OREGON HEALTH & SCIENCE UNIVERSITY

SCHOOL OF MEDICINE – GRADUATE STUDIES

STRAD PSEUDOKINASES ARE CRITICAL IN EMBRYONIC BRAIN DEVELOPMENT AND IN REGULATING LKB1 STABILITY

By

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CERTIFICATE OF APPROVAL

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List of Abbreviations

AD	Alzheimer's Disease
AMPK	adenosine monophosphate activated kinase
ANOVA	analysis of varitns
Atg5/7	autophagy protein 5/7
aPKC	atypical protein kinase C
BirA	bifunctional ligase/repressor A
BRSK	brain-specific kinase
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cdc37	mammalian ortholog of cell division cycle 37
cDNA	complementary deoxyribonucleic acid
CaMKKII	calcium/calmodulin dependent protein kinase kinase 2
ChIP	chromatin immunoprecipitation
CCM3	cerebral cavernous malformation 3
CMV	cytomegalovirus
Cos7	CV-1 Origin SV40 immortalized cell line
CP	cortical plate
CRMP2	collapsin response mediator protein 2
cTip2	chicken ovalbumin upstream reporter transcription factor-interacting protein 2
DNA	deoxyribonucleic acid

E9.5	embryonic day 9.5
E12.5	embryonic day 12.5
E14.5	embryonic day 14.5
E16.5	embryonic day 16.5
E18.5	embryonic day 18.5
ERα	estrogen receptor alpha
Emx1	empty spiracles homeobox 1
FGFR	fibroblast growth factor receptor
GABAb	gamma-aminobutyric acid b
GATA6	GATA binding factor 6
GCKIII	germinal center kinase 3
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GM130	Golgi matrix protein 130kD
GR ratio	green:red ratio
GSK3β	glycogen synthase kinase 3β
HEK cells	human embryonic kidney cells
Het	heterozygous
HIER	heat-induced epitope retrieval
Hsp70/90	heatshock protein 70/90
IP	immunoprecipitation
IZ	intermediate zone
JNK	c-jun N-terminal kinase

KO	knockout
Ldb1	lim domain binding 1
LMO4	lim domain only 4
LNA	locked nucleic acid
MADM	mosaic analysis of double markers
MARK	MAP/microtubule affinity regulating kinase
MCMC	Markov Chain Monte Carlo
MEK	mitogen activated protein kinase kinase (aka mapkk)
MELK	maternal embryonic leucine zipper kinase
MEM	Minimum Essential Medium Eagle
υg	microgram
mg	milligrams
mL	milliliter
mM	millimolar
MO25	mouse protein 25 kD
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
MUSCLE	multiple sequence comparison by log-expectation
NaCl	sodium chloride
NES	nuclear export signal
NLS	nuclear localization signal

NYAP	Neuronal tyrosine-phosphorylated phosphoinositide-3-kinase adapter 1
OB	olfactory bulb
OSVZ	outer subventricular zone
P1	postnatal day 1
PAK	p21-activate kinase
Par	partitioning-defective gene family
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	the measure of acidity or basicity of an aqueous solution
PH3	phospho-(ser10)histone H3
PI3K	phosphoinositide 3 kinase
PJS	Peutz-Jeghers Syndrome
PKA	protein kinase A
PMSE	Polyhydramnios Megalencephaly and Symptomatic Epilepsy
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PSRF	Potential Scale Reduction Factor
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene difluoride
RMS	rostral migratory stream
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction

S6K	S6 kinase
SADK	synapses of amphids deficient kinase
shRNA	small hairpin ribonucleic acid
Stk25	serine/threonine kinase 25
SVZ	subventricular zone
TBS	tris buffered saline
TBST	tris buffered saline with triton
TSC	tuberous sclerosis complex
TGFβ	transforming growth factor β
VZ	ventricular zone
WT	wild type
YFP	yellow fluorescent protein

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Abstract

The STe20-Related ADapter (STRAD) pseudokinases are highly evolutionarily conserved regulators of the protein kinase LKB1, but the roles of the vertebrate paralogs STRADα and STRADβ in the developing nervous system are not fully defined, nor is it known whether they serve distinct functions given their high degree of homology. Our phylogenetic analysis indicates that STRADα is the primal STRAD gene with STRADβ appearing following a gene duplication event solely in vertebrate species. The discovery and description of a hereditary developmental epilepsy syndrome known as Polyhydramnios, Megalencephaly, and Symptomatic Epilepsy (PMSE) Syndrome caused by homozygous deletion of part of the STRADα gene (Puffenberger et al., 2007) indicates that this family of proteins plays a key role in development. I conducted biochemical and genetic analyses to better understand the contribution of the STRAD pseudokinases to vertebrate brain patterning.

Here I report a novel STRADα splice variant, STRADα-7, as well as assigning tissue specificity to a previously reported splice variant, STRADα-1. Each splice form of STRADα and STRADβ was sufficient to potentiate axogenesis, and both STRADs promoted cell survival in the developing cortex. We also found a reciprocal protein-stabilizing relationship *in vivo* between LKB1 and STRADα, whereby STRADα specifically maintains LKB1 protein levels via cytoplasmic compartmentalization and control of nuclear export. We demonstrate for the first time, that STRADβ is sufficient for axogenesis, and that STRADα, but not STRADβ, is responsible for LKB1 protein stability *in vivo*. We also examined the regulation of the STRAD proteins by another

protein, Cerebral Cavernous Malformations 3/ProgrammeD Cell Death 10 (CCM3/PDCD10). CCM3 had differential effects on STRADa and STRADβ, suggesting that it acts to fine-tune LKB1 activity.

There are myriad causes of developmental epilepsy, both environmental and genetic. Hereditary epilepsies can be studied to better understand the genetic mechanisms underlying these diseases, and to perhaps generate effective interventions. We generated knockout mice of STRADα and its family member, STRADβ, as mouse models for this human disease, and to begin to outline the epileptic mechanism.

We also examined the timing of STRADα and STRADβ deletion and their effects on gross corticogenesis and show that timing and mode of deletion of STRADα determined its effects on cortical development, indicating tight developmental control of STRAD expression. A partial redundancy between STRADα and STRADβ was observed in the context of cortical lamination. Furthermore, loss of both STRADα and STRADβ did not phenocopy LKB1. Taken together, our data provide a richer understanding of the expression and function of the STRAD pseudokinases in the developing brain.

Chapter One: Introduction

A. Cortical Development

1. Overview

Cerebral cortex development is a highly stereotyped and complex process that is shared across mammalian species. Mouse cortical development has been extensively studied and has been complemented by comparative studies with gyrencephalic mammals (Jones and Rakic, 2010; Kriegstein and Noctor, 2004; Lui et al., 2011). Early in neocortical development, progenitor cells set up a scaffold along which later-born neurons migrate in an inside-out fashion to generate a 6-layered structure (Alvarez-Buylla, 1990; Alvarez-Buylla et al., 1988; Kriegstein and Noctor, 2004; Lui et al., 2011). During this time of cortical development, cell fate and position are determined in a complex interplay of positioning and cell cycle length within the cortex (Lui et al., 2011; Polleux et al., 1997). For a specific period during corticogenesis, progenitor cells give rise to populations of daughter cells of similar functions, destined for discrete cortical layers that migrate radially toward the pial surface, resulting in a columnar organization in addition to the 6 distinct layers of cells.(Jones and Rakic, 2010; Kriegstein and Noctor, 2004; Nauhaus et al., 2012). In gyrencephalic mammals, there is a population of intermediate progenitor cells that reside in the outer subventricular zone (OSVZ), which consist of both intermediate progenitors and radial-glial-like epithelial cells (Rakic, 1995; 2009; Wang et al., 2011). These cells allow the large surface area of the gyrencephalic cortex to form in a relatively short developmental time window and their proliferation is thought to contribute to the relatively large number of excitatory neurons in the human cortex (Lui et al., 2011). This cell type has also been identified in mouse neocortex, though in much smaller numbers (Lui et al., 2011; Wang et al., 2011).

2. Neuronal Polarity and Axon Formation

During cortical development, the progenitors in the ventricular zone divide either symmetrically or asymmetrically, giving rise to two progenitors or a progenitor and a daughter cell (Lui et al., 2011). Once daughter neurons divide from their mother, they take on a multipolar stellate morphology while moving along the radial glial fiber (Barnes and Polleux, 2009; Noctor et al., 2004). These multipolar daughter neurons appear to respond to extracellular cues and signaling gradients, and become bipolar, extending a leading process and trailing process along the radial glial fiber (Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009). This step is tightly controlled, as it is critical for subsequent migration and connectivity (Barnes and Polleux, 2009; Courchet et al., 2013). The extent to which daughter cells inherit polarity from their parent is unknown, but there is crosstalk between extracellular and intracellular signaling cascades, (Arimura and Kaibuchi, 2007). Among the intracellular signals, the PAR proteins (PARtitioning defective) are required for this symmetry-breaking and were first discovered in *C. elegans* mutant screens (Kemphues et al., 1988). PAR proteins are evolutionarily conserved and necessary for asymmetric cell division in neurons as well as in epithelial cells (Insolera et al., 2011). Many parallels have been drawn between epithelial and neuronal polarity. LKB1/PAR4, one of the key polarity proteins in both cell types (Baas et al., 2003; Barnes et al., 2007), interacts with the Par3/Par6/aPKC complex as well as PKA and the SADKs and MARKs (mammalian Par1 homologs) to effect axon specification (Insolera et al., 2011). The activity of LKB1/PAR4 is regulated by the STRAD pseudokinases (Baas et al., 2003; Boudeau et al., 2003a; Zeqiraj et al., 2009b). In this thesis, I examined the necessity of the STRAD proteins for appropriate cortical

development and axogenesis, and explored intracellular signaling pathways possibly affected by perturbation of the STRAD proteins.

Symmetry-breaking in neurons has been closely studied because axon-dendrite polarity is a defining feature of a neuron. Neuronal polarity arises in a stepwise fashion, sometime after daughter neurons become post-mitotic and begin migrating up the radial glial scaffold (Barnes and Polleux, 2009). At this point, newly formed neurons extend both a leading and trailing process, which will eventually become the axon and apical dendrite (Barnes and Polleux, 2009). Seminal studies performed in cultured mouse hippocampal neurons established the stages in early polarity (Dotti et al., 1988). These studies and many others found that PAR proteins are indispensible for this symmetry-breaking process, and Par3/Par6/aPKC and Par4/LKB1 in particular, are required (Barnes and Polleux, 2009; Insolera et al., 2011). Many signaling cascades are activated during the establishment of polarity and axogenesis, and extracellular signals, such as TGFβ, growth factors, and Wnts initiate downstream signals (Insolera et al., 2011). Par3/Par6/aPKC form a complex and signal through a number of downstream molecules, including GSK3β/CRMP2, the MARKs, and PAK1 to drive polarization by modulating microtubules and F-actin (Barnes and Polleux, 2009; Yokoyama et al., 2011). It is thought that Ras/Raf/MEK/Erk also affect neuronal polarity, though this pathway has not been entirely mapped out (Barnes and Polleux, 2009)

3. LKB1 in Neuronal Polarity

As mentioned above, LKB1/Par4 is critical in neuronal polarization and for development in general, as both mice and humans lacking LKB1 do not survive (Hemminki et al., 1998; Ylikorkala et al., 2001). During the polarization process, PI3K

and PKA signals converge on LKB1, which signals to SAD and Tau to effect axogenesis (Barnes and Polleux, 2009).

Human LKB1 is highly evolutionarily conserved, displaying an 81% similar kinase domain to *D. melanogaster* LKB1, and a 66% similar kinase domain to the *C. elegans* ortholog, Par4 (Martin and St Johnston, 2003). LKB1 is vital throughout development, as deletion of *C. elegans* Par4 causes misdistribution of Par3/Par6, failure of cleavage planes to form correctly, and a failure of oogenesis (Kemphues, 2000; Kemphues et al., 1988). In *C elegans*, Par4 interacts with PIG/MELK to drive polarity and cell survival, in this way regulating neuronal lineage (Chien et al., 2013). In *D. melanogaster*, LKB1 interacts with Par1/MARK 1-4 to drive both anterior-posterior polarity and apicobasal polarity (Martin and St Johnston, 2003). Early research in mammalian cells showed that activation of LKB1 was sufficient to induce apico-basal polarization of colonic epithelial cells, as evidenced by the appearance of a brush border upon its induction (Baas et al., 2004a). A similar role has been shown in bronchial epitheilia independent of LKB1 kinase activity (Xu et al., 2013). Despite this finding, for most epithelial cells polarity is thought to be mainly driven by activation of AMPK (Lee et al., 2007; Mirouse and Billaud, 2011; Zheng and Poo, 2007). In an analogous role, LKB1 is required for neuronal polarization and axon elongation (Barnes et al., 2007; Shelly et al., 2007), through, at least in part, SADK and Tau (Kishi et al., 2005). Conversely, overexpression of LKB1 along with its coactivator, STRAD, is sufficient to induce multiple axons in cultured neurons (Barnes et al., 2007; Shelly et al., 2007). This activity is dependent on phosphorylation of LKB1 at Serine 431 and is thought to be mediated in part by phosphorylated Tau (Barnes et al., 2007; Kishi et al., 2005).

However, once polarity is established, LKB1 is not required for its maintenance (Courchet et al., 2013; Lo et al., 2012). Beyond its role in early polarity, LKB1 also functions in dendritic branching through its interaction with the downstream kinase, NUAK (Courchet et al., 2013) in collaboration with Stk25 and PAK (Deguchi et al., 2010; Matsuki et al., 2010)

B. LKB1

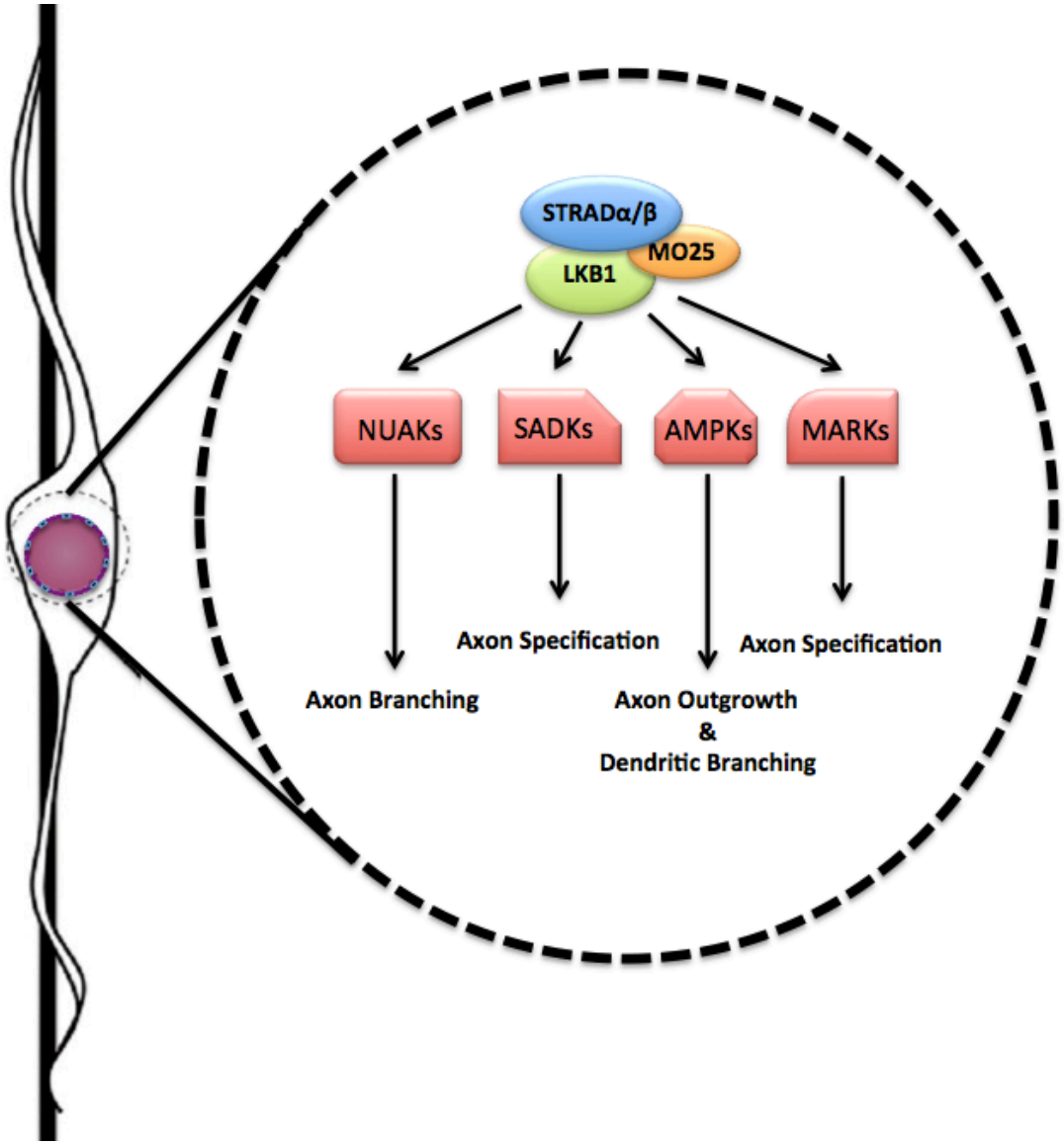
The previous section reviewed the importance of LKB1 in neuronal development, and that LKB1 is also required for brain patterning events such as axon outgrowth and branching. LKB1/Par4 is a tumor suppressor serine/threonine kinase that controls the activity of at least 13 downstream kinases, including AMPK, BRSK/SADK, MARKs, and NUAKs (Baas et al., 2004b; Jaleel et al., 2005; Lizcano et al., 2004). LKB1 activates all of these kinases by phosphorylating the T-loop (Alessi et al., 2006; Boudeau et al., 2004; Zeqiraj et al., 2009a), but the downstream signaling cascades are still not fully mapped.

Homozygous loss of LKB1 is embryonically lethal in mice and humans (Barnes et al., 2007; Hemminki et al., 1998). It is also haploinsufficient, as one mutated copy leads to Peutz-Jeghers Syndrome (PJS), which consists of hamartomatous polyps and gastrointestinal tract cancers (Hemminki et al., 1998). PJS patients are at risk for other cancers as well, and must undergo twice annual screenings for GI tumors (Hemminki et al., 1998; Smith et al., 1999). However, one functional copy of LKB1 is apparently sufficient for polarity and early development, as PJS symptoms begin to emerge in childhood (DORMANDY, 1958; Hemminki et al., 1998). A number of LKB1 mutations can lead to PJS with many affecting the kinase domain (Boudeau et al., 2004). The

interesting mutation SL26 renders LKB1 unable to bind its co-activator STRADα (Boudeau et al., 2004; Hemminki et al., 1998; Xie et al., 2009).

LKB1 in the absence of STRAD binding is relatively inactive and the active pool of LKB1 is part of a heterotrimeric complex consisting of LKB1, either STRADα or β, and one of two Mouse Protein 25kDa, α or β (MO25α or β). MO25 stabilizes the LKB1:STRAD dyad by binding to both proteins (Boudeau et al., 2003a; Zeqiraj et al., 2009b). LKB1 requires binding by a STRAD protein to expose its catalytic domain leading to autophosphorylation on threonine 189 and activation of the kinase (Baas et al., 2003; Zeqiraj et al., 2009a). This catalytically active heterotrimeric complex phosphorylates and activates the aforementioned downstream kinase cascades leading to various cellular outputs. Some of the downstream cascades relevant for neuronal function include the AMPK-like superfamily of kinases that are implicated in a number of processes including cell metabolism and polarity (Alessi et al., 2006; Jansen et al., 2009; Lizcano et al., 2004; Shackelford and Shaw, 2009). Interestingly, several members of the AMPK-like family regulate various aspects of axon formation: SAD-A/B (also known as BRSK1/2) in the developing cerebral cortex (Barnes et al., 2007; Kishi et al., 2005), AMPK-α1/α2 for axonal growth under metabolic stress (Williams et al., 2011) and neuronal polarity (Amato et al., 2011) as well as NUAK1/2 regulation of axonal branching (Courchet et al., 2013). (Introductory Figure)

Introductory Figure



Introductory figure.

LKB1 Signaling relevant in neural development. The activated LKB1/STRAD/MO25 complex signals to downstream kinases to drive neural development.

1. LKB1 Regulation

LKB1 is tightly regulated, as are most tumor suppressor proteins. Its folding is chaperoned by Heat shock proteins (Hsp's) 70 and 90 that, along with cdc37, confer some stability to the LKB1 protein (Boudeau et al., 2003b; Gaude et al., 2012). The STRAD proteins and MO25 act as both activators of LKB1 kinase activity and stabilizers of LKB1 protein (Boudeau et al., 2003a; Zeqiraj et al., 2009b). When expressed alone, LKB1 is found in the nucleus, where its functions are not well-defined (Smith et al., 1999). However, it can interact with the ERα promoter, as well as the transcription factors LMO4, GATA6 and Ldb1 (Linher-Melville et al., 2012; Nath-Sain and Marignani, 2009; Setogawa et al., 2006) and possibly exerts its cell cycle effects through these and other interactions. STRADα and STRADβ can be found in the nucleus as well as the cytoplasm (See Chapter 5), but only STRADα has the ability to export LKB1 from the nucleus, and to inhibit its active import to the nucleus (Dorfman and Macara, 2008). STRADα enhances this export via Exportin7 and CRM1, and by blocking the binding of LKB1 to Importinα (Dorfman and Macara, 2008). STRADα and STRADβ bind to and activate LKB1, but they are differentially expressed during brain development (Barnes et al., 2007). Whereas LKB1 mRNA can be found throughout the cortex at E15.5, STRADα is most highly expressed in the VZ/SVZ, where progenitors exist, STRADβ mRNA is mostly found in the cortical plate, where mature neurons finish their migration (Barnes et al., 2007). Though mRNA level is not a definitive indicator of protein level, this localization implies that these two STRAD proteins fulfill unique developmental roles. In this thesis, I address both the unique and complementary roles of STRADα and STRADβ in regards to LKB1.

2. LKB1 in Cell Cycle/Progenitor Maintenance

Because LKB1 is a tumor suppressor protein kinase, it is not surprising that it acts as a regulator of cell cycle in many contexts. LKB1 deletion causes tumors in many tissues (Hezel and Bardeesy, 2008) and can also cause unchecked proliferation (Lo et al., 2012). Shan et al. (2014) found LKB1 to be necessary to control proliferation and differentiation in muscle through AMPK, mTOR and GSK3β, and that there was limited regeneration potential when LKB1 was deleted by Pax7-Cre (Shan et al., 2014). Its function in causing G1 cell cycle arrest is kinase-dependent, p53-independent, and p21 functioning with LMO4, GATA6, and Ldb1 have been implicated in this role in cos-7 cells and intestine (Setogawa et al., 2006). LKB1 also has p53-dependent roles in cell cycle arrest as a result of its kinase activity and cytoplasmic localization. This effect is mediated by p21waf/cip1 and p53, and can be overcome by overexpression of Cyclin E or Cyclin D1 (Tiainen et al., 2002). In a complementary role, LKB1 interacts with p53 to regulate apoptosis (Karuman et al., 2001). *C. elegans* Par4/LKB1, is required along with PIG1/MARK 1-4 and STRD1/STRAD for correct neuronal/daughter cell lineage, as loss of any of these genes causes overproliferation of daughter cells of the Qp lineage (Chien et al., 2013). Loss of human LKB1 causes uncontrolled cell growth, hamartomatous polyps and other gastrointestinal tumors (Hemminki et al., 1998). Furthermore, LKB1 dysregulation is thought to lead to changes in cell size and morphology. This effect probably does not occur through AMPK signaling, at least in brain, as loss of LKB1 has no effect on pospho(T)172-AMPK in brain lysates (Barnes et al., 2007; Kishi et al., 2005). LKB1 loss leads to a disinhibition of mTORC1 signaling, and thus a change in (P)S6K, can cause increased cell size (Thomanetz et al., 2013). In a complementary

finding, Yokoyama et al reported that NYAP and PI3K signal through mTOR to regulate brain size (Yokoyama et al., 2011), pointing to a role for LKB1 in progenitor maintenance/control of survival. It is unknown whether, and if so, how LKB1 drives neural progenitor maintenance/stem cell renewal in the developing cortex. We hypothesized that loss of LKB1 activity through STRAD deletion would lead to aberrant cortical proliferation, and we examined this question in Chapter 5.

3. LKB1 in Migration

In addition to its roles in polarity and neuronal identity/morphology, LKB1 is critical for cell migration in a number of contexts. It helps in directional persistence via FAK inhibition in a wound-healing assay along with STRADα (Kline et al., 2013). NUAK1/2 inhibition has also been reported to increase migration in the wound-healing assay (Banerjee et al., 2013), and NUAK1/2 are targets of LKB1 (Lizcano et al., 2004). LKB1 also inhibits cancer cell migration via PAK1 inhibition (Deguchi et al., 2010). In a neuronal context, LKB1 may act to modulate radial migration in concert with PI3K, a kinase upstream of LKB1, and independently of Reelin and Akt (Jossin and Goffinet, 2007). Acute knockdown of LKB1 during development causes stalling of migrating neurons before they enter the subplate, whereas overexpression of LKB1 has no effect on migration (Asada et al., 2007). Further upstream, the $GABA_B$ receptor regulates migration and polarity by phosphorylation of LKB1 via the cAMP/PKA pathway (Bony et al., 2013).

4. LKB1 in Synapse Formation/Function

LKB1 is a critical upstream regulator of the SAD kinases, which are required for synaptogenesis as well as axogenesis (Barnes et al., 2007; Kishi et al., 2005). In *C.*

elegans, deleting sad-1 causes diffuse and disorganized presynaptic vesicles, and a failure of sensory axons to terminate while overexpression of SAD1 caused vesicle proteins to be mislocalized to dendrites (Crump et al., 2001; Kim et al., 2010). Deleting the mammalian SAD kinases also leads to perturbed nerve terminal maturation in both CNS and PNS (Lilley et al., 2014). SADKs are also important for synaptic function. In mammalian neurons, SAD-B overexpression increases the frequency, but not the amplitude, of mEPSC's, while overexpression of the c-terminal conserved domain of SAD-B reduces the readily releasable pool of vesicles (Inoue et al., 2006). Furthermore, spine density can be modulated by other LKB1-controlled kinases, as AMPK-CaMKKII overactivation causes a loss of spines (Mairet-Coello et al., 2013). These roles for the LKB1 pathway place LKB1 firmly in the family of kinases critical for both neuronal development and maintenance.

5. LKB1 in Aging/Degeneration

LKB1 is not only important for neuronal development, but also plays a critical role in aging neurons, and particularly synaptic health. Many neurodegenerative diseases have synaptic loss and demyelination as hallmarks, including Alzheimer's Disease (AD) and Multiple Sclerosis (MS). LKB1 is necessary for activating Par-1 and thus effecting phosphorylation of Tau in *Drosophila*, and LKB1 drives an increase in p-Tau in response to cellular stress (Wang et al., 2007). LKB1 is also required for axonal maintenance in rod photoreceptors and sensory neurons, via AMPK (Samuel et al., 2014; Sun et al., 2011). Furthermore, LKB1 is required for initial myelination and maintenance of myelin and therefore axonal health during aging, in a PTEN-dependent manner (Pooya et al., 2014; Shen et al., 2014; Snaidero et al., 2014; Sun et al., 2011) .

C. The STRAD proteins

The STRAD proteins are members of the STE family of kinases and are highly evolutionarily conserved (Boudeau et al., 2006). The most closely related family members, OSR1 and SPAK, are active kinases whereas STRAD proteins lack catalytically critical residues (Boudeau et al., 2006). STRADα and STRADβ are therefore classified as pseudokinases (Boudeau et al., 2006), but that does not mean they are nonfunctional. In fact, a STRAD protein is indispensible in LKB1 activation, and this activity is bolstered by a third protein, MO25 (Baas et al., 2003; Boudeau et al., 2003a; Neumann et al., 2007; Rajakulendran and Sicheri, 2010; Zeqiraj et al., 2009b). Whereas most kinases are activated by phosphorylation of the T-loop, LKB1 lacks this site for activation and is instead allosterically activated by STRAD binding resulting in an altered LKB1 conformation (Zeqiraj et al., 2009a; 2009b). Although STRADs differ from kinases in their ability to phosphorylate substrates, they are similar in that they maintain their ability to bind ATP, and this function aids in activating LKB1 (Zeqiraj et al., 2009b). The LKB1/STRAD interaction is so important in the LKB1 pathway, that LKB1 mutants that cannot associate with a STRAD are catalytically inactive (Baas et al., 2004a; Boudeau et al., 2004). These mutants are unable to accomplish the majority of functions associated with LKB1 (Baas et al., 2003; Boudeau et al., 2004; Hemminki et al., 1998). Interestingly, STRADα associates more strongly with kinase-dead LKB1 than with wild-type LKB1 in immuno-precipitation assays (Boudeau et al., 2003a).

Much work has been done to understand the biochemistry of the STRADα/LKB1 relationship. When LKB1 binds STRADα, it phosphorylates STRADα on T329 and T419, and also undergoes autophosphorylation on T189 (Baas et al., 2003). LKB1 that

has been activated by STRADα is also phosphorylated on T185, T402, T336/363, but these phosphorylations do not seem to change LKB1 substrate specificity (Baas et al., 2003). STRADα point mutants at T329 and T419 appear to retain their ability to activate LKB1, thus phosphorylation at these residues is of uncertain significance (Baas et al., 2003). However a kinase domain mutant of LKB1 that is unable to phosphorylate either STRADα or itself, is unable to mediate G1/S cell cycle arrest. Furthermore, a STRADα association mutant of LKB1 (SL26) is also unable to be activated by STRADα and cannot arrest the cell cycle (Baas et al., 2003)

STRADβ can bind and activate LKB1, but it does so to a lesser extent than STRADα, and requires MO25 to strengthen the association (Brajenovic et al., 2004). STRADβ and STRADα are expressed in differing levels across tissues and cell types (Boudeau et al., 2003a), implying that they have unique functions. For example, overexpression of STRADβ uniquely causes phosphorylation of JNK, via an unknown mechanism (Boudeau et al., 2004).

1. STRADs in LKB1 Activation/Subcellular Localization

Not surprisingly, a fair number of studies have explored LKB1 regulation given the various roles of LKB1 in tumor suppression and development.

LKB1 has a unique activation scheme that requires one of the STRAD pseudokinases to bind and allosterically expose its catalytic domain (Boudeau et al., 2004; Zeqiraj et al., 2009a). Notably, STRADα increases LKB1 catalytic activity more robustly than STRADβ (Baas et al., 2003).

As previously mentioned, both STRAD proteins can activate LKB1 kinase activity, but only STRADα has the ability to export LKB1 from the nucleus as STRADβ lacks a nuclear export signal (Dorfman and Macara, 2008; Orlova et al., 2010). STRADα also inhibits LKB1 translocation to the nucleus by obstructing the LKB1 Importin α3 binding site (Dorfman and Macara, 2008). When LKB1 and STRADβ are overexpressed together, they are mostly found in the cytoplasm (Baas et al., 2003), implying that LKB1 binds STRADβ after STRADα effects its nuclear export, or that newly synthesized LKB1 protein binds STRADβ before entering the nucleus.

LKB1 has diverse functions throughout development and with aging, and STRADs are the necessary accomplices in all of these functions. The active STRADα/LKB1 complex localizes to adherens junctions, as well as to the cytoplasm (Baas et al., 2004a; Liang et al., 2014; Sebbagh et al., 2009), and cytoplasmic localization of LKB1 is sufficient to inhibit G1/S transition (Boudeau et al., 2004; Tiainen et al., 2002).

2. STRAD/LKB1 Signaling Pathways

The role of LKB1 in energy homeostasis has been widely studied, and specifically, its regulatory role of AMPK has been well defined. LKB1 requires a STRAD and MO25 bound to phosphorylate AMPK on threonine 172, and this site can be dephosphorylated by protein phosphatases PP1 and PP2A (Hawley et al., 2003; Zhu et al., 2010), perhaps as a consequence of LKB1 dephosphorylation of S431. Pathophysiological conditions often provide insight into signaling pathways. For example, hyperglycemia seems to modulate the LKB1/AMPK pathway by changing STRADα and MO25 levels (Kundu et al., 2014). Specifically, during hyperglycemia in

mouse glomerular endothelial cells, STRADα mRNA is downregulated, reducing STRADα protein levels and effectively suppressing LKB1 signaling, which can be rescued by H_2S (Kundu et al., 2014). In this situation, STRADα association with MO25 is also reduced, further destabilizing STRADα (Boudeau et al., 2003a) and dampening LKB1 signaling, leading to higher levels of mTOR and the autophagy proteins Atg5 and Atg7 (Kundu et al., 2014).

During hypothyroidism, STRADα mRNA levels increase in skeletal muscle (Branvold et al., 2008). Paradoxically, when thyroid hormone levels are abnormally elevated, LKB1 and MO25 levels were reduced in this model system (Branvold et al., 2008). It is interesting that in this case, STRADα and MO25 levels are oppositely affected. Since STRADβ more strongly requires MO25 to activate LKB1, this finding offers the possibility that STRADβ could be playing a role with LKB1 during hypothyroidism. Interestingly, other disease conditions, such as ischemia, change the expression of exportin7 in particular cell types, thereby changing the LKB1 cytoplasmic:nuclear ratio (via STRADα) and increasing the amount of active LKB1 complex (Liang et al., 2014). This represents another level of fine-tuning of this critical signaling cascade.

3. STRADs in Polarity

The STRADα/LKB1 complex is capable of polarizing many cells including neurons (Baas et al., 2004a; Barnes et al., 2007; Shelly et al., 2007). The STRADα/LKB1 duo is both sufficient for axogenesis *in vitro* as well as for normal cortical axogenesis *in vivo* (Barnes et al., 2007; Shelly et al., 2007). Acute knockdown of STRADα stunts

neuronal migration, perhaps by disrupting polarity or by affecting cell motility (Causeret et al., 2009; Orlova et al., 2010; Parker et al., 2013). The extent to which STRADβ can and does contribute to LKB1-driven neuronal polarity is unknown. I aim to address this question in this work.

4. PMSE Syndrome

Although relatively little is known about STRADβ function *in vivo*, homozygous deletion within the human LYK5 (STRADα) locus results in the syndromic condition Polyhydramnios, Megalencephaly, and Symptomatic Epilepsy (PMSE) (Puffenberger et al., 2007). These patients have craniofacial dysmorphology, cognitive deficits, intractable infantile-onset epilepsy as well as muscle dystonia and often atrial-septal defects (Puffenberger et al., 2007). If protein is produced from this mutant locus, the PMSE-causing deletion of STRADα would result in a protein that is less able to bind LKB1 (Puffenberger et al., 2007; Zeqiraj et al., 2009a), which presents the possibility that PMSE is primarily caused by LKB1 misregulation. In other studies, loss of STRADα causes aberrant LKB1 localization, disinhibition of mTOR, and a subsequent increase in phospho-S6K, supporting the notion that PMSE syndrome is mainly caused by deregulation of LKB1 (Orlova et al., 2010). There has been moderate success in reducing seizure frequency in these patients using the mTOR inhibitor Rapamycin/sirolimus (Parker et al., 2013). Perhaps consistent with this treatment, a recent study reported an increase in mTORC1 activation due to lack of LKB1 inhibition, using phospho-S6/S6 as a gauge (Orlova et al., 2010). Neuronal migration in the developing cortex is also affected by acute knockdown of STRADα (Orlova et al., 2010) consistent with an observed periventricular heterotopia in one PMSE patient (Puffenberger et al., 2007), due

either to aberrant migration or overproliferation. There is some evidence for both these possibilities. Regarding the migration hypothesis, Parker et al found that knocking down STRADα acutely in mouse neuronal progenitor cells inhibits mTORC1 signaling and migration by a wound-healing assay (Parker et al., 2013). Another group attributed the migration phenotype to STRAD association with Stk25 and subsequent effects on gm130 and Golgi polarization (Matsuki et al., 2013). There is also evidence that STRADα may play a role in proliferation in mammalian cells and in nematodes (Guerreiro et al., 2011; Narbonne et al., 2010). Aberrant proliferation caused by STRADα loss and subsequent deregulation of LKB1 could cause heterotopias in the developing organism.

Notably, PMSE syndrome is quite different from the syndrome caused by hemizygosity of LKB1, Peutz-Jeghers Syndrome (PJS) (Hemminki et al., 1998). Homozygous loss of LKB1 is embryonic lethal. PJS patients exhibit gastrointestinal polyps and a general predisposition to tumors, but are developmentally normal, whereas the main symptoms in PMSE are developmental and confined to brain morphology/activity, craniofacial defects, glucose management problems, and loss of muscle tone (DORMANDY, 1958; Hemminki et al., 1998; Puffenberger et al., 2007). These differences imply that misregulation of LKB1 is distinct from a lowered dosage of LKB1, and that STRADα is critical during development. This begs the question: is STRADα working solely through LKB1 or can it also act independently of LKB1? An LKB1-independent role for STRADα in regulating invasion via PAK1 has been reported in cancer cell lines (Eggers et al., 2012), and in *C. elegans* STRD1/STRADα is sufficient to induce polarity via SAD in the absence of PAR4/LKB1(Kim et al., 2010). Furthermore, it appears that the role of LKB1 in proliferation may be independent of

STRAD. This view is bolstered by evidence in *C. elegans* (Narbonne et al., 2010) so it is possible that STRADα and LKB1 can act both together and independently. We aim to address this question herein.

Although mutation of LKB1 causes Peutz-Jeghers Syndrome, STRADα mutation is not required for PJS symptoms (de Leng et al., 2005; Hemminki et al., 1998), supporting the primacy of LKB1 in this signaling cascade, and the potential redundancy between STRADα and STRADβ. Interestingly, loss of LKB1 destabilizes STRADα, so it is possible that some PJS symptoms could be attributed to decreased STRADα protein (Eggers et al., 2012). Furthermore, most PJS-causing LKB1 mutations yield LKB1 protein that is unable to bind to STRAD and is mislocalized (Baas et al., 2004a; Tiainen et al., 2002), giving more credence to the idea that STRAD regulation of LKB1 is critical. Given the clear impact on human health and brain development, further insights into STRAD pseudokinases are needed to clarify their contributions to nervous system development and disease.

5. STRAD Expression Patterns

Developmentally, STRADα is expressed throughout the mouse cerebral cortex, whereas STRADβ is found predominantly in post-migratory neurons of the cortical plate (Barnes et al., 2007), suggesting that these proteins may serve distinct functions during cortical development. Many critical questions remain unaddressed regarding the roles of STRAD pseudokinases during cortical development. Importantly, it is unclear whether STRADα and STRADβ are functionally redundant during neuronal development and what distinct roles they might serve. Chicken STRADα is only expressed in brain, hypothalamus, heart and skeletal muscle, whereas STRADβ is present in all tissues,

supporting the possibility that the two STRAD proteins have some differing functions (Proszkowiec-Weglarz et al., 2006). Mouse tissues also display varying degrees of expression of each STRAD protein by Northern blot. Furthermore, STRADα is highly spliced in human cancer lines, although the functions of these splice variants are unclear (Boudeau et al., 2003a; Marignani et al., 2007). Here, we address these questions and provide new insights into the molecular mechanisms of STRAD protein function during neuronal development.

