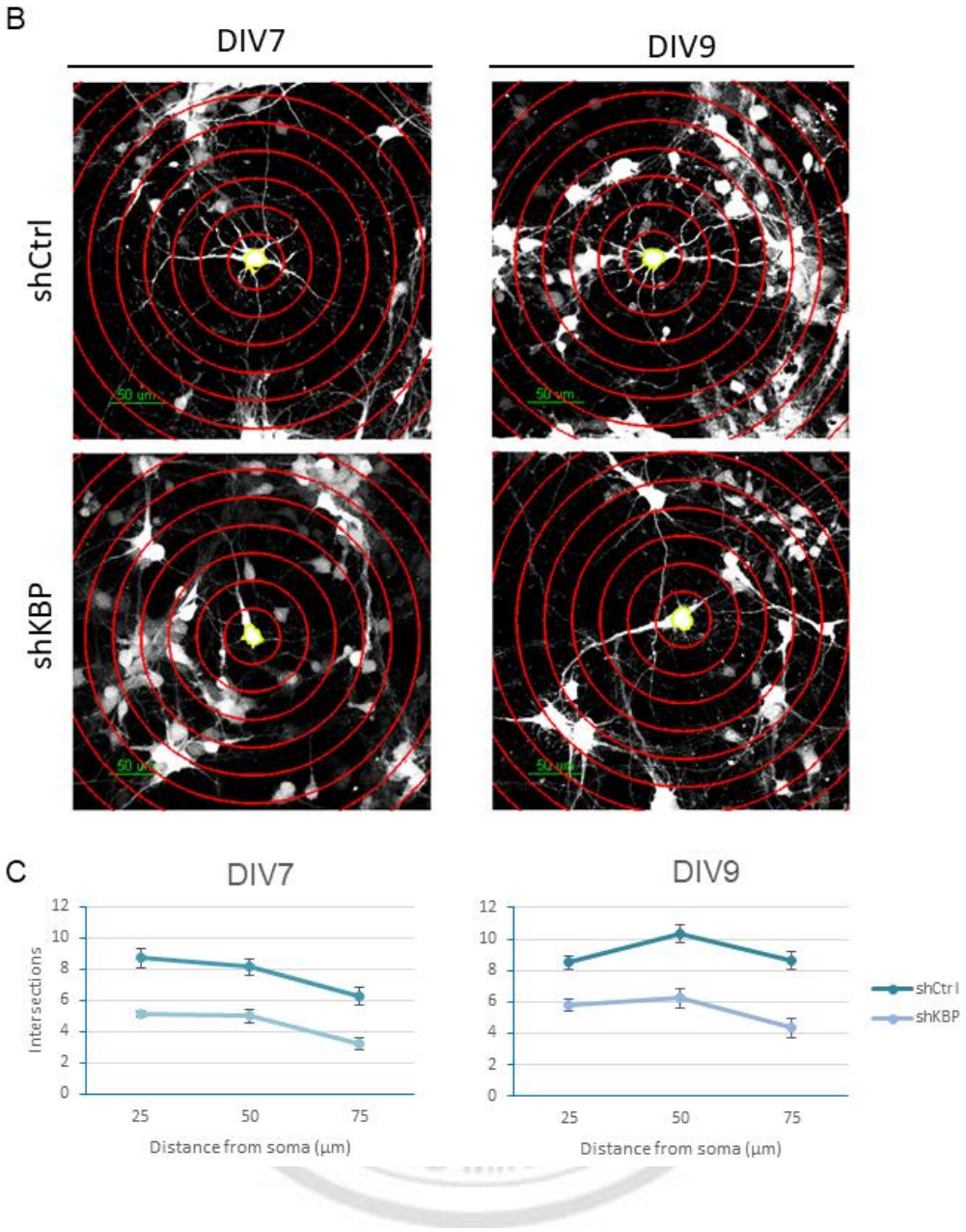


Figure 5. KBP knockdown reduced neuritic complexity in vitro. (A) Mouse cortical neurons isolated at E14.5 introduced with shCtrl and shKBP were fixed at DIV7 and DIV9 following by immunostaining. GFP+ cells co-localize to neuron-specific marker MAP2 (red) are identified as neurons and were randomly selected for morphology analysis. Left panel shows higher magnification of GFP+ neurons pointed out by arrowheads in right panels. Scale bar = 50μm.



