Cell

LKB1 and SAD Kinases Define a Pathway Required for the Polarization of Cortical Neurons

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DOI 10.1016/j.cell.2007.03.025

SUMMARY

The polarization of axon and dendrites underlies the ability of neurons to integrate and transmit information in the brain. We show here that the serine/threonine kinase LKB1, previously implicated in the establishment of epithelial polarity and control of cell growth, is required for axon specification during neuronal polarization in the mammalian cerebral cortex. LKB1 polarizing activity requires its association with the pseudokinase Stradα and phosphorylation by kinases such as PKA and p90RSK, which transduce neurite outgrowth-promoting cues. Once activated, LKB1 phosphorylates and thereby activates SAD-A and SAD-B kinases, which are also required for neuronal polarization in the cerebral cortex. SAD kinases, in turn, phosphorylate effectors such as microtubule-associated proteins that implement polarization. Thus, we provide evidence in vivo and in vitro for a multikinase pathway that links extracellular signals to the intracellular machinery required for axon specification.

INTRODUCTION

Polarization lies at the heart of several aspects of neural development. As the brain forms, cortical neurons born at the apical ventricular zone undergo a vectorial migration toward the basal pia, where they form and populate specific layers. Then, as neurons extend neurites, one differentiates to become an axon and the others acquire distinct molecular, histological and electrophysiological properties to become dendrites. This cellular polarization underlies directional flow of information within neurons, from dendrites to soma to axon. Finally, processes contact each other and form synapses, which are asymmetric, polarized junctions in which the presynaptic terminal is specialized to release neurotransmitter and the postsynaptic membrane is specialized to respond to it. Accordingly, major questions in developmental neuroscience are how neuron polarization is initiated and implemented (Arimura and Kaibuchi, 2005; Wiggin et al., 2005). The earliest aspect of neuronal polarization (the specification and differentiation of axons and dendrites) has so far been studied most extensively in cultured rodent hippocampal and cortical neurons. In the past few years, these studies have implicated numerous proteins in the establishment of neuronal polarity including multiple kinases, phosphatases, small GTPases, microtubule-associated proteins, and scaffolding proteins (Chen et al., 2006; Da Silva et al., 2005; Inagaki et al., 2001; Jiang et al., 2005; Menager et al., 2004; Schwamborn and Puschel, 2004; Shi et al., 2003, 2004; Yoshimura et al., 2005, 2006; Yu et al., 2000). It seems likely that among these proteins are critical determinants of polarization as well as factors that modulate cellular differentiation in diverse tissues, epochs of development, and contexts. Current challenges include determining which of these proteins play major roles in vivo and how they link extracellular determinants of polarity to intracellular responses.

Here, we address these issues with respect to a serine/threonine kinase, LKB1 (also known as STK11). We focused on this enzyme for three reasons. First, LKB1 is a critical regulator of cellular polarity in nonneural tissues of vertebrates, insects and nematodes (Baas et al., 2004; Martin and St Johnston, 2003; Watts et al., 2000; reviewed by Alessi et al., 2006). Second, the *C. elegans* ortholog of LKB1, *Par4*, is one of a group of six genes identified in a screen for determinants of early embryonic polarity (Kemphues et al., 1988) and later implicated in multiple other aspects of polarization. Recently, vertebrate orthologs of *Par1*, *Par3* and *Par6* have been shown to regulate polarization of rodent neurons in vitro (Chen et al., 2006; Shi et al., 2003). Third, LKB1 is capable of phosphorylating and activating a set of at least 14 serine/threonine kinases related to *Par1* (Lizcano et al., 2004);

these include the kinases SAD-A and SAD-B (also called Brsk2 and Brsk1), orthologs of a *C. elegans* gene implicated in synapse formation and neuronal polarity (Crump et al., 2001; Hung et al., 2007). SAD kinases are the only vertebrate proteins shown to date to be required for neuronal polarization in vivo (Kishi et al., 2005). Based on these results, we hypothesized that LKB1 might regulate the polarization of neurons, and might do so by activating SAD kinases.