D. MO25

There is a third member of the LKB1/STRAD active complex, MO25, which has 2 isoforms, MO25α and MO25β (Boudeau et al., 2003a). Both isoforms are expressed in brain, but have unique expression patterns in other tissues (Boudeau et al., 2003a). Based on the crystal structure, MO25 binds STRAD at two critical residues on MO25 – R227 and M260, whereas STRADα binds it at four sites – E105, I138, Y185, and the C-terminal WEF domain(Filippi et al., 2011; Mehellou et al., 2013; Zeqiraj et al., 2009b). STRADβ also has a WEF motif and strongly binds MO25 (Boudeau et al., 2004). Although MO25 has been shown to bind STRAD, and not LKB1, binding of STRAD and LKB1 increases the affinity of MO25 for STRADα (Boudeau et al., 2004). The interaction between STRADα and LKB1 further stabilized by R240 on MO25, within the WEF binding pocket, as it only binds STRADα when it is complexed with LKB1 (Boudeau et al., 2004). Conversely, knocking down MO25 reduces the catalytic activity of the LKB1/STRAD complex, as determined by reduced P-Erk1/2 levels(Boudeau et al., 2003a). Furthermore, MO25 has been found to bind and activate other Germinal Center Kinase III (GCKIII) kinases with conserved WXF or WEW motifs, including SPAK,

OSR1, MST3, MST4, and YSR1 (Filippi et al., 2011), placing it in a potentially regulatory position in multiple cellular processes. There also exists a concave binding site on MO25 not bound by STRAD, which may act as a scaffold for yet-to-be-identified interactors (Milburn et al., 2004). MO25 levels are self-regulating, and association of MO25 with STRADα is reduced in hyperglycemia (Kundu et al., 2014). MO25 increases the catalytic activity of each LKB1/STRAD complex, and is especially important for LKB1/STRADβ catalytic activity (Boudeau et al., 2003b; 2004). MO25 also increases the cytoplasmic localization of the LKB1/STRAD complex (Boudeau et al., 2003b). MO25 also interacts with MST3 and MST4, and this is necessary for LKB1-dependent apical brush border formation (Klooster et al., 2009). Additionally, MO25 regulates other members of the GCKIII family kinases (Filippi et al., 2011), and activates each protein it binds, going through a transitional state on the way to activation (Hao and Zhou 2014) (Hao et al., 2014). This MO25 interactions add yet another layer of temporal and spatial regulation onto STRAD/LKB1 signaling.

Chapter Two: Materials and Methods

A. Phylogenetic analysis

Sequences were aligned using multiple sequence comparison by log-expectation (MUSCLE). Phylogenetic analysis was performed using MPI version of MrBayes 3.1.2 with MPICH2 installed by running in parallel on eight nodes (Ronquist 2003; Altekar). Bayesian trees with posterior probabilities were constructed with mixed amino acid models, a gamma distribution for rate variation among sites and a propor- tion of invariable sites. MrBayes was executed with two runs (four chains for each run), four million generations of Markov Chain Monte Carlo (MCMC) analyses, with 1,000 as the sample frequency and with a temperature parameter 0.2. The number of MCMC generations assured convergence of the two runs by having a standard deviation of split frequencies less than 0.005. The posterior probability of each split was estimated by sumt with 1,000 trees discarded as burnin based on the plot of 'generation vs. log probability'. Tree with branch lengths and posterior probabilities is shown in Additional file 1: Figure S1. Parameters were summarized by sump with 1,000 burnin, and values for the Potential Scale Reduction Factor (PSRF) were all close to 1.0 for all parameters. Scale bar represents amino acid replacements/site/unit evolutionary time.

B. Animals

All mouse experiments in this study were performed using methods and protocols reviewed and approved by the Oregon Health and Science University Institutional Animal Care and Use Committee (protocol number IS00001565) or the Utrecht University Institutional Animal Care and Use Committee (protocol number HL05.1010 and governmental approval number E17) and were carried out in accordance with National Institutes of Health standards and following established guidelines of the Public

Health Service. The STRADα gene trap mice were generated by random insertion of a virally-encoded splice acceptor cassette into mouse embryonic stem cells (Lexicon Genetics, see Figure 4.2) and STRADβ-floxed transgenics bear Cre recombinase recognition sites flanking exon 2 which encodes the start codon of the open reading frame (Figure 4.2). For conditional and germ-line deletion, the STRADβ conditional line was mated to mice carry cre re- combinase under the control of either the empty spiracles homeobox 1 (Emx1) or cytomegalovirus (CMV) pro- moter. LKB1 floxed mice are available from the National Cancer Institute Mouse Repository (strain number 01XN2).

C. Constructs and reagents

1. RNA

Total RNA from mouse tissues was extracted using Trizol reagent (Life Technologies, Grand Island, NY, USA) alone or in combination with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). A total of 2 μg RNA was used as a template with Superscript VILO cDNA Synthesis Kit (Life Technologies). Alternatively, we employed a polyadenylated mRNA-derived cDNA tissue panel (Clontech, Mountain View, CA, USA). Splice forms of STRADα were amplified using Taq polymerase (Qiagen) with the following primers of the STRADα message in mouse. The sequences for 5' segments were as follows: 5'-TGCGCTCTGACTCCTA GACC-3' and 5'-GCTGCTCATCATCTCTGGTTT-3'. To detect 3' splicing, primers targeting this region were: 5'-TACGGCTCTGCAAGGATCT-3' and 5'-AGTTGGTG ATGGGAGTGACTG-3'.

STRADβ was amplified using the following primers:

5’-TCTGCACCAAAATGGCTGTA-3’ and 5’-ACATCC AGTGGGCTATACGG-3’. Amplicons were purified and sub-cloned into either the TOPO-TA pCR 2.1 or pCR Blunt vector (Life Technologies) and sequenced using M13 forward or reverse primer. Quantitative RT-PCR was carried out based on the manufacturers protocol (Applied Biosystems – Life Technologies, Grand Island, NY) using total RNA extracted as described above.

D. Electroporation and Neuron Culture

Ex utero electroporation and primary neuron culture *ex utero* electroporation was performed as described previously using a 2 µg/µL final concentration of cDNA in STRAD/LKB1 overexpression experiments (5). After electroporation, cortices were dissected, dissociated and plated on poly-D-lysine-laminin coated coverslips (BD Biosciences, San Jose, CA, USA), cultured for multiple days in vitro in Neurobasal media supplemented with B27 supplement, penicillin-streptomycin, and Glutamax (all from Life Technologies).

Neuronal transfection was carried out using Lipofectamine 2000 (Life Technologies) in Optimem media (Gibco) for a total period of 6 hours.

E. Astrocyte culture

Astrocytes were cultured according to the Banker method from P1 pups [48]. They were fed every third day with glial MEM (MEM, 20% glucose, penicillin/streptomycin, 10% heat-inactivated horse serum) – all from Life Technologies. RNA was collected once astrocytes reached confluency.

F. Immunohistochemistry

Cells and tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes at room temperature (cells) or overnight at 4°C (tissues) on a rotating shaker. Before immunostaining, they were blocked for one hour in 0.1% cold water fish skin gelatin/1%BSA/0.5% TritonX-100/0.01 M Tris-buffered saline (TBS) (all from Sigma St. Louis, MO), after which primary antibodies were added (see Additional file 1: Table S1) overnight at 4°C on a rotating shaker then washed 3 × 10 minutes in 1x PBS. Alexa-Fluor fluorescent secondary antibodies (Life Technologies) were applied at 1:1,000 in the same blocking solution with the addition of 5% goat serum for one hour at room temperature in the dark.

Primary antibodies that require antigen retrieval were used in combination with heat induced epitope retrieval (HIER) using 0.1mM sodium citrate pH 6.0 for 30 mins at 98°C after which sections were blocked and immunolabeled as described above.

G. Confocal Imaging

All fluorescent images were acquired on a Nikon A1 laser-scanning confocal microscope using identical excitation and detection conditions within each set of slides and running NIS-Elements imaging software (Nikon).

H. Cycloheximide experiments

Cycloheximide (Sigma) at 100 mg/mL was used at 50 µg/mL final concentration in protein stability experiments. All protein stability experiments were carried out approximately 16 hours after transfection in Optimem media (Life Technologies). For time-courses of LKB1 degradation, cells were treated at the indicated time point relative

to control, then lysed and analyzed by Western blot. The amount of LKB1 protein was normalized to actin in each sample, and compared with untreated cells from the same experiment to determine the relative amount of LKB1 protein remaining at each time point. Curves were determined by Prism5 software's nonlinear fit of exponential decay function.

I. shRNA-mediated Knockdown

ShRNA's were designed using combinations of the following website-based algorithms:

http://www.genelink.com/sirna/shrnai.asp

https://rnaidesigner.invitrogen.com/rnaiexpress/setOption.do?designOption=shrna&pid=-5669327684189176500

http://www.thermoscientificbio.com/design-center/?redirect=true

Oligos were designed with the following specifications:

Loop:

TTCAAGAGA

Forward Oligo cloning site:

5' GATCC

3' TTTTTTGGAAA

Reverse Oligo cloning site:

5' AGCTTTTCCAAAAAA

3' G

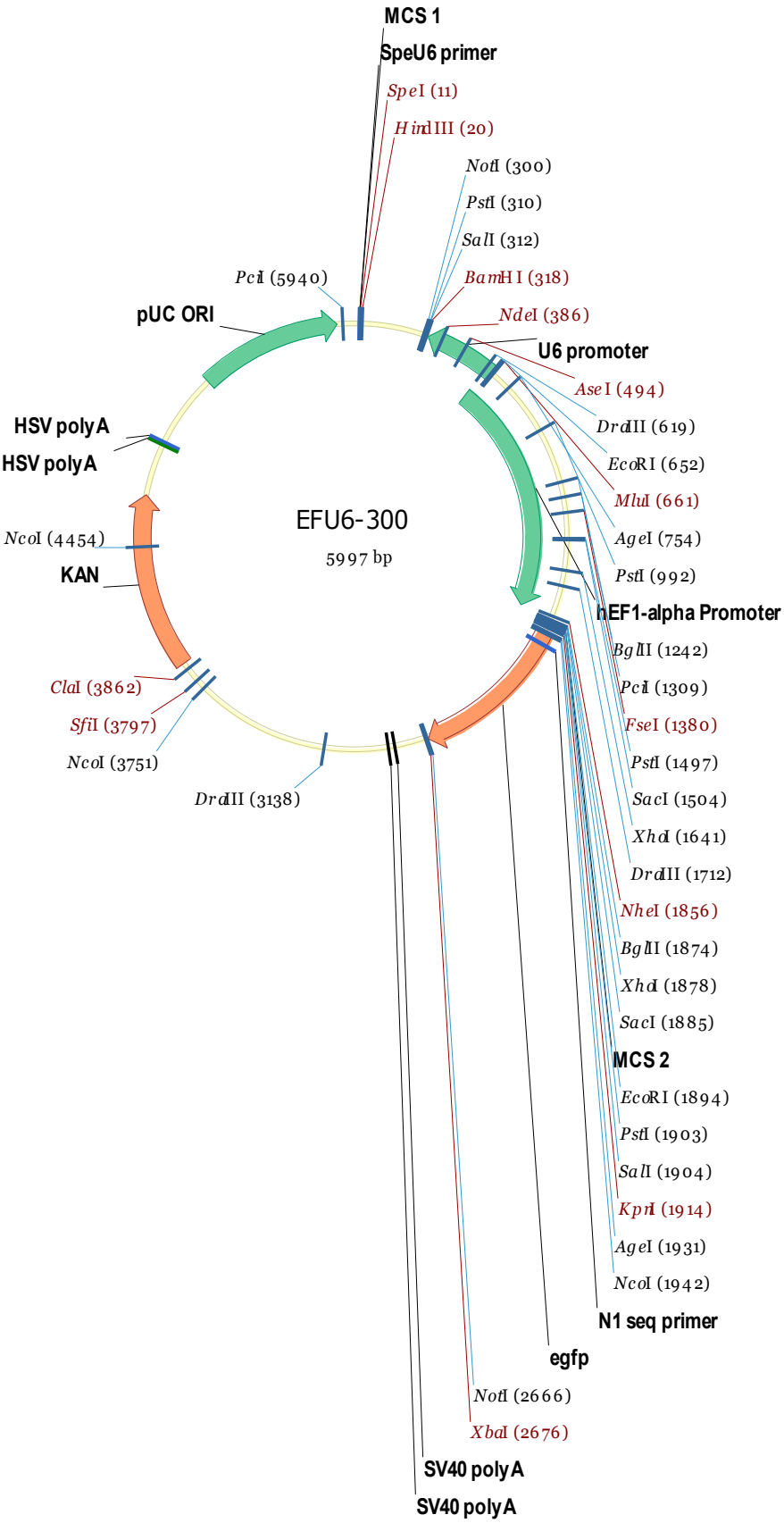
Forward Primer:

5' GATCC – Oligo seq –TTCAAGAGA – Antisense Oligo seq.– TTTTTTGGAAA 3'

Reverse Primer:

5’ AGCTTTTCCAAAAAA – Complementary sequence to sense primer – G 3’

The annealed oligomers were subcloned into a modified pSilencer 2.1 U6-Neo vector containing IRES-GFP for transfection efficiency determination using BamHI and HindIII restriction sites. The modified vector map is as follows:


MCS 1
SpeU6 primer
SpeI (11)
HindIII (20)
NotI (300)
PstI (310)
SalI (312)
BamHI (318)
NdeI (386)
U6 promoter
AseI (494)
DraIII (619)
EcoRI (652)
MluI (661)
AgeI (754)
PstI (992)
hEF1-alpha Promoter
BglII (1242)
PciI (1309)
FseI (1380)
PstI (1497)
SacI (1504)
XhoI (1641)
DraIII (1712)
NheI (1856)
BglII (1874)
XhoI (1878)
SacI (1885)
MCS 2
EcoRI (1894)
PstI (1903)
SalI (1904)
KpnI (1914)
AgeI (1931)
NcoI (1942)
N1 seq primer
egfp
NotI (2666)
XbaI (2676)
SV40 polyA
SV40 polyA
DraIII (3138)
NcoI (3751)
SfiI (3797)
ClaI (3862)
KAN
NcoI (4454)
HSV polyA
HSV polyA
pUC ORI
PciI (5940)
EFU6-300
5997 bp

J. Western Blotting

Tissues and cells were lysed in aqueous lysis buffer consisting of: 50 mM Tris-Cl pH 7.4 (Fisher); 150 mM NaCl (Fisher); 1% Triton (Sigma); protease inhibitor cocktail (Sigma)/phosphatase inhibitors 2 and 3 (Roche). Lysis volume was optimized to yield 1-2ug/mL protein concentrations for both tissues and cells. Lysates were kept at 4°C while being spun for 10 minutes at maximum speed in a cooled Eppendorf tabletop centrifuge. Supernatant was collected and boiled in Laemmli Buffer for 5 minutes at 98°C. Approximately 20ug of protein was loaded per lane (unless otherwise specified) of pre-cast NuPAGE Bis-Tris 4-12% gel (Fisher or Life Technologies) at a constant 60mA for two gels for one hour. Proteins were transferred a wet transfer system onto PVDF Immobilon FL membrane (Fisher Scientific), using 400mA for 80 minutes per two gels in a BioRad transfer apparatus (Mini Trans-Blot® Electrophoretic Transfer Cell #170-3930). After transfer, membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline with triton (TBST) for a minimum of one hour, and then primary antibodies were added at the concentrations listed in Table 1 on a rotating shaker for either one hour at 23°C or overnight at 4°C. Membranes were washed 3X10 minutes in TBST and then membranes were transferred to 5% BSA/TBST. Fluorescent secondary antibodies were added at a concentration of 1:10,000 for one hour at 23°C (Cell Signaling). Membranes were washed 3X10 minutes in TBST and then transferred to phosphate-buffered saline (PBS) for imaging. Imaging was carried out on an Odyssey Original imager per manufacturer's guidelines (Li-cor). Densitometry was measured using the Odyssey 2000 software, and results were normalized to Actin densitometry from the same lane (unless otherwise specified).

K. Glutathione Sepharose or Streptavidin Pulldown

Tissues or cells were lysed as previously described and then exposed to either Glutathione Sepharose coated agarose beads (GE Healthcare) or streptavidin-coated agarose beads (GE Healthcare), depending upon the experiment. Lysate was incubated with 30uL of the appropriate beads for one hour at 4°C on a rotisserie shaker. The volume was brought up to 500 uL with IP wash (described above). Samples were then spun at 5000Xg for one minute in a 4°C cooled centrifuge. Supernatant was removed and replaced with fresh IP wash, and samples were placed on the rotisserie shaker for 10 minutes at 4°C. This step was repeated three times. After the third wash, all the supernatant was removed using a flat gel loading tip and 30 uL 5X Laemmli buffer (described previously) was added to each sample. Beads were boiled for 5 minutes at 98°C and then spun down at maximum speed for one minute on a tabletop centrifuge (Eppendorf). Laemmli buffer was collected with a flat gel loading tip and the whole volume was run on a Western blot, as described above.

L. Migration analysis/Cell quantification

Migration of electroporated or PH3 immunolabeled cells was done relative to the ventricular and pial surfaces. Specifically, using NIS Analysis software (Nikon), each surface was traced with a guideline. Then, the distance of each positive cell (GFP or PH3) from the ventricle and from the pial surface was measured. These two values were summed to determine the total cortical thickness at that particular point, and the cell's distance from the ventricle was represented as a percentage of that cortical thickness. Cortical zones (VZ/SVZ, IZ, CP) were determined by taking multiple measurements

within each cortex and calculating the average percentage of total cortical thickness at which each zone began/ended, by nuclear density within each region.

Total PH3+ cell numbers were counted using the same software, by quantifying all positive cells within the outlined cortical region, across multiple similar sections from each animal.

For MADM experiments, the green to red ratio (GR ratio) was determined by quantifying all green cells and all red cells within the region of interest, and by dividing the number of green cells by the number of red cells. Cells containing both green and red were excluded.

Chapter Three: Splicing and Expression of the STRAD proteins

I. Introduction

Brain development is driven by many series of genes, and their varying expression patterns contribute to regulating each step of corticogenesis (Jones and Rakic, 2010; Kriegstein and Noctor, 2004; Noctor et al., 2004; Sidman and Rakic, 1973; Tissir and Goffinet, 2003). During development, gene expression must be quickly and accurately regulated, and transcription and splicing are two of the ways to accomplish this feat. Since the developing the brain is a highly dynamic environment, we were interested in understanding the regulation of the STRAD mRNAs and transcripts across developmental time. In this section, we will explore the relative expression patterns of the STRAD transcripts during brain development in greater detail than has previously been reported (Barnes et al., 2007).

Mammals have very complex brains, but do not have a coordinately larger genome when compared with many invertebrate species, such as C. elegans. To achieve this observed level of complexity, the relatively few genes must be utilized in a large variety of ways. One strategy to accomplish this task is alternative splicing (Starokadomskyy, 2007). Many studies have shown cell type specific splicing, and this seems to be especially relevant in neurons (Li et al., 2007; Licatalosi et al., 2012; Lipscombe, 2005; Makeyev et al., 2007). Other groups have reported multiple splice variants of both STRADs and of LKB1 (Boudeau et al., 2004; Branvold et al., 2008; Marignani et al., 2007), but their relevance has not yet been explored in primary tissues. We aim to elucidate the expression patterns of these splice variants throughout cortical development and to determine the extent to which they have unique functions related to neuronal development.

II. Results:

A. STRADα is the most evolutionarily conserved STRAD homolog among metazoans.

The conservation of sequences and traits can be extremely informative to the functions of a protein. Since there are two very closely related STRADs, we were curious as to their evolution, and the extent of their relatedness. To address this question, we examine their phylogeny in a number of invertebrate and vertebrate species.

Significant biochemical characterization has been conducted using vertebrate STRADs, yet few studies have addressed their function *in vivo* using genetic loss-of-function approaches. Conversely, a number of phenomenological studies, but few biochemical analyses, have been performed using invertebrate STRADs (Chien et al., 2013; Denning et al., 2012; Kim et al., 2010; Narbonne et al., 2010). We conducted a phylogenetic analysis of the STRAD proteins to contextualize the relationship and functional contributions of the vertebrate paralogs and better understand how the invertebrate data sets inform vertebrate functions. We find that metazoan STRAD proteins exhibit a high level of structural and sequence similarity within a species, such as mouse (Figure 3.1A), and also between species (Figure 3.1B Figure 3.2). Our analysis further suggests that a single STRAD gene duplication event occurred sometime between the origin of, and the last common ancestor for, the vertebrate lineage (Figure 3.1B and Figure 3.2). The phylogenetic pattern indicates that the vertebrate STRADα is more similar to the single invertebrate STRAD ortholog than is STRADβ. This primacy is paralleled in our loss of function studies and by previous studies demonstrating the profound effects of STRADα loss-of-function mutations in human patients (Puffenberger

et al., 2007) and these observations will be discussed in further detail in Chapters 4 and 5.

Figure 3.1

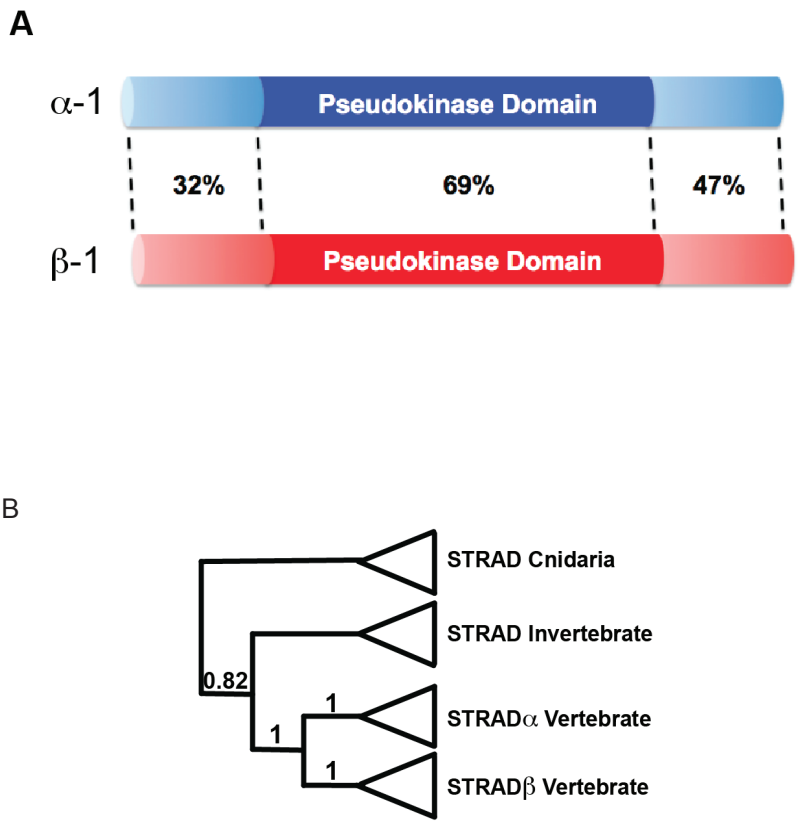


Figure 3.1 - STRAD splice forms are expressed in a tissue-specific manner. (A) Schematic of mouse STRADα and STRADβ proteins indicating percent similarity between the two proteins. (B) Schematic of phylogenetic tree based on the STRAD gene from Bilaterians using Cnidaria as the outgroup with posterior probabilities indicating support for nodes (0–1, 1 being the strongest support) labeled on nodes of interest. The tree is based on MrBayes phylogenetic analysis (see Figure 1.2).

Figure 3.2

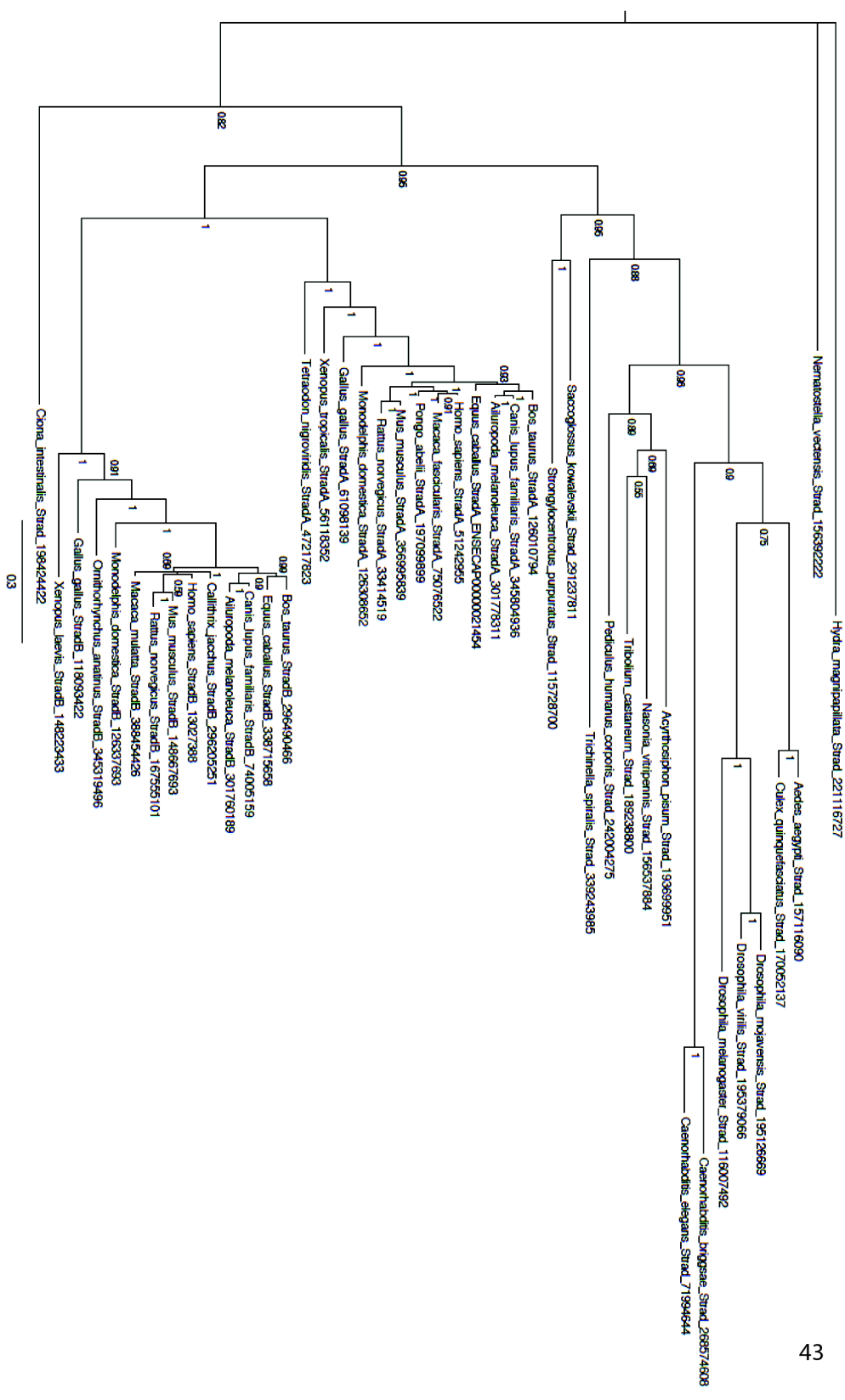

Nematostella_vectensis_Strad_156392222
Hydra_magnipapillata_Strad_221116727
Aedes_aegypti_Strad_157116090
Culex_quinquefasciatus_Strad_170052137
Drosophila_mojavensis_Strad_195126669
Drosophila_virilis_Strad_195379066
Drosophila_melanogaster_Strad_116007492
Caenorhabditis_briggsae_Strad_268574608
Caenorhabditis_elegans_Strad_71994644
Acyrthosiphon_pisum_Strad_193699951
Nasonia_vitripennis_Strad_156537884
Tribolium_castaneum_Strad_189238800
Pediculus_humanus_corporis_Strad_242004275
Trichinella_spiralis_Strad_339243985
Saccoglossus_kowalevskii_Strad_291237811
Strongylocentrotus_purpuratus_Strad_115728700
Bos_taurus_StradA_126010794
Canis_lupus_familiaris_StradA_345804936
Ailuropoda_melanoleuca_StradA_301778311
Equus_caballus_StradA_ENSECAP00000021454
Homo_sapiens_StradA_51242955
Macaca_fascicularis_StradA_75076522
Pongo_abelii_StradA_197098899
Mus_musculus_StradA_356995839
Rattus_norvegicus_StradA_33414519
Monodelphis_domestica_StradA_126308652
Gallus_gallus_StradA_61098139
Xenopus_tropicalis_StradA_56118352
Tetraodon_nigroviridis_StradA_47217823
Bos_taurus_StradB_29649466
Equus_caballus_StradB_338715658
Canis_lupus_familiaris_StradB_74005159
Ailuropoda_melanoleuca_StradB_301760189
Callithrix_jacchus_StradB_296205251
Homo_sapiens_StradB_13027388
Mus_musculus_StradB_148667693
Rattus_norvegicus_StradB_167555101
Macaca_mulatta_StradB_38845426
Monodelphis_domestica_StradB_126337693
Ornithorhynchus_anatinus_StradB_345319496
Gallus_gallus_StradB_118093422
Xenopus_laevis_StradB_148223433
Ciona_intestinalis_Strad_198424422
03

Figure 3.2 - MrBayes tree of STRAD gene in Metazoan using Hydra magnipapillata as the outgroup (46,47). The taxon name is composed of the species name, gene name (STRAD, STRADα, or STRADβ), and gene ID (NCBI GI numbers and one Ensembl protein ID for Equus caballus STRADa). Scale bar represents amino acid replacements/site/unit evolutionary time.

B. A developmentally-regulated, tissue-restricted form of STRADα is expressed exclusively by neurons in the CNS.

Previous work established that the STRAD genes are expressed in developing cortex (Barnes et al., 2007), yet the distribution and developmental expression patterns needed clarification. We approach this question using quantitative PCR as a way to assess STRAD mRNA levels. Our analysis indicates that STRADα levels decrease slightly prenatally, while STRADβ mRNA levels increase throughout cortical development (Figure 3.3 A-B). Additionally, an *in situ* hybridization study found STRADα mRNA highly expressed throughout the E15.5 mouse cortex, and particularly in the ventricular zone, while STRADβ mRNA is expressed more highly in mature neurons in the cortical plate (Barnes et al., 2007). While informative, these studies do not differentiate splice variants of the STRADs, so we aimed to elucidate this. Our examination of the STRAD expression patterns reveals the relative contributions of STRADα and STRADβ splice variants (Figure 3.4 A and B) and finds that they change across developmental time. A number of STRADα isoforms result from differentially utilized exons near the translation initiation site in normal adult mouse tissues (Figure 3.4 C), some of which are observed in Western blots of rat tissue (Filippi et al., 2011) (Figure 3.4 C for nomenclature). Despite the fact that multiple STRADα splicing events have been described in human tumor cell lines it is likely that they reflect aberrant splicing relative to normal tissues (Marignani et al., 2007). With this in mind, we aimed to determine the *in vivo* expression pattern of STRADα mRNA isoforms in adult tissues and during brain development. The amino terminal of STRADα is highly spliced and these small amino acid differences are difficult to detect using Western blot. To circumvent this, we characterized the expression

of the STRADα variants relative to one another using reverse transcriptase PCR, and specifically amplified the N-terminus of STRADα. Our amplicon sequence analysis indicates that the largest STRADα variant (STRADα-1) results from the use of an alternate splice donor site and the inclusion of exon IV, along with a second nuclear export signal (NES) previously reported to be functional (Dorfman and Macara, 2008), but the inclusion of this second NES is of unknown significance (Figure 3.4 A, C). Previous studies have shown human cells lines exhibit splicing in the exons encoding carboxyl regions of the protein (Marignani et al., 2007), but we do not detect these splice forms in mouse tissues (Figure 3.4 A'). STRADα-4 generates a larger amplicon, yet smaller predicted protein due to a frame shift resulting in a stop codon just following the typical translation start site, forcing translation to initiate further in the mRNA (Figure 3.4 A, C). This isoform has previously been reported in human tumor cell lines (Marignani et al., 2007), and we detect it in most tissues (Figure 3.4 A), but it is essentially absent from neurons (Figure 3.5 A, asterisk). Of the N-terminal splice variants, STRADα-1 appears most abundantly in the nervous system, and to a much lesser extent in skeletal muscle and testes (arrowhead Figure 3.4 A). Within the CNS, it is exclusive to neurons (Figure 3.5 A) and is developmentally regulated (Figure 3.5 B). This protein level trend holds embryonically as well as postnatally (Figure 3.6 A), and multiple splice variants are observed in human tissue lysates (Figure 3.6 B), in agreement with previous studies (Marignani et al., 2007). By RT-PCR, we observe three additional splice variants previously seen and a novel fourth isoform, STRADα-7, a predicted protein in humans (GenPep EAW94290).

To test the functionality of N-terminal STRADα nuclear export signal, we

transfected this neuronal N-terminus of STRADα tagged with YFP into primary cultures of E15.5 WT cortical neurons. We find that it is effectively targeted to the cytoplasm but was not further targeted within any subcellular domain in these cells (Figure 3.7).

Previous work has detected a single splice variant of STRADβ owing to a skipping of exon 8 and premature stop codon (Nishigaki et al., 2003; Sanna et al., 2002). Characterization of STRADβ indicated broad expression in adult tissues and within brain, it was found most highly expressed in the cortical plate along with MO25α (Barnes et al., 2007). This co-expression of MO25α along with STRADβ is not surprising, as MO25α has been found to significantly increase the catalytic activity of the LKB1/STRADβ complex. Our results detect STRADβ-1 (full length protein) in all tissues probed and STRADβ-2 (truncated splice variant) is seen in all except skeletal and cardiac muscle (Figure 3.4 B). Unlike STRADα, transcriptional levels and splicing of STRADβ appear to vary less in cortical tissue and neurons across developmental time in vivo and in vitro (Figure 3.5 C, D).

Figure 3.3

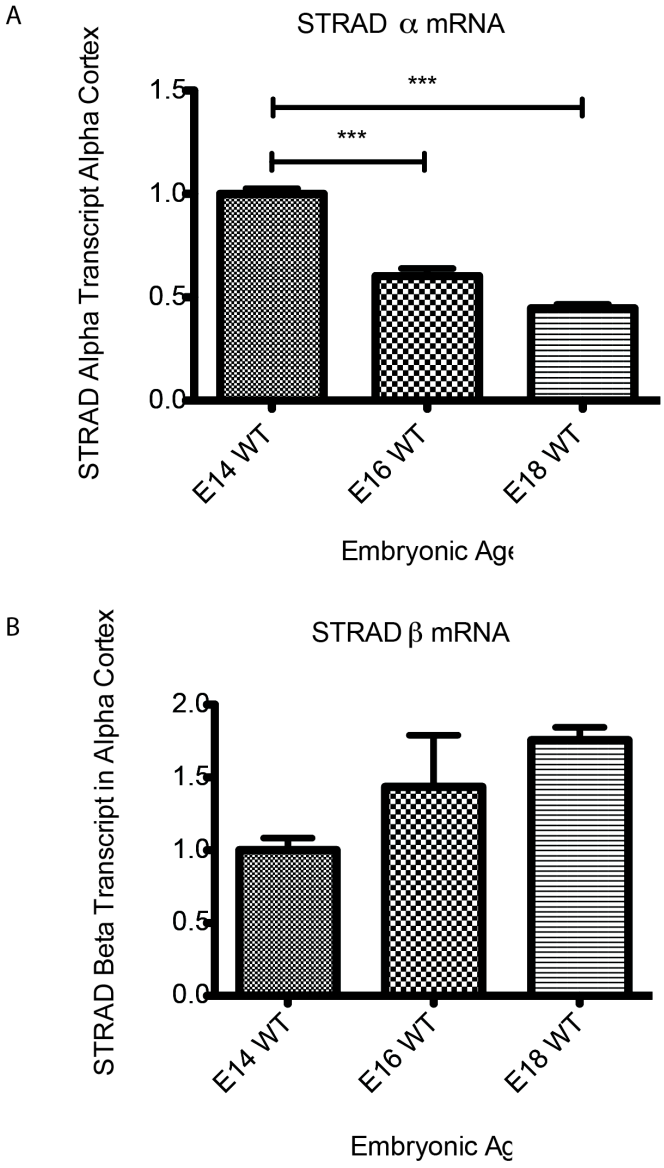


Figure 3.3 - Quantitative real-time-PCR of STRADα and STRADβ across developmental time, normalized to GAPDH. (A) STRADα mRNA decreases across developmental time in the developing cortex. (B) STRADβ mRNA does not increase significantly across cortical development, but trends towards an increase. In all cases, significance was determined by One-way ANOVA with Dunnett's multiple comparisons test, using E14 as the control group. *** P<0.001

Figure 3.4

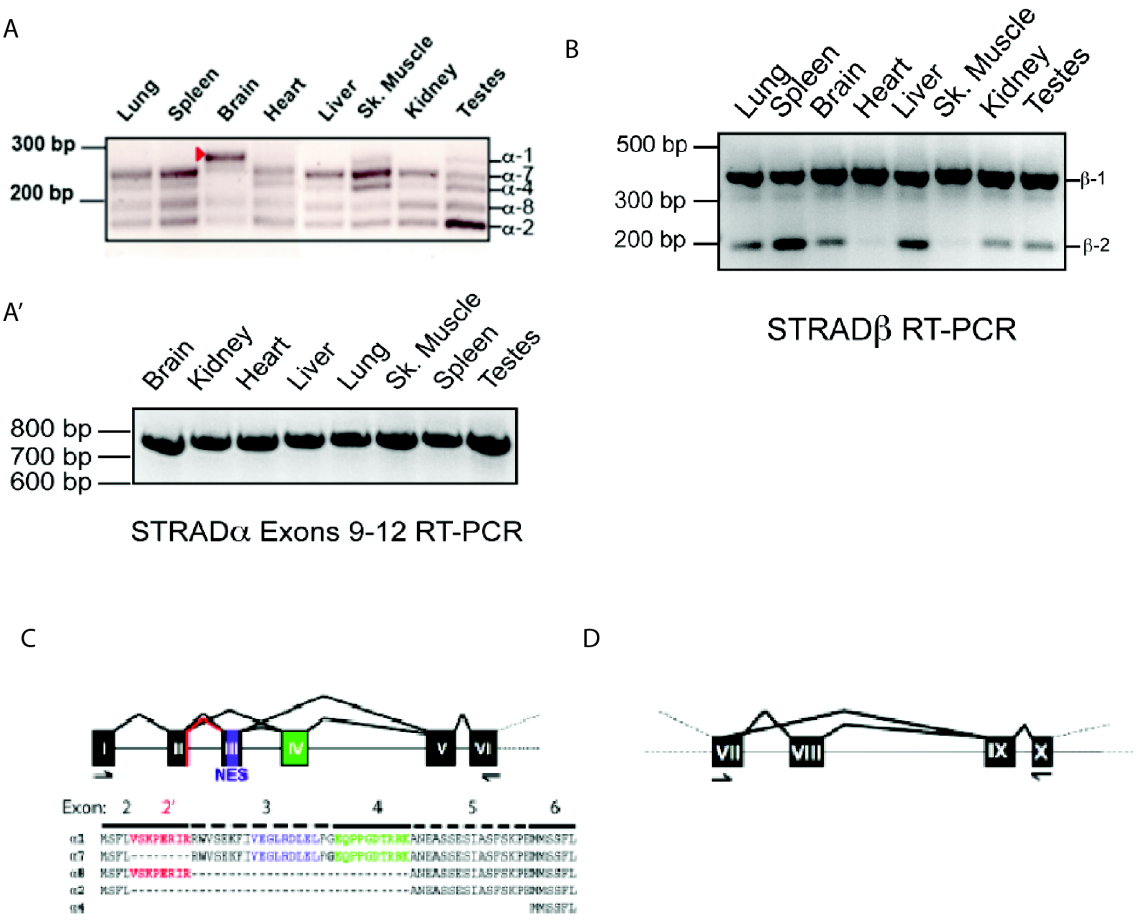


Figure 3.4 - (A) Reverse transcriptase-PCR (RT-PCR) of STRADα indicates that multiple variants exist in distinct tissue types. In particular, the largest species (arrowhead) appears to be specific to brain, skeletal muscle and testes. (A') RT-PCR of C-terminal STRADα splicing (primers span exons 9-12) of STRADα in multiple tissues. (B) the only reported splicing variant for STRADβ indicates that this splicing event occurs in all tissues except skeletal and cardiac muscle. (C) Amplicon sequencing indicates that at least five variants arise in the amino terminal domain of STRADα. Arrows indicate RT-PCR primer placement. (D) STRADβ splicing diagram in which arrows denote primers. Exon 8 is skipped in STRADβ-2 creating a premature stop codon and truncated mRNA.