(B) Matlab program for sholl analysis was used to quantify neuronal complexity. Export annotated images shows intersection-analyzing process set with 25µm radius (red concertric circles; green circles: labeling somas). (C) Line graphs shows intersections respectively. KBP knockdown reduces intersections indicating KBP is required for neuritic complexity. (DIV7: n=8; DIV9: n=10)

Figure 6

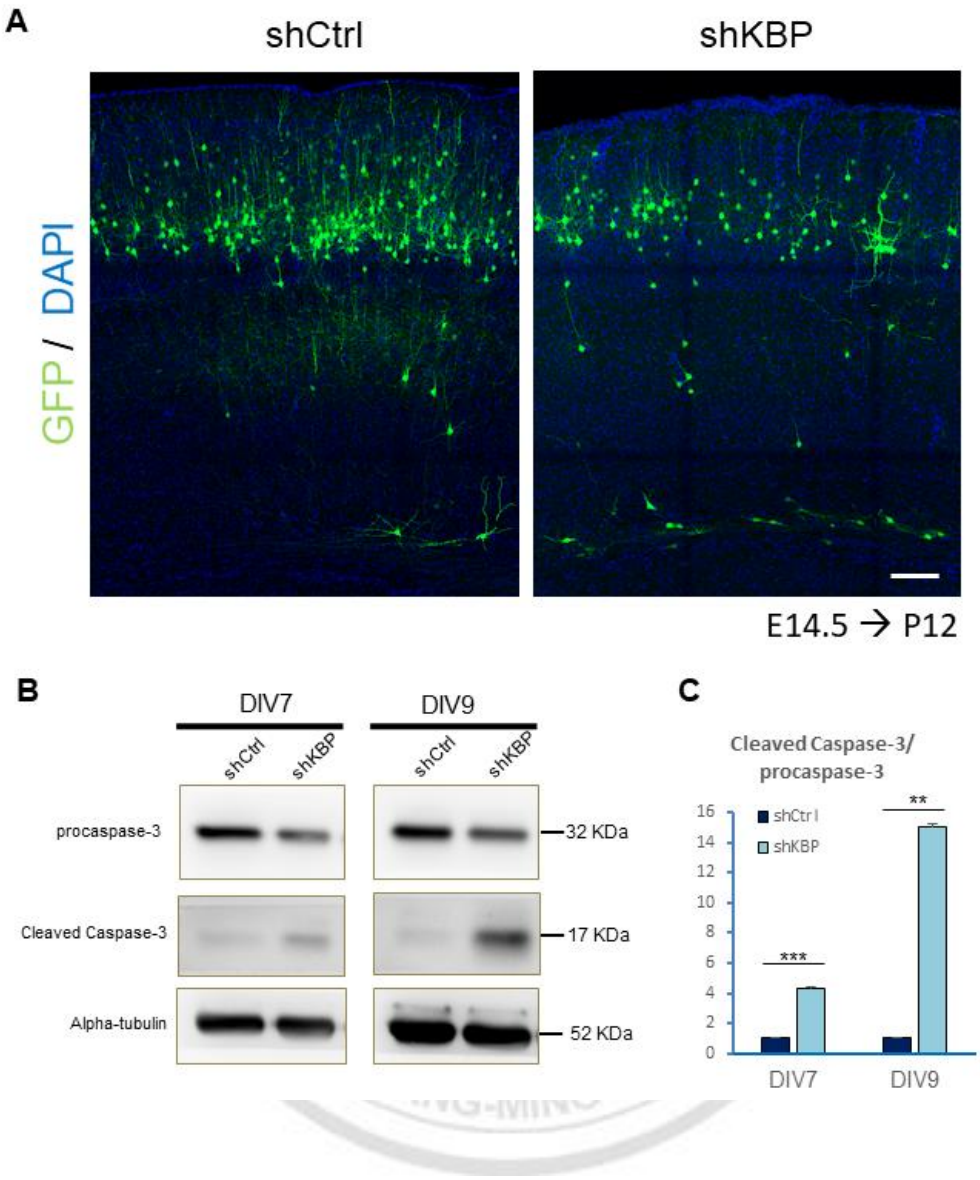
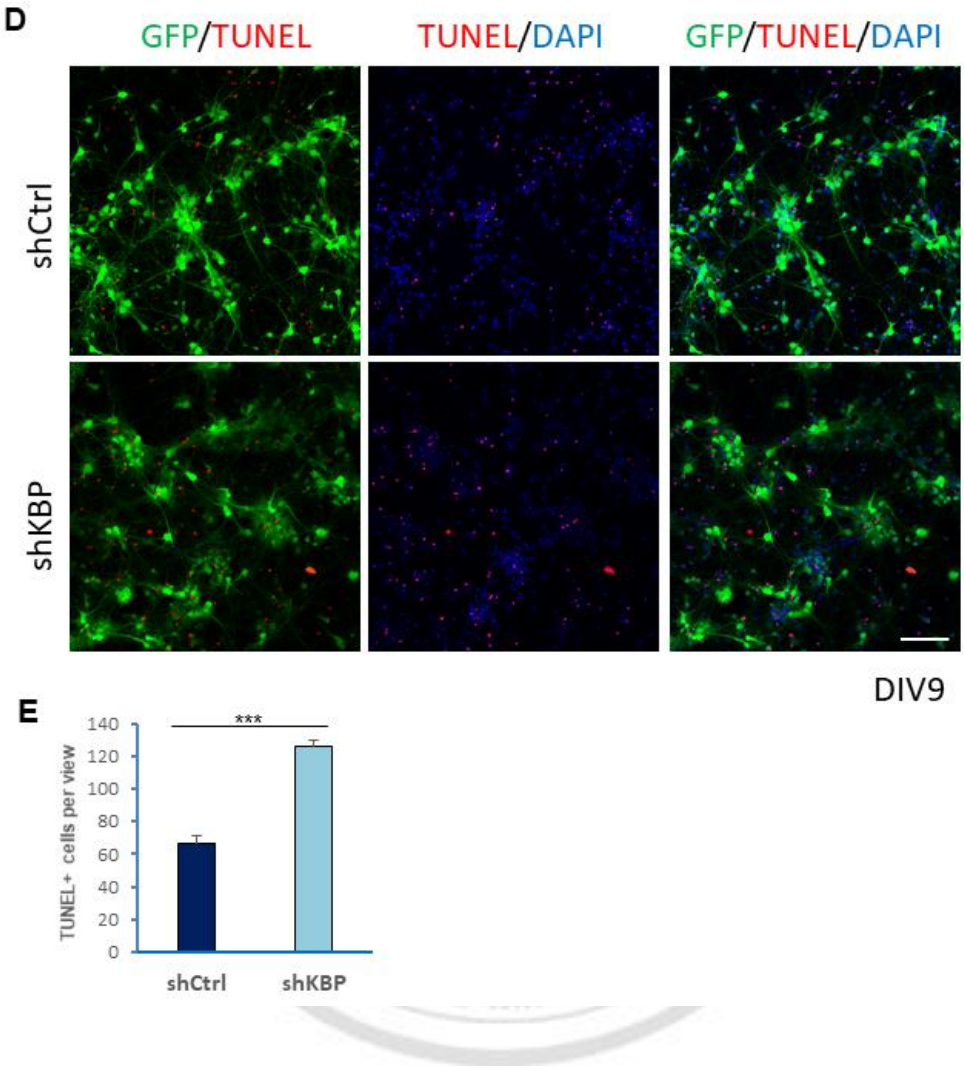