Here, we show that LKB1 is required for axon initiation during neuronal polarization in the embryonic cortex in vivo and in vitro. The polarizing activity of LKB1 is enhanced by phosphorylation at a specific site, Serine 431 (S431), previously shown to regulate oocyte polarity in *Drosophila* (Martin and St Johnston, 2003). This residue is the substrate for kinases such as protein kinase A (PKA) and p90 Ribosomal S6 Kinase (p90RSK), (Collins et al., 2000; Sapkota et al., 2001; Su et al., 1996) which mediate effects of extracellular cues that promote axon growth (Kao et al., 2002; Lebrand et al., 2004; Ming et al., 1997; Wong et al., 1996). Once activated, LKB1 phosphorylates SAD-A and B, a modification required for activation of their catalytic activity. LKB1 is the major activator of SAD kinases in the developing cortex, and effects of LKB1 on neuronal polarity are mediated to a large extent by SAD kinases. Finally, activated SAD-A/B, but not LKB1, phosphorylates microtubule-associated proteins such as Tau that are involved in the growth and differentiation of axons and dendrites. Together with findings reported in a companion article (Shelly et al., 2007), these results provide evidence for a pathway that links extracellular determinants of neuronal morphogenesis through a cascade of at least five kinases -TrkB, PKA, LKB1, SAD-A, and SAD-B- to cytoskeletal effectors that polarize neurons in vivo.

RESULTS

Neural Expression of LKB1 and Its Coactivators

To begin this study, we mapped expression of *LKB1* in the developing nervous system. *LKB1* was broadly expressed in the central nervous system (CNS) at E15.5, with highest levels in the forebrain (Figures S1A and S1B in the Supplemental Data available with this article online). It was also expressed by peripheral nervous structures such as dorsal root ganglia (Figure S1C). Within the forebrain, *LKB1* mRNA was present both in the ventricular zone (VZ), which contains neural progenitors, and in the cortical plate (CP), which contains postmitotic neurons (Figure 1A). Expression was maintained through embryogenesis and postnatal life (Figures S1E and S1F).

LKB1 is active only when complexed with a pseudokinase protein called STRAD, and an armadillo-like protein called MO25 (Boudeau et al., 2003). The mouse genome contains two *Strad* genes, (*Strad*α and *Strad*β) as well as two *MO25* genes (*MO25*α and *MO25*β). At E15.5, *Strad*α is expressed relatively uniformly throughout the forebrain, but *Strad*β and *MO25*α are selectively expressed in the CP (Figures 1B–1D). Thus, LKB1 might be differentially activated in postmitotic neurons and dividing progenitors. Like *LKB1,* expression of *Strad*α, *Strad*β and *MO25*α in the cortex is maintained throughout embryogenesis and postnatal life (Figures S1D and S1G–S1L).

We next assessed the distribution of LKB1 protein in the developing cortex. At E15.5, LKB1 was found in the VZ and the CP (Figures 1E and 1I). Ventricular labeling was associated with nestin-positive radial glia and neural progenitors (Figures 1E–1H). LKB1 in the CP and the marginal zone (MZ) was present in postmitotic neurons, as shown by double-labeling with an antibody to the neuron-specific MAP2(a-b) isoforms (Figures 1I–1L). From E15.5 until birth, LKB1 was enriched in the nucleus of neurons in the deepest portion of the CP (Figures 1E, 1M, 1Q, and 1R) suggesting a progressive nuclear enrichment in the most differentiated neurons.

Within cortical neurons, LKB1 was concentrated in nuclei, but also present at low levels in axons, as shown by coincidence with the corticofugal axon marker, TAG1 (Figures 1M–1P); (Kawano et al., 1999). To more clearly visualize neuritic LKB1, we dissociated E14.5 cortical progenitors, cultured them for 7 days, then stained with the axonal marker, Tau-1 and the dendritic marker, MAP2. In vitro as in vivo, LKB1 was enriched in the nucleus, as previously shown in nonneuronal cells (Baas et al., 2003), but was also present in neurites. Immunoreactivity was present in both axons and dendrites (Figures 1S–1V). Because levels of immunoreactivity were low, we also coexpressed an LKB1-EGFP fusion and a cytoplasmic red fluorescent protein (tdTomato) in cortical progenitors which confirmed that LKB1 is concentrated in nuclei but also present in both axons and dendrites (Figures 1W–1Y).