Figure 3.5

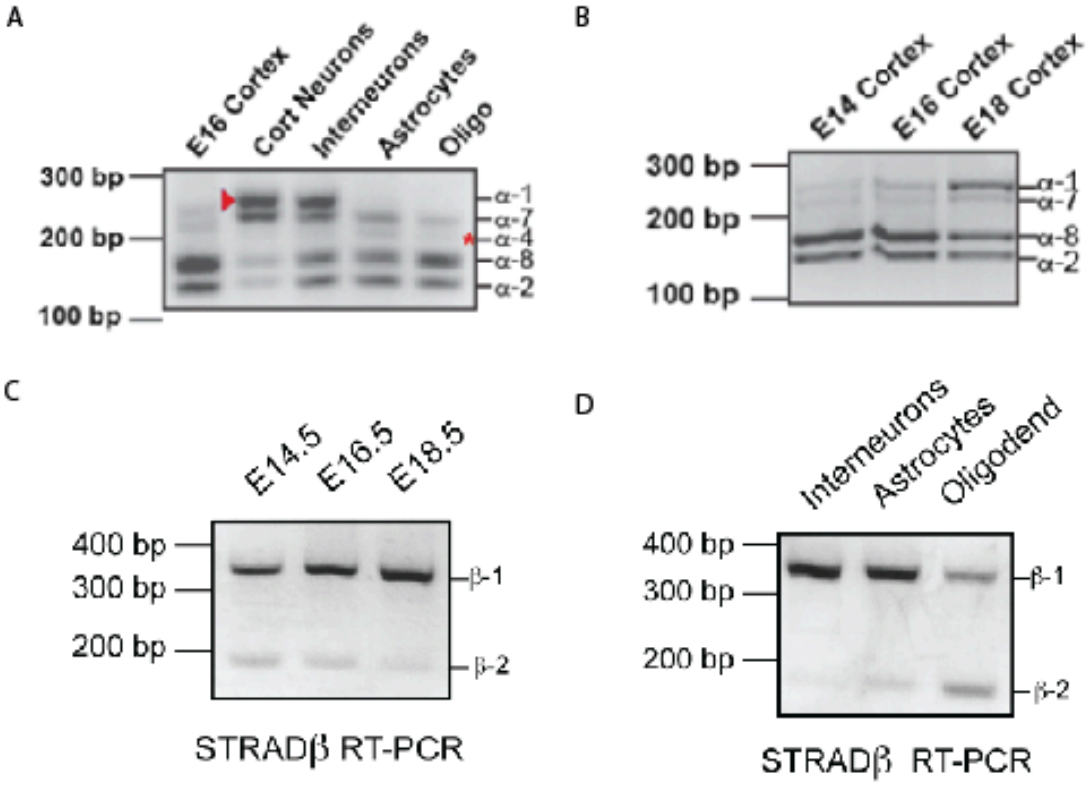
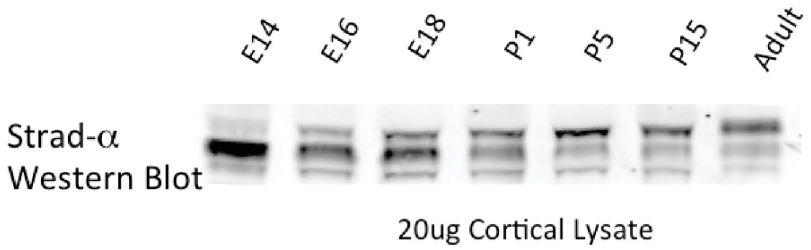


Figure 3.5 - A) RT-PCR products from enriched cultures of the dominant CNS cell types. (STRADα-1 isoform – arrowhead; STRADα-4 isoform - asterisk). Cort neurons – cerebral cortex primary neurons; Interneurons – medial ganglionic eminence primary neurons, Astrocytes – primary post-natal day 1 astrocytes, Oligo – primary oligodendrocyte cultures. (B) RT-PCR of STRADα isoforms across developmental time in the cerebal cortex. CNS, central nervous system. (C) STRADβ splicing across developmental time in E14.5-E18.5 cortex. (D) STRADβ splice variant expression in primary cultured interneurons, astrocytes and oligodendrocytes.

Figure 3.6

A



B

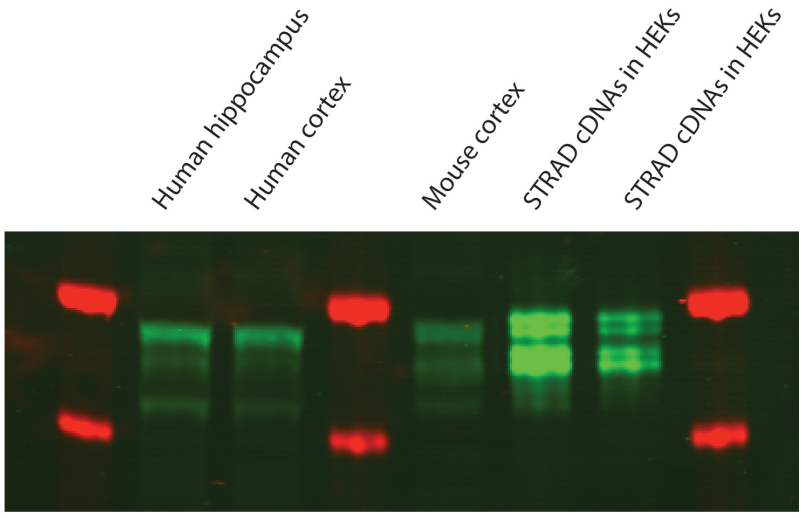


Figure 3.6 - (A) Western blot for STRADα across developmental time reveals multiple splice variants that are developmentally regulated. (B) Western blot for STRADα reveals expression in human brain lysate, as well as antibody specificity as determined by overexpression of STRAD α cDNA in HEK cells.

Figure 3.7

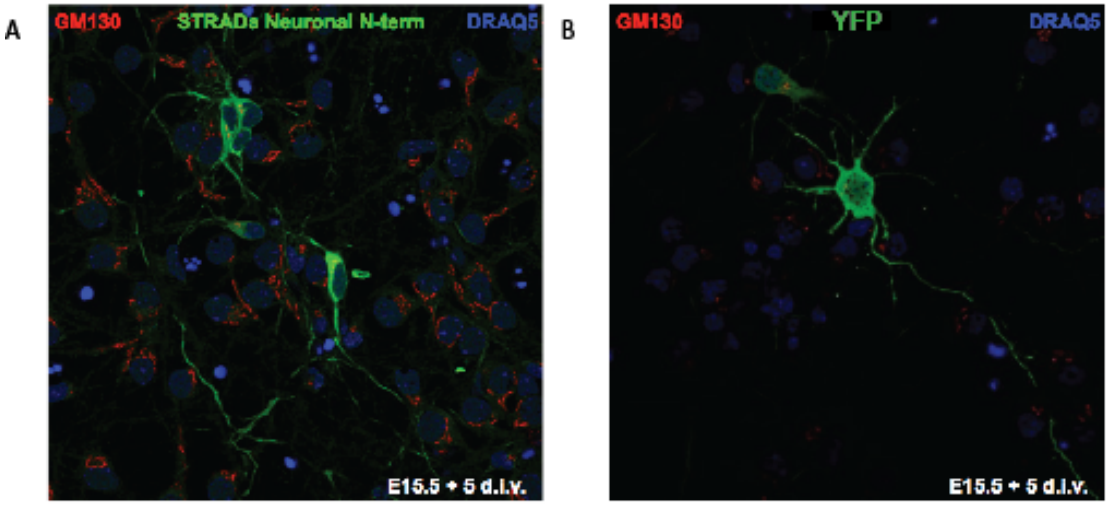


Figure 3.7 - Neurons were transfected with vector containing the cDNA of interest tagged with YFP. Transfection took place a day after plating and neurons were fixed after 5 days in vitro. (A) The neuron-specific N-terminus of STRADα is excluded from the nucleus. (B)YFP protein can be found throughout the neuron. Cultures were immunostained for GFP, the golgi marker GM130, and the DNA marker DRAQ5.

C. STRADα splice forms and full-length STRADβ exhibit similar competence to drive axon formation.

One of the hallmarks of neuronal development is the establishment of polarity and subsequent axogenesis. LKB1 is required for this process, so we examined its regulators, the STRAD pseudokinases, during brain development. The observation that these two very closely related genes have developmentally-regulated, tissue-specific, and cell-type specific splicing led us to question the functions of these splice variants. We hypothesized that the splice variants would have different effects on developing neurons. We chose to assay their function in axogenesis, as previous work has shown the ubiquitous variant of STRADα is capable of eliciting multiple axons with LKB1, and because this is a defining characteristic of neurons. To address the significance of each STRADα and STRADβ splice form in neurons we used an *ex utero* electroporation gain-of-function approach (Figure 3.8) (Hand et al., 2005). We and others have previously shown the ubiquitous STRADα-2 isoform to be capable of driving the formation of multiple axons when expressed alone in cultured neurons or with LKB1 in neural progenitors (Barnes et al., 2007; Shelly et al., 2007). Here, we quantified the proportion of green fluorescent protein positive (GFP^+) cells with one or more axons after overexpression of each STRAD splice form with or without LKB1. We found that when LKB1 or each STRAD isoform was individually expressed with GFP-expressing control vector, no significant increases in axon number per neuron were observed (Figure 3.9 A-G, H), consistent with prior observations (Barnes et al., 2007).

When paired with LKB1, we found that all tested STRADα splice forms are capable of eliciting multiple axons in a significant proportion of neurons, compared with

the STRAD isoform + GFP-expressing vector (Fig 3.9 B, B'-E'). In contrast, the full-length form of STRADβ was able to induce a similar phenotype (Fig 3.9 2F', G') while STRADβ-2 only slightly but significantly affected axogenesis (Fig 3.9 2G, G).

Figure 3.8

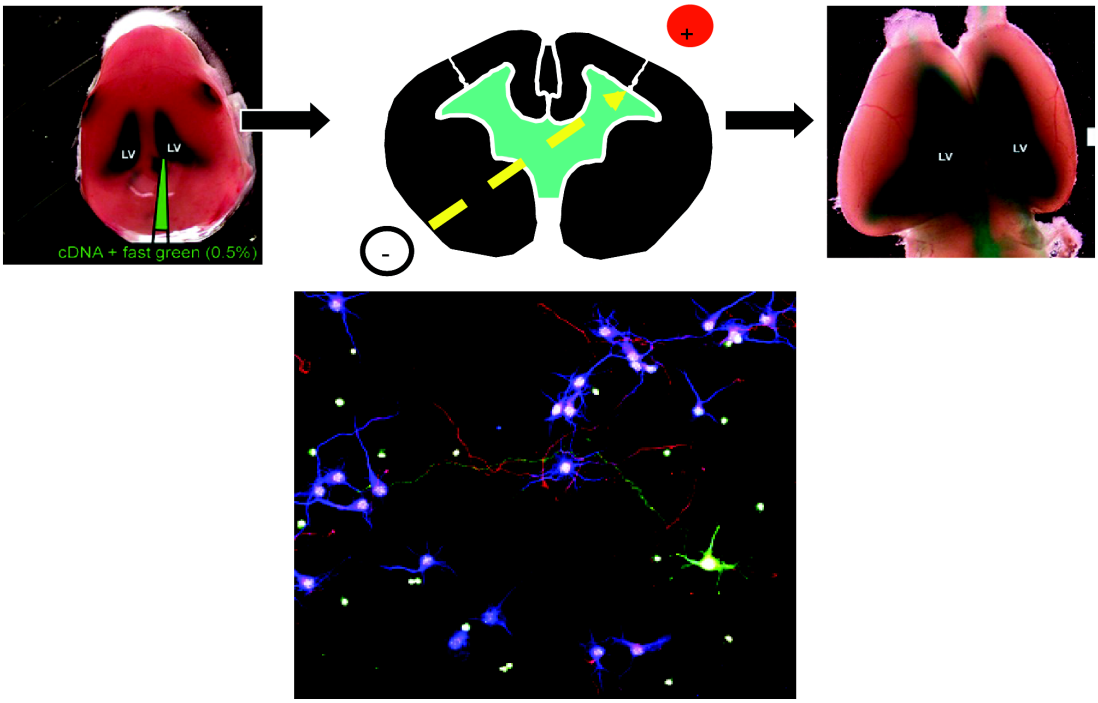


Figure 3.8 - Ex vivo cortical electroporation was used to target a relatively homogeneous population of neural progenitors in the ventricular zone. Embryos are dissected in cold complete HBSS and heads are removed, then injected with cDNA of interest and FastGreen Dye. A current is applied across the head, targeting DNA to the ventricular zone. Brains are dissected from the skulls and cortical regions are further dissected from the brains. Cells are dissociated according to Hand et al..

Figure 3.9

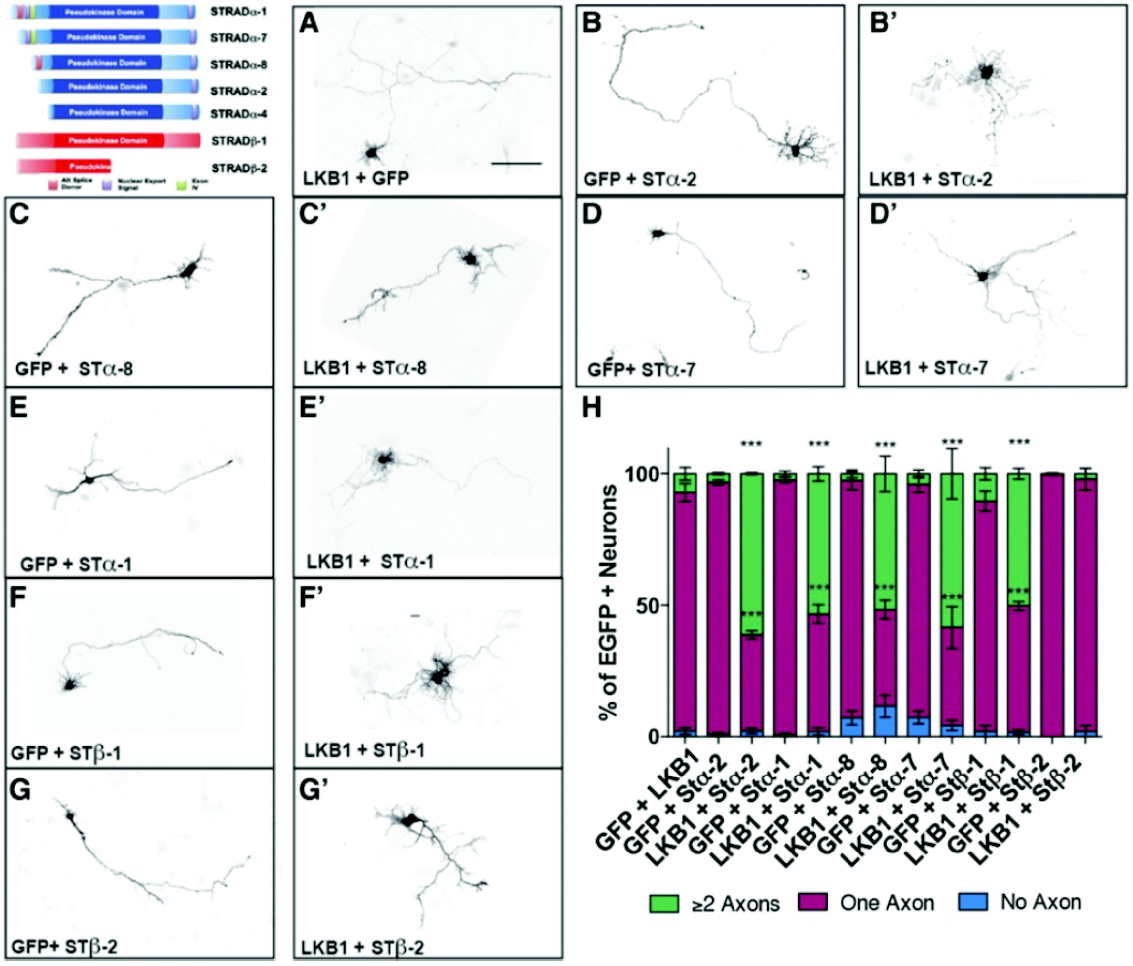


Figure 3.9 - Over-expression of STRAD isoforms leads to the initiation of multiple axons. Multiple splice variants of each STRAD gene were tested for their ability to elicit supernumerary axons alone or with LKB1. Wild-type E15.5 cortices were electroporated with designated constructs and axon number, as determined by MAP2/Tau immuno-labeling, was quantified in GFP+ cortical neurons after five days in vitro. Representative neurons from each condition are shown. Neither LKB1 nor individual STRADα splice forms exhibit a significant increase in multi-axon cells (A-G). When co-expressed with LKB1, all forms of STRAD are sufficient to significantly drive the formation of multiple axons (B'-F') except the truncated form of STRADβ, (G, G'). Scale bar = 50 μm. ***P <0.001 (two-way ANOVA with Bonferroni's post-test comparing the STRAD isoform + LKB1 with the STRAD isoform + GFP). (H) Quantification of N = 3 to 6 independent experiments for each set of constructs. >300 GFP+ cells counted in each condition. ANOVA, analysis of variance; E, embryonic day; MAP2, microtubule associated protein 2.

III. Discussion

STRADα and STRADβ are very closely related, with STRADα appearing first in evolutionary time (Figure 3.2). There are two STRAD proteins in all vertebrates we examined, and only one in invertebrates, indicating a gene duplication event occurred somewhere during the divergence of vertebrates from invertebrates. Gene duplication events are not uncommon during evolution and often yield expanded functionality within a single gene family. Duplicated genes can provide redundancy, and sometimes affect the dosage of their proteins. Alternatively, gene duplications can have positive effects and yield a survival advantage. One example of this is the gene duplication event that yielded 3 opsins in Homo sapiens and Old World Primates with the effect of expanding the range of color vision (Dulai et al., 1999; Nathans et al., 1986). Each opsin gene only differs from other opsins by a few amino acids and is flanked by Alu repeats, allowing for a relatively high rate of mutation/duplication/loss of these genes (Dulai et al., 1999). This enhanced functionality allowed primates to differentiate red and green, and was useful in identifying edible and inedible food sources. While we have not identified the selective advantage for having two STRAD genes in terms of axogenesis, we have shown a level of redundancy that could be advantageous in mammalian species (Figure 3.9).

Our findings on phylogeny and axogenesis imply that STRADβ may have emerged in early vertebrates to serve a distinct purpose. PJS arises from a heterozygous LKB1 mutation, yet differs from PMSE Syndrome, which is caused by a homozygous mutation in STRADα (Hemminki et al., 1998; Puffenberger et al., 2007). The differing symptoms and severity of PJS and PMSE Syndrome speak to this expanded STRADβ functionality, and also imply that the tissue-specific splicing we observe is likely critical

for complete functionality of the STRAD/LKB complexes. Coinciding with the invertebrate to vertebrate expansion of the STRAD genes, STRADα and LKB1 also evolved. Invertebrate STRAD lacks a nuclear export signal and seems to have LKB1-independent functions (Chien et al., 2013; Narbonne et al., 2010). Conversely, LKB1 in invertebrates has also been shown to have STRAD-independent functions in proliferation (Narbonne et al., 2010). Invertebrate forms of LKB1 have no nuclear localization sequence and are found distributed throughout the cell, so do not require STRAD for nuclear export (Martin and St Johnston, 2003; Watts et al., 2000). The fact that only STRADα exports LKB1 from the nucleus could indicate that STRADβ is primarily responsible for regulating LKB1 nuclear functions, while STRADα drives LKB1 cytoplasmic signaling (Baas et al., 2003).. On the other hand, STRADβ is observed predominantly cytoplasmically when overexpressed in HEK cells (Figure 4.4), despite having no nuclear export signal. It is intriguing that STRADβ mRNA levels are higher in mature cortical plate neurons compared with the progenitors of the ventricular and subventricular zones (VZ and SVZ). This could indicate that STRADβ takes on unique roles in mature neurons, such as regulating dendritic branching, and this assertion remains to be tested. Perhaps STRADβ can bind LKB1, forming a weakly active complex wherever it is found, and this may represent a way to grade LKB1 signaling. Furthermore, the balance of STRADα and STRADβ could determine the relative activity of LKB1 in various developmental stages, acting to fine-tune the kinase.

As previously mentioned, another mode of quickly regulating protein expression is alternative splicing. Splicing acts as a form of specialization of a gene and allows it to be multifunctional across various tissues and developmental time (Maniatis, 1991). This

mechanism of regulation allows for the limited number of genes to be capable of executing diverse functions to generate and control the complex development of the brain (Lipscombe, 2005). While splicing exists in all tissues, it is highly prevalent in the nervous system and has been shown that certain proteins drive neuronal-specific splicing (Cao et al., 2007; Li et al., 2007; Licatalosi et al., 2012; Lipscombe, 2005; Mazin et al., 2013). Many studies have found this mechanism to be especially important in the calcium channels of sensory neurons, reflecting the complexity required for such specializations (Lipscombe, 2005). Developmental splicing mechanisms have also been well documented and are thought to help drive cell fate decisions (Mazin et al., 2013).

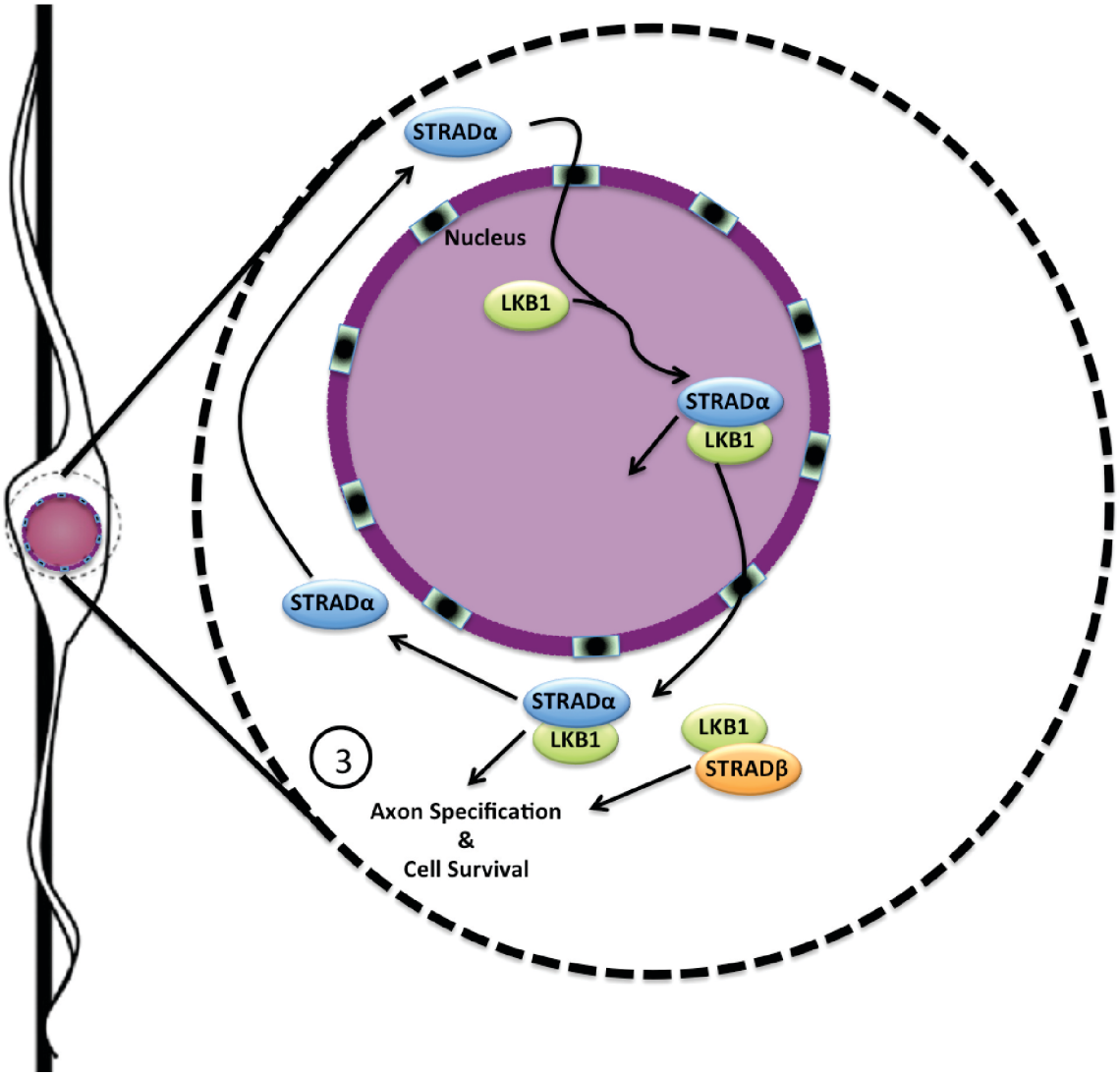
We observe that STRADα is highly spliced *in vivo* and *in vitro,* with unique splicing patterns in each tissue we probed. However, the detailed distribution of these splice variants is unknown, and future work should address this question. One possibility is to visualize splice variant distribution using locked nucleic acid (LNA) in situ probes (Kumar et al., 1998). LNA technology is normally used to visualize miRNA distribution and allows probing for very short nucleotide sequences, so could easily be adapted for this task. This information would be useful in teasing apart the many functions of LKB1/STRADα during brain development. The significance of the multiple N-terminal STRADα splice variants is unknown, but the existence of a neuron-specific splice variant implies a specialization of STRADα/LKB1 signaling critical to neurons. This longest form of STRADα has two NES's, perhaps representing more efficient nuclear-cytoplasmic shuttling of LKB1, or a shuttling that is independent of C-terminal modification events. This high level of developmentally regulated and tissue-restricted splicing is striking, but not unique to STRADα. Other genes that are similarly regulated

include the FGFRs (Emmenegger et al., 2012) and hrpf-1 (Barberan-Soler and Zahler, 2008).

The short form of STRADβ is also poorly understood, as it does not seem to act in a dominant negative fashion in terms of axogenesis, but only elicits a slight increase in multiple axons when overexpressed (Figure 3.9). This ability of the truncated STRADβ to bind and activate LKB1 indicates that a truncated form of STRADα, as found in PMSE patients, if expressed, could have similar function activity. A partially functional truncated STRADα may be functional enough for development and could be the reason human PMSE patients often survive into adolescence. STRADα and STRADβ are very tightly regulated, but maintain some redundancy throughout development, similar to the Importins and Exportins (Liang et al., 2014). There are many members of these gene families, and they have both overlapping and unique functions, and are also tissue- and developmentally-restricted (Liang et al., 2014).

While we did not find differential functions for each STRAD splice variant, the axogenesis assay is relatively qualitative. Future work should address the function of each splice variant in terms of axon and dendrite branching, synaptogenesis, proliferation, cell fate, and other neuronal functions. It is likely that this high level of temporal and cell-type specific control is important and could inform neurodegeneration as well as neurodevelopment.

Chapter Three Summary Figure



Chapter 3 Summary Figure. Schematic of STRAD/LKB1 signaling within the developing neuron. STRADα and STRADβ both complex with LKB1 and are capable of eliciting axogenesis in primary cultured neurons (3). Only STRADα can export LKB1 from the nucleus.

Chapter Four: Loss of Function of STRADα

I. Introduction:

Having established the spatiotemporal pattern of expression for each STRAD protein in the developing brain, we next turned our attention to addressing the *in vivo* and *in vitro* effects of STRADα loss. In the previous chapter, we found that each STRAD isoform can drive axogenesis by examining gain-of-function of the STRAD proteins. Additionally, our phylogenetic analysis indicates STRADα is the main evolutionary STRAD gene, so we geared our initial *in vivo* studies towards STRADα. Since overexpression can have off-target effects, we thought it necessary to also address STRADα loss of function.

Tightly controlled proliferation, polarity, cell survival, and cell fate are critical processes for successful corticogenesis. Previous studies have found LKB1 to be necessary for axogenesis and cell survival during cortical development but its role in progenitor balance in the brain has yet to be defined (Barnes et al., 2007). However, LKB1 has been shown to drive the G1/S transition in some cell types (Tiainen et al., 2002), so modulating its main regulator, STRADα, could affect cell cycle length in the developing cortex. Others have also found that STRADα plays a role in neuronal migration, and LKB1 has been shown to have roles in cellular proliferation and cell cycle, as well as cell fate (Chien et al., 2013; Lai et al., 2013; Orlova et al., 2010; Parker et al., 2013). Since a STRAD protein is indispensible for LKB1 kinase activity (Baas et al., 2003; Boudeau et al., 2004; Zeqiraj et al., 2009a), and STRADα is the primary evolutionary STRAD (Chapter 3), its loss could lead to aberrant proliferation and confused cell identity, as well as aberrant migration. However, the existing STRADα

studies have used PMSE patient-derived tissues or shRNA-mediated knockdown in rodent tissues (Orlova et al., 2010; Parker et al., 2013). Here I will describe experiments that advance the field by *in vivo* loss-of-function studies using two novel STRADα mouse lines, as well as shRNA-mediated STRADα knockdown. These tools allow us to address both acute and chronic reduction of STRADα expression, and complement our gain-of-function studies from Chapter 3. Furthermore, we aimed to create a model of human PMSE Syndrome to begin to elucidate the mechanistic basis of these patients' symptoms.

II. Results:

A. *Ex utero* knockdown of STRADα does not perturb cortical migration.

Human PMSE patients have been reported to suffer cortical heterotopias, and acute knockdown of STRADα by *in utero* electroporation of shRNA has been shown to cause migratory arrest of cortical neurons in mice (Matsuki et al., 2013; Orlova et al., 2010; Parker et al., 2013; Puffenberger et al., 2007). In our hands, shRNA knockdown of STRADα in organotypic slice cultures at embryonic day 15.5 (E15.5) had no effect on neuronal migration compared with a control shRNA (N=1 and N=3, respectively) (Figure 4.1 A-C.) However, our shRNA elicited ~80% knockdown of STRADα (Figure 4.1 C), and it is conceivable that even 20% of STRADα is sufficient for normal neuronal migration. Alternatively, our differing results could be due to different timing of knockdown and the system used.

To complement the extant *in* vitro studies and to address the *in vivo* complementation of the STRAD paralogs, we received two novel mouse lines from Hans

Clevers and generated a third line (a STRADα-null line, a conditional STRADβ line), and generated a third line (a STRADα-conditional line (Figure 4.2)). The STRADα constitutive null animals do not produce STRADα protein (Figure 4.3 A) and expire perinatally of unknown etiology (Figure 4.3 B), while the STRADβ null mice have no overt phenotypes. We were unable to confirm loss of STRADβ protein in these mice due to lack of an effective antibody against STRADβ. Additionally, our results from analyzing axogenesis in these mutants is consistent with STRADβ is indeed disrupted, and will be discussed later.

Often, acute knockdown and chronic loss of a protein have very different effects, so we examined two aspects of migration in the STRADα constitutive null mouse brain. The first method directly examined migration using *ex utero* electroporation of a GFP-expressing vector at E16.5 in organotypic brain slices (as in (Hand et al., 2005)). Using this technique, we qualitatively assessed migration and saw no effect in the STRADα constitutive null mice, mirroring the non-effect in LKB1 conditional null mice (Figure 4.4) (Barnes et al., 2007) . The second aspect of migration we assayed was cortical lamination, and we find normal layers in the STRADα$^{-/-}$ cortex at E18.5, as was previously observed in the LKB1 conditional knockout brain (Figure 4.5 and(Barnes et al., 2007)). Taken together, these findings indicate that acute reduction or chronic deletion of STRADα has no discernible effect on neuronal migration, but they do not rule out possible effects on migration of a more complete acute knockdown of STRADα.

Figure 4.1

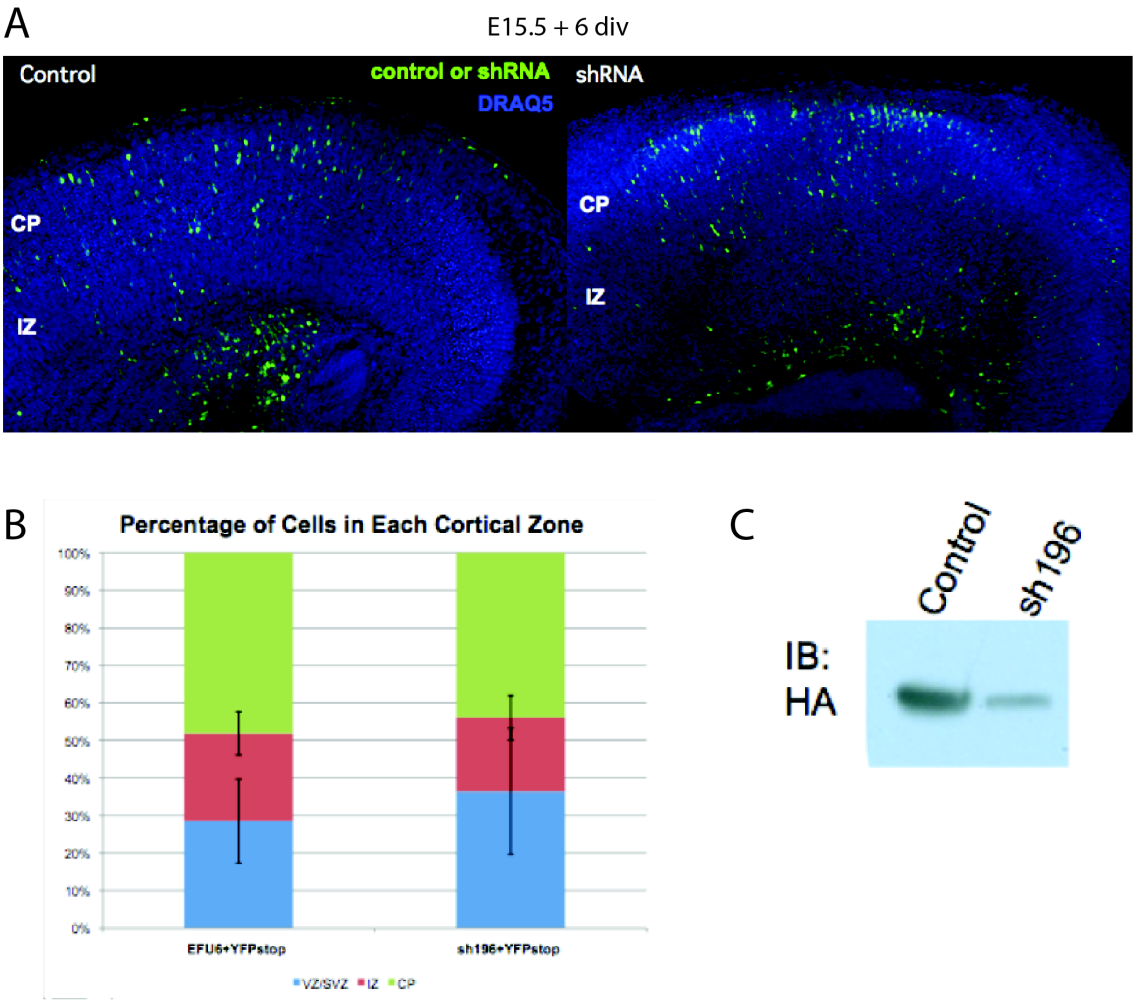


Figure 4.1 - A) WT E15.5 organotypic brain slices electroporated at E15.5 and cultured for 6 days.GFP-containing control shRNA vector or vector with shRNA against STRADα (sh196) was electroporated. Nuclei are labeled with DRAQ5. B) GFP+ cells were quantified and scored for presence in one of three cortical zones, as determined by nuclear density and morphology. There was no significant difference in proportion of cells in each zone between control shRNA and sh196. C) The efficacy of sh196 against HA-tagged STRADα protein was determined by Western blot against HA. Knockdown efficiency is about 86% by densitometry normalized to Actin..

Figure 4.2

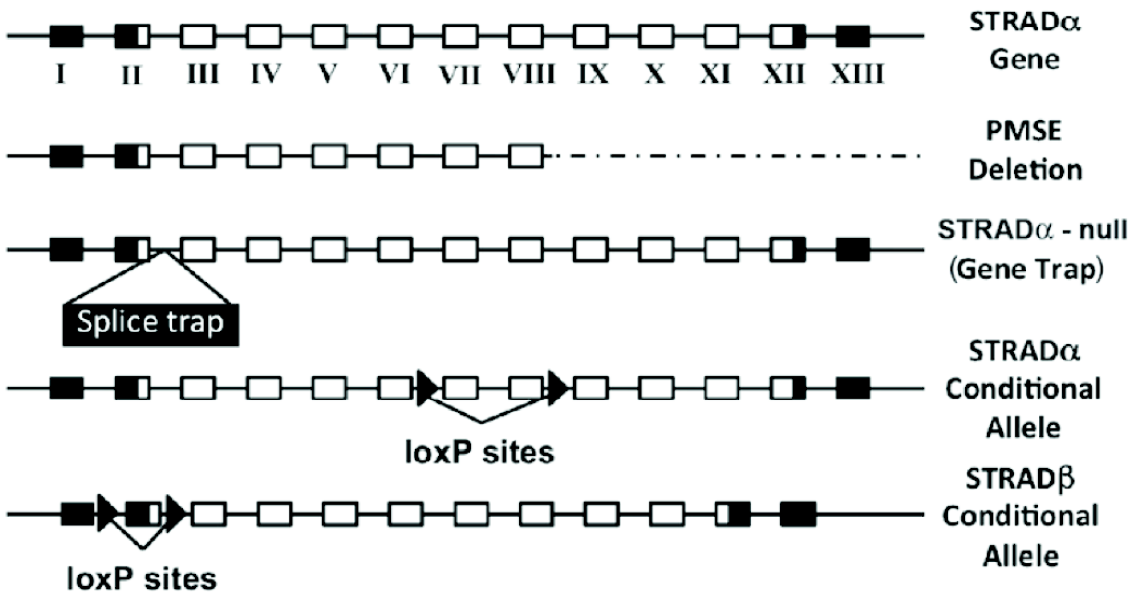
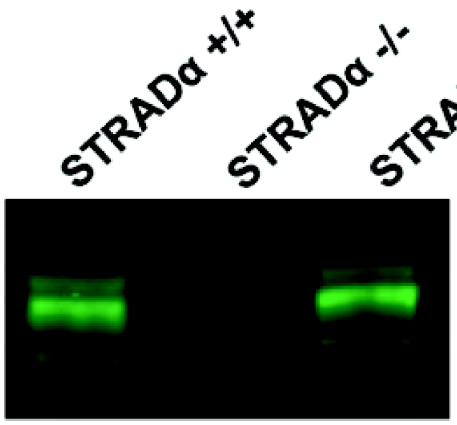


Figure 4.2 - (A) The human and mouse STRADα genes consist of 13 exons and STRADβ has 12. Schematic of the human PMSE Syndrome deletion eliminating exons 9-13. Schematic of the mouse STRADα locus disrupted via insertional mutagenesis of a splice trap construct that eliminates gene expression and the conditional allele in which exons 7 and 8 are flanked by loxP sites. Schematic of the STRADβ conditional allele in which exon 2 (which includes the start codon) is flanked by loxP sites. Black boxes indicate untranslated regions of exons and white boxes indicate the open reading frame.

Figure 4.3

A



B

Prenatal Litters

	+	α
+	29.1% n=67	23.5% n=54
α	23.5% n=54	23.9% n=55

Weanlings

	+	α
+	33% n=59	33% n=59
α	33% n=58	0% n=0

Figure 4.3 - A) Western blot demonstrating a loss of STRADα expression in STRADα-/- E16.5 cortex, while protein level is unchanged in STRADα+/- cortex. B) STRADα-/- mice are not viable postnatally, while almost normal Mendelian ratios exist prenatally.

Figure 4.4

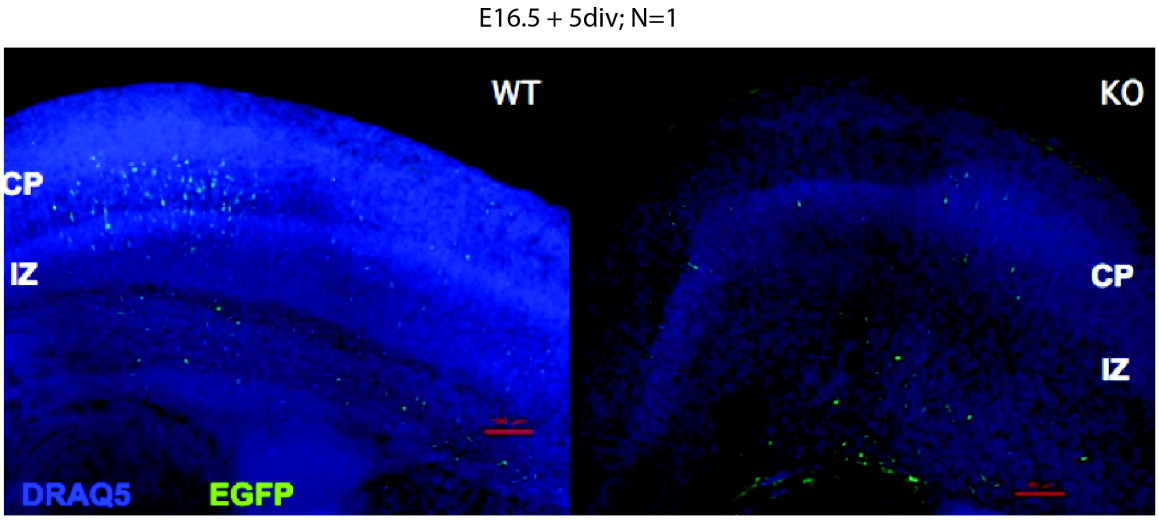


Figure 4.4 - STRADα +/+ or STRADα -/- E15.5 organotypic brain slices electroporated at E16.5 and cultured for 5 days.One brain of each genotype was electroporated with GFP-containing vector. Nuclei are labeled with DRAQ5 and GFP is in green. GFP+ cells were qualitatively analyzed and there was no obvious migration defect in the STRADα -/-, as seemingly equal proportions of neurons made it to the cortical plate by day 5.

