The Functions of LKB1 in the Development of Inhibitory Interneurons in the Cerebral Cortex

by

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ABSTRACT

LKB1/STK11 is a serine/threonine kinase first identified in *C.elegans* as a gene important for cell polarity and proliferation. Mutations in LKB1 are the primary cause of Peutz-Jegher's cancer syndrome, an autosomal dominantly inherited disease, in which patients are predisposed to benign and malignant tumors. Past studies have focused on defining LKB1 functions in various tissue types, for example LKB1 regulates axonal polarization and dendritic arborization by activating downstream substrates in excitatory neurons of the developing neocortex. However, the implications of LKB1, specifically in the developing cortical inhibitory GABAergic interneurons is unknown. LKB1 deletion was achieved by using Cre-lox technology to induce LKB1 loss in cells localized in the medial ganglionic eminence (MGE) that express Nkx2.1 and generate cortical GABAergic neurons. In this research study it is suggested that LKB1 plays a role in GABAergic interneuron specification by specifically regulating the expression of parvalbumin during the development of fast-spiking interneurons. Preliminary evidence suggest LKB1 also modulates the number of Nkx2.1-derived oligodendrocytes in the cortex. By utilizing a GABAergic neuron specific LKB1 deletion mutant, we confirmed that the loss of parvalbumin expression was due to a GABAergic neuron autonomous function for LKB1. These data provide new insight into the cell specific functions of LKB1 in the developing brain.

TABLE OF CONTENTS

Page

LIST OF FIGURES

1. INTRODUCTION

PAR Proteins

The *Par*tition Defective (PAR) genes were first identified by Ken Kemphues and colleagues in 1998 by screening for genes in *C.elegans* that were involved in mitotic cell division of fertilized zygotes (Diane G. Morton 1992). Mutations in these *Par* genes have led to lethality in *C.elegans* due to the disruption in cell-cycle asymmetries established during embryogenesis (J.L. Watts 2000). The Par genes are highly conserved that its activities have been identified in many different cell types of the same organism as well as distantly related species (Shelly and Poo 2011). Today, six proteins have been identified to be in the Par protein family that have different roles in cell generation and maintenance; Par1-4. The cells produced in Par-4 mutants via synchronous cell division were identical in size to the wild-type and did not produce early lethality in embryos of *C.elegans*. Additionally, no defects were detected in mitosis and cytokinesis. This demonstrated that Par-4 is not required for general cellular metabolism and proliferation during early embryogenesis in *C.elegans*. However, the lack of Par-4 resulted in undifferentiated intestinal cells in embryos.

Liver Kinase B1

Liver Kinase B1 is a serine/threonine kinase also known as STK11, is the human analog of the Par-4 protein family. LKB1 is activated by allosterically binding to STRAD (STE20 pseudokinase) and MO25 (a scaffold protein) to form a heteromeric complex in the cytoplasm. STRAD being a pseudokinase lacks intrinsic catalytic activity (Baas AF

2003). However, acting as an upstream activator of LKB1 together with MO25, aids LKB1 to localize and anchor itself from the nucleus to the cytoplasm(Baas AF 2003). LKB1 is ubiquitously expressed in all fetal and adult tissues. The activated form of LKB1 phosphorylates primarily AMPK (AMP-activated protein kinase) and 14 other related kinases, such as NUAK-1/2, SAT- A/B, and MARK 1-4. LKB1 phosphorylates these threonine kinases at the T-loops of their catalytic domains in order to activate them (Zeqiraj, Filippi et al. 2009). AMPK is an important kinase in the regulation of metabolic pathways and cell polarity under stress environments. This is due to its ability to act as a glucose sensor and regulate lipid metabolism which is necessary in deciding if a cell continues to proliferate or undergo apoptosis(Shaw, Kosmatka et al. 2004). Therefore, LKB1's interaction with AMPK kinases is highly important.

LKB1 as a Tumor Suppressor

The LKB1-AMPK interaction gained much interest to cancer researchers when it was discovered to inhibit tumor growth. LKB1 inhibits the growth of tumor by activating AMPK which then inhibits growth and proliferation when a nutrient deficiency is present. More specifically, LKB1/AMPK signaling suppresses tumor growth by inhibiting the G1 phase in the cell cycle which then promotes cell apoptosis (Shackelford and Shaw 2009, Fogarty, Ross et al. 2016). The *Lkb1* gene maps to the human chromosomal region 19p13, which is responsible for Peutz-Jeghers syndrome, which is a form of inherited cancer disease. Peutz-Jegher's syndrome (PJS) is an autosomal dominantly inherited syndrome, that is characterized by the development of benign harmartous polyps in the gastrointestinal tract. Patients with this rare disease are often susceptible to developing cancers of the gastrointestinal tract, ovaries, testis and breast.

These patients also often developed dark pigmentation around and inside their lips, mouth and eyes. Additionally, due to the formation of the polyps in the gastrointestinal tract, PJS patients experience symptoms such as bowel obstruction, chronic bleeding and abdominal pain (Resta, Pierannunzio et al. 2013, GHR 2019). However, up to date there are no studies showing neurological defects related to LKB1.

To better understand LKB1's function, a germline knockout mouse model was generated, which resulted in embryonic lethality between embryonic day 8.5 (E8.5) to 9.5 (E9.5) due to a variety of developmental abnormalities. However, mice that were heterozygous for the *Lkb1* deletion developed gastrointestinal polyps and tumor, similar to PJS patients. This study provided a great insight that LKB1 is needed for survival at embryonic stages as well as a tumor suppressor at postnatal stages (Jishage, Nezu et al. 2002). Since then it has been shown that LKB1 particularly modulates cell growth, proliferation and survival in response to stress. This explains why mouse fibroblasts lacking LKB1 readily undergo cell apoptosis under stress, and fail to senescence in culture medium (Bardeesy, Sinha et al. 2002, Shaw, Kosmatka et al. 2004). While much has been learned, many questions remain about LKB1's functions in specialized cell types. In this project, we are particularly interested in LKB1's functions in neuronal cells during development.

BRAF Negatively Regulates LKB1 in Tumor Cells

Genetic studies have shown that AMPK is directly phosphorylated at Thr-172 by LKB1 in order to activate it. The activation of AMPK by LKB1 is necessary because it regulates the fate and polarity of dividing cells and the loss of function in LKB1 often results in tumor or cell death as discussed previously. BRAF, a member of the RAF

kinase family, also a serine/threonine kinase has been shown to contribute to several types of human cancer including thyroid, ovarian and colorectal cancer (Hussain, Baig et al. 2015). BRAF is a key component of the Ras/RAF/MEK/ERK (ERK/MAPK) signaling pathway, a canonical signaling pathway downstream of receptor tyrosine kinases (RTKs) and multiple other ligand/receptor complexes. The ERK/MAPK signaling pathway is essential in regulating cell growth, proliferation and survival (Chong, Vikis et al. 2003). A recent study demonstrated that constitutively activated V600E BRAF, a mutation linked to cancer, leads to ERK and Rsk hyper activation, which ultimately inhibits LKB1's activity via phosphorylation at two different sites. The inhibition of LKB1 suppressed the ability of LKB1 to bind and activate AMPK which is necessary to induce apoptosis under stress. This in turn contributes to the continuous growth of tumor cells (Zheng, Jeong et al. 2009).