LKB1 Is Necessary for Axon Formation in Cortical Neurons In Vivo

Mice lacking LKB1 die between E8 and E11, before the cerebral cortex forms, reflecting roles of LKB1 in mesenchymal and vascular development (Jishage et al., 2002; Miyoshi et al., 2002; Ylikorkala et al., 2001). To assess the roles of LKB1 in cortical development, we used a conditional allele ($LKB1^{F}$), which generates a null allele upon Cre-mediated recombination (Bardeesy et al., 2002). We limited LKB1 inactivation to dorsal telencephalic progenitors by using the $Emx1^{Cre}$ transgenic mice (Gorski et al., 2002) which drives efficient recombination in progenitors of at least 95% of all pyramidal neurons in the cerebral cortex (Bareyre et al., 2005; Gorski et al., 2002). Loss of LKB1 from $Emx1^{Cre/+}$;$LKB1^{F/F}$ cortex was confirmed by immunostaining and immunoblotting (Figure S2). Residual LKB1 expression may reflect its presence in nonpyramidal interneurons, which do not express *Emx1* (Gorski et al., 2002).

The cortex was roughly normal in size and shape in $Emx1^{Cre/+}$;$LKB1^{F/F}$ mice, but had a thinner cortical wall and larger lateral ventricles than in controls (Figures 2A–2D). Cortical thinning reflected, in part, death of postmitotic neurons, as shown by double-labeling with anti-activated caspase 3 and the neuronal marker anti-β-tubulin III

(Figure S3). In contrast, labeling with the mitotic marker anti-phospho-Histone H3 provided no evidence for strong defects in neurogenesis (Figure S4). We used a panel of markers to assess the cytoarchitecture of mutant cortex, including nuclear (Draq 5) and cytoplasmic dyes (Neurotrace), general neuronal markers (NeuN and β-tubulin III), a dendritic marker [MAP2(a-b)], and layer-specific neuronal markers for layer 1 (reelin and Tbr1; [Hevner et al., 2001; Ogawa et al., 1995]), layer 2-4 (Cux1; [Nieto et al., 2004]) and layer 6 neurons (Tbr1; [Hevner et al., 2001]). This analysis revealed subtle defects in formation of discrete laminae, confinement of specific cell types to appropriate layers, and radial orientation of dendrites (Figures 2E–2N). Nonetheless, by all these criteria, cytoarchitecture was largely preserved in the absence of LKB1, suggesting that it is dispensable for proper radial migration and lamination during cortical development.

In contrast, staining with axonal markers revealed a dramatic defect in $Emx1^{Cre/+}$;$LKB1^{F/F}$ cortex. In control embryos, corticofugal axons, selectively labeled with antibodies to TAG-1 (Kawano et al., 1999), grow laterally toward the internal capsule, *en route* to subcortical target structures (arrows in Figure 2A). In contrast, few TAG1-positive axons were observed in $Emx1^{Cre/+}$;$LKB1^{F/F}$ embryos (Figure 2B). Similarly, callosal axons labeled with antibodies to L1 were nearly absent from $Emx1^{Cre/+}$;$LKB1^{F/F}$ cortex, resulting in agenesis of the corpus callosum (Figures 2C and 2D). Labeling with a general axonal marker, NF165 kDa, confirmed the drastic reduction of cortical axons in the absence of LKB1 expression, both at E15.5 and at birth (Figures 2C and 2D and data not shown).

The lack of axons in $Emx1^{Cre/+}$;$LKB1^{F/F}$ cortex could result from an early failure to initiate axon growth, impairment of axon elongation, or degeneration following initial extension. To distinguish these possibilities, we examined the morphology of individual neurons during radial migration. Recent studies have shown that many cortical neurons initiate axon outgrowth during radial migration, before they reach the CP (Hatanaka and Murakami, 2002; Noctor et al., 2004). Cells were labeled by electroporation of EGFP ex vivo at E14.5 (Figure 3A); this method selectively marks nestin-positive radial glial progenitors in the VZ, which predominantly give rise to layer 5 pyramidal neurons at this stage (Angevine and Sidman, 1961; Hand et al., 2005; Polleux et al., 1997). Following electroporation, the cortices were maintained as organotypic slice cultures for 3 to 5 days then imaged using confocal microscopy. After 3 days in vitro, many electroporated neurons in both control and mutant slices had migrated through the IZ and reached the CP (Figures 3B and 3C), confirming that LKB1 is dispensable for proper radial migration. In control slices, most labeled neurons in the IZ (43/52 or 82.7%) and all of those that had reached the CP (34/34) had extended a single >100 microns long axon (Figures 3D and 3F). In contrast few of the labeled neurons in the IZ (5/57 or 8.8%; $p < 0.001$ compared to control) or CP (3/41 or 7.3% $p < 0.001$ compared to control) of $Emx1^{Cre/+}$; $LKB1^{F/F}$ slices had a >100 μm long axon (Figures 3E and 3G–3I). Even after five days in vitro, when numerous EGFP-labeled axons had grown toward the midline or internal capsule in control slices, only few labeled axons were present in mutant cultures (Figures 3J–3M). Thus, LKB1 is required for axon initiation when neurons engage radial migration through the intermediate zone.