Figure 4.5

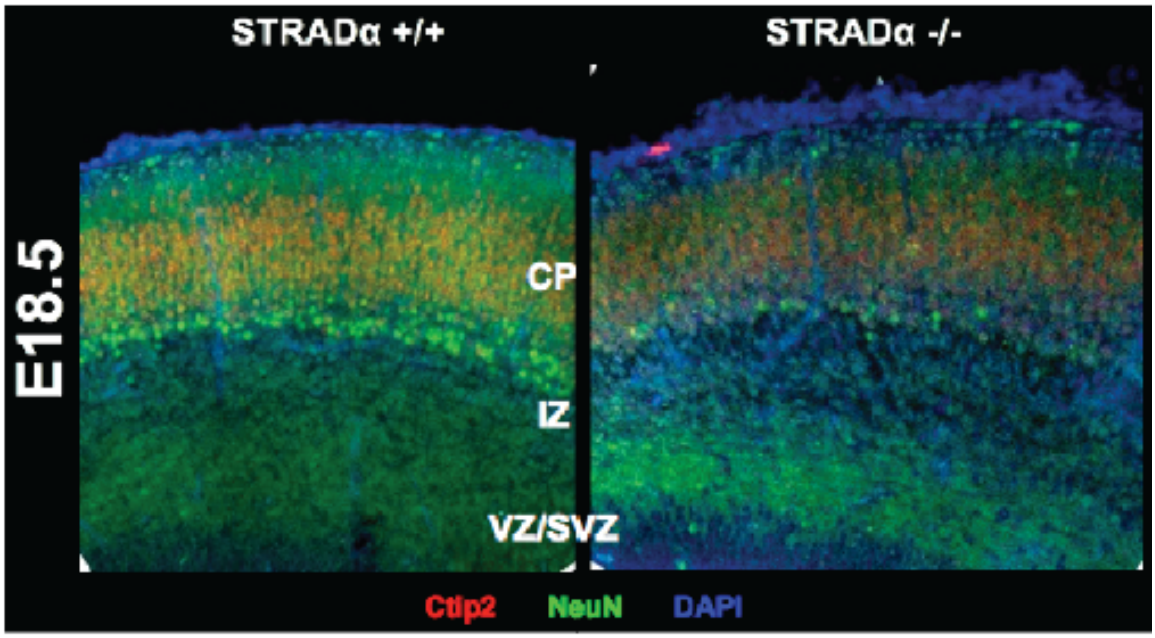


Figure 4.5 - STRADα +/+ or STRADα -/- brains fixed at E18.5 immunolabeled for the layer 5/6 marker Ctip2, the subplate and general neuronal marker NeuN, and DNA (DRAQ5). There is no obvious difference in cortical lamination in the STRADα -/- brain.

B. Constitutive loss of STRADα and Emx1^{cre}-mediated conditional deletion are not equivalent.

We began our analysis of STRADα loss using a constitutive null STRADα line (Figure 4.2), but these pups did not survive postnatally (Figure 4.3 B). In an attempt to circumvent this limitation, we generated a mouse line with a floxed allele of STRADα and crossed it with a line harboring Emx1^{cre}, which targets dorsal telencephalic progenitors cells in the developing cortex and hippocampus beginning at E9.5 (Gorski et al., 2002). While we see no overt brain morphology changes following constitutive deletion of STRADα, conditional deletion of STRADα by Emx1-cre, reliably yields cortical malformations (Figure 4.6). While this evidence is preliminary, we consistently observe this result in STRADα^{ff}; Emx1^{cre+} brains at E16.5, and do not observe this in heterozygous or wild-type littermates harboring Emx1^{cre} (N=3, N=4, N=3, respectively, Figure 4.6). We visualized these cortical malformations using DRAQ5 labeling of DNA along with Tag1 and L1, labeling cortical axons (Figure 4.6). It is interesting that the axon tracts are not perturbed in these brains even though heterotopias are present (Figure 4.6), and this could be informative of the timing of the malformation.

Figure 4.6

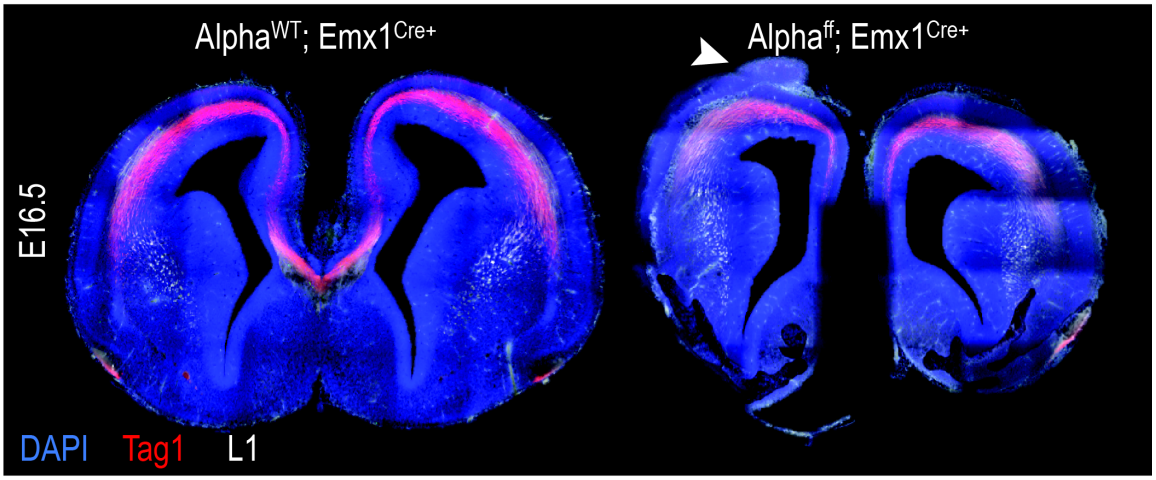


Figure 4.6 - Acute loss of STRADα perturbs cortical patterning. STRADα-fl/fl; Emx1-Cre+ brains display aberrantly localized cells (arrowhead) of unknown origin/composition compared with STRADα-WT; Emx1-cre+ controls at E16.5. Axons were immunolabeled with Tag1 (cortical projection axons) or L1 (thalamocortical inputs) and DNA was visualized with DAPI.

C. Loss of STRADα may affect cell cycle/progenitor balance.

Regulated cell proliferation is critical to corticogenesis (Polleux et al., 1997), and understanding the mechanisms underlying this regulation is necessary. LKB1 occupies a unique position at the center of a number of pathways critical to both development and tumor suppression (Hawley et al., 2003; Lizcano et al., 2004). Among these pathways, LKB1/STRAD have been found to drive G1/S cell cycle arrest (Tiainen et al., 2002). Given this role, we hypothesized that loss of STRADα could remove this cycle arrest and yield increased proliferation in the developing cortex. We visualized proliferating cells using P-Serine10-Histone H3 (PH3) immunolabeling, which labels cells in M1 of prophase (Wang et al., 2005). We find no overt effect on the number of PH3+ cells across three developmental ages (Figure 4.7). However, we also assayed the relative position of each PH3+ cell within the cortex relative to the ventricular and pial surfaces. Using this measure, we find a modest change in their positioning within the cortex at E17.5 (Figure 4.8). There is a shift in the proportion of proliferative cells away from the VZ and towards the SVZ/OSVZ boundary (WT N=3, KO N=2; Figure 4.8 A, B). These analyses were performed in multiple sections from one set of littermates, and need to be repeated in more litters before firm conclusions can be drawn. However, if real, this shift could indicate that STRADα regulates the coupling of neuronal migration and proliferation, or it could represent a different cell type that is aberrantly proliferating in this region of the STRADα knockout brain.

Figure 4.7

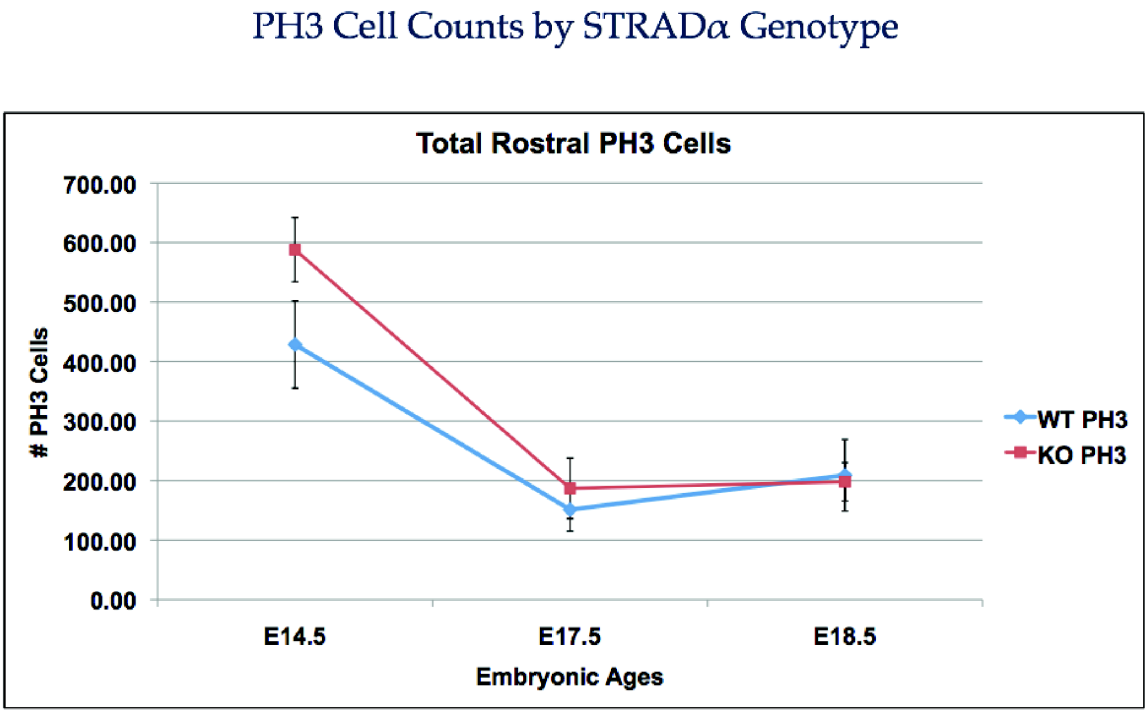


Figure 4.7 - Quantification of P-Histone H3 positive cells in STRADα WT (blue) or STRADα constitutive null (red) embryonic cortex across developmental time. PH3+ cells were quantified across multiple cryosections from the cortex of each animal. E14.5: N=3 WT, N=2 KO; E17.5: N= 3 WT, N= 3 KO; E18.5: N=2 WT, N= 2 KO

Figure 4.8

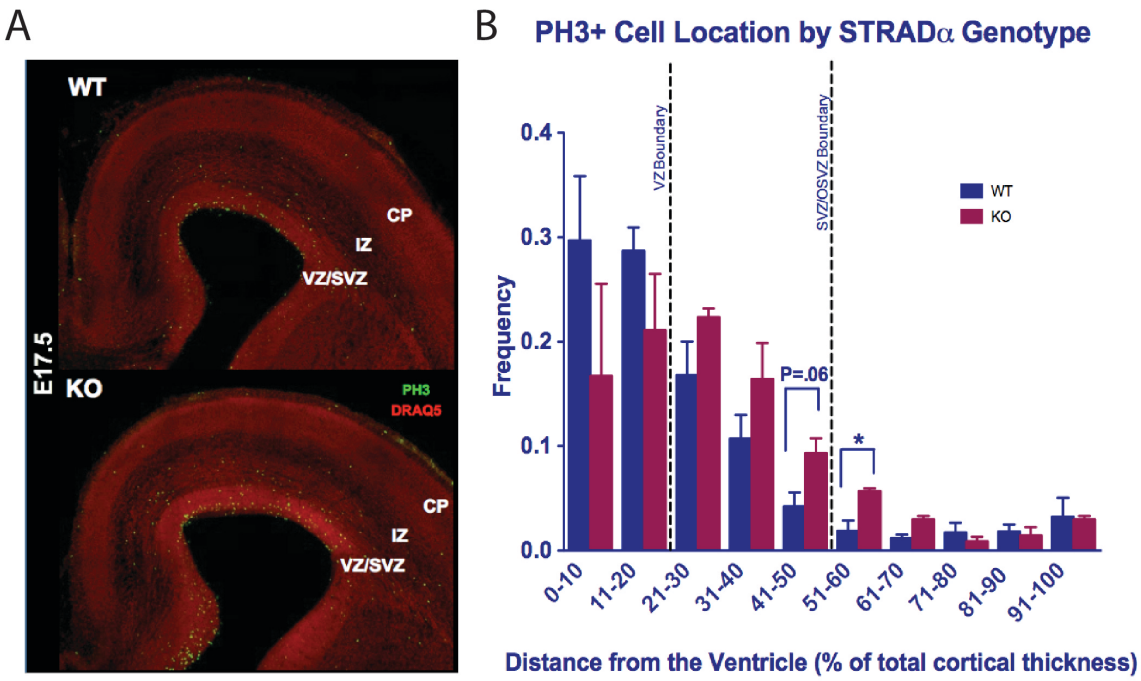


Figure 4.8 - A) Representative images of cortical sections analyzed for P-Histone H3 (PH3) immunolableing. PH3 (green) and DNA (red). VZ/SVZ = ventricular zone/subventricular zone; IZ = intermediate zone; CP = cortical plate B) Quantification of P-Histone H3 positive cells in E17.5 STRADα WT (blue) or STRADα constitutive null (red) embryonic cortex, measured in 10% increments and represented as % total cortical thickness. PH3+ cells were quantified across multiple cryosections from the both sides of cortex of each animal. N= 3 WT, N= 3 KO *P<0.05

D. STRAD signaling during cortical development

The STRAD/LKB1 dyad has been reported to regulate and or modulate AMPK, BRSK1/2, NUAK1/2, MARKs 1-4, TSC1/2, and GSK3β, among others at various stages of development and aging (Boudeau et al., 2004; Courchet et al., 2013; Lehtinen et al., 2011; Lizcano et al., 2004; Orlova et al., 2010). To address the impact of STRADα loss in these pathways during brain development, we conducted Western blot analyses from constitutive STRADα$^{-/-}$ knockouts and STRADα$^{+/- \text{ or } +/+}$ controls. A schematic of these proteins and their actions within dividing progenitors is provided in Figures 4.9-4.11 A. Since LKB1 has been shown to modulate GSK3β signaling during neuronal polarization and migration (Asada et al., 2007; Lehtinen et al., 2011; Ossipova et al., 2003; Shan et al., 2014; Shelly and Poo, 2011), we examined the effect of STRADα loss on known upstream and downstream components of GSK3β signaling (Figure 4.9A). Using lysates from all but cortex (rest of brain, ROB), we compared P(S9)-Akt/total Akt ratios in STRADα constitutive null mice, and find no difference between WT, heterozygous, and knockout mice (Figure 4.9 B). However, there is a trend towards a decrease in the inactivating P(Ser9)-GSK3β/totalGSK3β, indicating that in STRADα knockout cortex, GSK3β may be more active (Figure 4.10 B). While GSK3β signaling trends towards a change, downstream β-catenin signaling is unaffected by STRADα loss (Figure 4.11 B). This implies that either there are compensatory mechanisms at work, or that the change in GSK3β activity is not biologically significant, or is affecting pathways other than β-catenin.

Figure 4.9

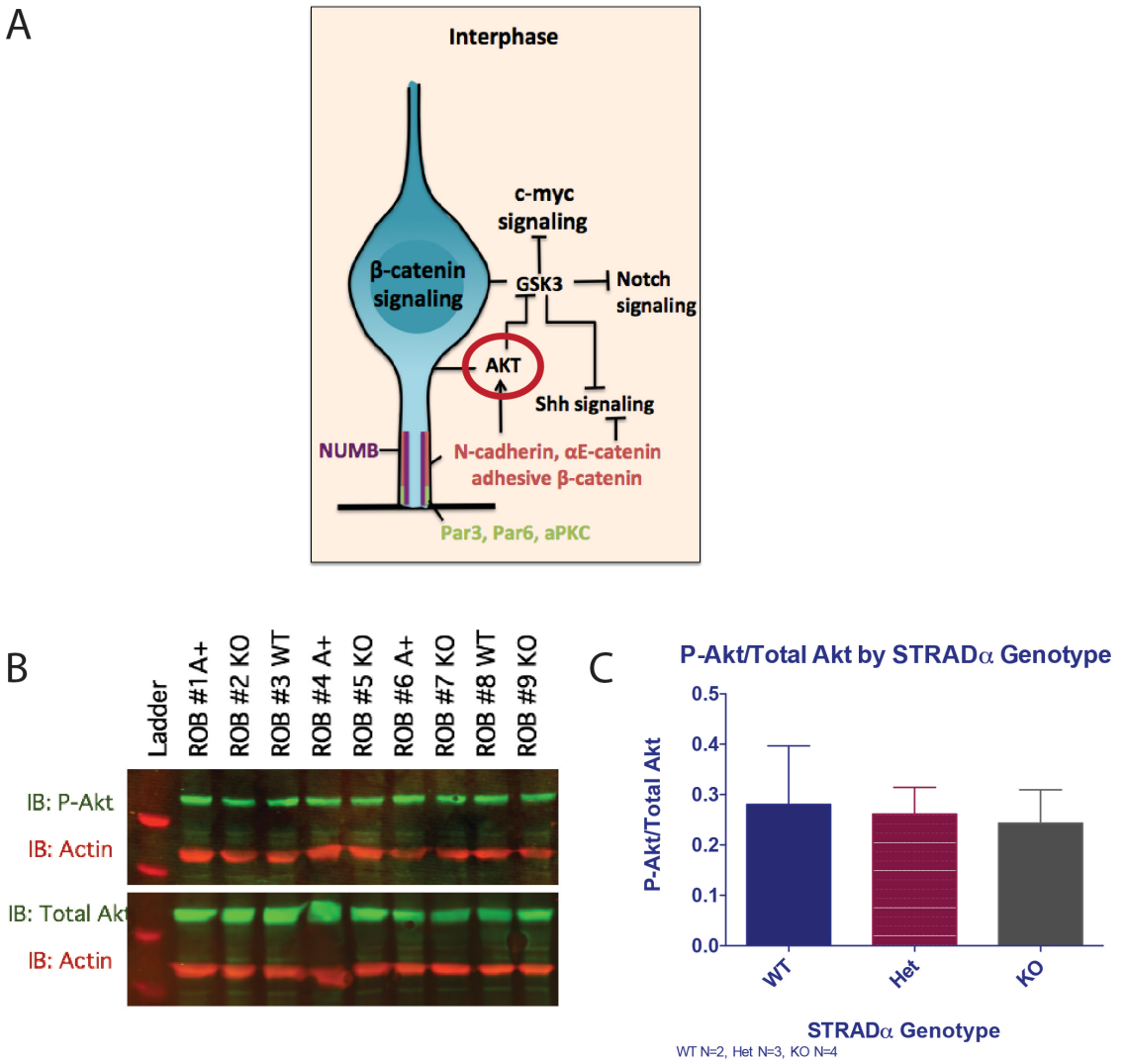


Figure 4.9 - A) Schematic of signaling relevant in dividing neural progenitors, adapted from Lui et al. 2011. B)Western blot of P(S)9 Akt and Total Akt in STRADα constitutive KO mouse line. Lysates were made from "rest of brain", meaning all but the cortex, at embryonic day 16.5 C) There is no significant difference in the P-Akt/total Akt ratio in between STRADα+/+, STRADα +/- and STRADα-/- brains, as determined by One-way ANOVA with Dunnett's post test.

Figure 4.10

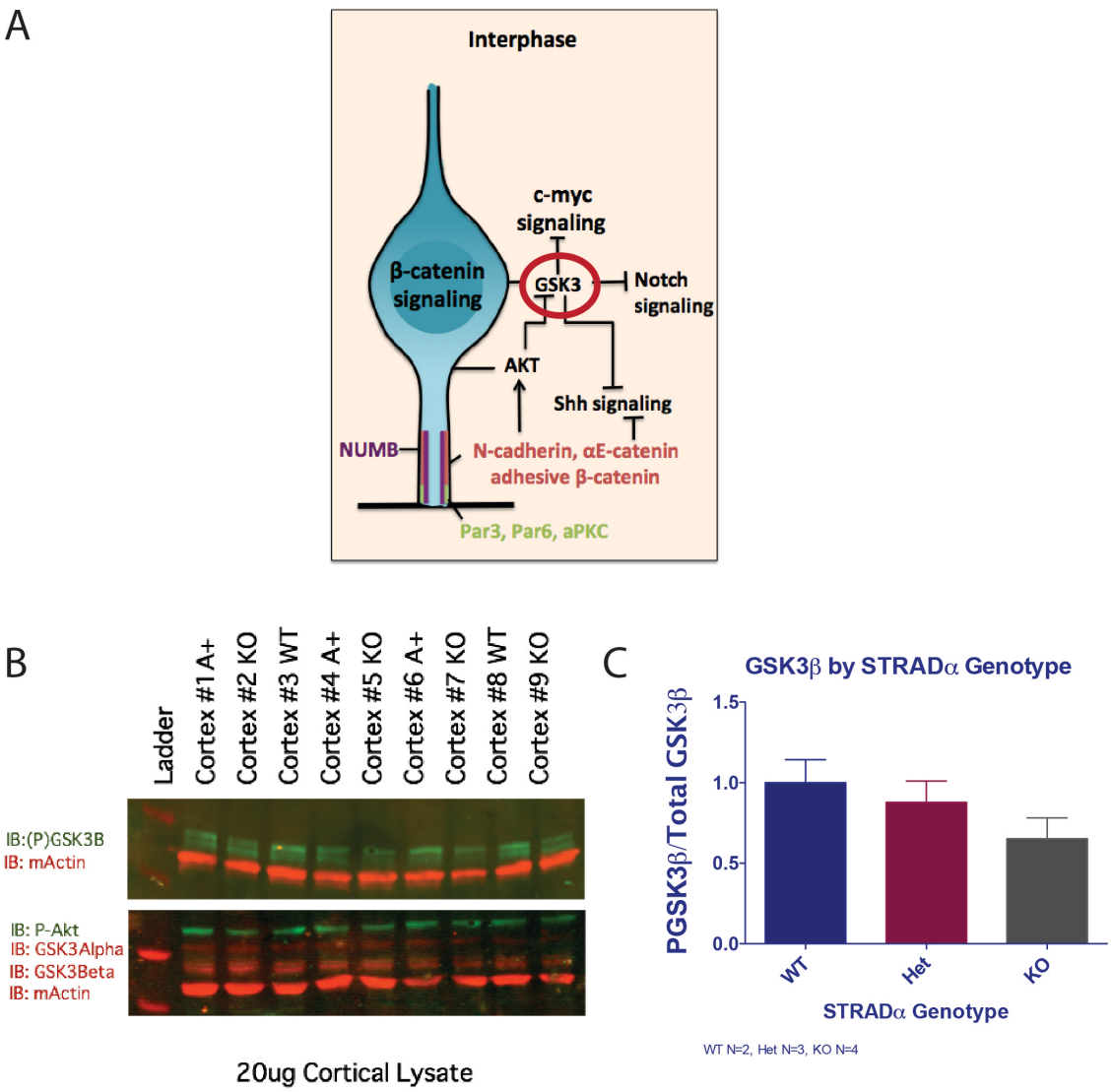


Figure 4.10 - A) Schematic of signaling relevant in dividing neural progenitors, adapted from Lu et al. 2011. B)Western blot of P(S)9 GSK3β and total GSK3α and β and in STRADα constitutive KO mouse line. Lysates were made from cortex at embryonic day 16.5 C) There is no significant difference in the P-GSK3β/Total GSK3β ratio in STRADα WT, STRADα Het, or STRADα KO brains, as determined by One-way ANOVA with Dunnett's post test.

Figure 4.11

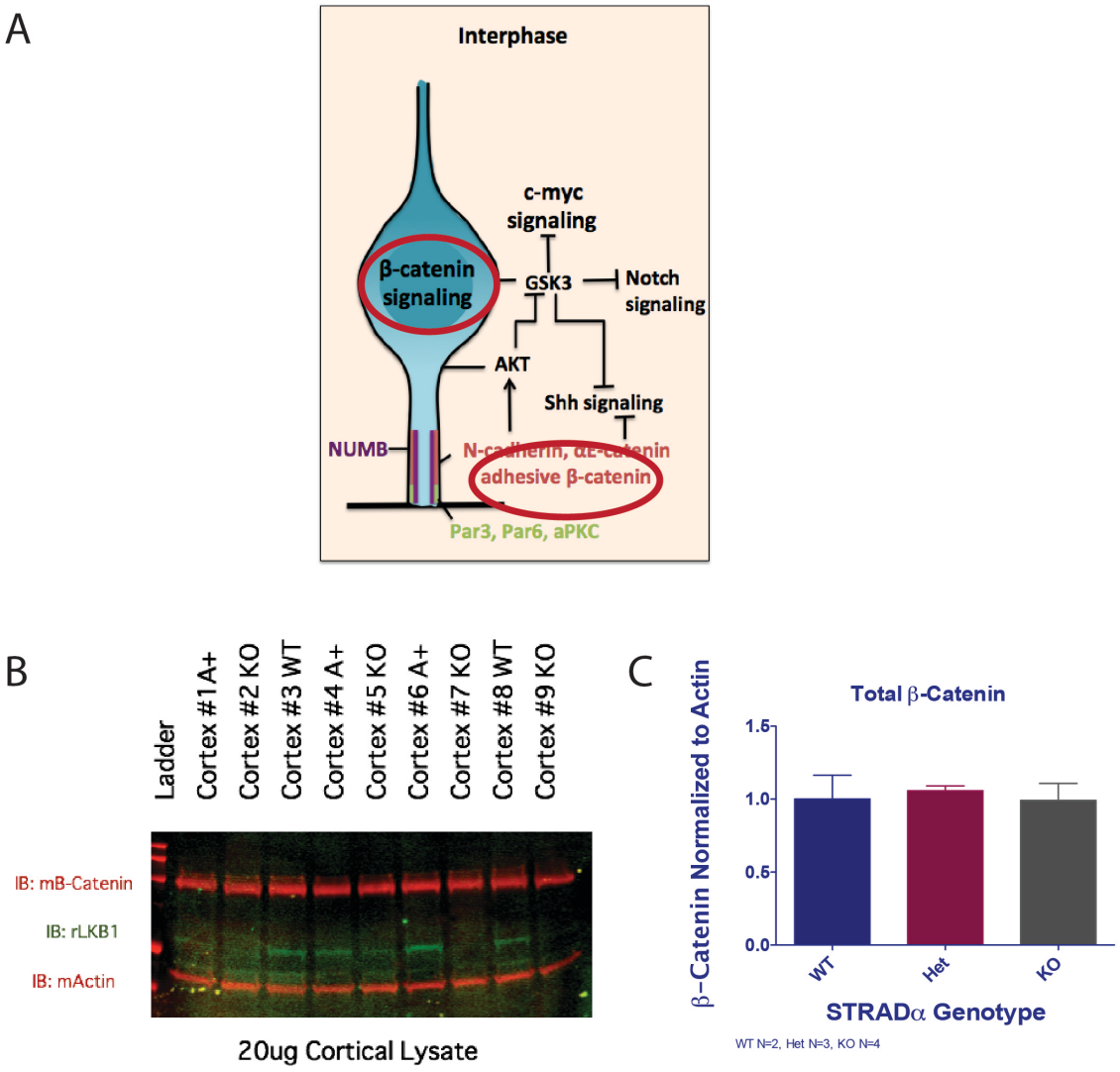


Figure 4.11 - A) Schematic of signaling relevant in dividing neural progenitors, adapted from Lu et al. 2011. B)Western blot of β–Catenin and total LKB1 in STRADα constitutive KO mouse line. Lysates were made from cortex at embryonic day 16.5 C) There is no significant difference in total β–Catenin in STRADα WT, STRADα Het, or STRADα KO brains, as determined by One-way ANOVA with Dunnett's post test.

E. STRADα in Proliferation

Human PMSE patients have intractable epilepsy of unknown origin. Many epilepsies are driven by overproliferation or increased dendritic arborization of hippocampal cells (Amiri et al., 2012; Neugebauer and Susser, 2009; Puffenberger et al., 2007; Theodore et al., 2006). Additionally, LKB1 has been shown to affect progenitor cell cycle (Tiainen et al., 2002), so we hypothesized that STRADα could play a role in driving cell proliferation via LKB1. We were specifically curious about this role in the dentate gyrus because STRADα mRNA is highly expressed there (©2013 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas [Internet]. Available from: http://developingmouse.brain-map.org.). Since the STRADα$^{-/-}$ mouse is perinatal lethal, we were only able to begin addressing this question in the STRADα$^{fl/fl}$; Emx1^{cre+} mouse. We examined GFAP immunolabeling as a marker of cells in the astrocyte lineage (Middeldorp and Hol, 2011). This work is preliminary, but there is a striking increase in GFAP^{+} cells in the dentate gyrus of P28 STRADα$^{fl/fl}$; Emx1^{cre+} brains compared with a heterozygous control (Figure 4.12). It remains to be determined whether these cells are reactive astrocytes or if they represent an increased pool of adult neural progenitors.

Our findings that proliferation may be subtly affected by constitutive STRADα loss (Figure 4.8) and our results in the dentate gyrus led us to question the cell-autonomy and the timing of this effect. Serendipitously, mouse STRADα is on chromosome 11, so to address this question and circumvent the perinatal lethality of STRADα$^{-/-}$ pups, we used the MADM-Ch11 mouse line ((Hippenmeyer et al., 2013; 2010; Zong et al., 2005)Figure 4.13 A, B). We crossed the heterozygous STRADα-null mouse strain to the

MADM11-TG line, and Emx1^{cre} to the MADM11-GT line, yielding green STRADα$^{-/-}$ cells, red STRADα$^{+/+}$ cells, and yellow STRADα$^{+/-}$ cells on a STRADα$^{+/-}$ background. This allowed us to quantify the number of cells of each genotype, and to determine the extent to which STRADα drives proliferation and/or survival. Most postnatal proliferation takes place in the subgranular zone of the hippocampus and the subventricular zone, with newly born cells migrating along the rostral migratory stream (RMS) to the olfactory bulb (OB) (Curtis et al., 2007; Lennington et al., 2003). Since STRADα mRNA is highly expressed in the hippocampus, and especially the dentate gyrus (©2013 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas [Internet]. Available from: http://developingmouse.brain-map.org, Figure 4.14), we compared the number of green (KO) and red (WT) cells in the dentate gyrus of the hippocampus at postnatal day 21. We then determined the green:red (GR) ratio in both STRADα$^{+/-}$; MADM-11 mice and in control STRADα$^{+/+}$; MADM-11 mice as in (Hippenmeyer et al., 2010; 2013). While there was no significant difference between the experimental and control animals, there was a trend towards higher GR ratio in the experimental animals (N=3 of each genotype, figure 4.15). Taken together, these data point to a regulatory role for STRADα in proliferation, probably through its control over LKB1 activity.

Figure 4.12

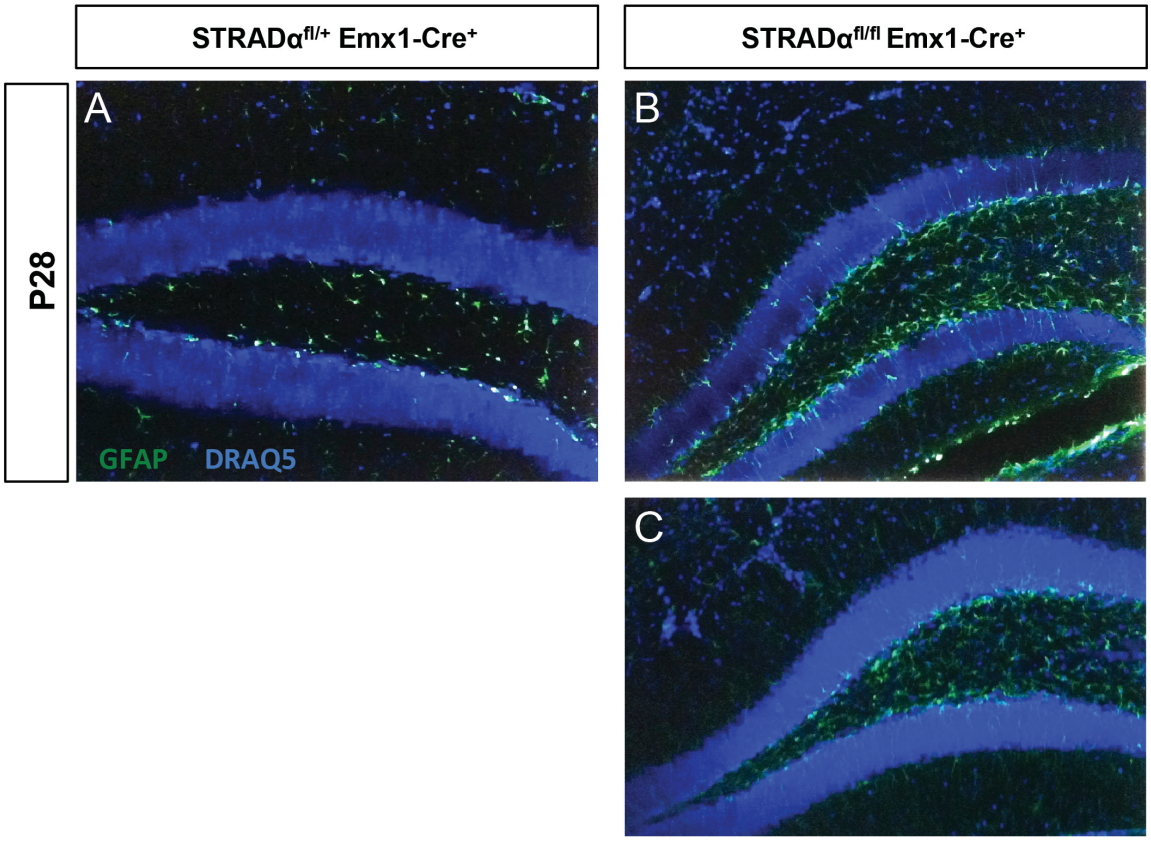


Figure 4.12 - Analysis of postnatal day 28 STRADα floxed hippocampus. GFAP is represented in green and DNA is in blue (DRAQ5). A) STRADα fl/+; Emx1-Cre+ control dentate gyrus shows sparse GFAP immunolabeling. B) and C) STRADα fl/fl; Emx1-Cre+ dentate gyrus exhibits dense GFAP signal, with radial glia spanning the granular layer staining positive for GFAP. N=1 control, N=2 cKOs

Figure 4.13

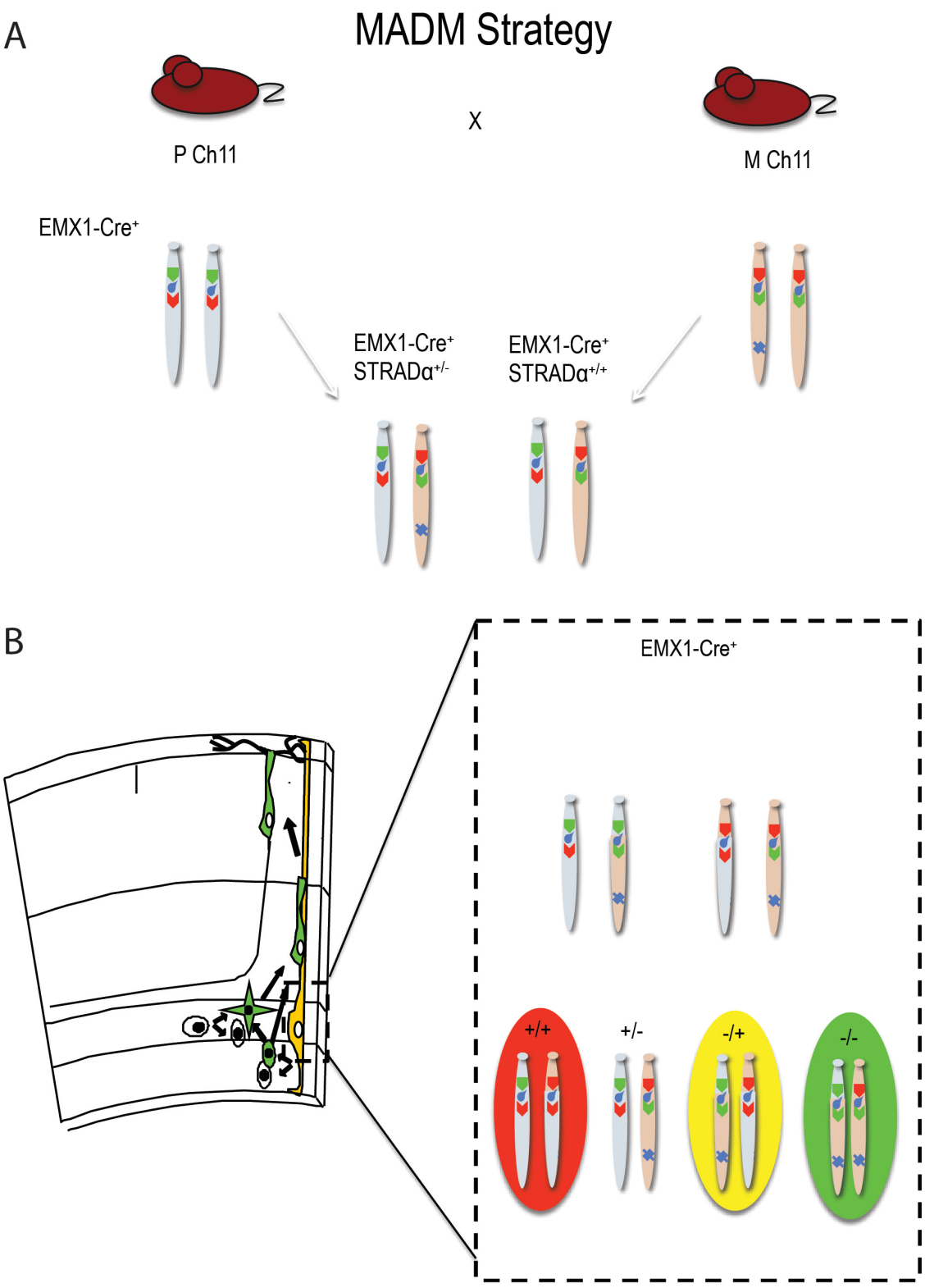

A
MADM Strategy
P Ch11
X
M Ch11
EMX1-Cre+
EMX1-Cre+
STRADα+/-
EMX1-Cre+
STRADα+/+
B
EMX1-Cre+
+/+
+/-
-/+
-/-

Figure 4.13 - A) Example Mosaic Analysis of Double Markers (MADM) breeding strategy. In this case, the sire is Emx1-Cre+ and is homozygous for the GT allele on chromosome 11. The dam is homozygous for the TG allele and heterozygous for the STRADα gene trap. Emx1-Cre+ offspring will be TG/GT and either STRADα+/+ or STRADα+/-. B) Cross-section of embryonic cortex. Radial glial fiber is yellow, neural progenitor is white and daughter neurons are green. During mitosis, the chromosomes are copied, and some recombine at the loxP sites between the split TdTomato and split GFP, in Emx1-Cre+ cells, yielding one of 4 possible outcomes in the daughter cell. Red cells are STRADα +/+, non-fluorescing cells are STRADα +/-, as are neurons expressing both GFP and TdTomato (yielding yellow), and green fluorescing neurons are STRADα -/-.