The ERK/MAPK is critical in brain development (Newbern, Zhong et al. 2008, Samuels, Saitta et al. 2009, Xing, Larsen et al. 2016). In the mature brain it is important for activity dependent plasticity. While in the developing cortex ERK/MAPK has been shown to be necessary for mediating neuronal responses to receptor tyrosine kinases (RTKs) and neurotransmitters (Thomas and Huganir 2004, Samuels, Saitta et al. 2009). We currently have preliminary data to suggest that the upregulation of ERK1/2 signaling results in altered GABAergic neuron development in the cerebral cortex (unpublished data, Holter, Newbern et.al).

Role of LKB1 in Neuronal Development

Neuronal cells are post-mitotic once differentiated and, operate mechanistically differently from mitotic cells. While, LKB1's ability to regulate polarity and growth in

mitotic cells has been well established, its functions in neuronal cells have only recently been investigated. A few studies have emerged showing that some of the cellular processes in the central and peripheral nervous system are mediated by LKB1 during neuronal development.

LKB1's Activities in the Central Nervous System

LKB1's role in neuronal development was spearheaded by two studies demonstrating that axons fail to form in the hippocampus and no signs of axon formation were detected in cortical excitatory neurons in LKB1 null mice (Kuwako and Okano 2018). The first study, showed that PKA (cAMP protein kinase) phosphorylates the activated form of LKB1 (LKB1/STRAD/MO25 complex) to induce axon initiation (Shelly, Cancedda et al. 2007), and the second study further emphasized this phenotype and pointed out that LKB1 is a master regulator of SAD-A/B kinases (Barnes, Lilley et al. 2007). SAD-A/B kinases are an important regulator of neuronal polarity (Masashi Kishi and Sanes 2005). Moreover, LKB1 regulates axonal branching by activating NUAK1, an AMPK related kinase. LKB1 or NUAK1 knockout mice had a reduction in axonal branching due to the inability of mitochondria to immobilize itself at nascent presynaptic boutons (Courchet, Lewis et al. 2013). This was determined when Synthaphilin a mitochondria protein marker that's essential for mitochondria immobilization (Kang, Tian et al. 2008), was seen in low levels coupled with a high rate of mitochondria motility. When, NUAK1 and/or LKB1 was overexpressed the mitochondria motility rate was reestablished, contributing to normal axonal branching in vivo, indicating that the LKB1-NUAK1 interaction is essential to mobilize mitochondria for terminal axon branching (Courchet, Lewis et al. 2013). In addition to regulating axon

specification and branching, LKB1 also mediates dendritic branching. It was first identified in *C.elegans* that the Par4 protein mediates dendritic branching in the DA9 motor neurons(Teichmann and Shen 2011). Then it was shown that the deletion of *Lkb1* from developing Purkinje fibers in mice drastically disrupted "dendrite self-avoidance", a characteristic of many developing neurons in which dendrites from the same parents avoid overlapping in order to ensure a full coverage of any area for optimal synaptic input. Therefore, through these studies it is clear that LKB1 is essential for axonal polarity as well as in axonal and dendritic branching in hippocampal and cortical excitatory neurons. Additionally, it has also been shown that LKB1 is required for neuronal migration. Knockdown of LKB1 in immature cortical neurons, impaired proper centrosomal positioning, thus impairing migration drastically (Asada, Sanada et al. 2007). Altogether, these studies reflect the varied mechanistic functions of LKB1 in the developing central nervous system.

LKB1's Activity in the Peripheral Nervous System

LKB1's role in the developing central nervous system raised questions regarding its functions in the peripheral nervous system (PNS). A study by Lilley and colleagues in 2007, addressed that question by inactivating both LKB1 and SAD-A and SAD-B (SAD A/B) kinases, since these kinases are widely expressed in both the CNS and PNS. Surprisingly, LKB1 and SAD A/B were not required for axon formation in the spinal cord and brainstem in early developmental stages (Lilley, Pan et al. 2013). This contradicted the large defects that were found in cortical and hippocampal tissues when LKB1 and the SAD kinases were inactivated (Barnes, Lilley et al. 2007, Shelly and Poo 2011). However, the SAD A/B kinases are required for axon specialization in sensory

neurons responsive to neurotrophin-3 (NT-3) during a later phase of axonal development. Where else, the inactivation of LKB1 alone did not contribute to any significant defects.

Next, they investigated the signaling pathway downstream to TrkC, the receptor for NT-3. They identified the ERK/MAPK signaling pathway to be highly responsive to the loss of SADA/B and NT-3/TrkC signaling. This was evident when BRAF V600E was constitutively activated, it increased ERK1/2 levels, which led to the rescue of SAD protein levels in sensory neurons (Lilley, Pan et al. 2013). This is interesting, because a similar constitutive activation of BRAF, followed by ERK activation in tumor cells inhibited LKB1 instead. Subsequently, AMPK was inhibited in these cells resulting in tumor growth. Overall, these data show that sensory neurons do not utilize LKB1 to mediate developmental processes and clearly show that LKB1's functions are dependent upon the specific cell type under examination. Indeed, LKB1's function in the development of cortical GABAergic neurons in the CNS is unknown.

GABAergic Interneurons in the Cerebral Cortex

GABAergic interneurons are a subset of neurons expressing the neurotransmitter, GABA (γ-aminobuytric acid). GABA is synthesized by the enzyme GAD (Glutamic Acid Decarboxylase) which converts glutamic acid into GABA at pre-synaptic terminals (Ben-Ari, Gaiarsa et al. 2007). In the mature cortex, GABAergic interneurons tend to synapse onto principal neurons and other interneurons (Kirkcaldie 2012). The GABAergic interneurons are highly diverse morphologically as well as physiologically. Some of the morphology that these neurons exhibit include Basket cells which are the most common form (Kirkcaldie 2012). Other morphologies of inhibitory neurons include Chandelier cells, and Martinotti cells that are often found in deeper layers 2/3 to 6 and are known to

send long axons into layer 1 (Kirkcaldie 2012). Besides their morphological classification, GABAergic neurons are also largely classified by the expression of cytoplasmic proteins that are thought to in part contribute to their physiological activity. These proteins include, Parvalbumin, Somatostatin, Calretenin, Calbindin, Neuropeptide-Y and 5HT3A.

GABAergic interneurons are important in establishing a normal balance of synaptic inhibition during brain development. Thus, any abnormality in the GABAergic circuitry leads to dysregulation in the excitatory to inhibitory balance, which may contribute to a genesis of neurodevelopmental disorders, including epilepsy and Autism Spectrum Disorders (Lu 2013).

Development of the Cerebral Cortex

The key functions of the cerebral cortex include processing of sensory information, controlling motor outputs and mediating complex cognitive functions. The cerebral cortex carries out these functions by communicating via thalamic relays and controlling the outputs of other CNS structures (Kirkcaldie 2012). The cerebral cortex is dominated by two major classes of neurons; interneurons that are inhibitory (GABA) and projection neurons that are excitatory (Glutamate; glutamate neurotransmitter carrying interneurons). Approximately 80% of the cortex consist of projection neurons and 20% are inhibitory (Tremblay, Lee et al. 2016). Although inhibitory neurons compose a smaller population in the cerebral cortex, the inhibitory activity elicited by these neurons working together with the projection neurons creates a balanced circuit in the cortex.