Because the electroporation method only labels a subpopulation of layer 5 neurons, we microinjected Biotinylated Dextran Amine (BDA) into neonatal cortex which provides full anterograde tracing of cortical axons throughout cortical layers (Chang et al., 2000). In control mice, BDA-labeled corticofugal axons projected to the internal capsule (double arrows in Figure 3N) and toward the corpus callosum (arrow in Figure 3N). Similar injections in the cortex of $Emx1^{Cre/+}$;$LKB1^{F/F}$ mice labeled neuronal somata, but fail to label substantial numbers of efferent axons (Figure 3O). A few single axons were occasionally found deep in the cortical wall close to the ventricle (Figure 3P). Altogether these results show that LKB1 expression is required for axon extension by cortical neurons in vivo.

Cell-Autonomous Role of LKB1 in Axon Specification

For detailed analysis of neuronal differentiation in the absence of LKB1, we marked cortical progenitors at E14.5 by ex vivo electroporation as described above (Figure 3A), then dissociated the cortices and cultured the cells. In this method, electroporated progenitors are cultured before they have undergone any overt polarization or differentiation (Hand et al., 2005; and data not shown). Most EGFP-labeled neurons differentiating in control or mutant cultures extended multiple processes (Figures 4A and 4B). In control cultures, the longest process was Tau1-positive but MAP2-negative, marking it as an axon (double arrows in Figures 4A and 4G–4J), whereas the shorter processes were MAP2-positive but Tau1-negative, marking them as dendrites (arrowhead in Figures 4A and 4C–4F). In contrast, EGFP-labeled cortical mutant neurons failed to polarize properly. Neurites were relatively uniform in length (Figure 4B), and contained both MAP2 and Tau-1 (Figures 4K–4N). Nearly all (98%) of control neurons but only 30% of mutant neurons bore an axon as defined by being more than 100 microns long, Tau-1-positive, and MAP2-negative (Figure 4O). Moreover, the average length of the longest neurite in LKB1 mutant neurons was about one-fourth that in control neurons, and therefore intermediate between that of axons and dendrites in control neurons (Figure 4P). Taken together, these results show that LKB1 is required for neuronal polarization of cortical neurons.

Results presented so far do not allow us to distinguish whether LKB1 is part of the cellular machinery required for neuronal polarization to occur, or whether it plays a critical regulatory role in this process. To address this issue, we performed a gain-of-function experiment. We used ex vivo electroporation to overexpress LKB1 and/or