Figure 4.14

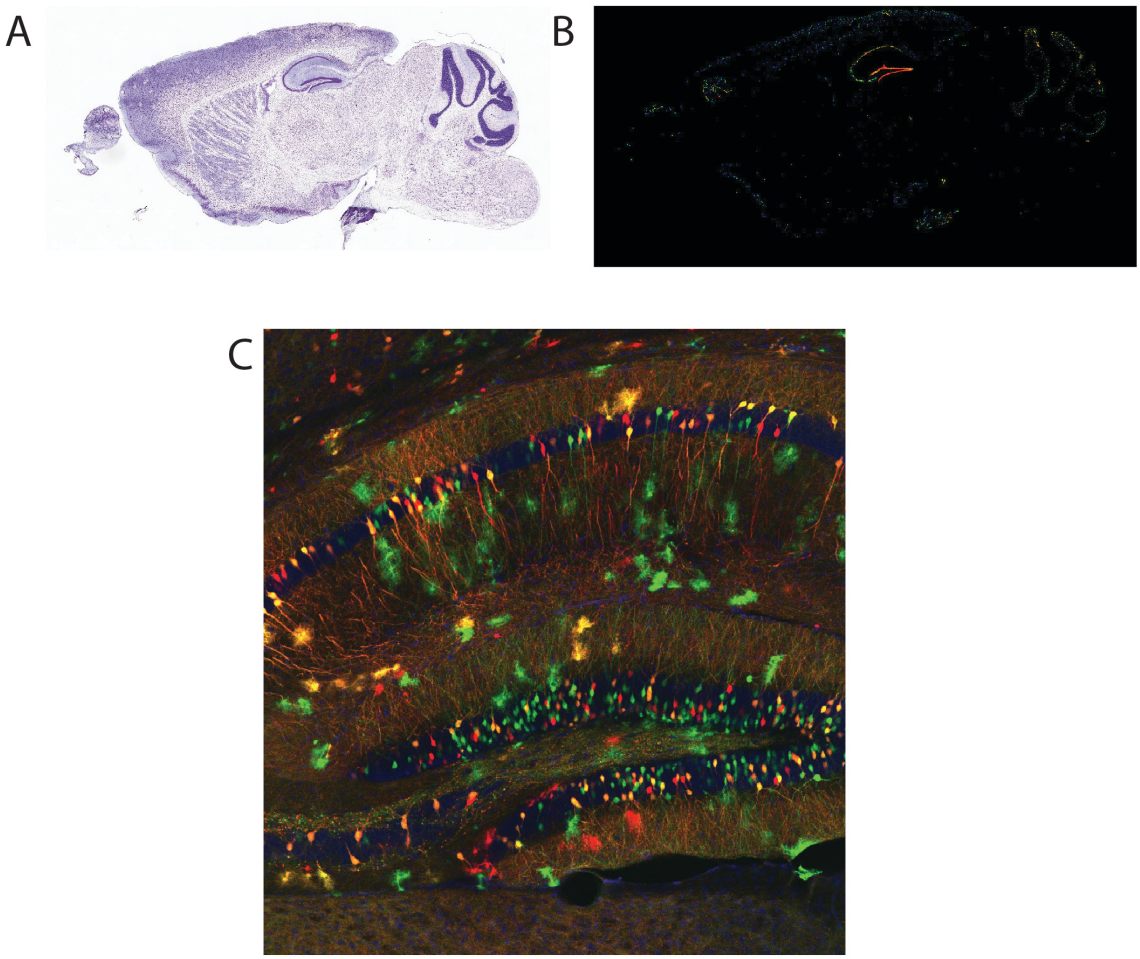


Figure 4.14 - A) In situ hybridization for STRADα in adult saggittal section from Allen Brain Atlas. STRADα is highly expressed in cerebellar granule cells as well as hippocampus. B) Fluorescent in situ hybridization for STRADα in adult saggittal section from Allen Brain Atlas. STRADα is highly expressed in hippocampus, especially in dentate gyrus. C) Representative image of a TG/GT Emx1-Cre+ hippocampus at P7 showing effective recombination in this structure, and especially in the dentate gyrus.

Figure 4.15

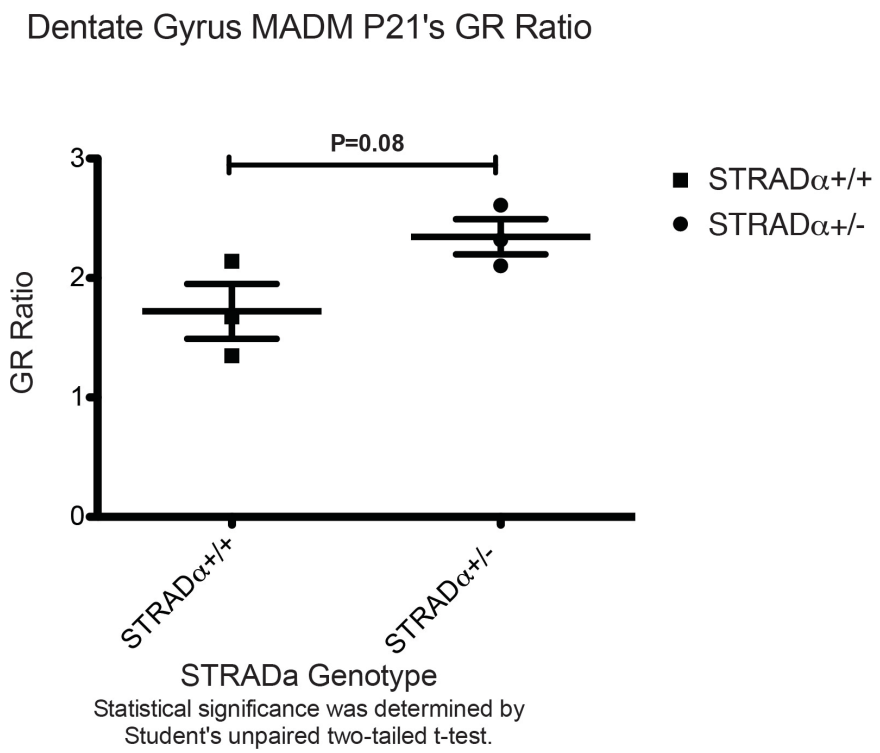


Figure 4.15 - MADM Green:Red (GR) ratio analysis in the P21 dentate gyrus reveals a trend towards increased proliferation in STRADα-null cells. All mice analyzed were Emx1-Cre+; MADM-TG/GT and either STRADα+/+ (WT) or STRADα+/- (Het). Cells were counted in multiple sections from 3 animals of each genotype, and statistical significance was tested using Students unpaired two-tailed t-test.

III. Discussion

The work in this chapter is focused on cortical development and the multiple signaling pathways converging on corticogenesis. However, the genetic mechanisms regulating all of these pathways are beginning to be elucidated. In this chapter we have discussed the role of STRADα along with LKB1 in migration, proliferation, and signaling in the developing mouse brain. One way in which mice and humans differ is our relative brain size. The cortex of gyrencephalic animals, including humans, is folded due to the large number of cells that must fit in a small space. In order to generate this large number of cells in a short time, gyrencephalic organisms utilize intermediate progenitors in the outer SVZ, also known as oRG (outer radial glia), and these cells are thought to maintain many of the characteristics of traditional radial glia (Wang et al., 2011). These cells have also been observed in the rodent cortex, but in much lower numbers (Hansen et al., 2010; Wang et al., 2011). OSVZ cells are thought to retain progenitor potential, and act as transit amplifying cells, sharing many characteristics with VZ progenitors (Wang et al., 2011). The molecular mechanisms driving these oRG cells are still being investigated, but it is thought that many of the same pathways are active in both RG and oRG cells (Wang et al., 2011). Our work suggests that STRADα may be critical for the proliferation control of OSVZ cells (Figure 4.8). We were unable to determine the molecular pathways driving this perturbation, perhaps due to the relatively small numbers of OSVZ cells requiring STRADα. Future work should focus on this population of cells, as it could be the origin of the heterotopias seen in human PMSE patients, or of their epileptic symptoms.

It is curious that human PMSE patients survive into early adolescence while the

STRADα$^{-/-}$ mice die perinatally. This could be due to expanded roles of STRADβ in the human, allowing for greater compensation upon loss of STRADα. Alternatively, it could be that the human PMSE deletion leaves some marginally active form of STRADα, as was previously postulated. Finally, PMSE symptoms could actually be due to the effects on LIMD2, whose promoter is affected by the PMSE deletion ((Puffenberger et al., 2007)and http://www.ncbi.nlm.nih.gov/gene/92335). While this is a possibility, it is unlikely in light of the Crino lab findings that inhibiting mTOR via rapamycin treatment ameliorates the PMSE patient seizures (Parker et al., 2013). These results would indicate that STRADα is indeed playing into the TSC1/2-mTOR-S6K pathway, or that LIMD2 is also a part of this pathway, and the combinatorial effects of STRADα and LIMD2 promoter disruption cause the symptoms seen in PMSE.

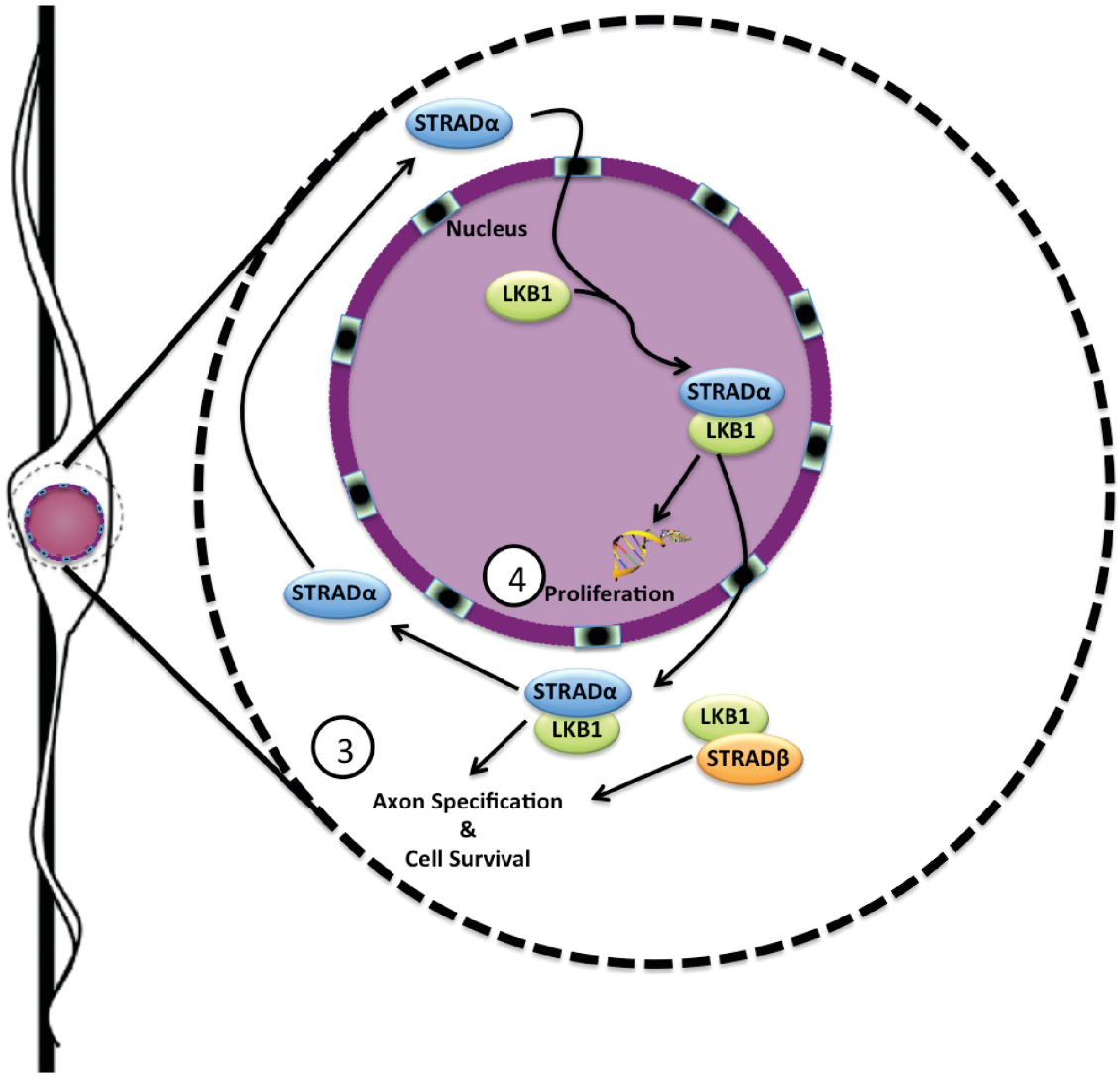
The findings discussed in this chapter suggest that STRADα is required during brain development for normal corticogenesis. STRADα is the main activating protein of LKB1, and is necessary to export it from the nucleus. The Nestin-Cre-mediated deletion of STRADα and constitutive deletion of STRADα have no effect on gross morphology of the developing cortex (not shown), but the Emx1-Cre-mediated homozygous deletion of STRADα yields a partially penetrant cortical heterotopia phenotype. This implies that STRADβ can compensate for loss of STRADα at certain times, but that deletion of STRADα at other developmental stages or in other cell types is detrimental. It is important to note that the Nestin-cre mouse line has varying penetrance and efficacy and this could have skewed our findings (Liang et al., 2012). However, it is also possible that timing of STRADα deletion could explain the discrepancy between our finding that

neuronal migration is unaffected by STRADα knockdown at E15.5, and the previously published finding that STRADα knockdown at E14.0 disrupts neuronal migration (Orlova et al., 2010). Futhermore, we used *ex utero* electroporation, while the Orlova study utilized *in utero* electroporation to deliver the shRNA, which also could have influenced the outcome (Orlova et al., 2010). Both shRNA vector promoters, U1 and U6, are PolIII promoters, and have been found to express well in neurons, and can persist for months in the mouse brain (Kügler et al., 2003; Mäkinen et al., 2006), making it unlikely that the difference in promoter influenced the outcome of the migration studies. This strengthens the assertion that there is a discrete developmental window in which STRADα is absolutely required to guide brain patterning. Further work should focus on determining the origins and fates of these aberrantly localized cells. Furthermore, it would be important to investigate whether mice harboring these malformations survive into adulthood, or whether they represent non-survivors. Ideally, these studies would be bolstered by behavioral analyses and observation for seizures.

Since STRADα is the main activator of LKB1, and LKB1 can cause cell cycle arrest (Tiainen et al., 2002), it stands to reason that loss of STRADα could perturb cell cycle. One hypothesis is that losing STRADα would cause LKB1 to be inactive or less active, and thus unable to effect G1/S cycle arrest, causing an over-proliferation of cells missing STRADα. Our preliminary findings of GR ratio in the STRADα$^{-/-}$ MADM-Ch11 mice support this possibility. These mice also speak to the cell-autonomous nature of this effect, as the mouse is heterozygous for STRADα, and cells missing STRADα outnumber those with WT STRADα. Another possibility is that these cells do not over-

proliferate, but rather survive when they should not. To address this question definitively, BrdU/EdU labeling studies should be carried out along with phospho-histone H3/Ki67/activated Caspase3 immunolabeling studies. This would determine cell cycle length, and whether STRAD$\alpha^{-/-}$ cells are proliferating more, or persisting longer in the brain. It would also allow for postnatal analysis of these questions, and perhaps speak to the epilepsy mechanism in human PMSE patients.

Chapter 4 Summary Figure



Chapter 4 Summary Figure. Schematic of STRAD/LKB1 signaling within the developing neuron. (3) STRADα and STRADβ both complex with LKB1 and are capable of eliciting axogenesis in primary cultured neurons. Only STRADα can export LKB1 from the nucleus. (4) STRADα may help regulate proliferation with LKB1.

Chapter Five: Axogenesis requires the STRAD proteins

I. Introduction:

The establishment of polarity and subsequent axogenesis is required for neuronal function. Many of the genes driving this process are highly evolutionarily conserved, and are, in many cases, the same genes that drive symmetry-breaking in other cell types. LKB1 is one of these highly conserved and necessary genes and has been shown to be both necessary and sufficient to drive polarity and axogenesis when activated (Baas et al., 2004a; Barnes et al., 2007; Shelly et al., 2007). The role for LKB1 in these processes has been extensively studied, but how its activators, STRADα and STRADβ, are regulated is less well understood. In this chapter, we examine the necessity and sufficiency of each STRAD protein during cortical development

While cortical development is extremely complex and nuanced, it is also quite robust, often retaining the capability to compensate following gene loss. Our results from Chapter 3 and those from previous studies clearly show that LKB1 is necessary for axogenesis, and that its activation by either STRAD protein can drive this function *in vitro* (Barnes et al., 2007; Kishi et al., 2005; Shelly et al., 2007). However, the *in vivo* complementation and redundancy of the STRAD proteins was unknown, here I will describe my efforts to elucidate the extent to which each STRAD protein can compensate for the other's loss.

Previous work on LKB1 has shown that a STRAD protein is necessary to allosterically activate the kinase by exposing its T-loop (Baas et al., 2003; Boudeau et al., 2004; Zeqiraj et al., 2009a). LKB1 that cannot bind a STRAD, such as the SL26 mutant, is incapable of causing G1/S cell cycle arrest, and is constrained to the nucleus (Baas et al., 2003; Boudeau et al., 2004; Tiainen et al., 2002). Since STRADα but not STRADβ

can export LKB1 from the nucleus, it is likely that each STRAD is responsible for unique LKB1 functions. We aimed to delineate the overlapping and unique roles of each STRAD protein using novel mouse models and various modes of deletion. The STRAD proteins have been studied most extensively in terms of their roles regulating LKB1. However, the question of whether or not LKB1-independent functions exist for the STRAD proteins has not been explored. We approached this question using novel STRAD mouse lines and assaying for phenotypes previously seen in the LKB1 cortical conditional knockout mouse (Barnes et al., 2007).

In vitro work by other groups has attributed to STRADα an LKB1-stabilizing function (Baas et al., 2003). However a similar function for STRADβ has not been tested, nor has the effect of STRADα and STRADβ deletion on LKB1 stability *in vivo*. We address this question over the next two chapters to better understand the molecular mechanisms at work and ultimately to better understand PMSE Syndrome.

II. Results

A. Genetic elimination of both STRAD genes in the cerebral cortex disrupts the formation of projection axons.

Our finding that constitutive STRADα loss has no effect on gross brain morphogenesis (Figure 5.1 B), coupled with the fact that either STRADα or STRADβ is sufficient to drive axogenesis when overexpressed in neurons (Figure 3.9), led us to delete both genes simultaneously in an attempt to phenocopy LKB1 loss (Barnes et al., 2007), or to reveal distinctions between these scenarios. The oldest age we could reliably examine was E18.5 due to the perinatal lethality of STRADα constitutive null mice.

Histologic characterization of cerebral cortices at E18.5 indicated that elimination of either STRADα (Figure 5.1 B, B') or STRADβ (Figure 5.1 C, C') alone is not sufficient to disrupt axogenesis. However, deletion of both STRAD genes caused a profound loss of corticofugal TAG1-positive projection axons (Figure 5.1 D, D'), mirroring the effect observed following conditional deletion of LKB1(Barnes et al., 2007). Similarly, neuronal polarization defects (indeterminate neurites) were observed in primary cortical cultures of STRADα/β double KO cortices compared to controls when immunostained for the axon/dendrite markers Tau1 and microtubule associated protein 2 (MAP2), respectively (Figure 5.1 E-G). This indicates that either STRADα or β is sufficient to drive axogenesis during corticogenesis, and that this effect is cell-autonomous.

Figure 5.1

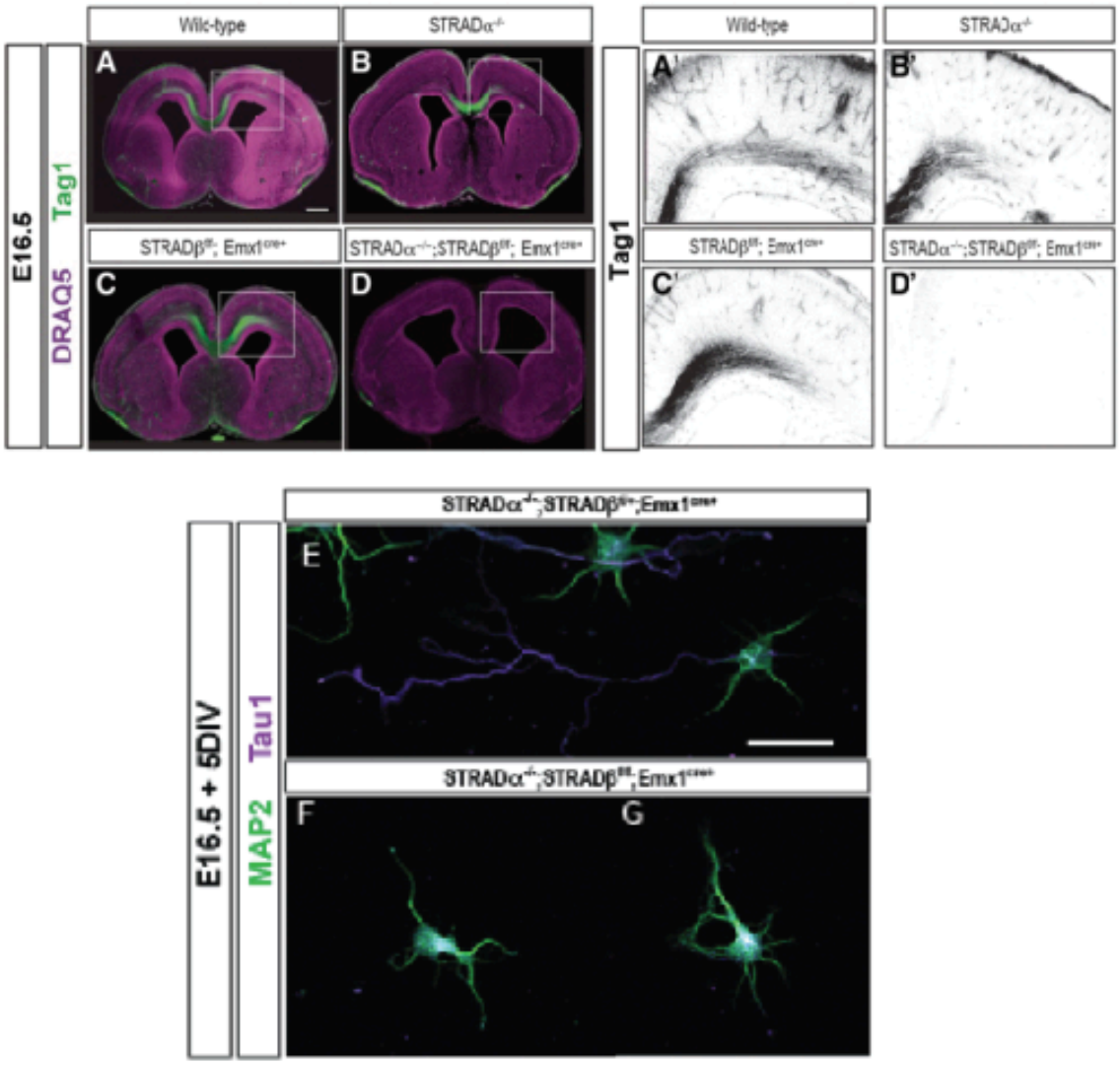


Figure 5.1 - STRADα and STRADβ are redundant in axogenesis and limiting programmed cell death. Embryonic day 18.5 coronal sections immunolabeled for corticofugal fibers (Tag1, green) (A-D, A'-D') and a nuclear marker (DRAQ5), magenta. Axons are normal in wild-type, STRADα KO and STRADβ cKO brains, but completely gone in the STRADα/STRADβ double KO brain (A-D). Scale bar = 100 µm. Higher magnifications of cortical regions of TAG1 immunolabeling are shown in grayscale to the right (A'-D'). (E-G) Primary culture cortical neurons from embryonic day 16.5 mouse cortex grown for 5 days in vitro and immunolabeled for MAP2/Tau. Indeterminate neurites are seen in (F-G) STRADα-/-;STRADβfl/fl;Emx1-cre+ neurons but not in (E) STRADα+/-;STRADβfl/fl;Emx1-cre+. Scale bar=20um.

B. Deletion of both STRAD paralogs results in programmed cell death in the cerebral cortex.

Another phenotype associated with LKB1 loss is an increase in cell death in the cortical plate, as evidenced by activated caspase 3 immunolabeling (Barnes et al., 2007). To test whether eliminating either or both STRAD genes parallels the cortical loss of LKB1 in the context of programmed cell death, we examined activated caspase3 in cortical tissue. Immunostaining of either the single constitutive STRADα KO (Figure 5.2 A, A') or STRADβ cKO (Figure 5.2 B, B') cortices indicates no change in apoptosis relative to wild- type control (Figure 5.2 C, C'). In contrast, the STRADα/β double KO cortex displays substantial activated caspase3 immunolabeling, an obvious thinning of the cortical wall, and enlarged ventricles (Figure 5.2 D, D'). These results indicate that loss of both STRADα and STRADβ is required to phenocopy loss of LKB1, demonstrating the functional redundancy between these LKB1-activating pseudokinases, and establishing the sufficiency of STRADβ in cell survival and axogenesis during corticogenesis.

Figure 5.2

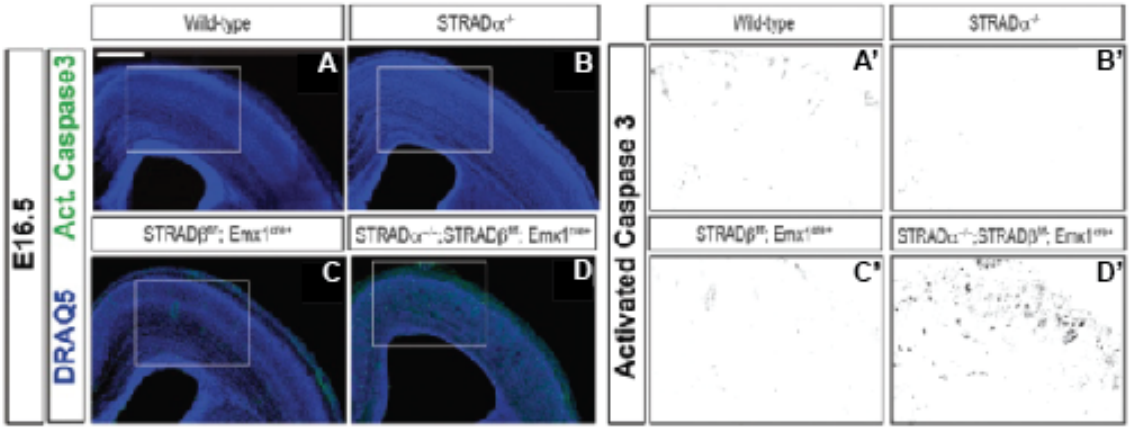


Figure 5.2 - STRADα and STRADβ are redundant in axogenesis and limiting programmed cell death. Embryonic day 18.5 coronal sections immunolabeled for activated caspase3 (Act-Casp3, green) (A-D, A'-D') and a nuclear marker (DRAQ5, blue in A-D). Activated caspase3 is largely absent from wild-type, STRADα KO and STRADβ cKO brain, but drastically increased in STRAD α/β double KO cortex (A-D, A'-D'). Scale bar = 100 μm. Higher magnifications of cortical regions of activated caspase 3 are shown in grayscale to the right (A'-D').

C. Loss of both STRADs does not completely phenocopy loss of LKB1.

To date, the only function associated with STRADα and STRADβ in vertebrates is to regulate LKB1 protein, as independent functions not been observed or tested. However, C. elegans STRD1 and PAR4 do have some unique roles (Eggers et al., 2012; Kim et al., 2010). Given this evidence, we set out to determine the extent to which STRAD and LKB1 deletion are similar in the mouse cortex. Conditional deletion of LKB1 protein by $Emx1^{cre}$-mediated recombination does not affect cortical lamination (Barnes et al., 2007). While the cortex is thinner and ventricles are larger in these mutants, this is likely due to the loss of projection axons and subsequent reduction in cortical volume, as this phenotype is not recapitulated when LKB1 is postnatally deleted (Courchet et al., 2013). We hypothesized that loss of the STRAD proteins would also not affect cortical lamination. We chose a marker of early-born cortical neurons, cTIP2 (layer 5/6), in each of the LKB1 cKO, STRADα KO, STRADβ cKO, and STRADα KO/β cKO cortices since the oldest age we could study was E18.5. These neurons are especially important, as early-born neurons act as guides for neurons born later in development (Kriegstein and Noctor, 2004; Lui et al., 2011). We find that cTIP2 is distributed normally in all knockouts except the STRAD double knockouts. In the $STRADα^{-/-}$;$STRADβ^{fl/fl}$; $Emx1^{Cre+}$ brain, cTIP2 is more sparse, and $cTIP2^{+}$ nuclei were found scattered throughout the cortical plate (Figure 5.3 A-G). We also observed this same pattern of staining in the $STRADα^{fl/fl}$; $STRADβ^{fl/fl}$; $Emx1^{Cre+}$ cortex (Figure 5.4 A, B). This implies that either the STRAD proteins have functions distinct from LKB1 in mouse brain, or that in the absence of STRAD proteins, LKB1 acts in a deleterious manner.

Figure 5.3

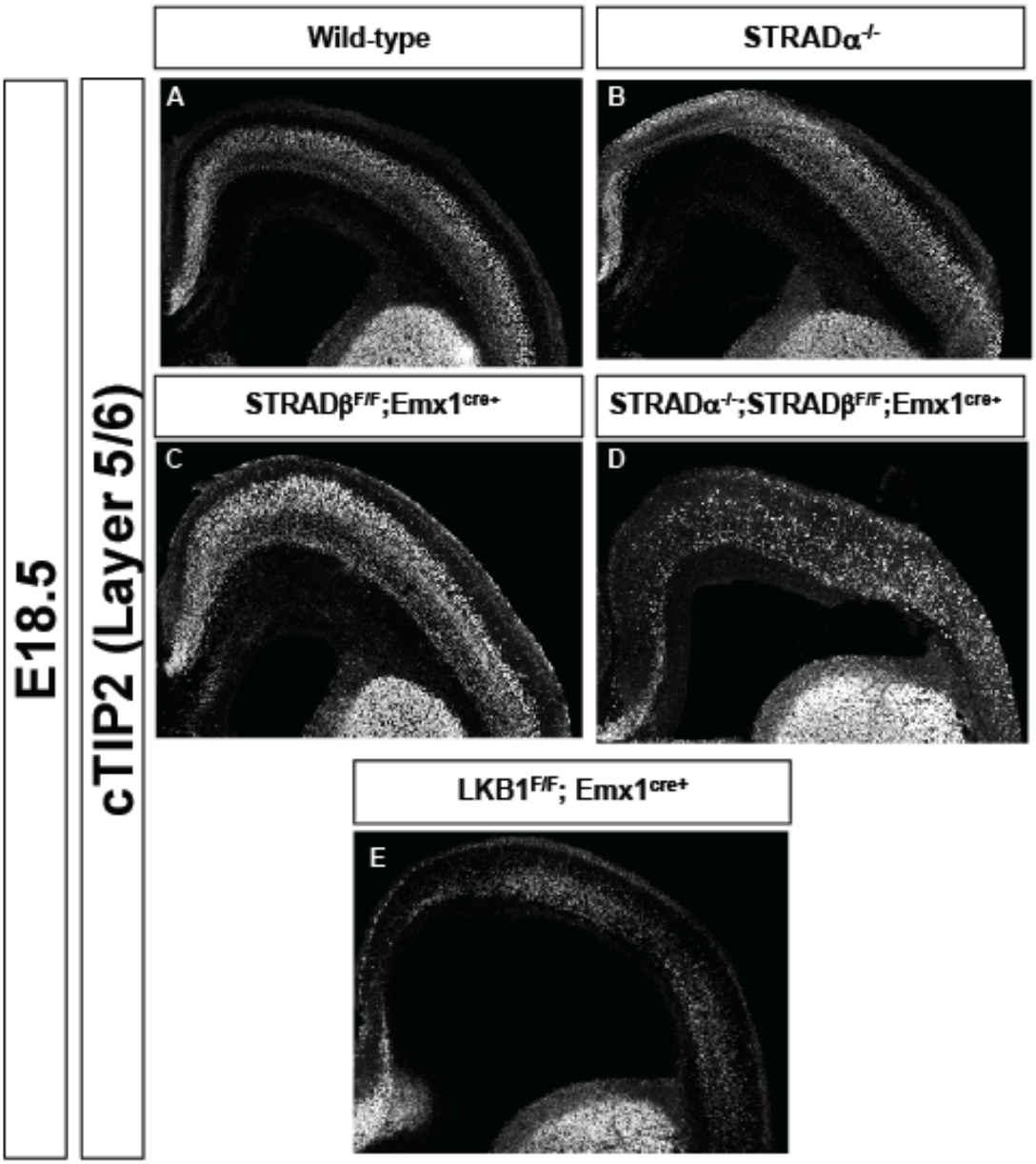


Figure 5.3 - STRADα and STRADβ are redundant in lamination. Embryonic day 18.5 coronal sections immunolabeled for cTIP2, a layer 5/6 marker (A-E). cTIP2 labeling is bright and present in two layers in wild-type, STRADα KO and STRADβ cKO brain, but the intensity is reduced and the pattern is perturbed in STRADα/β double KO cortex (A-D). However, the signal in LKB1 cKO cortex appears similar to each single STRAD knockout, in that it is bilayered and organized.

Figure 5.4

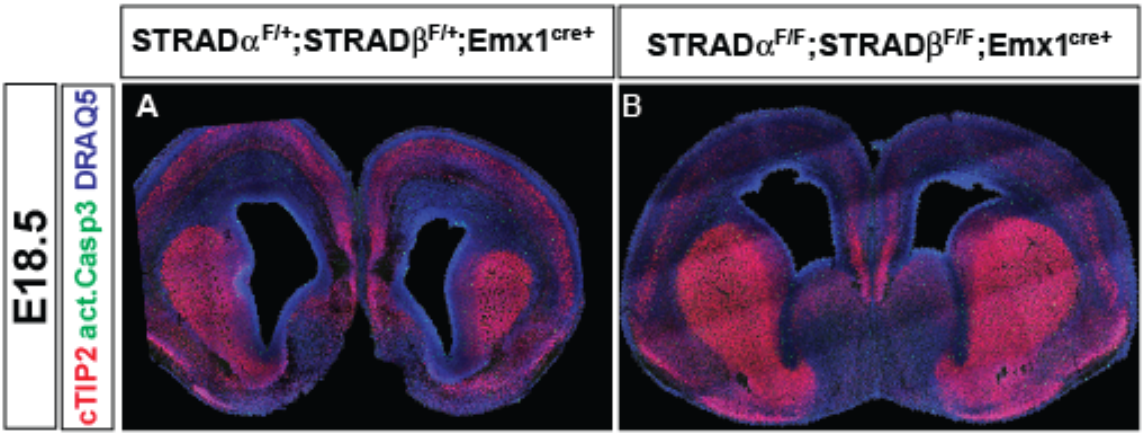


Figure55.4 - STRADα and STRADβ are redundant in lamination. Embryonic day 18.5 coronal sections immunolabeled for cTIP2, a layer 5/6 marker (red), activated caspase 3 (green) and DRAQ5 (DNA, blue). cTIP2 labeling is bright and presents in two layers in STRADα/β double heterozygotes (A) but in STRADα/β double conditional KO cortex, the intensity is reduced and the pattern is perturbed (B). This phenocopies the observations in STRADα constitutive null/STRADβ contidional null cortex.

D. Loss of STRADα, but not STRADβ, reduces LKB1 stability in vivo.

Prior work has implicated STRADα in stabilizing LKB1 *in vitro,* and opposing relationships between the chaperone Hsp90and STRADα have been reported regarding LKB1 activity (Boudeau et al., 2003b; Gaude et al., 2012; Nony et al., 2003). Given these links, we tested to what extent endogenous LKB1 expression is affected by loss of either or both STRAD proteins *in vivo*. We find loss of STRADα leads to a significant decrease (approximately 85%) in LKB1 protein levels in the embryonic (E16.5) cerebral cortex (Figure 5.5 A, B), a result mirrored in other tissues (Figure 5.6 C). However, MO25 levels were unchanged in the STRADα constitutive KO (Figure 5.6 A, B). In contrast, STRADβ deletion does not have the same effect on LKB1 protein levels, nor is the effect exacerbated by loss of both pseudokinases (Figure 5.5 A, B). To determine whether this destabilization of LKB1 was a posttranslational phenomenon, we examined the level of LKB1 mRNA using quantitative RT-PCR (qPCR) and observe a significant increase in LKB1 transcription (Figure 5.5 C). This finding implies that LKB1 is regulated post-transcriptionally, and that perhaps there is a feedback mechanism driven by LKB1 or STRADα protein level. Surprisingly, this reduction in LKB1 levels impairs neither axogenesis nor cell survival (Figure 5.1), and the phosphorylated form of LKB1 important in axogenesis is relatively preserved (Figure 5.7), at least in neuronal culture.

The fact that this dramatic reduction in LKB1 proteins levels in the STRADα KO did not affect axon formation led us to explore how this alteration in protein expression impacts LKB1 phosphorylation at serine 428 (S431 in murine LKB1), a post-translational modification of LKB1 that we and Shelley et al. have shown to be critical for axogenesis (Barnes et al., 2007; Shelly et al., 2007). Interestingly, total LKB1 levels are only

decreased by 50% in dissociated neurons, compared with the 85% reduction in cortical lysates (Figure 5.7). This could be due to the differential presence of growth factors and other nutrients in culture media as compared with the *in vivo* environment. Western blot analysis indicates that the proportion of LKB1 phosphorylated on S431 increases relative to total LKB1 in STRADα KO dissociated neurons, but that the absolute amount of P(S)431 is essentially unchanged (Figure 5.7). This result implies that LKB1 P(S)431 must exist at a certain level to effect axogenesis, and that this function is prioritized in the developing neuron. This finding speaks to the multifactorial nature of axogenesis, and gives an insight into the interplay between cell-intrinsic and cell-extrinsic mechanisms.

Given the role of STRADα in LKB1 stability in E16.5 cortex, we tested how this impacts LKB1 expression at various postnatal timepoints. We took advantage of the floxed allele of STRADα that we had generated and crossed this line to a Nestin-cre mouse line, deleting STRADα from neural progenitors and their offspring starting at embryonic day 12.5 (see Figure 4.2). This developmental analysis indicates that cortical LKB1 expression is compromised by STRADα loss in all post-natal ages examined (Figure 5.8 A, B), indicating that STRADα is absolutely required to achieve normal levels of LKB1, implying that LKB1 protein and/or STRADα mRNA/protein levels drive this process. LKB1 transcription control is not entirely understood, and this result implies that LKB1 protein and/or STRADα mRNA or protein levels drive this process.

We observe a reciprocal in vivo stabilizing relationship between LKB1 and STRADα, as cortical lysates from $LKB1^{fl/fl}$; $Emx1^{Cre+}$ mice display a significant reduction in STRADα levels (Figure 5.9), extending observations of STRADα and LKB1 stability we and others

have previously made *in vitro* (Baas et al., 2004a; Cheng et al., 2011; Eggers et al., 2012; Hawley et al., 2003; Shelly et al., 2007). Taken together, these experiments provide the first evidence of an *in vivo* function unique to STRADα in our mouse models.