The development of excitatory, glutamergic cortical neurons is well-understood. The progenitor cells of these neurons, radial glia, are born in the dorsal region of the

brain lining the ventricular zone. Upon birth and specification, immature cortical neurons migrate radially into the cortical plate with the help of radial glial processes arising from the ventricular zone (Rubenstein 2011). In contrast, the inhibitory neurons are born in the ganglionic eminences of the subpallial region in the ventral forebrain. Namely, these are the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE). The LGE lies dorsal to the MGE, and the CGE lies more caudally and is where part of the MGE and LGE meet. The MGE is the largest contributor to the cortical GABAergic interneuron population, making up about 70% of all cortical interneurons (Lu 2013, Lim, Mi et al. 2018). The inhibitory subtypes that arise from the MGE include somatostatin, a quarter of calretinin and virtually all parvalbumin interneurons (Kirkcaldie 2012). MGE derived GABAergic interneurons are specified by the *Nkx2.1* homeobox gene which is driven by SHH signaling and later on by *Nkx6.2* and *Lhx6* transcription factors (Marin 2013). Upon specification they begin migrating dorsolaterally into the dorsal cortex via tangential migration. Once in the cortex, they begin radially migrating, in a similar fashion to the excitatory neurons. The migration pattern of both the excitatory and inhibitory post-mitotic cells gives rise to a laminar organization in the cortex (Rubenstein 2011). There are six laminas in most regions of mammalian cerebral cortex, with layer VI being established first and located deep in the cortex, while layer I is the outermost and youngest layer. Lamination allows for compartmentalization of diverse neuronal subtypes that give rise to a complex network of circuitry (Kirkcaldie 2012).

Research Statement

It is clear that kinases are important regulators of cell behavior in the developing nervous system. Kinases are necessary for regulating the catalytic activity of other kinases or molecules in a signaling pathway. For example, aberrant ERK/MAPK signaling is implicated in neurodevelopmental syndromes such as Autism, and other neurodevelopmental syndromes, such as the RASopathies (Samuels, Saitta et al. 2009). Interestingly, GABAergic circuit malfunctions are also thought to give rise to many of the same disorders. However, the functions of LKB1 in the development of GABAergic interneurons are poorly understood. LKB1 is a kinase that is shown to have important functions in the polarization and growth of axons and dendrites in the excitatory neurons of the developing neocortex. In past studies, LKB1 did not have any immediate effects on sensory neuron development, compared to the excitatory cortical and hippocampal neurons. This may be due to differential coupling with AMPK and its related kinases. Therefore, the functions of LKB1 in cortical inhibitory interneurons are difficult to predict, thus, we began our investigation by first asking, does LKB1 regulate the development of cortical GABAergic interneuron number and/or specifications. In order to understand this, we genetically deleted LKB1 in the developing cortex. Since LKB1 germline mutants are lethal, we employed a cell-specific deletion strategy using the Cre-loxp system that targets LKB1 deletion specifically to MGE progenitors or post-mitotic GABAergic neurons. Our data show that LKB1 does not appear to regulate the number of cortical GABAergic interneurons but may be important for modulating the number of cortical oligodendrocytes that arise from the MGE. However, later in GABAergic

development, LKB1 is crucial for the differentiation of a specific subtype of GABAergic neuron that expresses Parvalbumin.

2. MATERIALS AND METHODS

Cre-Lox Recombinase

The Cre-Lox technology is an efficient tool for disrupting the expression of a gene of interest in a specific cell type via DNA modifications. The Cre-Lox recombination uses a Cre-recombinase enzyme, derived from the bacteriophage P1 which cuts out DNA sequences that are flanked by the lox P sites at specific cite of interest.

Generation of LKB1 Nkx2.1: Cre and VGAT: Cre Mice

In order to study the specific activity of LKB1 in GABAergic interneurons, Nkx2.1:Cre was utilized to generate mutant mice to delete the expression of LKB1. The Nkx2.1 is a homeobox domain that is expressed in the MGE region of a developing brain, which gives rise to the majority of the interneurons in the cortex. Therefore, the Nkx2.1:Cre recombinase is utilized to flank out regions of interest between lox P cites in a LKB1 loxp/loxp homozygous mice. In these mice, exons 3 to 6 were flanked out by the loxp insertions, resulting in the disruption of LKB1's catalytic function. Similarly, the control mice utilized in this experiment were generated by crossing a mouse harboring the Nkx2.1: Cre with LKB1 floxed at one allele. Therefore, control mice carried one functional allele of *Lkb1* and exhibited haplosufficiency. Pups were generated at expected Mendelian ratios, and both control and mutants survived into adulthood. A similar, strategy was utilized to generate the LKB1 mutants with VGAT: Cre mice.

Genotyping and Gel Electrophoresis

Toe samples were collected from pups that were about P7-P8 for genotyping. The tissue samples were submerged in about 75µL of extraction buffer, and then heated on a heating plate at 80 degrees Celsius for 30 minutes. Once the DNA was extracted, 75µL neutralizing buffer was added to neutralize the extraction buffer, which concludes the extraction process. In order to know if the gene of interest was inherited, a PCR test was conducted to amplify the regions of interest in the extracted DNA sample. PCR steps in order are as follows; DNA denaturing at 95°C for 20 seconds, primer annealing at 60°C for 30 seconds, and primer extending at 72°C for 30 seconds. This cycle was then repeated 32 times and finally cooled to 4°C.

The primers used for PCR as follows (listed 5'-3'): LKB1 lox P sites-TCTAACAATGCGCTCATCGTCATCCTCGGC amplifies 300bp floxed allele, Forward-GGGCTTCCACCTGGTGCCAGCCTGT and Reverse-GAGATGGGTACCAGGAGTTGGGGCT amplifies a 220bp wild type allele, Cre-Forward- GCTAAACATGCTTCATCGTCGG and Reverse-GATCTCCGGTATTGAAACTCCAGC amplify Cre allele, R26- Forward-AAGGGAGCTGCAGTGGAGTA and Reverse-CCGAAAATCTGTGGGAAGTC amplify 297bp wild type allele for R26 (used as Ai9 control/wild-type) and, Ai9-Forward- ACATGGTCCTGCTGGAGTTC and Reverse-GGCATTAAAGCAGCGTATCC amplify 197bp Ai9 allele. The amplified DNA regions were fractioned via gel electrophoresis by running through a 1.9% agarose gel block that was conducted using 5X TBE (Tris/Borate/EDTA) buffer.

Perfusion, Cryopreservation, Cryosectioning

Pups that expressed the genes of interest, were perfused at P14. These pups were first anesthetized using Avertin, then cleared with approximately 5-10mL 1X PBS (or until liquid expelled from circulatory system was clear). This step was followed with a 15-30mL of 4% paraformaldehyde (PFA)/1X PBS perfusion. Upon perfusion brain samples were dissected out and post-fixed overnight in 4% PFA/PBS solution.