Figure 6. Loss of KBP leads to neuronal apoptosis. (A) P12 brain sections conterstained with DAPI introduced with shKBP (right panel) at E14.5 shows less total GFP+ cells than these introduced with shCtrl (left panel). Scale bar =100μm. (B) Western blot analysis shows decreased procaspase-3 protein level in KBP knockdown group both at DIV7 and DIV9. The cleaved caspase-3 protein level increases in KBP knockdown group, and increasing rate is higher at DIV9 than DIV7. Alpha-tubulin is used for loading control. (C) The bar graph shows normalized relative level of cleaved

Caspase-3 to procapase-3 in DIV7 and DIV9. KBP knockdown group shows significant increase in relative level. (n=3) **: p<0.01, ***: p<0.001, student's *t*-test.



(D) TUNEL staining with cultured cortical neurons fixed at DIV9. TUNEL (red) signals co-localized to DAPI (blue) and GFP (green) were analyzed as apoptotic neurons. Most GFP signals co-localize to TUNEL are blurred due to cell apoptosis. Upper panel is control group; lower panel is KBP knockdown group. Scale bar = 50μm. (E) Bar graph shows TUNEL+ cells in each randomly chosen view and shKBP shows significant increased. (shCtrl: 67 ± 11.3%, n=7 views; shKBP: 126 ± 10.9%, n=9 views). ***: p<0.001, student's *t*-test.