Figure 5.5

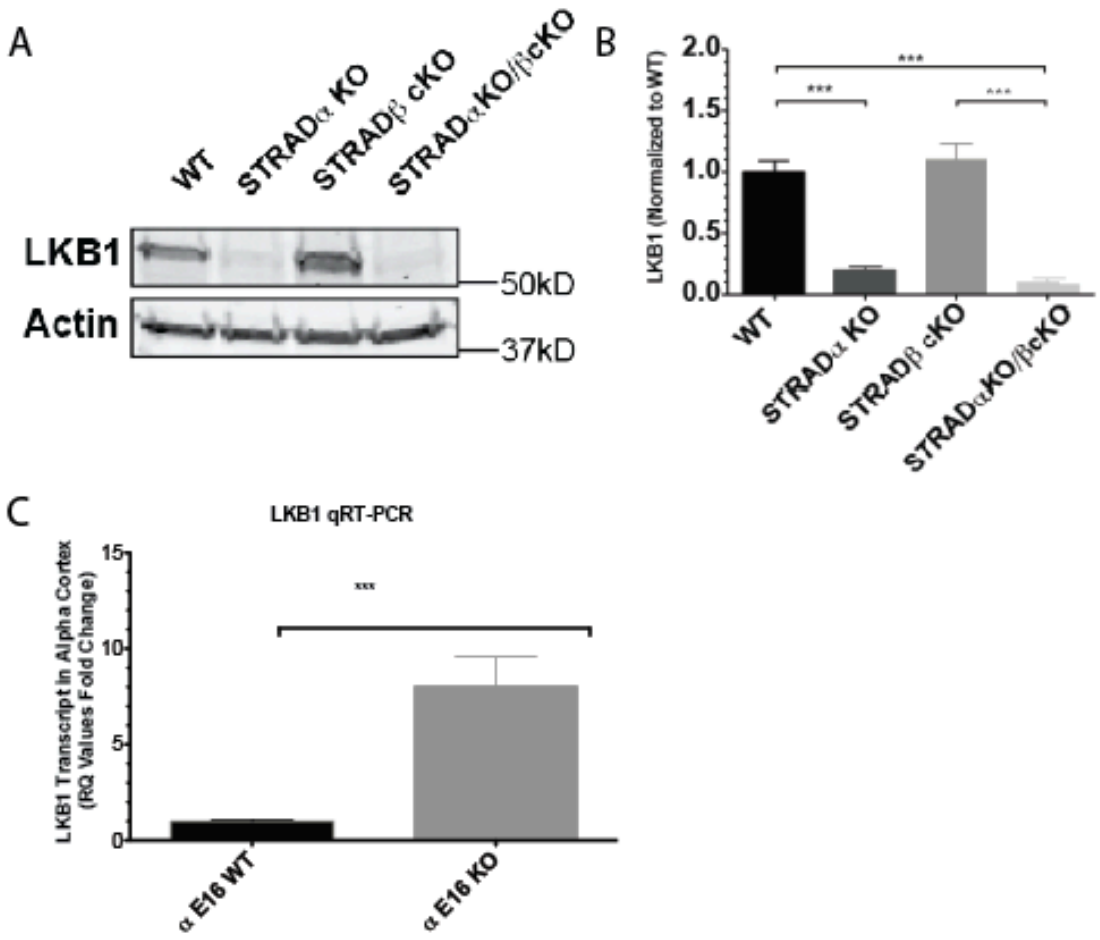


Figure 5.5 - STRADα, but not STRADβ stabilizes LKB1 protein. (A) Representative Western blot of lysates from wild-type, STRADα KO, STRADβ cKO, or STRADα/β double KO embryonic day 16.5 (E16.5) cortex. Actin is a loading control. (B) Quantification of LKB1 protein levels analyzed by Western blot, normalized to wild-type cortical lysate. N ≥15 cortices from at least three litters of each genotype. There is no significant difference between columns 1 and 3 or columns 2 and 4. ***P <0.0001 using one-way ANOVA with Bonferroni's multiple comparison test. (C) Up-regulation of LKB1 mRNA in STRADα KO E16.5 cortex relative to STRADα WT as assessed by qRTPCR. WT N=3, KO N=4 from 2 different litters. Values were normalized to GAPDH. Significance was determined using Student's unpaired two-tailed t-test. ***P<0.001

Figure 5.6

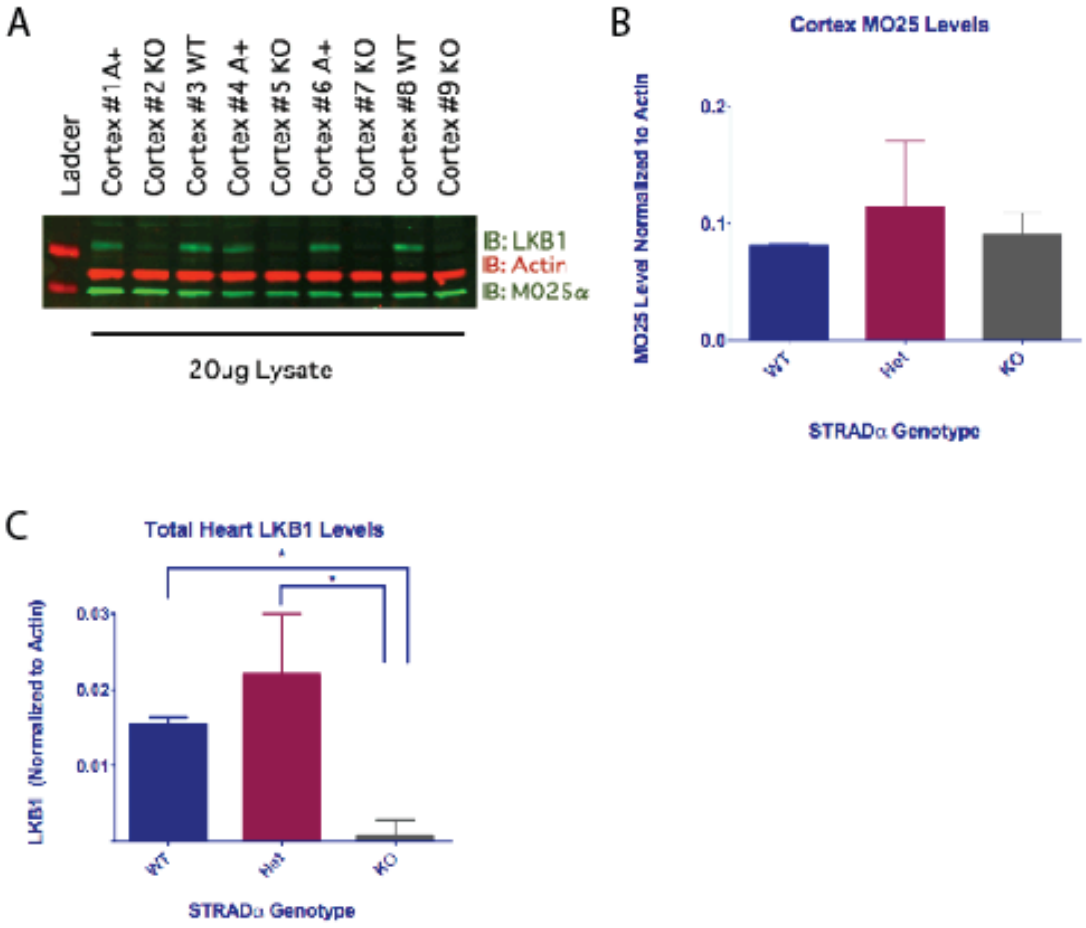


Figure 5.6 - MO25 protein level is unaffected by STRADα deletion (A) Representative Western blot of lysates from wild-type, STRADα KO, STRADβ cKO, or STRADα KO/β cKO embryonic day 16.5 (E16.5) cortex. Actin is a loading control. MO25 is the green band under Actin (B) Quantification of MO25 protein levels analyzed by Western blot, normalized to Actin. WT N=2, Het N=3, KO N=4. There is no significant difference between genotypes as determined by One-way ANOVA with Dunnett's post-test. LKB1 is destabilized by STRADα loss in multiple tissues. (C) Quantification of embryonic heart LKB1 levels by Western blot in the STRADα KO mouse line. WT N=2, Het N=3, KO N=4 All error bars represent SEM* P<0.05, ** P<0.01 by One-way ANOVA with Dunnett's post-test.

Figure 5.7

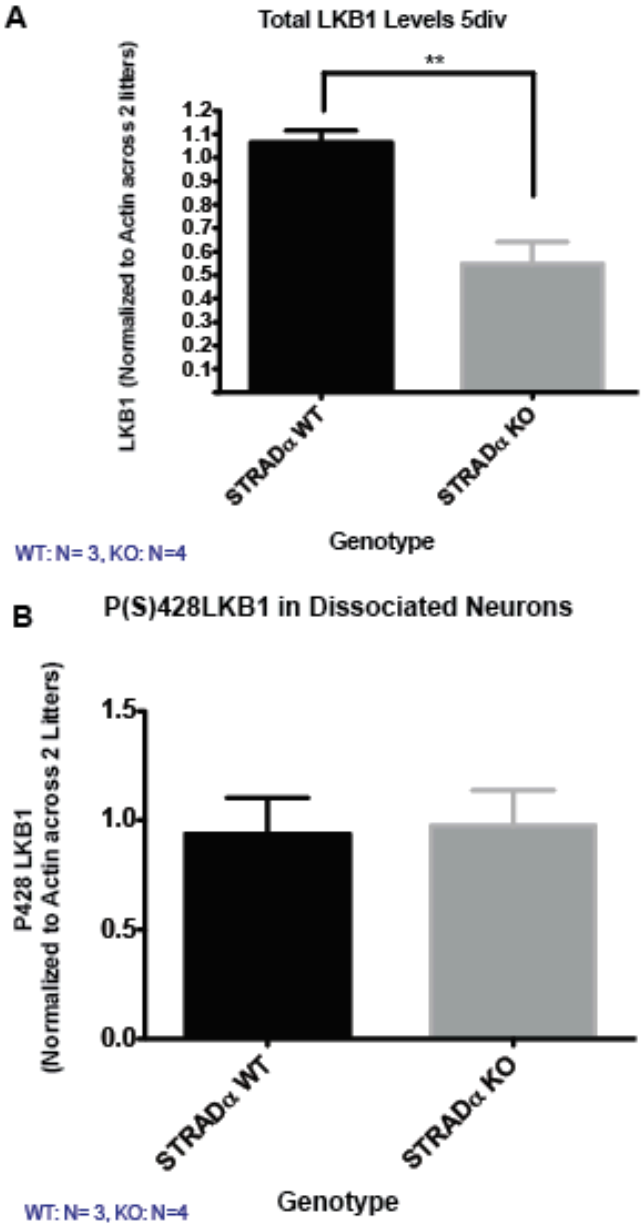


Figure 5.7 - (A) LKB1 is decreased in STRADα KO dissociated neurons relative to WT neurons. Values normalized to Actin. E15.5 + 5 div. (B) Quantification of western blot analysis of the levels of LKB1 phosphorylated at serine 428/431 in primary cultures derived from either control or STRADα-null mice. P(S)431 is relatvely preserved compared to total LKB1. All error bars represent SEM ** P<0.01 by Student's two-tailed unpaired t-test.

Figure 5.8

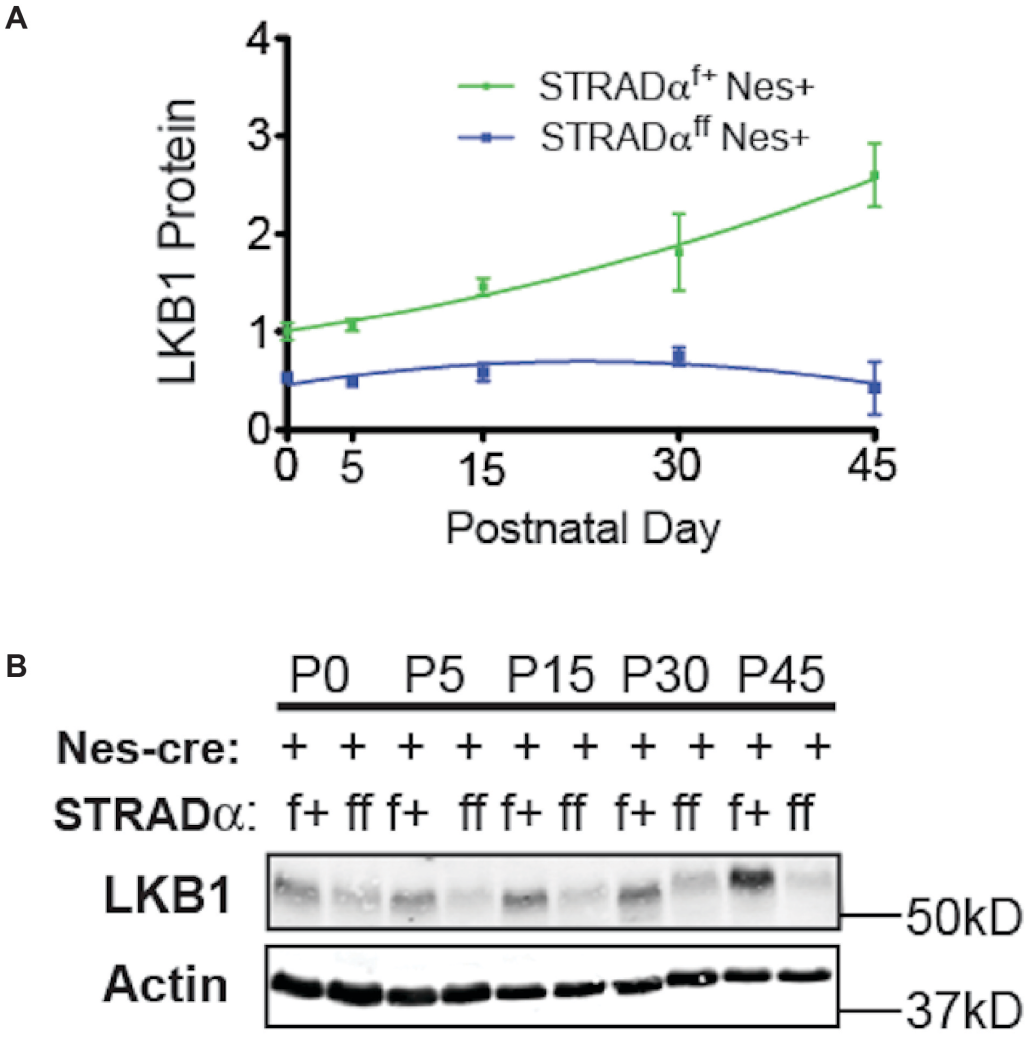


Figure 5.8 - STRADα is required for postnatal LKB1 protein accumulation. (A) (C) Western blot of Nestin-cre+; STRADαf+ or STRADαff lysates across developmental time. Quantification of LKB1 protein, normalized to postnatal day 0 (P0) STRADαf+; Nestin-cre+. N ≥3 brains of each genotype from three litters for each time-point.

Figure 5.9

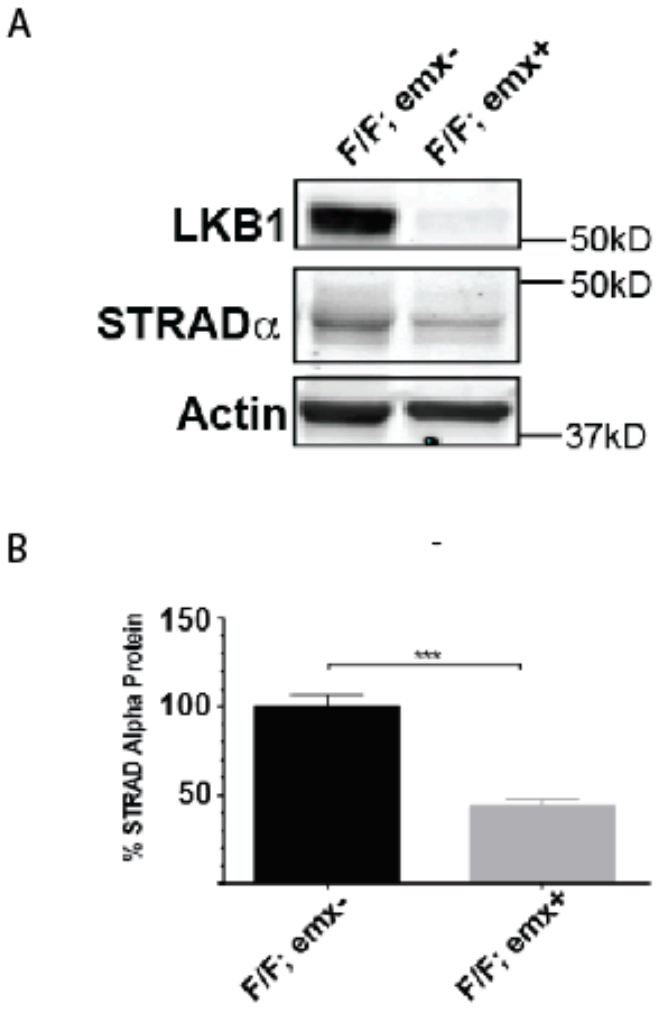


Figure 5.9 - LKB1 stabilizes STRADα. (A) Western blot of E16.5 cortical lysates showing STRADα protein is significantly reduced following conditional cortical loss of LKB1. (B) STRAD α protein is destabilized in LKB1ff; Emx1-Cre+ E16.5 cortex compared with littermate controls. N ≥ 10 cortices of each genotype from at least 3 litters. Significance was determined using Student's unpaired two-tailed t-test. ***P<0.001

III. Discussion

We have found that STRADα and STRADβ can compensate for one another in terms of axogenesis and cell survival, but not in terms of LKB1 stability. This is the first function unique to STRADα that has been described beyond the established role in conferring nuclear export to LKB1, and I will pursue the molecular mechanisms further in Chapter 6. While other studies have addressed the ability of the STRAD proteins to form complexes with LKB1 (Baas et al., 2003; Boudeau et al., 2003a) and to effect axogenesis (Barnes et al., 2007; Kishi et al., 2005; Shelly et al., 2007), we are the first to show *in vivo* redundancy between these two pseudokinases.

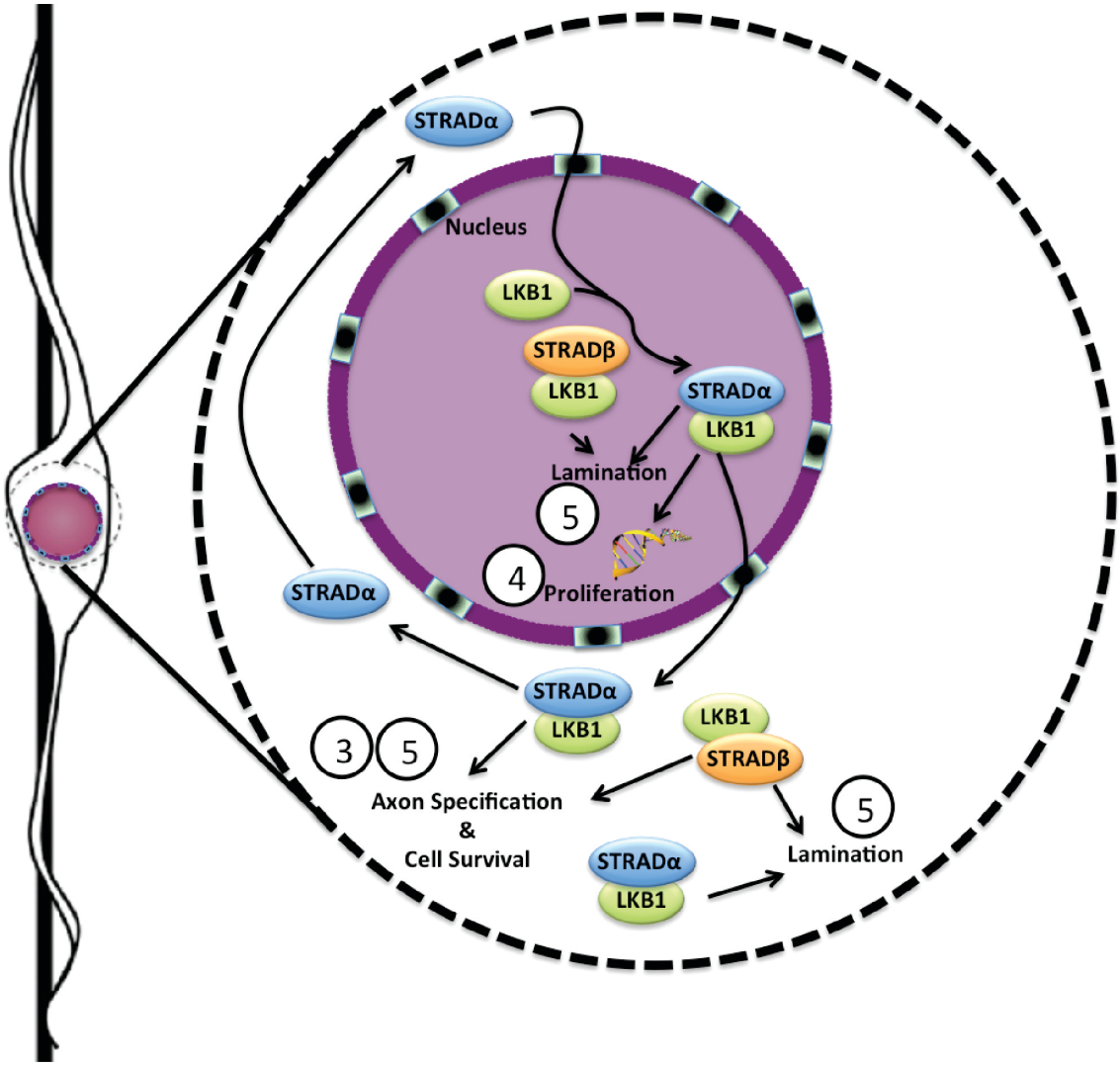
The lamination defect found exclusively in the STRADα$^{-/-\text{ or } fl/fl}$; STRADβ$^{fl/fl}$; Emx1^{Cre+} cortex is particularly intriguing. The mechanism behind this finding remains to be explored, and a couple of possibilities exist. While it is possible that the STRAD proteins have LKB1-independent functions that lead to this phenotype, more work will be required to determine the cause of the aberrant cTIP2 staining since the LKB1$^{fl/fl}$ Emx1^{Cre+} mouse has normal lamination by multiple markers including cTIP2 ((Barnes et al., 2007), Figure 5.3). It is more likely that LKB1 normally plays a nuclear role, and that, in the absence of both STRAD proteins, this role is deregulated. While LKB1 nuclear functions are not well described, recent studies have found that it interacts with p53 to bind the p21 promoter, interacts with LM04 and GATA6 to drive p21 in a p53 independent manner, and inhibits Yap, a transcriptional co-activator (Nguyen et al., 2013; Setogawa et al., 2006; Zeng and Berger, 2006). LKB1 gene expression has been shown to be regulated by ERα (Linher-Melville et al., 2012) and LKB1 interacts with LMO4, Ldb1, and GATA6 to drive G1/S cell cycle arrest, further supporting the idea of nuclear

roles for LKB1 (Setogawa et al., 2006). These results represent only the beginnings to understanding LKB1 nuclear functions and future work should focus on delineating these aspects further. Additionally, LKB1 cytoplasmic localization/activity has also been shown to be critical for its role in G1/S cell cycle arrest (Sapkota et al., 2002; Setogawa et al., 2006; Shan et al., 2014; Tiainen et al., 2002). The fact that STRADβ has been found both in the nucleus and cytoplasm ((Boudeau et al., 2004; Dorfman and Macara, 2008) Figure 6.3) further supports the assertion that LKB1 has a nuclear role for which the STRAD proteins are required. Experiments to test this assertion should include BioID (Roux et al., 2012) experiments with LKB1 and the STRAD proteins followed by cell fractionation to determine the unique binding partners of each protein within each cell compartment. Furthermore, these experiments could be carried out in cells with one or both STRAD proteins knocked down and LKB1-BirA overexpressed in order to find the unique binding partners in each condition. ChIP experiments of LKB1 could also be useful from single STRAD knockout mice and the double knockouts, to inform the extent to which STRAD presence or absence affects which area(s) of the genome LKB1 targets.

Caveats – Our work on the STRADα/STRADβ double knockouts and on the STRADα conditional knockouts suggests that cortical development is highly sensitive to timing of STRAD deletion. It is possible that we see no effect on the brain of STRADβ deletion because we have not examined a time point at which STRADβ is absolutely required. Since STRADβ mRNA levels increase over developmental time, it is likely that its main functions are in the adult brain. Future work should aim to determine the necessity and sufficiency of STRADβ for the functions of mature neurons. This work could be undertaken by crossing the STRADβ mouse line to an inducible Cre mouse line such as

Emx1-Cre^{ERT2} to address the long-term effects of STRADβ deletion in a discrete window of time. Furthermore, since STRADβ null mice are viable, they should be further characterized.

Chapter 5 Summary Figure



Chapter 5 Summary Figure. Schematic of STRAD/LKB1 signaling within the developing neuron. (3) STRADα and STRADβ both complex with LKB1 and are capable of eliciting axogenesis in primary cultured neurons. Only STRADα can export LKB1 from the nucleus. (4) STRADα may help regulate proliferation with LKB1. (5) STRADα and STRADβ can each participate in cortical lamination with LKB1.

Chapter Six: STRAD and LKB1 Protein Stability

I. Introduction

Our previous observations that STRADα mRNA is tightly controlled during development and that it is necessary to stabilize LKB1 led us to further investigate the question how LKB1 protein levels are regulated. In this chapter, I will examine the dynamics of LKB1 protein stability in HEK cells. In addition to protein synthesis control, protein stabilization/degradation are ways in which protein levels can be quickly and accurately controlled during development. These mechanisms contribute to the precise timing of protein expression in the developing organism. The main system for degrading proteins is the ubiquitin/proteasome system where proteins are targeted for degradation by poly-ubiquitin linkages on specific lysines within the protein (Komander and Rape, 2012). These linkages are created in a series of steps involving E1, E2, and E3 ubiquitin ligases, and the ubiquitin moiety itself, and ubiquitylation can have many functions other than proteasomal targeting, depending on the specific ubiquitin linkage (Komander, 2009). Generally, though, a chain of four or more ubiquitin moieties on a single lysine is sufficient to target said protein for degradation (Haas and Siepmann, 1997; Komander and Rape, 2012). Our *in vivo* findings from Chapter 5 indicate that STRADα is required to either affect LKB1 translation or protect LKB1 protein levels, while STRADβ is not sufficient to maintain normal LKB1 levels. The major known functional difference between STRADα and STRADβ is the ability of STRADα to export LKB1 from the nucleus, so it is possible that LKB1 is degraded there, or that the ubiquitination machinery required to target LKB1 for degradation is present. However, the subcellular dynamics of LKB1 degradation have not yet been determined, and we will begin to address them in this chapter.

A. CCM3

Much of the work in the previous chapters is aimed at understanding STRAD regulation of LKB1, but we were also curious about regulation of the STRAD proteins. A candidate that has recently emerged for this role is Cerebral Cavernous Malformations 3 (CCM3) - one of three CCM proteins required for normal vascular development of the brain (Faurobert and Albiges-Rizo, 2010). In collaboration with the laboratory of Dr. Brian Howell, we have begun to pursue the contribution of this protein to LKB1/STRAD stability. CCM3 mutations in humans cause debilitating migraines and malformations of brain vasculature (Faurobert and Albiges-Rizo, 2010). CCM3 has been shown to heterodimerize with GCKIII family kinases including Stk25 and MST3 during development (Faurobert and Albiges-Rizo, 2010; Shi et al., 2013). MO25 has also been shown to interact with the MST kinases as well as with the STRAD proteins, so it could act as a link between the two complexes (Filippi et al., 2011). Among its developmental roles, CCM3 has been found to regulate proliferation and apoptosis in various tissues, and has been shown to affect cell morphogenesis in the developing heart (Fuller et al., 2012) and has been shown to interact with Stk25 to modulate apoptosis under oxidative stress conditions (Zhang et al., 2012). Within neurons, overexpression of CCM3 was found to be sufficient to induce apoptosis (Lin et al., 2010). CCM3 has recently been implicated in neuronal migration during brain development (Louvi et al., 2014) and along these lines, Stk25 was recently found to interact with STRADα and LKB1 to control Golgi deployment during brain development (Matsuki et al., 2010). Since CCM3 and STRADα/LKB1 are all known to affect Golgi morphology, it is possible that CCM3 does so in a STRADα/LKB1-dependent manner.

II. Results

A. STRADα stabilizes LKB1 via direct contact through subcellular localization.

We find that HEK293 cells exhibit similar patterns of STRAD-dependent LKB1 stability (Figure 6.1 A, B), as was observed in prior studies (Cheng et al., 2011; Shelly et al., 2007). In these cells, we were able to replicate the stabilizing effects of STRADα on LKB1 by measuring protein turnover following cycloheximide-mediated protein synthesis inhibition (Figure 6.1). One of the main functional differences between STRADα and STRADβ is the ability of STRADα to effect export of LKB1 from the nucleus, so we were curious as to whether STRADβ could also stabilize LKB1. Our results show that STRADβ co-expression can preserve LKB1 protein levels, but to a lesser extent than does STRADα. This ability of STRADβ to slightly stabilize LKB1 could be explained by cytoplasmic sequestration of LKB1 by the predominantly cytoplasmic STRADβ if indeed localization is the key to the STRADα effect. The next question was whether LKB1 stabilization requires direct contact with STRADα. Using a previously validated LKB1-interaction mutant of STRADα-2 (YHF, Y185F/H231A/F233A) (Zeqiraj et al., 2009a; 2009b), we find that LKB1-binding by STRADα is required for this stabilizing effect (Figure 6.1 A, B; Figure 6.4). This is not surprising in the context that previous work has shown competitive binding of LKB1 by Hsp90/Hsc70 and STRADα (Boudeau et al., 2003b; Gaude et al., 2012). It supports the hypothesis that interaction with STRADα either recruits stabilizing factors or prevents binding of destabilizing factors to LKB1.

Previous biochemical and cell biological studies have specifically implicated

STRADα in shuttling LKB1 to the cytoplasm, as STRADβ lacks key residues involved in the interaction with the nuclear export machinery (Boudeau et al., 2003a; Dorfman and Macara, 2008). This suggests the possibility that STRADα-dependent cytoplasmic translocation of LKB1 prevents LKB1 degradation in the nucleus. To test this, we examined LKB1 stability in the context of a nuclear-localization signal (NLS) mutant of LKB1 (LKB1-ΔNLS) (Figure 6.2, also see Figure 6.3) (Tiainen et al., 2002). We find that LKB1-ΔNLS demonstrates a robust stabilization when expressed alone (Figure 6.2), and this increased stability is not significantly different from WT-LKB1 expressed in combination with STRADα-2, nor is the stability of LKB1-ΔNLS further enhanced by co-expression of STRADα-2 (Figure 6.2).

We confirmed the subcellular localization of the mutants described here in HEK293 cells (Figure 6.3) and also verified that each STRAD isoform/mutant except STRADαYHF can bind LKB1 (Figure 6.4). In preliminary stability studies, we found that the LKB1-SL26 mutant was fairly stable compared with WT-LKB1 four hours after cycloheximide-mediated translation inhibition (Figure 6.5). This was surprising given that this mutant does not strongly bind STRAD (Figure 6.4). Further work will address the mechanism of this stabilization, and determine whether STRAD binding of LKB1 both stabilizes the protein and initiates a degradation cascade, and where in the cell this takes place.

Given that nuclear export of LKB1 affects its stability and that splice forms of STRADα can contain an additional nuclear export signal (NES), we tested how these isoforms (STRADα-1 and −7) impact STRAD's ability to stabilize LKB1 but we do not

observe any significant differences in LKB1 stability conferred by these STRADα splice variants (Figure 6.6 A). We also tested the ability of a STRADα mutant missing its nuclear export signal (STRADα-2 ΔNES) to stabilize LKB1 protein, but these findings were inconclusive and will likely require additional mutagenesis of STRADα (Figure 6.6 B). This could be due to a cryptic NES within the STRADα sequence, or perhaps LKB1 that has been kept away from the nucleus is inherently more stable than LKB1 bound by STRADα within the nucleus. In any case, these findings argue for a nuclear role in LKB1 degradation.

We wanted to address the issue of LKB1 degradation while removing the confound of subcellular localization, so we undertook experiments using Leptomycin B as an inhibitor of active nuclear export. It is important to note that in the presence of Leptomycin B, passive diffusion to and from the nucleus still takes place. Interestingly, we find that simply inhibiting nuclear export slightly increases the half-life of LKB1 protein (Figure 6.7 A), though this difference is not statistically significant. This is unexpected given the finding that LKB1-ΔNLS is more stable than WT-LKB1, and implies that perhaps LKB1 is ubiquitinated in the nucleus, but is actually degraded in the cytoplasm. We also find that adding STRADα-2 in the presence of Leptomycin B trends toward an even longer LKB1 half-life (Figure 6.7 B), supporting the notion that STRADα sequesters LKB1 from ubiquitination machinery. Interestingly, the degradation curves of LKB1 in the presence of STRADβ-1 or alone with Leptomycin B treatment appear very similar (Figure 6.7 C). Furthermore, overexpressing STRADα-2 ΔNES in the presence of Leptomycin B stabilizes LKB1 to the same extent as WT-STRADα-2

without Leptomycin B (Figure 6.7 D). This finding further supports the previous assertion that STRADα stabilizes LKB1 by keeping it from ubiquitination. Intriguingly, we also find that STRADβ-1 is able to stabilize LKB1 similarly to STRADα-2 in the presence of Leptomycin B (Figure 6.7 E), and the timecourse of degradation of LKB1 with STRADβ-1 and Leptomycin B does not fit a single nonlinear regression (Figure 6.7 E). Notably, Leptomycin B treatment enabled STRADβ-1 to stabilize LKB1 significantly more than STRADβ-1 alone (Figure 6.7 E). Since Leptomycin B inhibits active nuclear export, these findings support a model in which nuclear LKB1 is tightly regulated, and targeted for degradation unless bound by its regulators and co-activators, the STRAD proteins. This is especially interesting in light of the finding that loss of both STRAD proteins perturbs both proliferation and cTIP2^{+} cell localization, and calls into question the exact roles of nuclear LKB1. An alternate model is that LKB1 is targeted for degradation in the nucleus, but is actually degraded elsewhere, and that Leptomycin B prevents this translocation. Future studies should directly address this question.

Figure 6.1

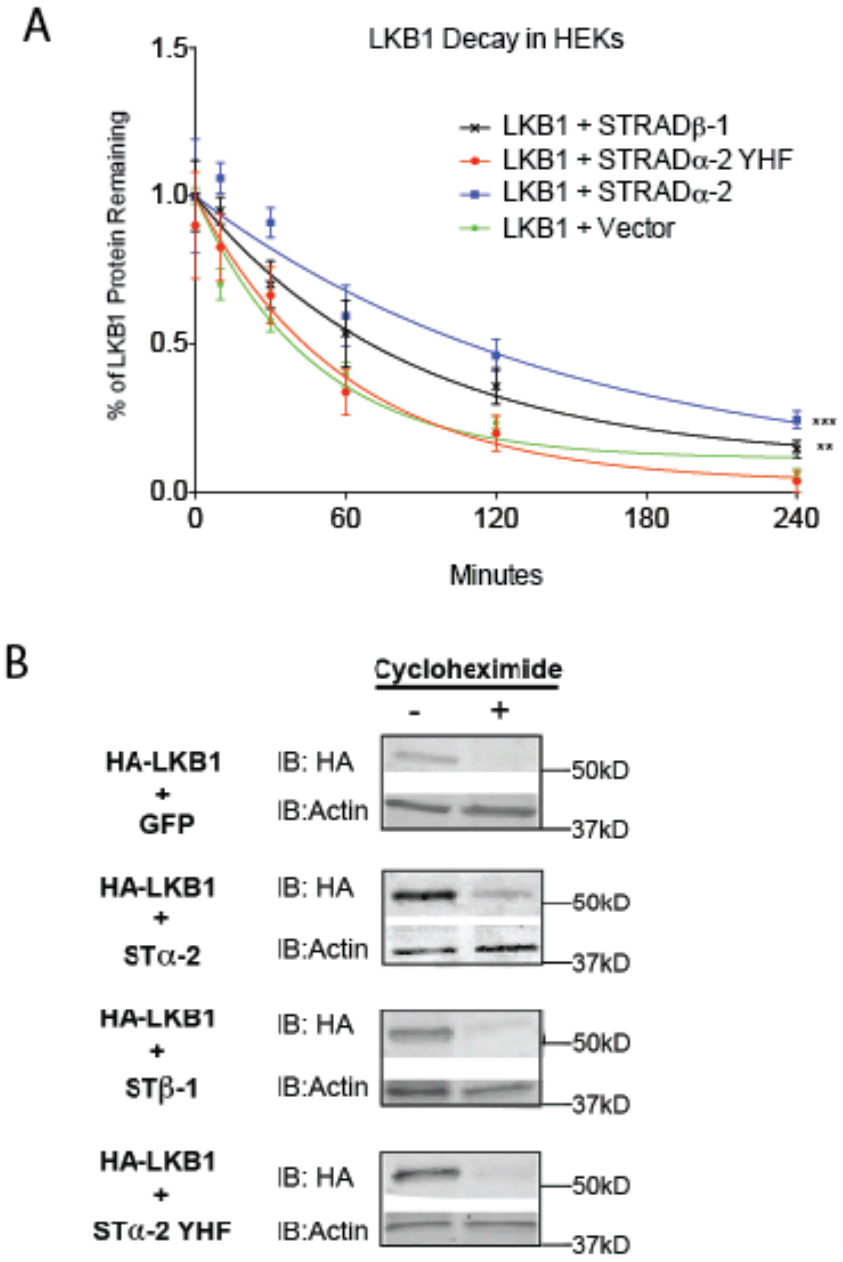
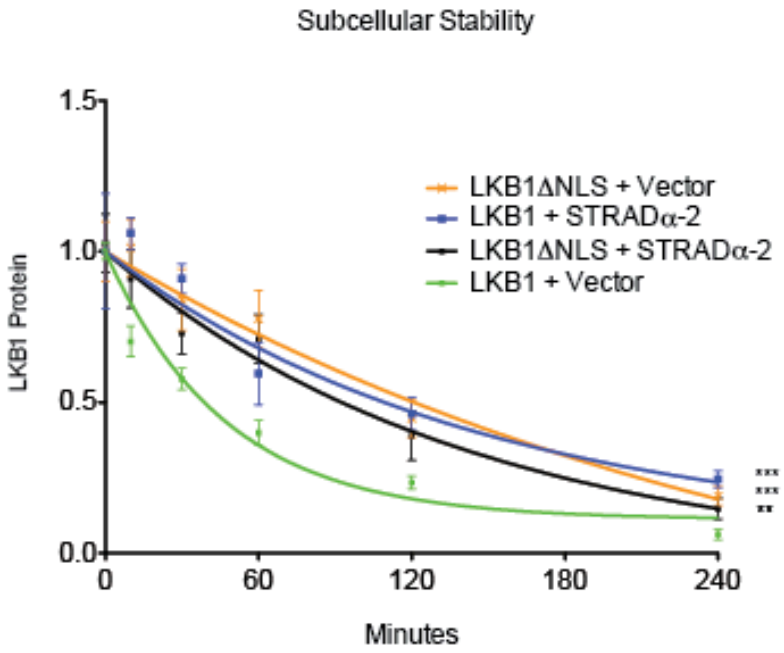


Figure 6.1 - (A) Quantification of N ≥4 independent LKB1 stability time-courses in HEK cells transfected with epitope-tagged LKB1 protein following cycloheximide-mediated translation inhibition, with three to six replicates of each condition in each experiment. STRADα-2 significantly increases LKB1 half-life, as does STRADβ-1, to a lesser extent. STRADα-2 YHF (STRAD-LKB contact mutant) does not significantly stabilize LKB1 protein. Error bars represent SEM. Repeated measures ANOVA with Dunnett's multiple comparison test with LKB1 + empty vector as the control was employed. ***P <0.001, **P <0.005. ANOVA, analysis of variance; KO, knockout; SEM, standard error of the mean. (B) HEK293 cells were transfected with HA-tagged LKB1 and STRAD constructs. Representative Western blot anti-HA of untreated and 4hrs 50μg/mL cycloheximide treated cells, with actin as a loading control.

Figure 6.2



Significance was determined by repeated measures ANOVA with Dunnett's post-test using LKB1 + Vector as a control **p<0.01 ***p<0.001

Figure 6.2 - Quantification of N ≥4 independent LKB1 stability time-courses in HEK cells transfected with epitope-tagged LKB1 protein following cycloheximide-mediated translation inhibition, with three to six replicates of each condition in each experiment. LKB1ΔNLS is as stable as LKB1 + STRADα-2 and LKB1ΔNLS + STRADα-2 is similar to LKB1ΔNLS + Vector. Error bars represent SEM. Repeated measures ANOVA with Dunnett's multiple comparison test with LKB1 + empty vector as the control was employed. ***P <0.001, **P <0.005. ANOVA, analysis of variance; KO, knockout; SEM, standard error of the mean; NLS, nuclear localization signal.