Once fixing is complete, brain samples were cryopreserved by freezing in OCT (Optimal Cutting Temperature compound). Prior to freezing, brain samples were immersed in 15% sucrose solution overnight, followed by 30% sucrose solution again overnight. This step was conducted to cryoprotect tissues from forming ice crystals when frozen. Upon the completion of graduate sucrose immersion, a cooling solution of 70% ethanol and dry ice was made. Brain samples are then submerged in OCT in an aluminum foil tray (2cm x 2cm x 2cm) and suspended in a cooling solution to freeze. Frozen brain samples were cryosectioned using a cryostat machine. All samples were sectioned at 55µm and then subjected to immunohistochemistry analysis.

Immunohistochemistry

Brain sections were first incubated in 1x PBS, 0.2% Triton with 5% normal donkey serum (blocking solution) for an hour. Then the sections were incubated with primary antibodies diluted in blocking solution overnight (24 hours). Primary antibodies utilized were goat anti-Parvalbumin (1:1000), chicken anti-RFP (1:1000), rabbit anti-somatostatin (1:1000), rabbit anti-Olig2 (1:1000) and DAPI. Tissue was then washed 3 x for 20 minutes in 1x PBS, 0.2% Triton. Washed tissues were incubated in Alexa Fluor

dye-conjugated secondary antibodies and DAPI in blocking solution overnight. Upon immunostaining, sections were imaged using a Zeiss LSM800 Confocal microscope. At least three sections from the primary sensory cortex of each animal were imaged.

Histochemical Analysis

In order to assess if there is a change in Nkx2.1-derived cells in the cortex, RFP labelled cells were counted in a selected area of similar size in the sensory cortex of both mutant and control mice. The counted cells were quantified for density by dividing against the count area. To assess the difference in the change of parvalbumin and somatostatin interneurons, cells labeled with the respective markers are counted in a selected area and taken as percentage against the number of RFP labelled cells. The significance of the change in density and percentage between controls and mutants were evaluated by doing a Student's *t*-test. A p-value of $P<0.05$ was considered statistically significant.

3. RESULTS

In order to assess if LKB1 plays a role in the development of cortical GABAergic interneurons, we developed a mouse model in which LKB1 was inactivated in all progenitor cells arising from the MGE that express *Nkx2.1*. This inactivation was achieved by crossing a mouse harboring the conditional allele for LKB1 ($LKB1^{fl/fl}$) with a mouse expressing Cre-recombinase driven by the Nkx2.1 promoter (Bardeesy, Sinha et al. 2002, Kessaris, Fogarty et al. 2006). This allows for cell specific ablation of LKB1 in cells derived from the *Nkx2.1* lineage, which in the cortex, will result in deletion only in GABAergic neurons. To indelibly label Nkx2.1: Cre derived cells in the brain during

development, we used a Cre-dependent, red fluorescent protein (RFP) reporter gene, Ai9 (Madisen, Zwingman et al. 2010). The Ai9 reporter allowed for definitive tracking of cells that express Nkx2.1 :Cre. This model provides, a unique tool to understand the cell autonomous functions of LKB1 in Nkx2.1-derived cells from ventral forebrain progenitors in the MGE and cortical GABAergic interneurons.

Activity of LKB1 in Nkx2.1: Cre Progenitor Cells

Confirmation of Mouse Model: LKB1 Deactivation and Ai9 Expression

To determine if the inactivation of LKB1 was successfully induced, genomic PCR was performed for the absence or presence of lox P flanked genomic sequence in LKB1 alleles as well as the inheritance of Nkx2.1-Cre and Ai9 insertion in the Rosa-26 alleles. Upon confirmation mice were sacrificed for sectioning at postnatal day 14 (P14) at which point most interneurons from the subpallial region have been specified and migrated into the cerebral cortex. The RFP expressing Ai9 reporter line can be directly visualized via fluorescent microscopy, however we immunolabeled the RFP protein to further enhance detection.

LKB1 is Not Required for Nkx2.1-derived GABAergic Interneuron Number

Upon performing immunohistochemical analysis, the number of RFP labelled cells in the cerebral cortex were counted in both control and mutant mice. There was no significant change in the number RFP labelled cells in the LKB1 mutant mice, compared to the control mice (Student's t-test, p-value = 0.196). Thus, suggesting that LKB1 does not regulate cortical GABAergic interneuron number.

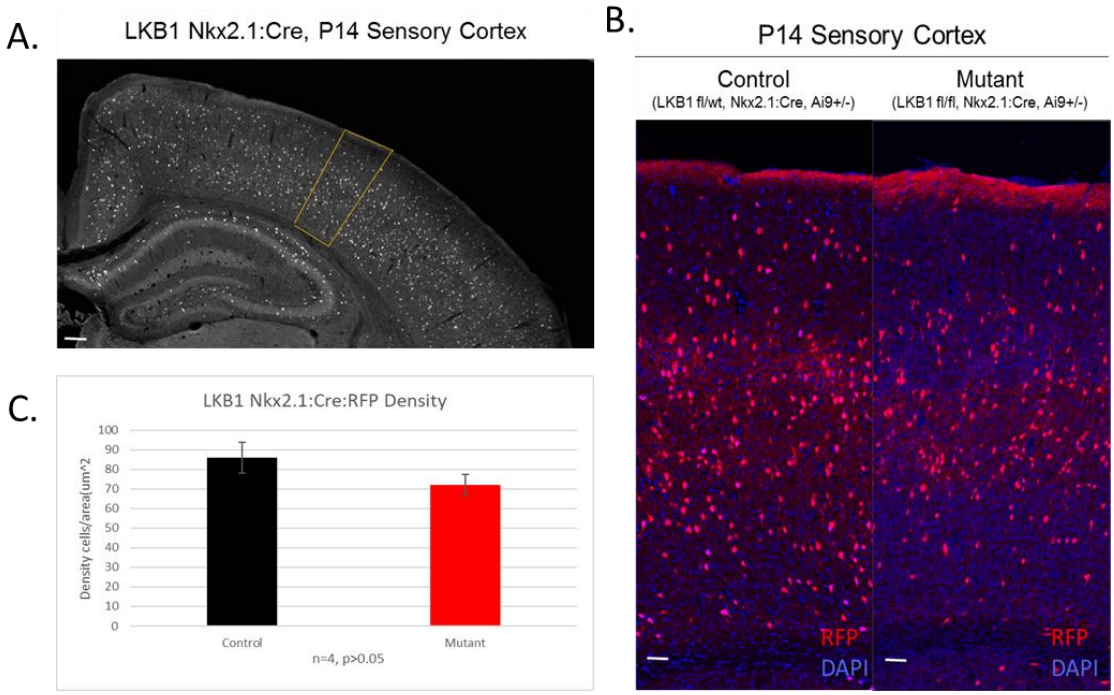

Figure1: RFP labelled cells. (A) Representative image of low magnification of cortical sensory region of cerebral cortex and example of area used in density and cell counts. (B) High magnification of 20x confocal image of cortical sensory area, showing decrease in RFP labelled cells. (C) Graphical analysis, for n=4 for RFP counts in control vs. mutants. Scale bar: 100µm (B).