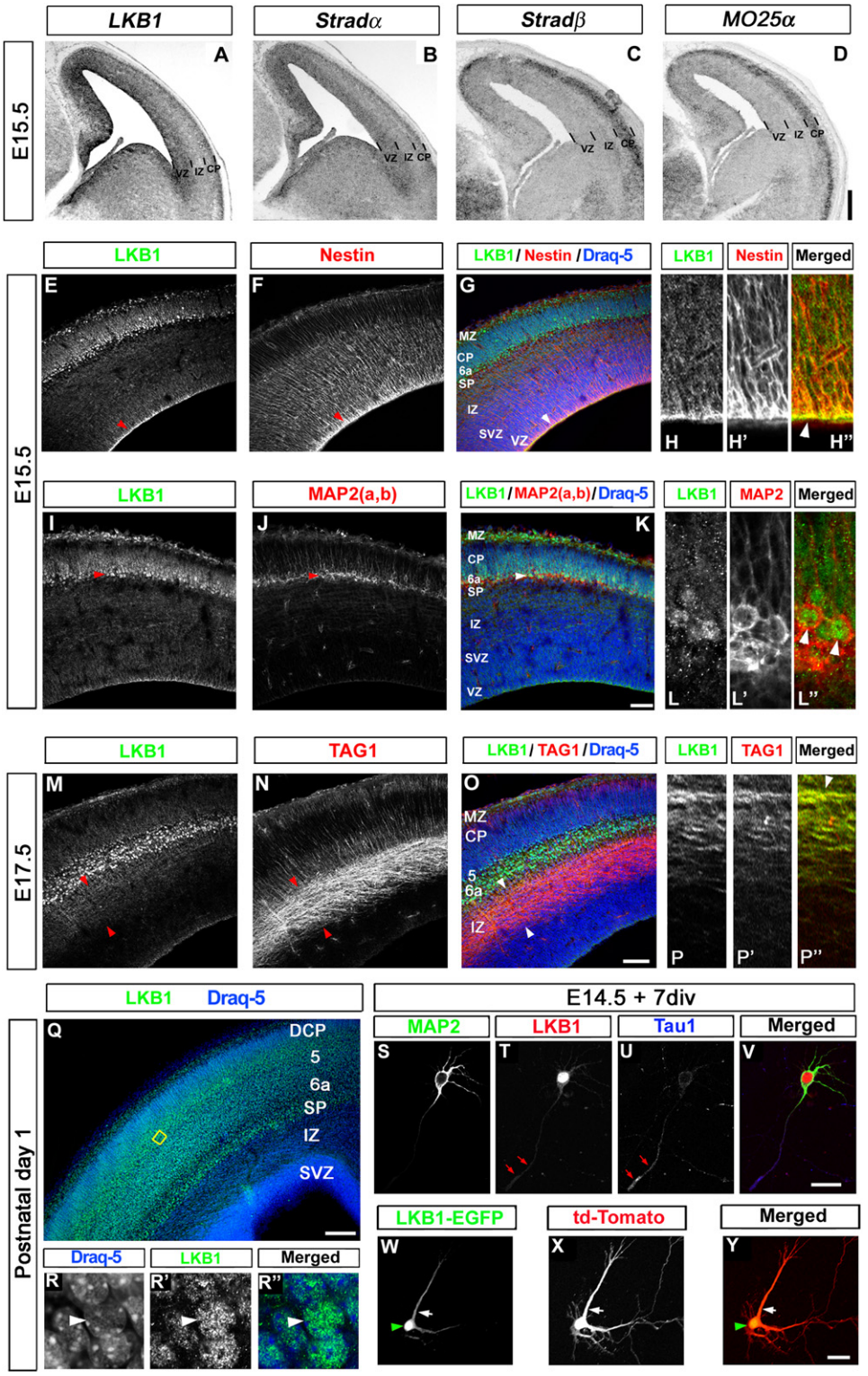

Figure 1. Expression of LKB1 Complex Components in Developing Cortex

(A–D) In situ hybridization for *LKB1* (A) and its coactivators *Stradα* (B), *Stradβ* (C), and *MO25α* (D) demonstrate that all four genes are expressed in the cortex at E15.5. The scale bar represents 150 microns.

(E–H'') LKB1 is present in nestin-positive radial glial neural progenitors at E15.5. H–H'' are high-magnification panels of areas marked by arrowheads in (E)–(G). The scale bar represents 60 microns.

Stradα in wild-type neurons. Coexpression of LKB1 and Stradα in E14.5 progenitors led to extension of multiple, long, Tau-1-positive axons from over half of the neurons examined (Figures 4Q–4X and 4Y). This result demonstrates a cell-autonomous and instructive role for LKB1 in axon specification. In contrast, overexpression of either LKB1 or Stradα had no significant effect on the number of Tau1-positive axons emerging from the cell body of cortical neurons (Figure S5 and Figure 4Y). This result suggests that endogenous LKB1 and Stradα are both present in limiting amounts in cortical neurons.

LKB1 Phosphorylation Is Required for LKB1-Dependent Neuronal Polarization

Although LKB1 is present in all neurites of cortical neurons (Figure 1), it appears to play a dominant role in specification of axons but not dendrites, raising the possibility that its activity is spatially regulated within neurons. One modification of LKB1 that regulates its function is phosphorylation at a conserved serine residue, S431 (Martin and St Johnston, 2003; Sapkota et al., 2001). Both PKA and p90RSK can phosphorylate LKB1 at this site (Sapkota et al., 2001). We examined phosphorylation of LKB1 at S431 in developing neurons, by using a phospho-specific antibody. Specificity of the anti-pLKB1 (S431) antibody was demonstrated by showing that it failed to stain cortical neurons isolated from $Emx1^{Cre/+}$; $LKB1^{F/F}$ mice (Figure S6). Immunoblotting showed that pLKB1 (S431) was present in the cortex at E15.5 and increased approximately 4-fold between E15.5 and P1, the interval during which newly-generated neurons extend axons (Figures 5N and 5O).