Figure 6.3

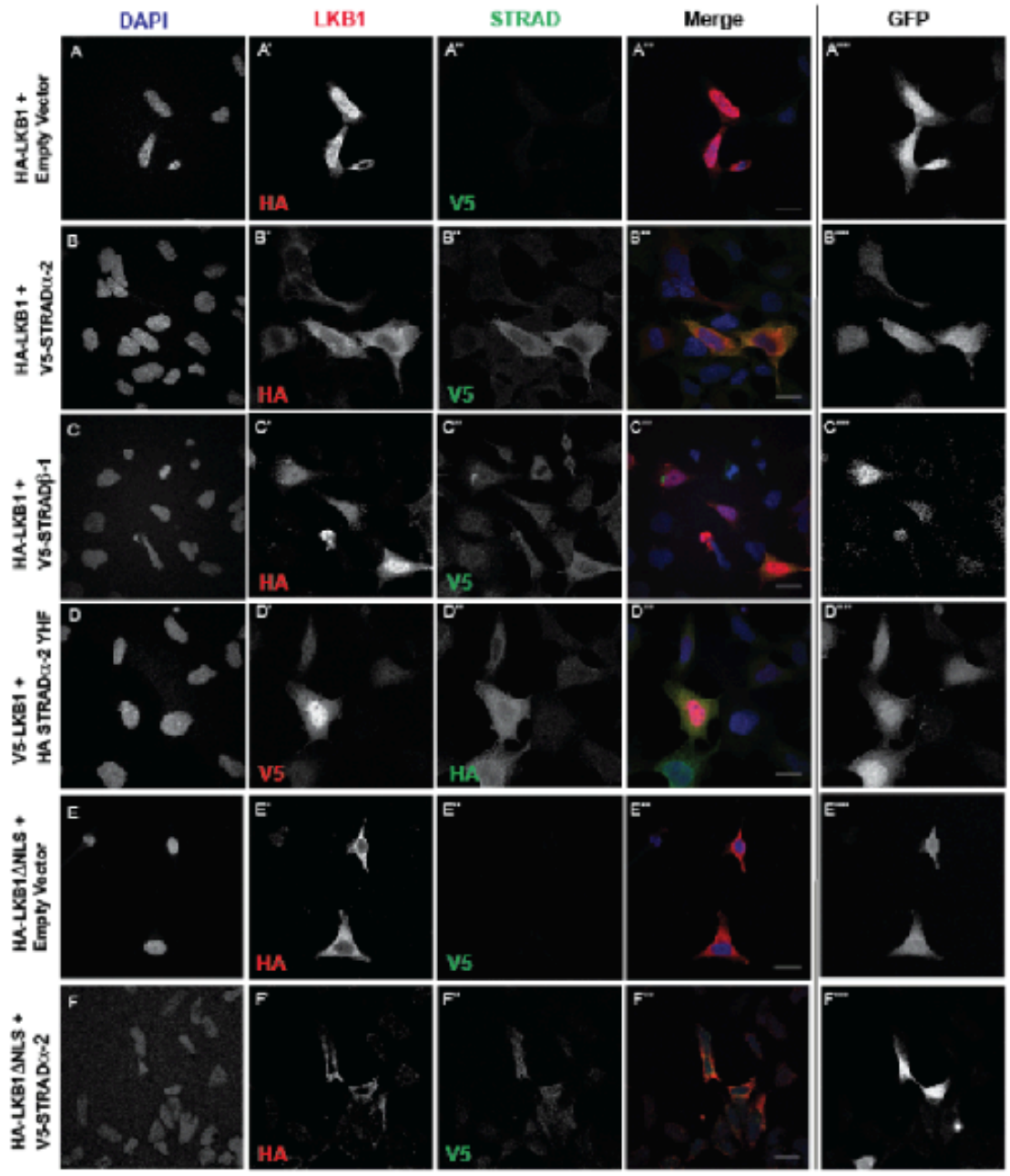


Figure 6.3 - Subcellular localization of epitope-tagged LKB1 and STRAD Variants. Verification of subcellular localization for epitope-tagged LKB1 and STRAD constructs transiently transfected in HEK293 cells. (A-A"") LKB1+GFP-expressing empty vector, (B-B"") LKB1+STRADα-2. (C-C"") LKB1+STRADβ-1, (D-D"") The LKB1+STRADα-2 YHF (LKB1 binding mutant), (E-E"") LKB1ΔNLS+GFP-expressing empty vector (F-F"") LKB1ΔNLS+STRADα-2.

Figure 6.4

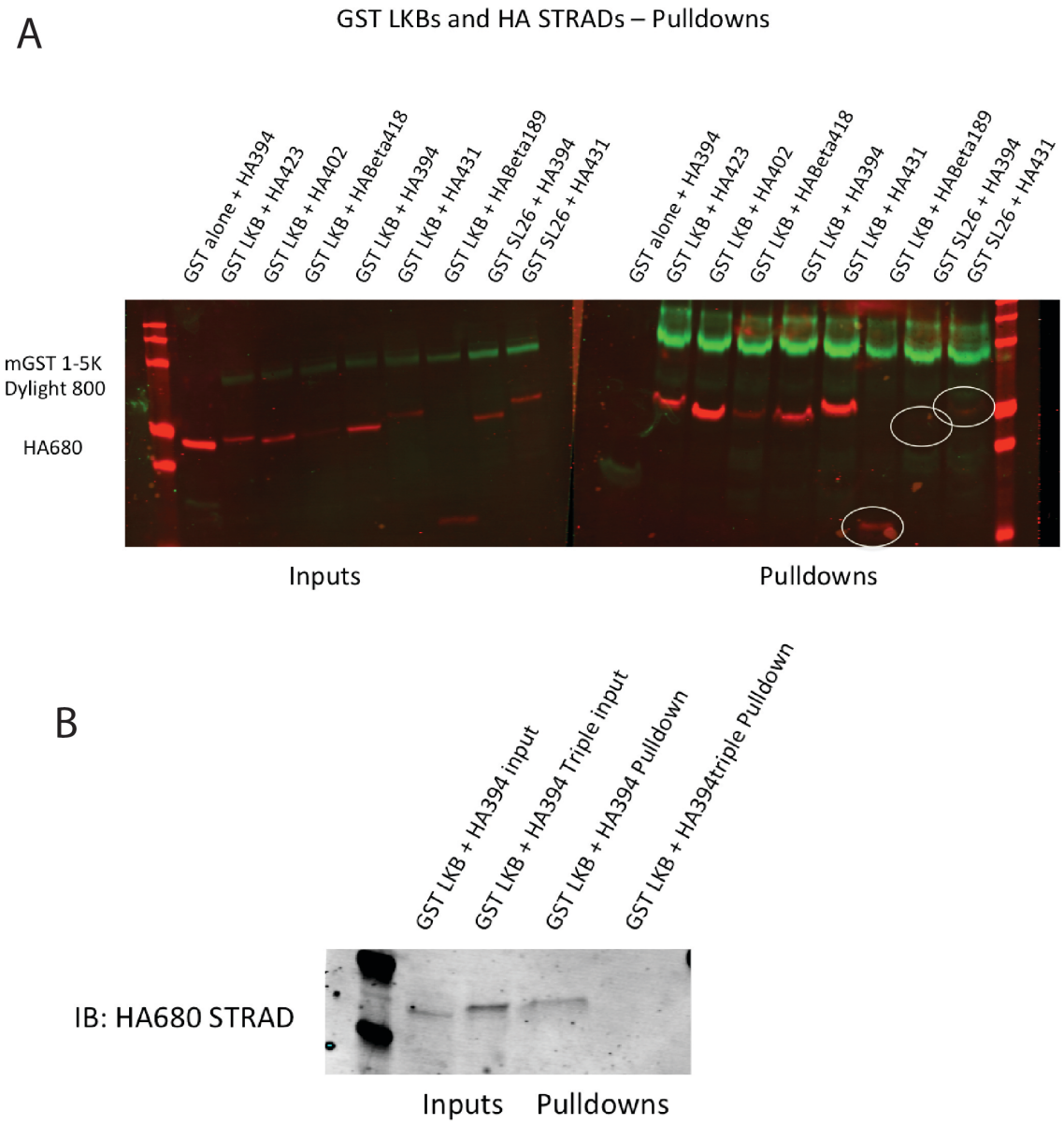


Figure 6.4 - GST-tagged LKB1 variants were co-transfected with STRAD variants and interaction was determined using pulldown with glutathione sepharose beads. All variants tested assocat with LKB1 except for the STRADα YHF mutant.

Figure 6.5

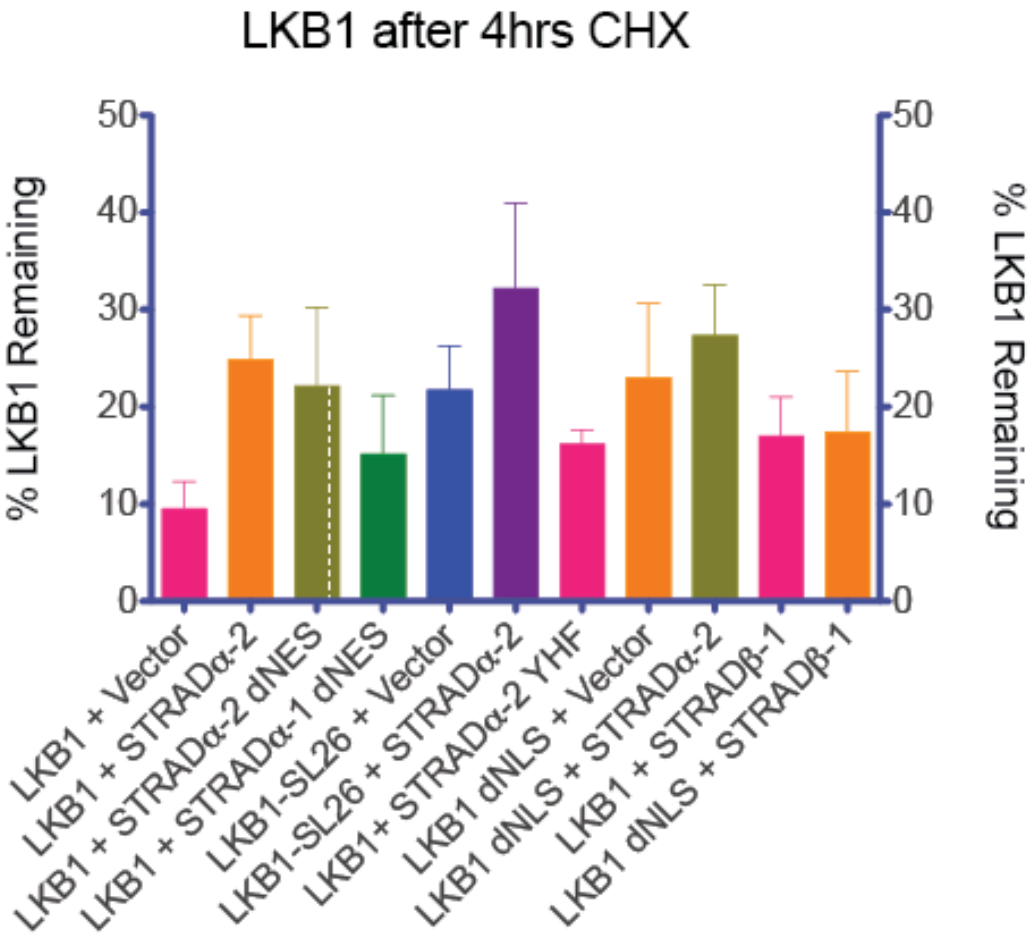


Figure 6.5 - Quantification of N = 3-10 independent LKB1 stability experiments in HEK cells transfected with epitope-tagged LKB1 and a second cDNA. Cells were treated with 50ug/mL cycloheximide for 4 hours and values are represented as treated/untreated, normalized to actin. Error bars represent SEM.; STRADα-2 YHF cannot contact LKB1; SEM, standard error of the mean; NLS, nuclear localization signal.

Figure 6.6

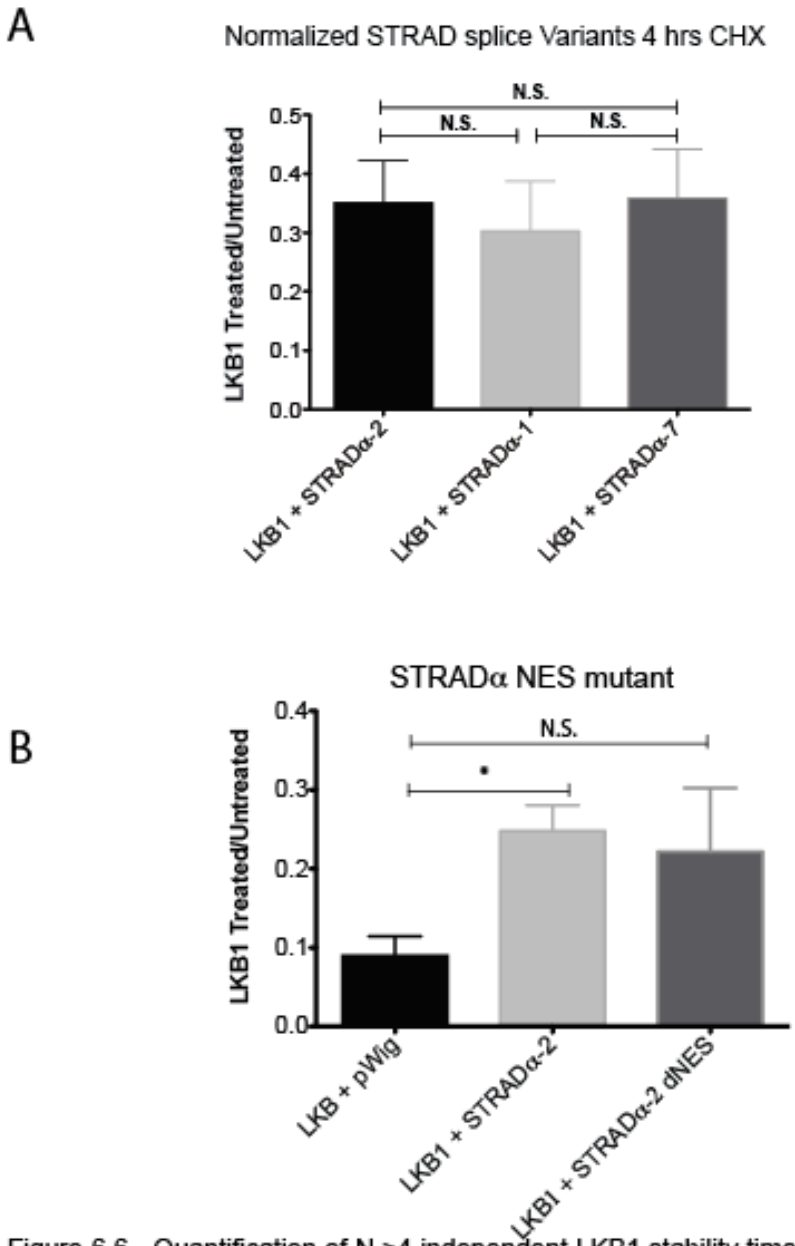


Figure 6.6 - Quantification of N ≥4 independent LKB1 stability time-courses in HEK cells transfected with epitope-tagged LKB1 protein following cycloheximide-mediated translation inhibition, Quantification and comparison of LKB1 stability following 50 µg/ml cycloheximide treatment when co-transfected with A) STRADα splice variants STRADα-2, -1 or -7 or B)Vector (pWig), STRADα-2, or STRADα-2 dNES. Repeated measures ANOVA with Dunnett's multiple comparison test with LKB1 + empty vector as the control was employed. ***P <0.001, **P <0.005. ANOVA, analysis of variance; KO, knockout; SEM, standard error of the mean; NES, nuclear export signal.

Figure 6.7

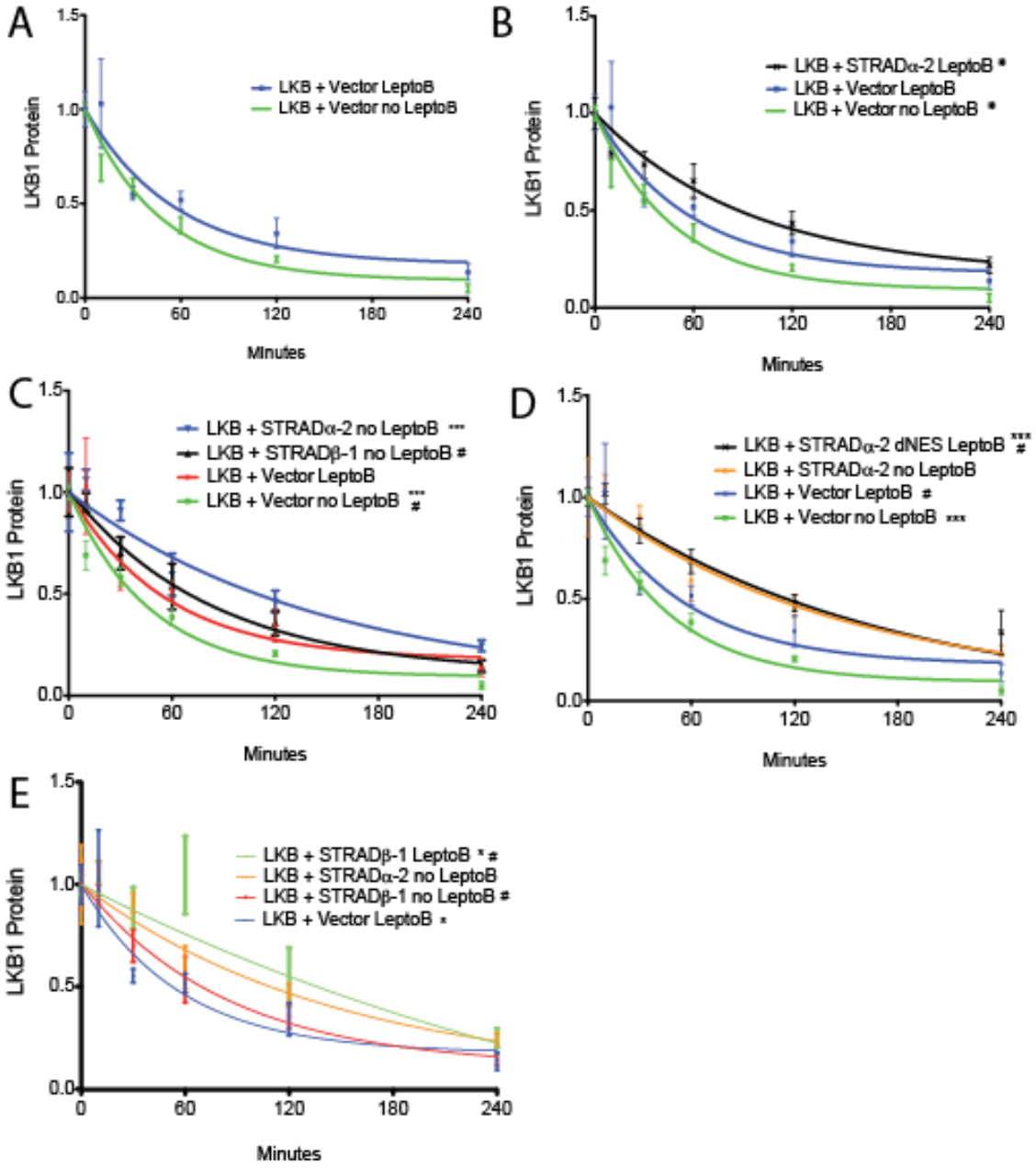


Figure 6.7 - Quantification of N ≥4 independent LKB1 stability time-courses in HEK cells transfected ith epitope-tagged LKB1 protein. LeptomycinB (LeptoB) was used to inhibit active nuclear transport. Decay curves of LKB1 protein following 50 µg/ml cycloheximide treatment for the amounts of time labeled when co-transfected with A) Nuclear export inhibition does not stabilize LKB1 significantly. B) STRADα-2 significantly stabilizes LKB1 in the nucleus. C) Blocking nuclear export somewhat stabilizes LKB1. D) STRADα-2 dNES with LeptomycinB stabilizes LKB1 to the same extent as does STRADα-2 without LeptomycinB. Both stabilize LKB significantly. Repeated measures ANOVA with Dunnett's multiple comparison test with LKB1 + empty vector as the control was employed. ***P <0.001, **P <0.005. ANOVA, analysis of variance; KO, knockout; SEM, standard error of the mean; NES, nuclear export signal.

B. LKB1 is degraded by the proteasome.

To determine whether LKB1 degradation is proteasomal, we generated a mutant of LKB1 in which all lysines, except two lysines critical for subcellular localization (Boudeau et al., 2004; Zeqiraj et al., 2009a) are mutated to arginines (LKB1 KR), thus preventing ubiquitination on those residues ((Komander and Rape, 2012)Figure 6.8). This LKB1 KR mutant is significantly more stable compared with WT LKB1 or LKB1-ΔNLS. However, the mutant is still gradually degraded (Figure 6.9), indicating there are other LKB1 degradation mechanisms at work, or that the two lysines remaining on LKB1 are sufficient to target LKB1 for degradation. We attempted to map the lysine on LKB1 targeted for ubiquitination by generating mutants of LKB1 with discreet regions of lysines mutated to arginines, as outlined in Figure 6.9 A-C. While the initial results implicated two stretches of lysines targeting LKB1 for degradation, the lysine mutant we generated for both these regions was no more stable than WT LKB1 (Figure 6.9 F). This could be due to a change in LKB1 protein folding, or an altered propensity to bind partner proteins that recruit the E3 ubiqutin ligase to LKB1.

Figure 6.8

A

LKB1-WT

MDVADPEPLGLFSEGELMSVGMDTFIHRIDSTEVIYQPRRKRAKLIGKYLM
GDLLGEGSYGKVKEVLDSETLCRRAVKILKKKKLRRIPNGEANVKKEIQLL
RRLRHRNVIQLVDVLYNEEKQKMYMVMEYCVCGMQEMLDSVPEKRFPVCQA
HGYFRQLIDGLEYLHSQGIVHKDIKPGNLLLTTNGTLKISDLGVAEALHPF
AVDDTCRTSQGSPAFQPPEIANGLDTFSGFKVDIWSAGVTLYNITTGLYPF
EGDNIYKLFENIGRGDFTIPCDCGPPLSDLLRGMLEYEPAKRFSIRQIRQH
SWFRKKHPLAEALVPIPPSPDTKDRWRSMTVVPYLEDLHGRAEEEEEEDLF
DIEDGIIYTQDFTVPGQVLEEEVGQNGQSHSLPKAVCVNGTEPQLSSKVKP
EGRPGTANPARKVCSSNKIRRLSACKQQ

LKB1-KR

PDVADPEPLGLFSEGELMSVGMDTFIHRIDSTEVIYQPRRRRARLIGRYLM
GDLLGEGSYGRVREVLDSETLCRRAVKILRRRRLRRIPNGEANVRREIQLL
RRLRHRNVIQLVDVLYNEERQRMYMVMEYCVCGMQEMLDSVPERRFPVCQA
HGYFRQLIDGLEYLHSQGIVHKDIRPGNLLLTTNGTLRISDLGVAEALHPF
AVDDTCRTSQGSPAFQPPEIANGLDTFSGFRVDIWSAGVTLYNITTGLYPF
EGDNIYRLFENIGRGDFTIPCDCGPPLSDLLRGMLEYEPARRFSIRQIRQH
SWFRRRHPLAEALVPIPPSPDTRDRWRSMTVVPYLEDLHGRAEEEEEEDLF
DIEDGIIYTQDFTVPGQVLEEEVGQNGQSHSLPRAVCVNGTEPQLSSRVRP
EGRPGTANPARRVCSSNRIRRLSACRQQ

B

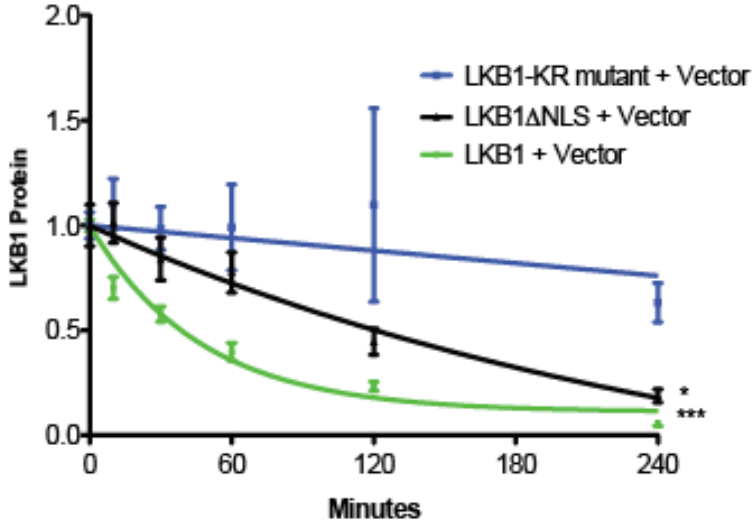


Figure 6.8 - A) Amino acid sequence of LKB1 protein, with all lysines highlighted in yellow (LKB-WT), and with lysines mutated to arginines highlighted in red (LKB1-KR). all but two critical contact point lysines were mutated to non-ubiquitinatable arginines in LKB1-KR. B) Quantification of N ≥ 2 independent LKB1 stability time-courses in HEK cells transfected with variants of epitope-tagged LKB1 protein. LKB1-KR mutant is significantly more stable than LKB1-WT or LKB1dNLS. Repeated measures ANOVA with Dunnett's multiple comparison test using LKB1-KR + empty vector as the control was employed. Error bars are SEM. ***P <0.001, *P <0.05. ANOVA, analysis of variance; SEM, standard error of the mean; NLS, nuclear localization signal.

Figure 6.9

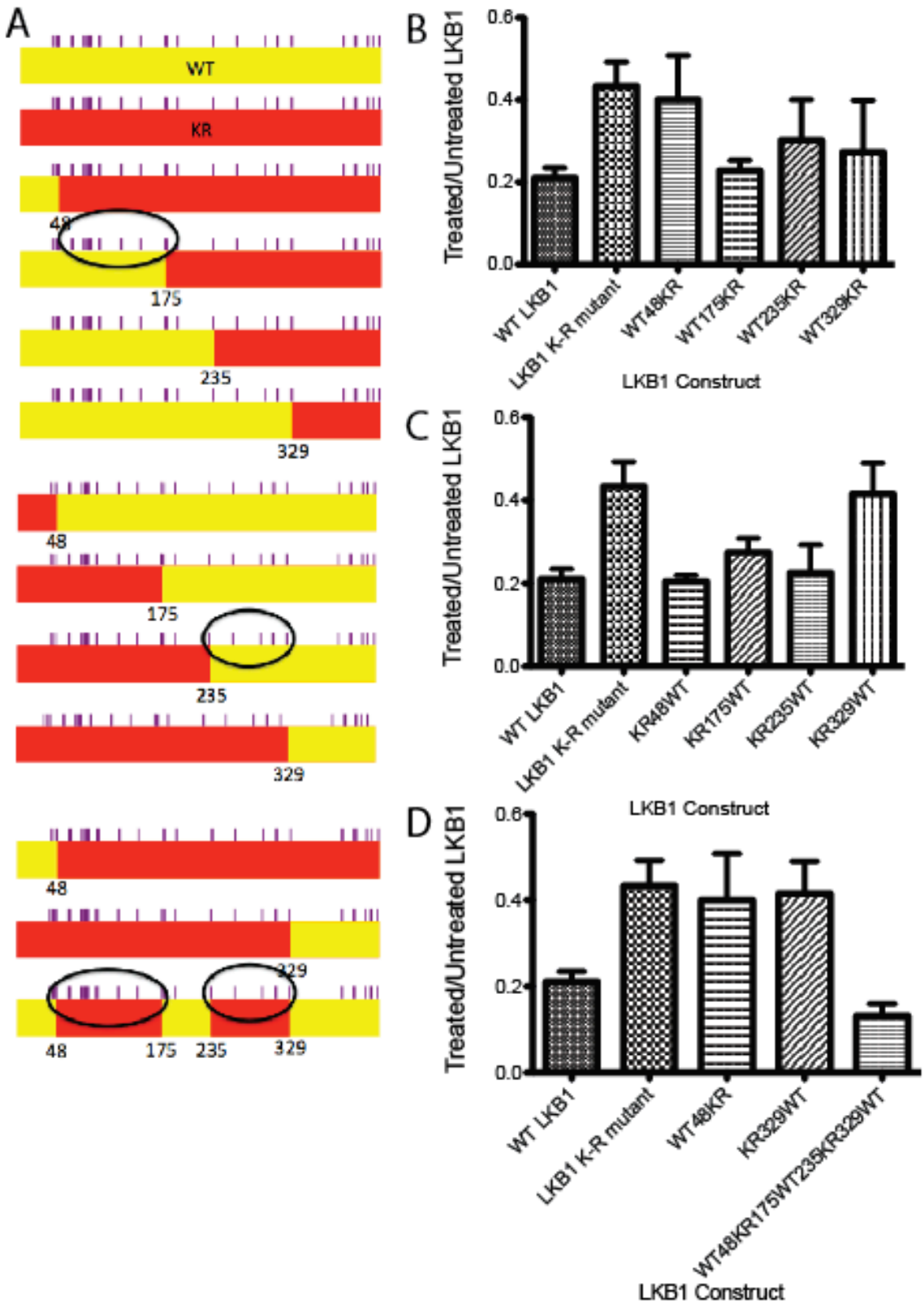

A
WT
KR
48
175
235
329
48
175
235
329
48
329
48
175
235
329
B
Treated/Untreated LKB1
0.6
0.4
0.2
0.0
WT LKB1
LKB1 K-R mutant
WT48KR
WT175KR
WT235KR
WT329KR
LKB1 Construct
C
Treated/Untreated LKB1
0.6
0.4
0.2
0.0
WT LKB1
LKB1 K-R mutant
KR48WT
KR175WT
KR235WT
KR329WT
LKB1 Construct
D
Treated/Untreated LKB1
0.6
0.4
0.2
0.0
WT LKB1
LKB1 K-R mutant
WT48KR
KR329WT
WT48KR175WT235KR329WT
LKB1 Construct

Figure 6.9 - A) Schematics of LKB1 protein with all lysines represented by tick marks. Areas of unmutated lysines are in yellow and areas of lysines mutated to arginines are in red. The only two lysines not mutated are represented in figure 4.7.Graphs represent HEK cells transfected with the construct of interest then treated with 50ug/mL cycloheximide for 4 hours. LKB1 is represented normalized to actin and treated/untreated. B) It appears as though there is a lysine between K48 and K175 imortant for LKB1 stability. C) There may be another lysine between K235 and K329 relevant for LKB1 stability. D) When lysines K48-K175 and K235-K329 were mutated to arginines, this decreased LKB1 stability at 4 hours, perhaps due to altered tertiatry structure or unfolded protein response. Error bars are SEM and N≥ 3 independent experiments per condition.

C. CCM3 can stabilize STRADα and is destabilized by STRADβ.

While we find that STRADα stabilizes LKB1 protein, the question of how STRADα itself is stabilized has remained open. A candidate for this role was Cerebral Cavernous Malformations 3 (CCM3), as it has been shown to interact with Stk25, which cooperates with STRADα during neuronal polarization and Golgi deployment (Matsuki et al., 2010). We show that CCM3 comes in close proximity to LKB1 in HEK293 cells by using the BioID proximity labeling technique ((Roux et al., 2012), Figure 6.10). We find that CCM3 is biotinylated by BirA-LKB1 both in the presence and absence of STRADα, indicating that it could be interacting directly with LKB1, or that endogenous STRADα levels are sufficient to facilitate this interaction. However, STRADα and LKB1 levels in stably transfected CCM3 shRNA HEK293 cells were no different from control shRNA HEK293 cells (Figure 6.11 A), indicating that CCM3 may not control STRADα/LKB1 stability. However, our collaborators find that STRADα upregulates LKB1 in W4 cells, in a CCM3-dependent manner (Howell Lab unpublished).. Our collaborators also find CCM3 levels unchanged in the STRADα constitutive KO cortex relative to WT (Figure 6.11 B), indicating that CCM3 may acutely control STRADα levels, but that chronic depletion of STRADα does not affect CCM3 levels.

Given these findings, we aimed to clarify the relationships between CCM3, LKB1, and STRAD. We find that CCM3 does not significantly stabilize LKB1 protein over baseline levels when the two were overexpressed in HEK293 cells (Figure 6.12). However, CCM3 does significantly stabilize WT-STRADα protein, but not a STRADα mutant that cannot bind LKB1 (STRADαYHF) (Figure 6.13). Interestingly, STRADα-

YHF is present at a higher baseline level (Figure 6.13). These findings indicate that CCM3 might stabilize STRADα via contact with LKB1, perhaps as part of the STRAD/LKB1 complex, and that STRADα that cannot bind LKB1 is inherently more stable. Interestingly, CCM3 does not stabilize STRADβ (Figure 6.13), and STRADβ destabilizes CCM3 protein (Figure 6.14). Since CCM3 stabilizes STRADα, which in turn stabilizes LKB1, this may represent a slow off-switch for LKB1 signaling when STRADβ is present. This could also explain why STRADβ does not stabilize LKB1 as much as does STRADα. These findings make possible a model of STRAD/LKB1 signaling that is self-regulating. In this model, CCM3, when present, stabilizes STRADα, which in turn stabilizes LKB1. When STRADβ is present, it depletes CCM3, causing STRADα to be destabilized by binding LKB1 and by lack of CCM3, and abrogating LKB1 signaling (Chapter 6 Summary Figure). This possibility is especially intriguing when subcellular localization is considered, as STRADα translocates LKB1 from the nucleus to the cytoplasm, where CCM3 and STRADβ can be found and this cycle can run its course.

Figure 6.10

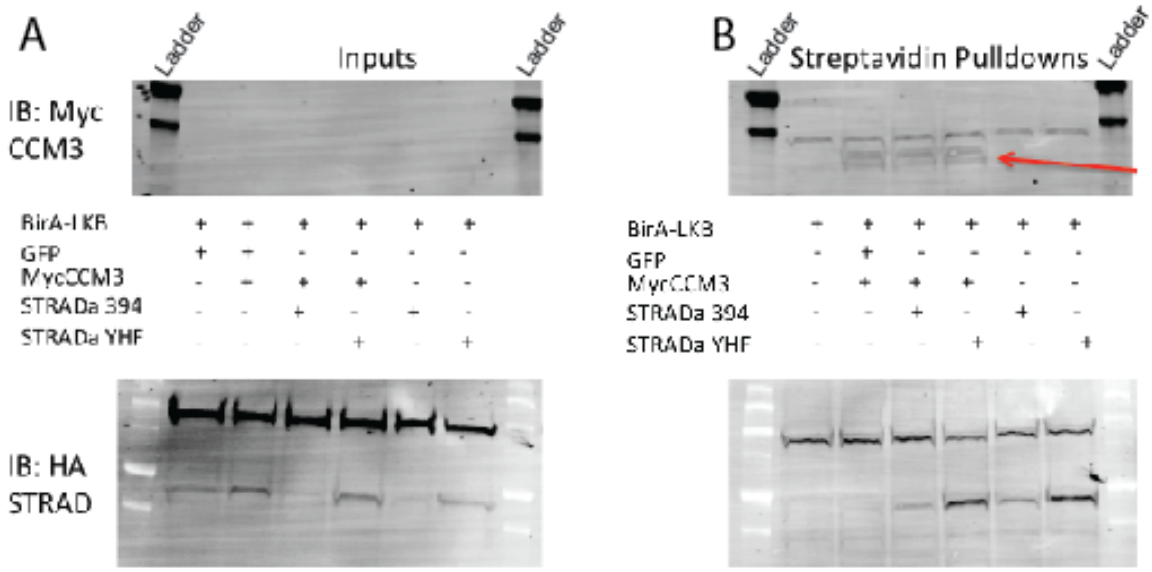


Figure 6.10 - HEK cells were transfected with the above constructs and treated with Biotin for 4 hours, after which lysates were collected and biotinylated proteins were pulled down using streptavidin beads. Myc-tagged CCM3 was seen to be biotinylated, as was STRADα. Whole cell lysates are in A while streptavidin-pulled down lysates are in B.

Figure 6.11

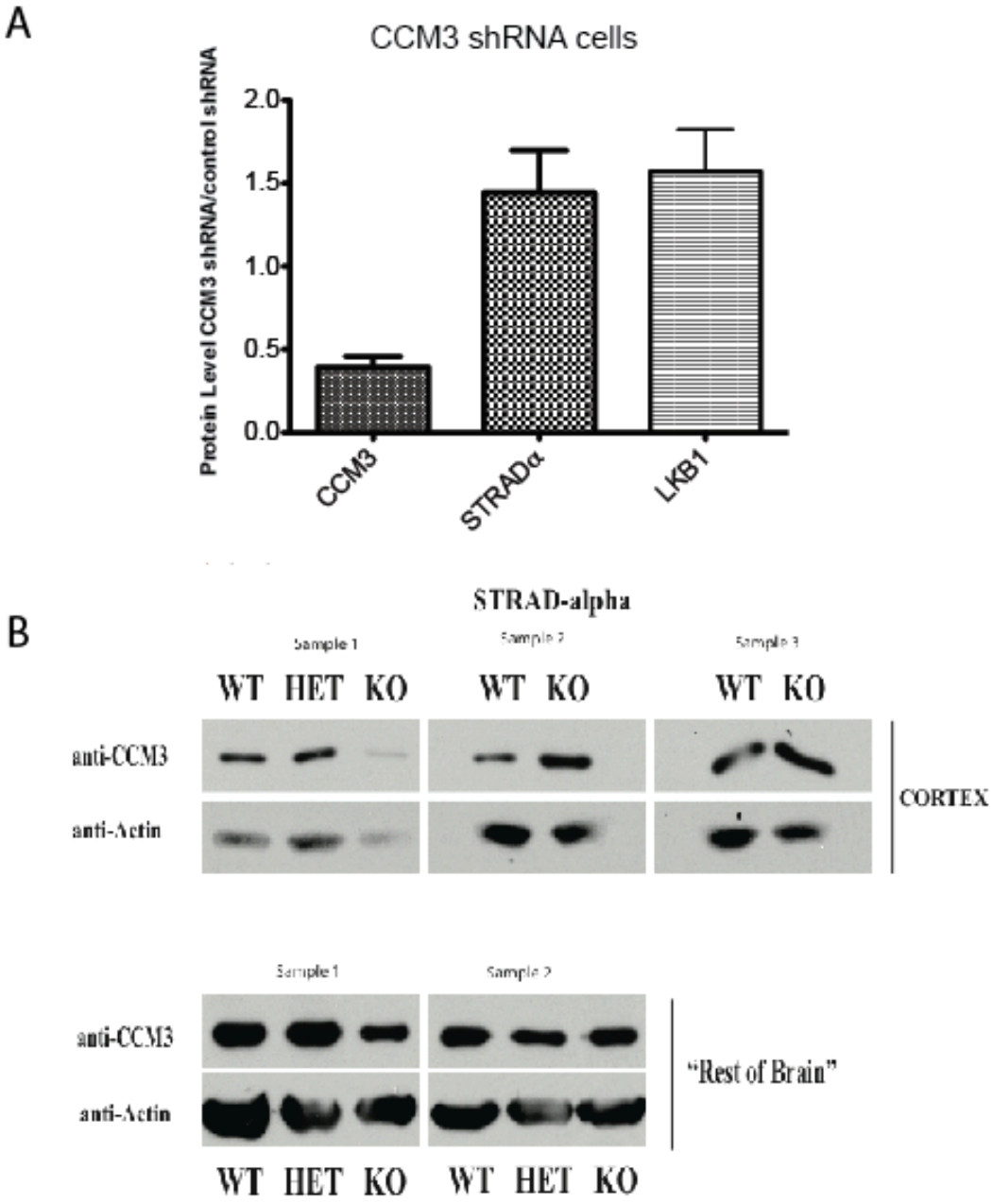


Figure 6.11 - (A) Chronic reduction of CCM3 in HEK cells does not affect LKB1 or STRADα protein level. Ratio of N = 6 independent experiments of HEK cells stably transfected with either CCM3 shRNA or a control shRNA. (B) CCM3 protein is not affected by constitutive deletion of STRADα in cortex or rest of brain at E16.5. Values were determined by densitometry and normalized to actin. Experiment was carried out by Zainab Mansaray in Dr. Brian Howell's lab at SUNY Upstate.