LKB1 Regulates GABAergic Interneuron Subtype

We next asked whether LKB1 was necessary for the specification of a particular interneuron subtype. We decided to first immunolabel with markers for the two largest GABAergic cortical subtypes; parvalbumin and somatostatin. This analysis revealed a striking decrease in the parvalbumin interneuron population that were co-labelled with RFP. In the $LKB1^{fl/fl}$, Nkx2.1:Cre mice, it was found that 15.56+/- 1.22% of cells expressing parvalbumin co-labelled with RFP, compared to 40.22+/- 3.81% of cells co-

labelled with RFP in controls (mean +/-SEM, n=4). Interestingly, we noted a modest, but statistically significant increase in the percent of RFP labeled cells that co-express somatostatin; 40.40+/- 3.33% in mutants compared to 29.61+/-1.34% in controls. Thus, this demonstrates that LKB1 plays a role in regulating the specification of GABAergic neuron subtype during early development, primarily toward the parvalbumin lineage.

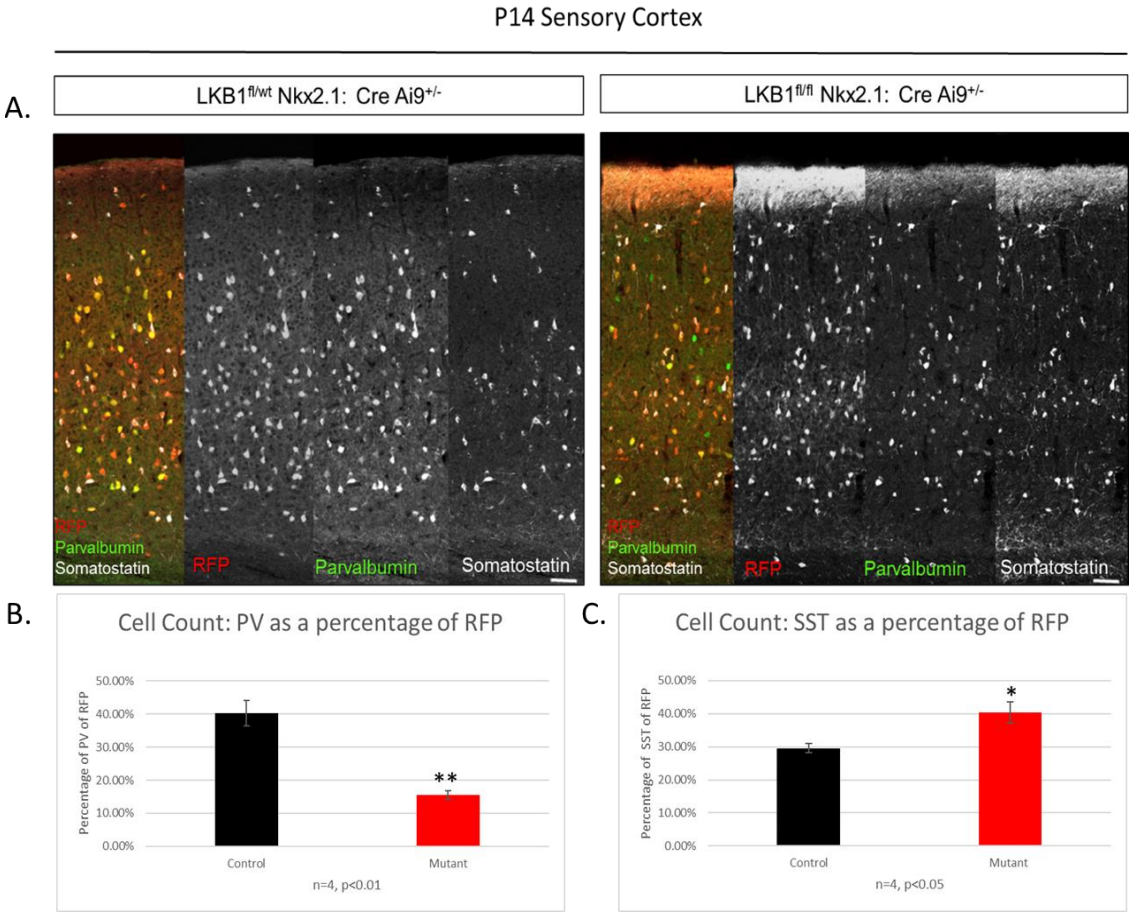

Figure 2: Reduced Number of Parvalbumin Labelled Cells in LKB1 Nkx2.1: Cre. (A) Cortical sensory area images of Parvalbumin, Somatostatin and RFP labelled cells in mutant and control. (B) Graphical depiction of percentage of Somatostatin cell count. (C) Graphical depiction of percentage of Parvalbumin cell count. Scale bars:100µm

LKB1 Inactivation does Not Exert Oxidative Stress on Parvalbumin Interneuron

Parvalbumin interneurons' fast spiking activity requires a high metabolic demand. In previous studies, it has been shown that increased oxidative stress and decrease in antioxidants are detected in the prefrontal cortex in schizophrenic patients (Yao and Keshavan 2011). Parallel to that, studies have shown that these Schizophrenia patients also exhibit changes in Parvalbumin expression (Steullet, Cabungcal et al. 2017). Additionally, environmental stresses could also contribute to an increase in oxidative stress and consequently result in Parvalbumin neuron impairments in the brain (Steullet, Cabungcal et al. 2017). Thus, given that LKB1 regulates parvalbumin expression in GABAergic neurons, we investigated if these neurons exhibited any oxidative stress. RFP expressing cells were co-labelled with 8-hydroxydeoxyguanosine (8-OHdG), an oxidative stress marker that binds to an oxidized nucleoside of DNA. However, our preliminary observation appears to indicate no detectable change in oxidative stress across the entire sensory cortex of the P14 mutant mice compared to the control. Further analysis will be necessary to confirm this preliminary observation.