Figure 7

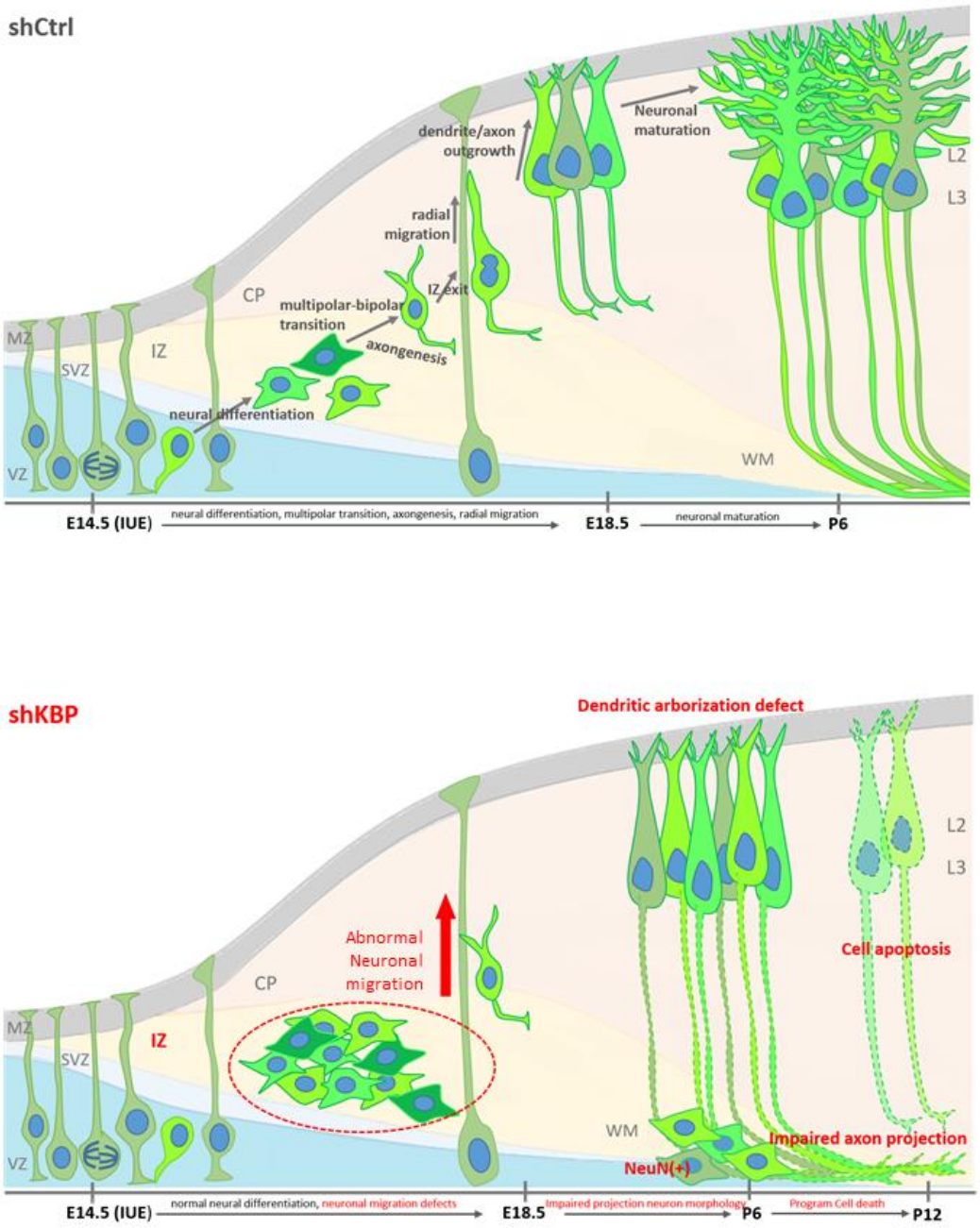


Figure 7. Schematic diagram of effects of KBP knockdown in cortical development. Normally (shCtrl, upper panel), neural progenitor cells will differentiate into neurons and undergo multipolar-bipolar transition in the IZ, and follow by radially migrating to the CP. During the migration and translocating to final position, post-mitotic neurons

undergo maturation process including dendritic arborization and axon outgrowth. In KBP knockdown group (shKBP, lower panel), neural progenitor cells normally differentiate into neurons while abnormally locate in the IZ four days after IUE (E18.5). At P6, one third of neurons (NeuN+) ectopically locate in the WM and layer II/III projection neurons show defects in dendritic arborization and impaired of axon projection. At P12, fewer cells observed in cerebral cortex implies neuronal apoptosis in KBP knockdown brain.