Figure 6.12

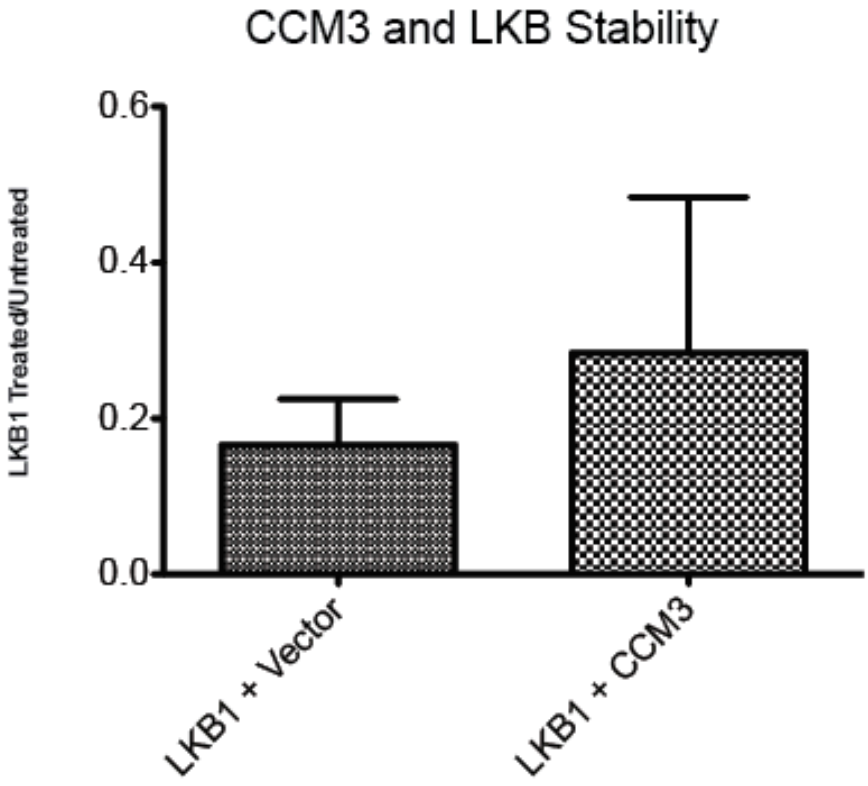


Figure 6.12 - LKB1 is not significantly stabilized by CCM3. LKB1 was transfected with empty vector or with a vector expressing CCM3. Cells were treated with cycloheximide for 4 hours the next day, and ratios are represented as treated/untreated LKB1 levels. Protein levels were determined by Western blot densitometry and normalized to actin for equal loading. Significance was determined using Student's unpaired two-tailed t-test.

Figure 6.13

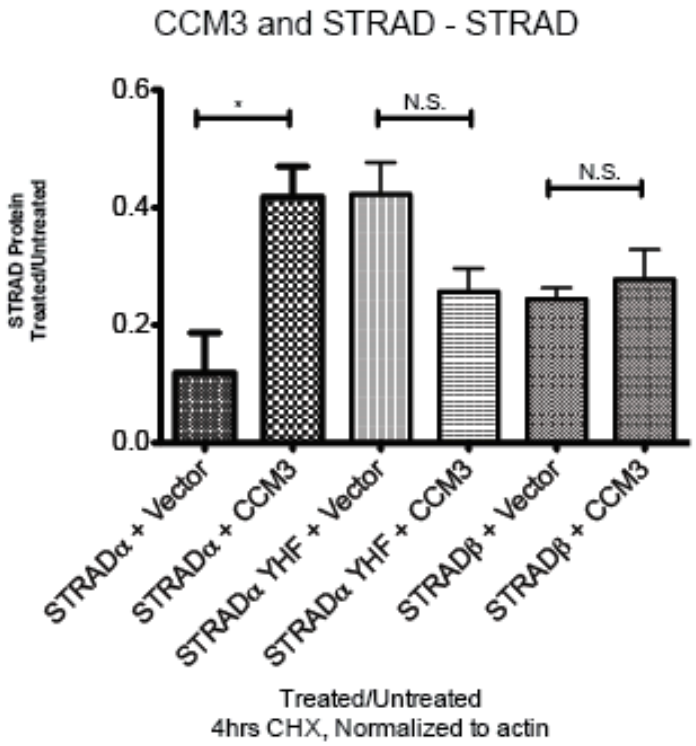


Figure 6.13 - Quantification of N ≥4 independent STRAD stability experiments in HEK cells transfected with epitope-tagged STRADα protein following cycloheximide-mediated translation inhibition. STRAD variants were cotransfected with either empty vector or CCM3, and stability was assayed after 4 hours of 50 µg/ml cycloheximide-mediated translation inhibition. Values are represented as ratio of treated/untreated STRAD protein, normalized to actin. CCM3 significantly stabilizes STRADα, but not STRADα YHF or STRADβ. Statistical significance was determined using one-way ANOVA with Bonferroni's multiple comparisons test (column 1 vs 2, 3 vs 4, 5 vs 6).

Figure 6.14

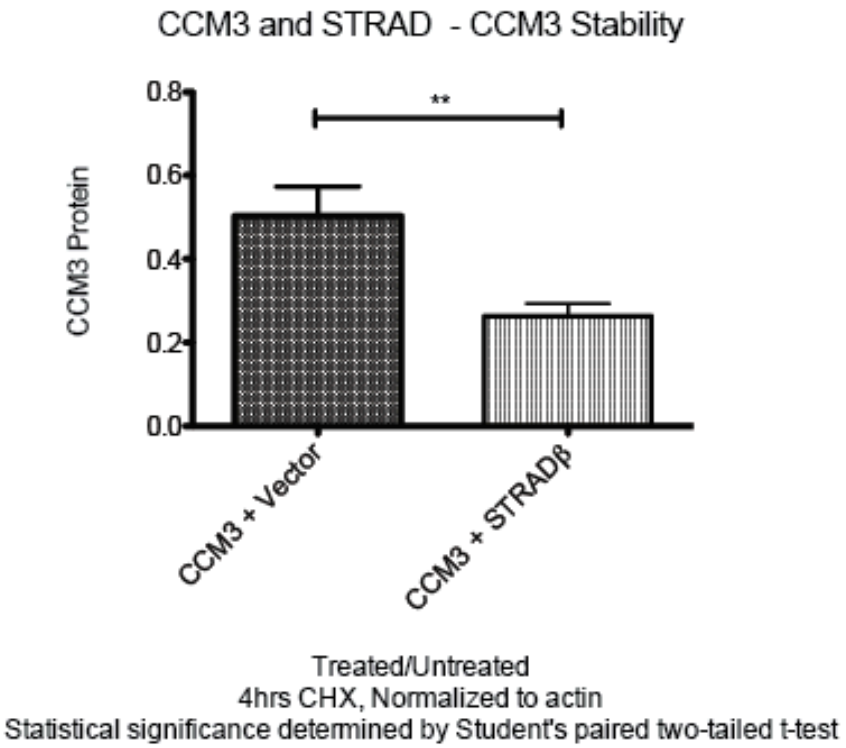


Figure 6.14 - Quantification of N ≥4 independent CCM3 stability experiments in HEK cells transfected with epitope-tagged CCM3 protein following cycloheximide-mediated translation inhibition. CCM3 was cotransfected with either empty vector or STRADβ, and stability was assayed after 4 hours of 50 µg/ml cycloheximide-mediated translation inhibition. Values are represented as ratio of treated/untreated CCM3 protein, normalized to actin. STRADβ significantly destabilizes CCM3. Statistical significance was determined using Student's paired two-tailed t-test. **P<0.01

III. Discussion

Our findings that STRADα can stabilize LKB1 in multiple cellular contexts imply that this mechanism is critical in controlling LKB1 activity and subcellular localization. The fact that this stabilization is STRADα/LKB1 contact-dependent further strengthens this assertion, and implies a direct mechanism of stabilization. While LKB1 mRNA is up-regulated in the STRADα KO, protein levels are still decreased, indicating a translational or posttranslational mechanism of stabilization, and our cycloheximide experiments indicate the latter is the case. The roles of the multiple splice variants of STRADα remain unclear, as each variant stabilizes LKB1 to an equal extent. LKB1 transcription has been found to be increased by FOXO3 and decreased by estrogen receptor α (ERα) (Linher-Melville et al., 2012; Lützner et al., 2012). Given that STRADα deletion causes increased LKB1 mRNA levels, it is possible that STRADα also interacts with these or other transcription factors.

LKB1 catalytic activity is enhanced by the STRAD proteins and by MO25 (Baas et al., 2003; Boudeau et al., 2003a; Hawley et al., 2003; Zeqiraj et al., 2009a). STRADα also stabilizes LKB1 protein *in vivo*, as well as *in vitro*, while STRADβ is unable to affect LKB1 protein level on its own, and requires MO25 to activate LKB1 to the same extent as does STRADα (Baas et al., 2003). Others have shown that STRADα and Hsp90/Hsc70 compete for LKB1 binding, and both stabilize the protein, but have opposite effects on its activity (Boudeau et al., 2003b; Gaude et al., 2012). It is possible that Hsp90/Hsc70 and the STRAD proteins cooperate to modulate LKB1 activity while keeping the protein level constant. Our finding that STRADα more robustly stabilizes

LKB1 than does STRADβ implies that LKB1 is degraded more quickly in the nucleus. However, the fact that LKB1 that has never been to the nucleus (LKB1dNLS) is as stable as LKB1 that has been exported from the nucleus (LKB1 + STRADα) raises the question of mechanism of LKB1 degradation. Further work should address the mechanism that makes STRADα a superior stabilizer, and should map the life cycle of LKB1 protein within the cell.

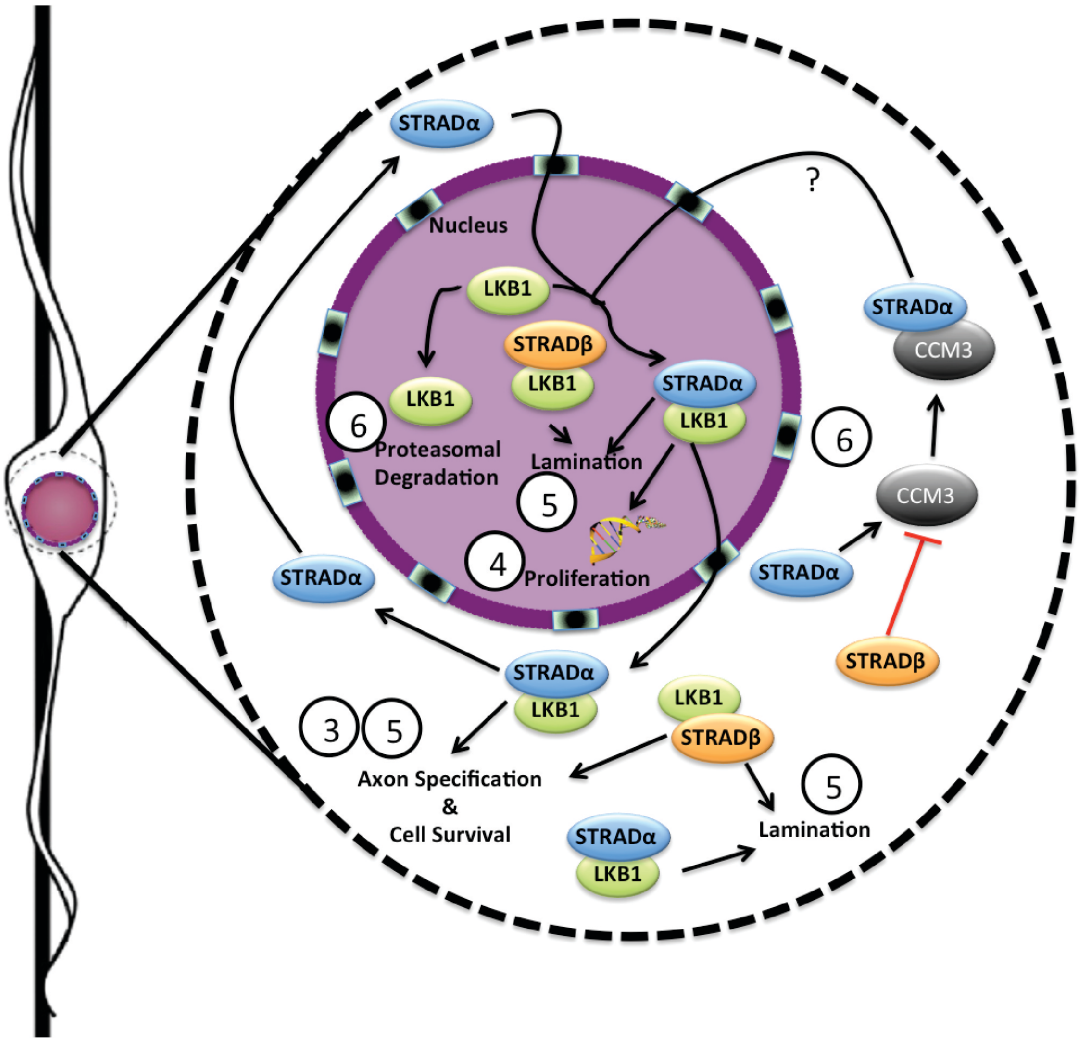
While we were unable to pinpoint the lysine(s) on LKB1 responsible for driving degradation, but our results point to two main stretches of lysines that may take part in this process. A more thorough approach to determining the degradation mechanism of LKB1 would include BioID mass spectrometry experiments, subcellular fractionation stability experiments, immunoprecipitation of various KR mutants with STRADs and other known players (such as Hsp70/90), and more detailed timecourses of KR degradation. In particular, these experiments could be useful in identifying the ubiquitin ligases driving LKB1 degradation, and could potentially identify a druggable target.

While LKB1 is expressed in almost all cell types in the body throughout development, its interactors and stabilizer proteins may be more tissue-constrained, thus conferring another level of control over LKB1 that is cell-type and developmental timepoint specific. In this aspect, CCM3 is an interesting candidate. It is expressed in many cell types, and appears to have varying functions regarding LKB1 within each cell type surveyed. In W4 cells, CCM3 is necessary to stabilize LKB1 upon activation of STRADα (Zainab Mansaray and Dr. Brian Howell, unpublished), while in HEK293 cells, LKB1 is not significantly stabilized by overexpression of CCM3. The ability of CCM3

to stabilize STRADα, but not STRADβ, could be another way to regulate LKB1 levels within the cells that these 4 proteins are expressed. Given that STRADβ mRNA levels increase in the cortical plate, while STRADα and LKB1 mRNA are particularly high in progenitors, it is plausible that CCM3 is playing a role in down-regulating LKB1 as cells mature and more STRADβ is present, which destabilizes CCM3. There are two more CCM proteins, and their relationships with STRAD and LKB1 have yet to be tested. This could act as a slow off-switch for LKB1 signaling, as neurons transition from migrating cells to mature cortical plate cells and their LKB1 needs change. Additionally, total LKB1 levels increase over cortical development and STRADα is required for this to occur (Figure 5.8), supporting the possibility that STRADβ could destabilize LKB1. From our data, it is plausible that the other CCM family members could compensate for CCM3, given enough time. Together, these findings imply that CCM3 can control LKB1 levels acutely, but that there may be compensatory mechanisms upon chronic CCM3 deprivation. Experiments to test this assertion would be useful, as would further work delineating the subcellular localization of the CCM3/(STRADα or STRADβ)/LKB1 complexes. Given the importance of CCM3 in early cell polarity, and specifically in orienting the Golgi apparatus, this timing of subcellular localization could be particularly informative (Matsuki et al., 2010).

LKB1 regulation is clearly tightly regulated in development and throughout life, so understanding these mechanisms will be critical for any potential therapies. I have discussed potential modulators of LKB1 activity and stability, and future experiments into understanding the details and time courses of this regulation will be necessary.

Chapter 6 Summary Figure



Chapter 6 Summary Figure. Schematic of STRAD/LKB1 signaling within the developing neuron. (3) STRADα and STRADβ both complex with LKB1 and are capable of eliciting axogenesis in primary cultured neurons. Only STRADα can export LKB1 from the nucleus. (4) STRADα may help regulate proliferation with LKB1. (5) STRADα and STRADβ can each participate in cortical lamination with LKB1. (6) LKB1 appears to be targeted for degradation within the nucleus, and STRAD association prevents this. CCM3 could act as a modulator of LKB1 activity via its differential effects on the STRAD proteins.

Chapter Seven: Summary of Results and Discussion

I. Summary of Results

LKB1/Par4 is indispensible in many aspects of embryonic development and in axogenesis in particular, and the STRAD proteins are necessary to activate LKB1 to effect polarity (Baas et al., 2003; Barnes and Polleux, 2009; Boudeau et al., 2004; Brajenovic et al., 2004; Shelly and Poo, 2011; Zeqiraj et al., 2009a). In this work, I further the field by investigating the *in vivo* expression patterns and establishing roles of each STRAD paralog in axogenesis and LKB1 stability *in vivo*. We find the STRAD proteins are highly spliced, with tissue-restricted and developmentally regulated splicing patterns. Splicing is particularly robust for STRADα splicing, and we are the first to report STRADα-1 as a neuron-specific splice variant. Most previous studies have used STRADα-1 in cell lines (Baas et al., 2003; 2004a; Dorfman and Macara, 2008; Shelly et al., 2007), but it was not known that this form is neuron-specific *in vivo*. All splice variants of STRADα and STRADβ were capable of eliciting multiple axons in our assays when expressed with LKB1. However, it remains to be determined what differing functions the splice variants may have.

Our findings indicate that STRADα is the primal STRAD paralog consistent with the fact that whole body deletion of STRADα is lethal, and in humans mutation, and presumed loss of function, of STRADα causes PMSE syndrome, whereas deletion of STRADβ has no overt phenotype. Additionally, whole body deletion of STRADα did not affect downstream signaling pathways in our studies. Interestingly, when we deleted STRADα$^{fl/fl}$ alleles using Emx1^{cre}, whose expression begins at E9.5, we frequently observed cortical heterotopias. The route of deletion of STRADα may also affect proliferation or migration within the cortex, which could explain the heterotopias. This

observation differs from the whole body deletion and the Nestin-cre mediated deletion. Perhaps this difference results from the differential timing and penetrance of STRADα deletion in these three mouse lines. It is possible that deleting STRADα either very early (constitutive null mouse) or relatively later (STRADα-Nestin Cre mouse) allows a window for STRADβ or some other pathway to compensate.

Deletion of both STRAD paralogs within the brain largely phenocopies LKB1 deletion in the brain, confirming that STRADα and STRADβ are indeed redundant for axogenesis. However, loss of both STRADs did not phenocopy loss of LKB1 in terms of the expression pattern of transcription factor cTip2^{+} cells, indicating that LKB1 either has an as yet unidentified nuclear function for which a STRAD is necessary or may be inactive and mislocalized leading to perturbations in neuronal specification or migration.

Although STRADα and STRADβ are redundant in many ways, we found that only STRADα has the capability to protect LKB1 from proteasomal degradation *in vivo*. Our evidence in HEK cells indicates that this disparity between STRAD proteins could be partially due to the ability of STRADα to export LKB1 from the nucleus. Future work should expand upon the different abilities of STRADα and STRADβ to stabilize LKB1, and to outline the lifecycle of LKB1 within subcellular compartments.

Much work examined the relationship of LKB1 and the STRADs, but it is unclear how the STRAD proteins are regulated. We found a relationship between the STRAD proteins and CCM3, another GCKIII family kinase. CCM3 stabilizes STRADα whereas STRADβ destabilizes CCM3. This finding hints at the complex interplay between LKB1 and each STRAD protein, and at the highly nuanced signaling made possible by tightly

controlled STRADα and STRADβ levels. Our results are the first report of STRAD stability control by a protein other than LKB1.

II. Discussion

The integral relationship between the two STRAD pseudokinases and their partner kinase is evidenced by the fact that all species bearing an LKB1 locus also contain at least one STRAD gene. Here, we provide evidence that STRADα is likely the phylogenetic ancestor of the STRAD paralogs in vertebrates, with the STRAD duplication event potentially providing selective advantage via genomic redundancy for this protein family. Furthermore, the high degree of amino-terminal splicing of STRADα hints at more cell type-specialized functions yet to be discovered.

Because PMSE patients survive into adolescence whereas our knockout mice die at birth, it is possible that human STRADβ has expanded functionality relative to mouse STRADβ, and can better compensate for STRADα. Another possibility is that the PMSE-truncated STRADα gene retains some basic functionality, which remains to be tested. The truncated protein could be produced, bind LKB1, and the amino-terminal NES could affect export. It is likely that a truncated STRADα/LKB1 complex would be highly unstable, as STRADα would retain little MO25 binding capability (Boudeau et al., 2003a; Zeqiraj et al., 2009b) and the truncated protein would likely be targeted for degradation. Further experiments to test this possibility could be carried out on human fibroblasts from PMSE patients. Specifically, it would be important to determine the protein levels of LKB1 in patient tissue relative to controls, and to ascertain the extent to which each STRAD protein is produced in these cells. If LKB1 destabilization is the

primary cause of PMSE Syndrome, alternative routes of LKB1 stabilization such as pharmacologic inhibition of specific ubiquitin ligases could be an avenue for treatment for these patients. Given our findings that loss of STRADα causes an increase in LKB1 mRNA, it is plausible that in human patients increased LKB1 protein could interact with STRADβ to accomplish many necessary functions.

It is not clear why STRADα constitutive null mice die perinatally given that normal Mendelian ratios exist essentially until term. It is likely that the perinatal lethality is due to a respiratory defect, cardiac or a feeding issue, as is often the case in perinatal lethality. This question could be determined by observing feeding, heart rhythms, and respiration patterns just after birth. Because we did not find any newly deceased pups, it is likely that they expired quickly after birth, and were eaten by the dam, implying a cardiorespiratory defect. If this is the case, there are three possibilities – 1) central respiratory dysregulation, 2) an inability of the diaphragm to effectively contract, or 3) compromised cardiac function. One could determine which of these scenarios is more likely by observing electrical activity in the brainstem respiratory control region of STRADα null mice, by watching diaphragm contraction, and by performing perinatal EKG. Theoretically, the brainstem circuitry could be examined at E18.5, as this connectivity should already exist (Takashima and Becker, 1986; Turgeon and Meloche, 2009). In any case, the fact that STRADβ null pups survive into adulthood highlights the fact that STRADα and STRADβ are not entirely redundant.

A. Splicing

Our data reveal a complex expression pattern of STRAD proteins in both

developing and adult tissues and identify a new form of STRADα (STRADα-7) not previously reported. We also establish that the STRADα-1 splice form is restricted to brain, skeletal muscle and testis in the mouse, and that within brain, it is uniquely expressed in neurons. Most intriguing is our discovery that a second nuclear export sequence within the amino-terminus of STRADα, which has been previously demonstrated to be functional for LKB1 export (Dorfman and Macara, 2008), is developmentally regulated by splicing in brain. Although the STRADα carboxy-terminal export signal contains a phosphorylation site targeted by LKB1(Baas et al., 2003), this N-terminal NES lacks any known post-translational modification, and may represent a less regulated form of STRADα nuclear export. This second NES may also represent a need for rapid shuttling of LKB1 from the nucleus in neurons. As this form increases in expression across neuronal developmental time, perhaps its function is to abrogate LKB1 nuclear functions and keep neurons post-mitotic. This is especially intriguing in light of the cTIP2 findings, which imply a STRAD-regulated nuclear role for LKB1. It is possible that full-length STRADα is needed to recruit LKB1 out of the nucleus to aid in axonal branching (Courchet et al., 2013). This idea could be tested with overexpression of each STRADα splice variant in STRADα/STRADβ null neurons. Each epitope-tagged splice variant in a GFP-expressing vector could be electroporated *in utero*, and brains examined postnatally to determine the capability of each splice variant to rescue axogenesis, and splice-variant specfic effects on gross neuronal morphology. For more detailed information about axonal branching, dendritic arborization, and synaptogenesis, *ex vivo* electroporation could be used, and cortical neurons could be cultured for 10-14 days *in vitro*. These experiments would address the cell-autonomy of STRAD-induced rescue

and the varying functions of each splice variant. To determine the optimal age at which brains should be electroporated, it would be useful to determine the distribution of each STRAD splice variant in the cortex across developmental time. Because STRADα splice variants only differ by small stretches of amino acids, one could use locked nucleic acids (LNA's) as *in situ* probes (Kumar et al., 1998). This system ensures a much greater binding specificity for short probes, and would allow analysis of each exon present within the tissue at any given time. Additionally, one could use RNA-Seq to detail the STRAD splice variants present at each developmental timepoint.

Furthermore, highly developmentally- and tissue- regulated splicing might be suggestive of unique LKB1 and STRADα functions in distinct tissues. If these nuances could be understood, it could lead to a system that can be manipulated in development, cancer, and neurodegeneration. One way to gain a deeper understanding of the STRAD splice forms' binding partners would be to use the Bio-ID (BirA*) approach previously described (Roux et al., 2012). One could express each BirA*-STRAD splice variant alone or in combination with LKB1, and then perform mass spectrometry on the pool of proteins pulled down by streptavidin. This approach would yield a list of candidate proteins that are differentially regulated by the various splice isoforms. These candidate proteins could serve as a starting point to understand the complex cell-type and developmental regulation of LKB1.

It is important to note that previous studies evaluating STRADα function biochemically and cell biologically often used the STRADα-1 form. Our results indicate that additional studies may be required to clarify the cellular context and specific roles of

STRADα depending on which isoforms are normally expressed in a given cell type or tissue. It is possible that amino- terminal variants of STRADα may affect recruitment of additional components to STRAD: LKB1 signaling complexes or result in alteration in localization or allosteric activation of LKB1, as previously suggested (Marignani et al., 2007).

Splicing is necessary for normal brain development, and the splicing of many genes changes across development (Dillman et al., 2013). Many genes that are most highly expressed in embryonic brain affect mitosis, M-phase, and general chromosomal organization, whereas adult brain generally exhibit high levels of genes involved in synaptic transmission and energy homeostasis (Dillman et al., 2013). Given this information, it is likely that STRADβ and STRADα-1 are involved in synaptic maintenance whereas the other STRADα isoforms are more involved in proliferation and mitotic processes. This would be an interesting hypothesis to test, and experiments to this end could include splice-form specific viral knockdown of STRAD variants in mouse brains of different ages. Behavioral experiments could be carried out to determine whether STRAD splice variants affect memory maintenance via synaptic stabilization. Interestingly, regulation of splicing and regulation of gene expression seem to be independent, suggesting, in the case of the STRADs, a layer of redundancy in their regulation (Dillman et al., 2013).

B. Axogenesis and cell polarity

Our work and that of others demonstrates LKB1 is a requisite component of the transduction machinery underlying axon formation (Barnes et al., 2007; Shelly et al.,

2007) and other cell polarity hallmarks (Jansen et al., 2009; Mirouse and Billaud, 2011). Acute knockdown of STRADα with small hairpin RNAs (shRNAs) leads to dysregulated mTOR signaling in the CNS (Orlova et al., 2010; Parker et al., 2013), supporting the idea that STRADα is also an important regulator of nervous system function. Biochemical characterization of the LKB1:STRAD complex indicates that its formation is required for allosteric activation of LKB1 (Baas et al., 2003; Filippi et al., 2011; Zeqiraj et al., 2009a). Previous neuronal loss-of-function studies have not distinguished between a requirement of LKB1 protein expression and kinase activity, but our *in vivo* data demonstrate that without these critical activator proteins, LKB1 is unable to elicit axon specification (Chapter 5). We demonstrate that either of the STRAD isoforms is sufficient to drive axogenesis, and report the first *in vivo* physiological contribution of STRADβ. Furthermore, we show that even the truncated form of STRADβ can drive axogenesis with LKB1 when overexpressed. This result indicates a high level of redundancy between the two genes, and supports the assertion that the STRAD kinase domain is not required for LKB1-dependent axogenesis (Boudeau et al., 2004).

Given that Nestin-cre-mediated deletion of STRADα or LKB1 does not affect cortical axon formation or lamination, but that Emx1-cre-mediated recombination causes mild cortical malformation, including misplaced cTIP2 positive-cells, it is possible that STRADβ can compensate for loss of STRADα, but only in certain developmental windows. The other possibility is that neither of the STRADs or LKB1 is necessary for brain morphogenesis beyond a certain developmental point. However, this is not likely, as LKB1 loss after axogenesis, mediated by Nex-cre, alters the branching of cortical axons (Courchet et al., 2013). This result could be a timing issue, or could indicate that

LKB1 activity is required for more subtle aspects of neuronal morphology. It is possible that high levels of LKB1 activity are required to set up the initial axon tracts in the cortex, and that later-born neurons can use cell contact cues to extend axons, or that cell-cell contact can help drive axogenesis, as seems to be the case in a proportion of STRADα$^{-/-}$; STRADβ$^{fl/fl}$; Emx1^{cre+} dissociated neurons (Chapter 5). It would be informative to know which cell surface molecules can mediate axogenesis in the absence of LKB1 signaling. This could be determined by isolating axons during development in culture using the Twiss preparation from wild-type and LKB1, STRADα, STRADβ and STRADα/β knockout neurons (Willis and Twiss, 2011), and then using mass spectrometry to isolate proteins of interest. Because STRADα-null cortices have axons regardless of mode of deletion of STRADα, STRADβ is sufficient to drive axogenesis at all developmental timepoints. This means that partner proteins for the STRADβ/LKB1 complex overlap significantly with that of the STRADα/LKB1 complex. It also implies that LKB1 nuclear functions could be at least partially responsible for axogenesis, as STRADβ cannot export LKB1 from the nucleus. This makes finding unique partners even more interesting and potentially informative.

C. Cell Survival

The increased apoptosis we observed following simultaneous inactivation of both STRAD genes provides another parallel with the LKB1 cKO (Barnes et al., 2007) and suggests that allosteric activation of LKB1 plays a significant role in cell survival. This apoptosis likely results from failed axogenesis and subsequent loss of trophic support as indicated by studies using conditional LKB1 mice and the post-mitotic cre recombinase regulated by the NEX (NeuroD6/MATH2) promoter (Barnes et al., 2007; Courchet et al.,

2013). Whether compromised axogenesis is the sole driver triggering this cell death remains unclear, but it is possible that additional survival pathways are affected by LKB1 inactivation or STRADα/β loss. We found some increased apoptosis at the midline in STRADα/STRADβ double heterozygous brains, but we did not determine the origins or types of these dying cells. Future studies exploring the nature and extent of this cell death, as well as the timing of its induction relative to when cortical axons encounter their intermediate and final targets will be useful. DiI labeling at various developmental stages to this end would be informative. CCM3 and Stk25 interact to regulate apoptosis (Zhang et al., 2012) and it is possible that this occurs through STRADα and STRADβ. This is an especially interesting possibility given the dichotomous effects of STRADα and STRADβ on CCM3 protein levels. It will be important to determine the molecular underpinnings of this cell death, as it could also be informative for neurodegenerative diseases involving demyelination and subsequent axon loss. Understanding the molecular pathways could yield a therapeutic target to arrest this apoptosis and widen a treatment window.

D. LKB1 in the Nucleus

The altered lamination we observed speaks to a nuclear role for LKB1 because each STRAD knockout line and the LKB1 knockout cortex have no known lamination issues ((Barnes et al., 2007), Figure 5.3). This finding implies that LKB1 has some nuclear role for which a STRAD protein is necessary. It is likely that a STRAD is required to sequester LKB1 from its nuclear role. The definitive proof of this would be to create a STRADα/STRADβ/LKB1 triple conditional knockout to survey the lamination in the cortex. If the lamination phenotype is rescued by deleting all three

proteins, this would bolster the idea that LKB1 nuclear functions are critical for lamination. Another piece of evidence for this line of thinking would be if LKB1ΔNLS failed to rescue this triple mutant lamination defect. Previous work has shown that LKB1 associates with LMO4, GATA6, and ERα, indicating a role for LKB1 in cell cycle arrest (Linher-Melville et al., 2012; Setogawa et al., 2006; Tiainen et al., 2002). LKB1 has also been associated with the centrosome, and has been shown to play a role in neuronal migration (Asada and Sanada, 2010; Asada et al., 2007). Perhaps LKB1 association with these nuclear factors drives cell fate decisions and accelerates cell cycle, and its nuclear localization causes mis-migration or confused neuronal identity. It is unknown whether these mislocalized cells form the correct neural networks, or whether their mislocalization disrupts this process. One could ask this question using retrograde labeling with rabies virus (Morcuende et al., 2002). Further studies should explore the timing of LKB1 and the STRAD proteins' roles in cell fate/proliferation/migration decisions using various Cre recombinase mouse lines that target a diversity of cell types across developmental times. This data would dovetail nicely with the systematic *in situ* hybridization approach previously outlined.

The cell autonomy of LKB1 nuclear roles has yet to be determined. One way to approach this question would be using the MADM approach described in Chapter 5. One could cross these lines with the STRADβ conditional line and an $Emx1^{cre}$ mouse line, thus generating STRADα/STRADβ dbKO cells in a STRADα heterozygous, STRADβ null background. The preliminary results from the STRADα MADM Ch11 mice hint at cell-autonomous effects on proliferation following loss of STRADα. It will be important to determine the extent to which total loss of LKB1 activity via deletion of both STRAD

proteins within a proliferating neuron might affect its migration and/or identity. It will also be possible to determine the cell autonomy of these processes using this double knockout MADM-Ch11 mouse model.

E. Stability of LKB1 and STRAD

It appears as though one of the main ways that STRAD proteins regulate LKB1 is by modulating protein level, as well as its subcellular localization. Another protein that stabilizes LKB1 is Hsp90, but it appears that both proteins cannot be bound to LKB1 at once (Boudeau et al., 2003b; Gaude et al., 2012; Nony et al., 2003). This may represent another form of LKB1 activity regulation, and could be subcellular compartment specific. We were unable to determine the lysine(s) on LKB1 important for its degradation, but future work should focus on finding both this/these lysine(s) and the ubiquitin ligase that targets it/them. These results would have much wider implications if they yield a druggable target, because LKB1 is a tumor suppressor, and stabilizing it selectively could have therapeutic effects in LKB1 pathway mutant cancers. It could also be helpful in PMSE Syndrome, as STRADβ could plausibly compensate for the other functions of STRADα if LKB1 could be stabilized in some other way.

A big unanswered question is how the mRNA levels of each of these genes might impact each other and themselves. There is clearly some possible compensation in the STRADα knockout mouse, as LKB1 mRNA levels increase in these mutants. However, the significance of this finding has yet to be determined, and it is unknown how other related mRNA levels might be changing. Future work should address this question, as it may provide clues to the relevant developmental pathways impacted by the STRADs and

LKB1.

II. Future Directions

The main implication of our work is that the STRAD proteins have redundancy terms of LKB1 regulation. Although STRADα seems to be the primary STRAD paralog, STRADβ has the capability of driving LKB1 in axogenesis and cell survival during development. Many open questions remain regarding the roles of STRADα and STRADβ relative to each other, especially the mechanism by which STRADα stabilizes LKB1. It is likely that this is a multifaceted regulation, as LKB1 mRNA increases in the STRADα knockout brain, but this is not sufficient to keep LKB1 levels up. Also, STRADβ is capable of stabilizing LKB1 to a degree when overexpressed in HEK cells, arguing for some redundancy in this role. It will be important to determine whether the ability of STRADα to export LKB1 from the nucleus is its main stabilizing characteristic, or if there is more to it than subcellular localization. One way to accomplish this would be to generate a LKB1ΔNLS knock-in mouse and to breed it with each of the STRADα and STRADβ floxed lines. If indeed subcellular localization is the key determinant of LKB1 stability, one would expect a LKB1ΔNLS; STRADα$^{fl/fl}$; Cre^{+} mouse to have normal LKB1 levels, and to resemble a wild-type mouse brain. This mouse line would speak to the nuclear functions of LKB1, as well as the sufficiency of STRADβ in cortical development. Along the lines of LKB1 stability, future work should focus on finding the lysine on LKB1 necessary and sufficient for proteasomal targeting. This could be done by continuing the work of LKB1 KR mutant mapping discussed herein. Efforts should also be made to find the E3 Ubiquitin ligase that targets LKB1 for degradation. One could

take a candidate approach to this, but that would likely be very expensive and time consuming. Alternatively, mass spectrometry could be used in conjunction with BirA* labeling of LKB1/STRADα/STRADβ interactors, and these data sets could be compared with each other to find unique partners, which could then be tested for this function.

Assuming that LKB1ΔNLS; STRADα$^{fl/fl}$; STRADβ$^{fl/fl}$; Cre^{+} mice lack axons various STRAD splice variants could *in utero* electroporated to test sufficiency in axogenesis without the confound of reduced LKB1 protein in double knockout brains. These experiments should also be done in the extant STRADα/STRADβ cKO mouse lines. It would be necessary to generate neuronal cultures from these experiments in order to carefully analyze each splice variant's effects on neurite length, neuronal morphology, axonal/dendritic branching, and potentially, connectivity. Additionally, crossing these lines with Cre driver lines targeting different cell types and at various ages will be very informative of the timing of STRAD necessity. Our findings from Chapter 5 indicate that the timing issue may be critical in terms of STRADα expression.

Future work should focus on outlining the pathways, both upstream and downstream, affected by STRADα and STRADβ. Specifically, it will be important to determine how the STRADs are regulated, and future experiments should expand on our preliminary finding that CCM3 differentially regulates STRAD stability. It will also be important to determine the extent to which pathways downstream of STRADα/STRADβ/LKB1 are affected by loss of each of these proteins. RNA-seq studies of each of these mutant mouse lines would be very informative and could uncover novel interactions between pathways.

Many of the STRAD studies thus far have only used the ubiquitous form of STRADα, and we are the first to show *in vivo* redundancy between all splice variants of STRADα and STRADβ. It will be important to determine the extent of this redundancy, including the timing of these effects. The finding that STRADα deletion affects corticogenesis only following deletion by Emx1-Cre and not constitutive deletion or Nestin-Cre mediated deletion points to a more complex regulation scheme, and implies that there are gradations of redundancy between these two genes. Future work should be aimed at outlining STRADα and STRADβ expression/splicing patterns at all stages of development/differentiation, especially within the brain, as this could give clues about the intractable epilepsy seen in PMSE patients. Elucidating this nuanced redundancy could be especially informative for patients suffering mutations in the STRAD/LKB1 pathways, as understanding STRAD splice variant functions could allow fine-tuning of LKB1 activity or localization to overcome patients' symptoms.

III. Concluding Remarks

LKB1 is a critical regulator of neuronal polarity and axogenesis, yet its nuanced regulation by the STRAD proteins had remained largely unexplored. Here we provide evidence of the evolutionary primacy of STRADα. We also demonstrate that messenger RNAs for the STRAD paralogs undergo substantial tissue-restricted and cell-type specific splicing. We show that either STRADα or STRADβ are necessary and sufficient for axon formation in the developing cortex, the first report of such functional redundancy between STRADα and STRADβ. We also found that multiple splice forms of each STRAD are each capable of effecting axogenesis with LKB1. Loss of STRADα has severe consequences for the developing organism, and that its timing of expression is

critical. Our results highlight a window from E9.5 until about E12.5 during which STRADα is absolutely required for cortical lamination and proliferation, although STRADβ can compensate for its other functions. Most importantly, we find that only STRADα can significantly stabilize LKB1 protein *in vivo* and that this stabilizing effect is required for up-regulation of LKB1 expression during brain development and maturation. LKB1 stabilizes STRADα *in vivo* as well, indicating a reciprocal stabilizing relationship. The normal axogenesis we observe in the absence of STRADα indicates that low levels of LKB1 are sufficient to provide the necessary signaling to permit axon specification in the developing cortex. In contrast, the perinatal lethality of these STRADα-null mice indicates a stronger sensitivity to LKB1 expression levels in other organ systems. Taken together, our data establish a previously unknown redundancy for STRADβ in axogenesis and demonstrate a unique role for STRADα in stabilizing LKB1 protein. Although this redundancy is robust, it is not complete, as STRADβ has differential effects on CCM3 protein and could represent another form of control of LKB1 signaling. We also describe a novel STRADα splice variant, and discover a new expression pattern for STRADα-1.

In conclusion, our findings lay the groundwork for understanding the complex control of a key tumor suppressor protein kinase required in development, and open the field to exploration of LKB1 control. Our findings bring to light the necessity of understanding the *in vivo* differences and redundancies of STRADα and STRADβ, and point future studies to elucidate the symptoms in PMSE patients.

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