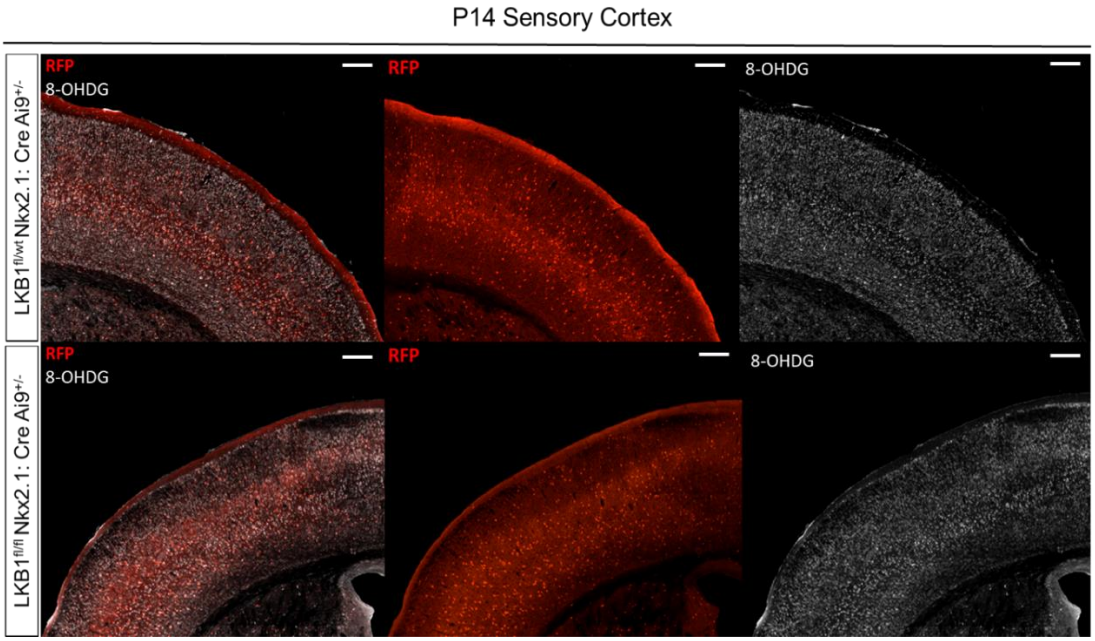

Figure 3: Preliminary Oxidative Stress Analysis. Scale bars:100µm

Perineuronal Net Formation is Not Dependent on LKB1

Perineuronal nets (PNN) are specialized neuronal extracellular matrix structures that forms around fast-spiking neurons in order to protect these neurons from oxidative stress as well provide structural support (Testa, Prochiantz et al. 2019). To further analyze if fast-spiking interneurons are disrupted in the cerebral cortex, WFA, a carbohydrate marker for PNN formation was utilized. Interestingly, we noted a substantial reduction in the number of WFA/ RFP co-labelled cells in LKB1 mutants. Since parvalbumin expression in interneurons is decreased in the cortex of these animals, the reduced number of cells labelled with WFA in the mutant further suggests that these neurons have lost properties associated with fast-spiking interneurons. However, multiple WFA labeled cells remain, demonstrating that the formation of PNN is not entirely dependent on LKB1.

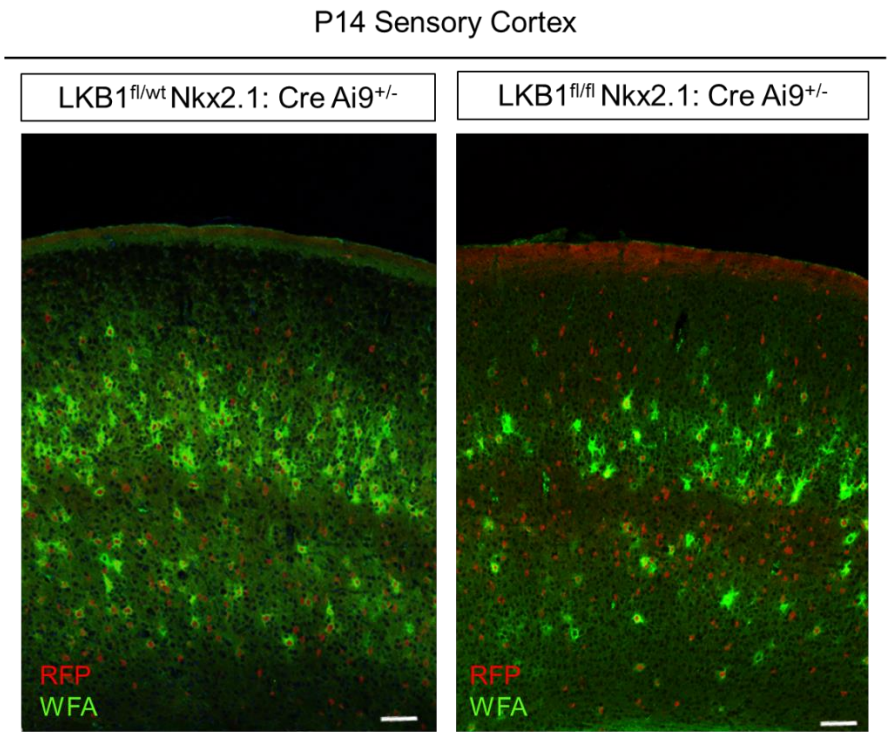

Figure 4: Preliminary Perineuronal Net Analysis. Scale bars:100µm

LKB1 Inactivation Promotes Presence of Oligodendrocyte in the Cortex

Having shown that LKB1 is necessary for the specification of different GABAergic neuron subtypes, we investigated whether if the specification of glia from Nkx2.1 derived progenitors was also affected. Oligodendrocytes are present throughout development and are detectable with markers for OLIG1 and OLIG2. Past studies have shown that astrocyte and oligodendrocyte progenitor cells also arise from the MGE region of the developing brain, and co-express Nkx2.1 (Kessaris, Fogarty et al. 2006, Minocha, Valloton et al. 2017)

However, ventral forebrain-derived oligodendrocytes are often not detectable in the dorsal cortex after P10, presumably due to programmed cell death after dorsally-derived oligodendrocytes populate the cortex (Kessaris, Fogarty et al. 2006). In our

investigation of P14 LKB1 mutants, immunostained with OLIG2 and RFP antibodies, we observed a number of OLIG2 cells that were co-labelled with RFP. This indicates that LKB1 deleted oligodendrocytes persist in the cortex after P10. This suggests that LKB1 is normally important for promoting neuronal specification from MGE derived progenitors, or that it plays an important role in driving the death of ventral derived oligodendrocytes. Further studies will be necessary to better understand these different mechanistic possibilities.

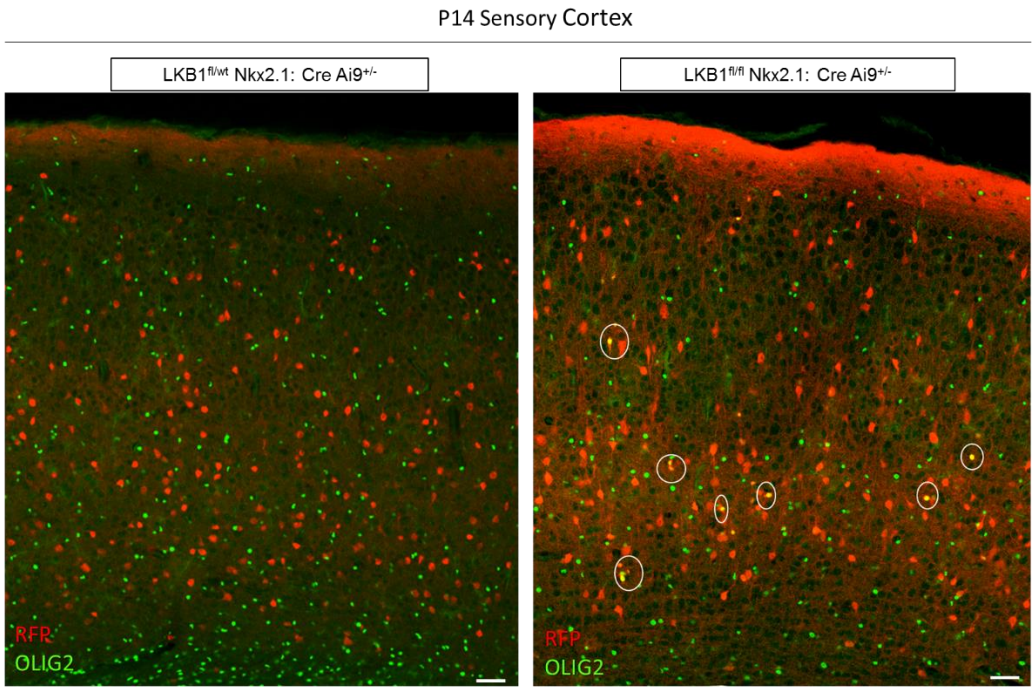

Figure 6: Labelling of LKB1 Nkx2.1-derived Oligodendrocytes. Scale bars: 50µm

GABAergic Interneuron Autonomous Activity of LKB1

Since the loss of LKB1 with Nkx2.1:Cre led to phenotypes in both neuronal and glial lineages, it was unclear whether LKB1 was regulating events in early progenitors or during later stages of neuronal specification. We developed an approach to only delete LKB1 in the postmitotic GABAergic neuronal lineage, in order to understand LKB1's

GABAergic neuron autonomous functions. The VGAT (Vesicular GABA transporter or SLC3A1): Cre line was utilized (Vong, Ye et al. 2011), generating mutant mice that were $LKB1^{fl/fl}$ VGAT: Cre $Ai9^{+/-}$ and control mice that were $LKB1^{fl/wt}$ VGAT: Cre $Ai9^{+/-}$. Methodically consistent to the Nkx2.1: Cre line, P14 brain sections were immunolabelled for RFP, Parvalbumin and Somatostatin markers. Interestingly, a similar phenotype to the Nkx2.1: Cre line was observed. In the cortex, there was a decrease in the Parvalbumin neurons that co-labelled with RFP and a slight increase in the Somatostatin neurons that co-labelled with RFP. This strongly suggest that LKB1 is necessary for the specification of a selective population of cortical Parvalbumin interneurons after committing to the neuronal lineage. Additional experiments are needed to understand the precise timing of LKB1 effects on GABAergic neuron differentiation.

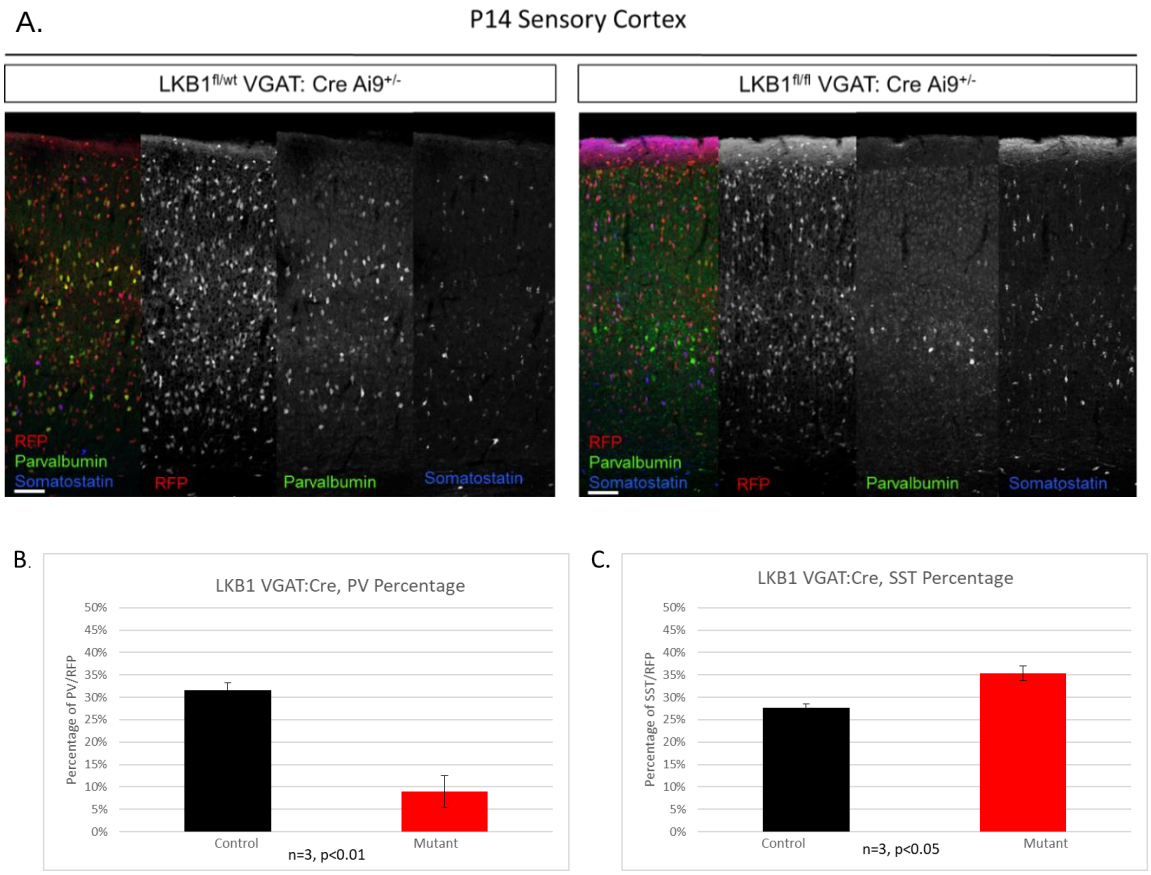

Figure 7: Reduced Number of Parvalbumin Cells in LKB1 VGAT:Cre mice. (A) Section of sensory cortex tissue showing RFP, Parvalbumin and Somatostain labelling. Significant decrease is seen in Parvalbumin labelling. (B) Graphical Depiction of percent of Parvalbumin labelling control vs.mutant. (C) Graphical Depiction of percent of Somatostatin labelling control vs.mutant. Scale bars:100µm. Work presented above is a contribution from Alexi Choueri.

4. DISCUSSION

We have demonstrated that LKB1 regulates the differentiation of distinct cortical cell populations that arise from the MGE. This conclusion is based on clear findings in genetically induced loss-of-function LKB1 mouse models. First, the population of

cortical cells derived from Nkx2.1-expressing MGE progenitors in *Lkb1* deleted mutants, showed no statistically significant change in number. But, further analysis in the same population of cortical cells was found to have a decrease in the number of the GABAergic interneuron subtype, parvalbumin, and an increase in somatostatin subtype. Third, the deletion of *Lkb1* in MGE-derived progenitors also led to the persistence of oligodendrocytes in the dorsal cortex, which is completely undetectable in controls. Finally, the GABAergic neuron-specific deletion of *Lkb1* in VGAT:Cre mice led to a decrease in the parvalbumin interneuron number.

LKB1's Cell Autonomous Functions in the Neocortex

Although LKB1 is ubiquitously expressed in all cells, our data provide further support for cell specific effects in the forebrain. In this study, our findings showed that LKB1 has a cell-specific function in cells arising from the MGE. By deleting LKB1 in the Nkx2.1:Cre and VGAT:Cre line, our data showed that all cells specified in these two lineages were present in the cortex at P14 and were not lost due to the deletion of LKB1. These data suggested that LKB1 does not regulate the number of cortical cells. However, our data for the deletion of LKB1 in the Nkx2.1:Cre line and VGAT:Cre line showed a significant decrease in the number of parvalbumin cortical interneurons. Therefore, suggesting that LKB1 regulates specific interneuron cell type in the cortex, specifically the parvalbumin subtype. A majority of parvalbumin expressing cortical interneurons are also classified as fast-spiking interneurons due to the high frequency of action potential firing. But how these interneurons specifically gain their identity is still poorly understood. LKB1's function as a master upstream activator of AMPK and AMPK-related kinases has been shown to be important in the regulation of cell death,

metabolism and proliferation of various cell types. In the peripheral nervous system, LKB1 is important for the activation of mitochondrial oxidative metabolism. This activation is critical for lipid generation in Schwann cells, in order to form myelin sheaths (Pooya, Liu et al. 2014). In the excitatory neurons of the cortex, LKB1 was shown to activate NUAK1 which is important for the activation of Synthaphilin, a protein necessary for mitochondrial arrest at presynaptic boutons of axons. Accumulation of mitochondria at presynaptic boutons are necessary for axonal arborizations (Courchet, Lewis et al. 2013). Since fast-spiking interneurons fire at a higher frequency, it requires a higher ATP demand to ensure that the Na+/K+ pump is constantly activated (Carter and Bean 2009). Therefore, we hypothesize that LKB1 regulates mitochondrial activity for the generation of ATP to power Na+/K+ pumps in fast-spiking, parvalbumin interneurons. This hypothesis provides a plausible explanation for the potentially parvalbumin-neuron specific effects of LKB1 deletion, without diminishing the expression of somatostatin. Further experiments will be necessary to examine other aspects of GABAergic neuron function to better understand the extent of LKB1 in different neuronal subtypes.

Moreover, transcription factors have been extensively studied in the development and specification of MGE-derived interneurons. The conditional loss of Nkx2.1 results in a reduction in both of parvalbumin and somatostatin expressing interneurons, demonstrating that Nkx2.1 is critical in the generation and specification of interneuron subtypes (Tremblay, Lee et al. 2016). However, high levels of Shh signaling in the MGE to activate Nkx2.1, which has been shown to preferentially give rise to somatostatin interneurons. Low levels of Shh signaling gives rise to parvalbumin interneurons (Marin

2013). It is therefore possible that LKB1 expression levels of Nkx2.1 in neuronal progenitors, which may lead to different biased toward parvalbumin interneurons.

LIM Homebox 6 (Lhx6) is another transcription factor that is critical for the specification of MGE-derived cortical interneurons and is the downstream target of Nkx2.1. In the absence of Lhx6, neural progenitors are still able to migrate to the ventricular zone, however most will fail to express somatostatin or parvalbumin (Marin 2013). Since our data only showed a decrease in parvalbumin, and the specification mechanism via Lhx6 is not well defined, it is possible that there could be an interplay between LKB1 and Lhx6 that targets parvalbumin interneurons specifically. In LHX6 mutants, that the somatostatin and parvalbumin expressing interneurons are not eliminated entirely. Implying that there may be other transcription factors and genes that are necessary for the specification of MGE-derived interneurons. Clearly, additional studies are necessary to discern the mechanism underlying LKB1 effect on cortical interneuron development. Further analysis of LKB1 function may aid in understanding a molecular signaling pathway for the specification of interneurons, especially for the specification of cortical parvalbumin interneurons.

LKB1's functions in MGE-derived Oligodendrocytes

Oligodendrocytes in the cerebral cortex are primarily derived from radial progenitor cells in the ventricular zone of the mature brain. Kessaris and colleagues have shown that in the developing brain oligodendrocytes migrate into the cortex in waves. Importantly, these waves of oligodendrocytes originate from other areas of the developing brain. One of these early waves of oligodendrocytes is seen to emerge from the MGE around E18 and are mostly lost by P10 (Kessaris, Fogarty et al. 2006). There is

an idea that large numbers of oligodendrocytes migrate into the cortex to support the larger number of neuronal cells present during early postnatal stages. In our findings we observed that the loss of LKB1 resulted in the presence of these oligodendrocytes past P10. This indicates that LKB1's functions are important for oligodendrocytes originating from the MGE in the cortex and might play an important role in the elimination of these oligodendrocytes. However, if LKB1 is needed for these oligodendrocytes to function or myelinate is unknown.

Typically, in the developing brain, neurogenesis occurs first and gliogenesis follows. Gliogenesis peaks around late embryogenesis in the mouse forebrain (Kessaris, Fogarty et al. 2006). Progenitor cells in the MGE region that express high levels of Nkx2.1 necessary for neuronal differentiation, also suppresses OLIG2, the transcription factor responsible for the differentiation of oligodendrocytes (Lu 2013). However, this suppression is lifted and the expression of OLIG2 is greatly enhanced to produce oligodendrocytes, upon the completion of neurogenesis (Lim, Mi et al. 2018). Since LKB1 is deleted in the Nkx2.1 cell lineage which comes on early around E9, it is also possible that the loss of LKB1 may have induced prematurel differentiation of progenitor cells into oligodendrocytes. However, if these oligodendrocytes persist until and throughout adulthood is unknown.

Translational Implications

LKB1 mutations cause Peutz-Jegher's syndrome (PJS) (Jishage, Nezu et al. 2002). A rare disorder affecting ~1 in 25000 to 300000 individuals (GHR 2019). Mutations on the chromosome at 19p13.3 is mapped to PJS and the generation of LKB1. As mentioned previously, LKB1 null mice died around E9 and LKB1+/- mice survived to

adulthood (Baas AF 2003). These mice developed gastrointestinal tumors similar to PJS patients which led to important discoveries regarding LKB1's role in tumor suppression. It is however important to note that the mutants generated in our study were homozygously deleted for LKB1. Therefore, our mouse model has a more severe effect on LKB1 expression than in PJS patients. Nonetheless, this mouse model provides a valid method to study LKB1's cell specific functions as well as an avenue to map out a molecular signaling pathway that regulates normal inhibitory cortical interneuron development. To date, no neurological defects have been clearly identified in patients with PJS. However, a recent study that aimed at screening for genes associated with epilepsy found LKB1 to be a potentially important candidate (Wang, Lin et al. 2017). Moreover, approximately 100 different mutations of *LKB1* have been reported (Jang, Lee et al. 2017), but the prevalence of these individual mutations are quite rare.

A recent study reported a clear case of heterozygous deletion of exons 1-10 in *LKB1* of a 11-year old boy. This boy exhibited mental retardation as well as recurring seizures (Jang, Lee et al. 2017). A malfunction in the inhibitory cortical circuit often results in epilepsy, schizophrenia and brain disorders such as ADD/ADHD. In our research focused on studying the deletion of LKB1 in cortical interneurons, we found a reduction in the number of parvalbumin interneurons, thus suggesting there might be a decrease in inhibition in the cortex. The mouse mutant we reported here would be an outstanding tool for asking whether altered LKB1 function in GABAergic neurons is sufficient to cause seizure activity or learning deficits. Understanding the normal functions of LKB1 could aid in developing therapies that target neurological defects

associated with some LKB1 mutations and possibly other, cortical inhibitory interneuron related brain disorders.

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