We then used this antibody to stain neurons dissociated and cultured from E15.5 cortex. We categorized neurons by the criteria proposed by Dotti et al. (1988) for cultured hippocampal neurons: Stage 2 neurons have multiple neurites but are unpolarized, whereas by Stage 3, one neurite has become substantially longer than the others, and begun to acquire axonal characteristics. pLKB1 (S431) was present at low levels in all neurites of Stage 2 neurons but became concentrated in the axon of Stage 3 neurons (Figures 5A–5F). Because total LKB1 was present at similar levels in all neurites at this stage (Figures 1S–1Y), this result suggests preferential phosphorylation and/or deficient dephosphorylation of LKB1 at S431 in axons.

To ask whether phosphorylation of LKB1 at S431 affects its ability to promote axon formation, we tested a mutant in which S431 was mutated to alanine ($LKB1^{S431A}$). As expected, the pLKB1 (S431) antibody recognized wild-type LKB1 but not $LKB1^{S431A}$ in immunoblots of transfected cells (data not shown). Coexpression of $LKB1^{S431A}$ and Stradα by ex vivo electroporation was significantly less effective in promoting formation of supernumerary axons than was coexpression of wild-type LKB1 and Stradα) (Figure 5M).

LKB1 Polarizes Neurons by Activating SAD-A/B Kinases

What are the critical substrates of LKB1 mediating its function during axon specification? Two observations suggested that they might include SAD kinases. First, the cortical phenotypes of $Emx1^{Cre/+}$;$LKB1^{F/F}$ mutants resemble those of SAD-A; SAD-B double mutant mice, both in vivo and in vitro (Kishi et al., 2005). Second, in a cell-free system, LKB1 has been shown to phosphorylate and activate several related kinases including the human orthologs of SAD-A and –B (Lizcano et al., 2004). This residue is T175 in SAD-A and T187 in SAD-B, and the two kinases show 100% identity over a stretch of 27 residues flanking this phorphorylation site (Kishi et al., 2005). To ask whether LKB1 phosphorylates SAD kinases in vivo, we generated and purified phospho-specific antibodies to the shared peptide centered around pT175/T187 of SAD-A and SAD-B, respectively. We refer to this phospho-specific antibody as pSAD (T-al), since it is directed to the phosphorylated Threonine residues of the activation loop of SAD-A and SAD-B. ELISA showed that the pSAD (T-al) antibody reacted >1000-fold more strongly with phosphorylated than nonphosphorylated peptide, and immunoblotting showed that it reacted well with both phospho-SAD-A and phospho-SAD-B, but not with SAD-A or -B mutants in which the activation loop threonine had been mutated to alanine (T175A or T187A; Figure 6A and data not shown).

To assay LKB1-dependent phosphorylation of SAD kinases, we used HeLa cells, because they express Stradα and MO25α but not LKB1, SAD-A or SAD-B (Figure 6A) (Boudeau et al., 2003). SAD-A was phosphorylated on T175 when coexpressed in HeLa cells with wild-type LKB1, but was not detectably phosphorylated on this residue when expressed alone or coexpressed with

(I–L'') LKB1 is present in MAP2a/b-positive cortical neurons at E15.5. (L)–(L)'' are high-magnification panels showing of areas marked by arrowheads in (I)–(K). The scale bar represents 60 microns.

(M–P'') LKB1 is present in somata of layer 5 and 6a neurons and in TAG-1 positive corticofugal axons at E17.5. (P)–(P)'' are high-magnification panels of areas marked by arrowheads in (M)–(O). The scale bar represents 45 microns.

(Q–R'') LKB1 is present in cortical neurons at P1. (R)–(R)'' are high-magnification panels of area boxed in (Q), showing enrichment of LKB1 in Draq-5-positive neuronal nuclei. The scale bar represents 150 microns.

(S–V) LKB1 is present in nuclei, MAP2-positive dendrites, and Tau1-positive axons of cultured cortical neurons at 5 days in vitro (div). The scale bar represents 20 microns.

(W–Y) Electroporation of LKB1-EGFP fusion (W) together with the red fluorescent protein tandem-dimer Tomato (for whole-cell filling; [X]) in E14.5 cortical neurons confirms enrichment of LKB1 in nuclei (arrowhead) and presence in all major neurites (arrows). The scale bar represents 20 microns.

Abbreviations are as follows: CP, cortical plate; DCP, dense cortical plate; IZ, intermediate zone; MZ, marginal zone; SP, subplate; SVZ, subventricular zone; VZ, ventricular zone; 5, 6a, cortical laminae 5, and 6a.

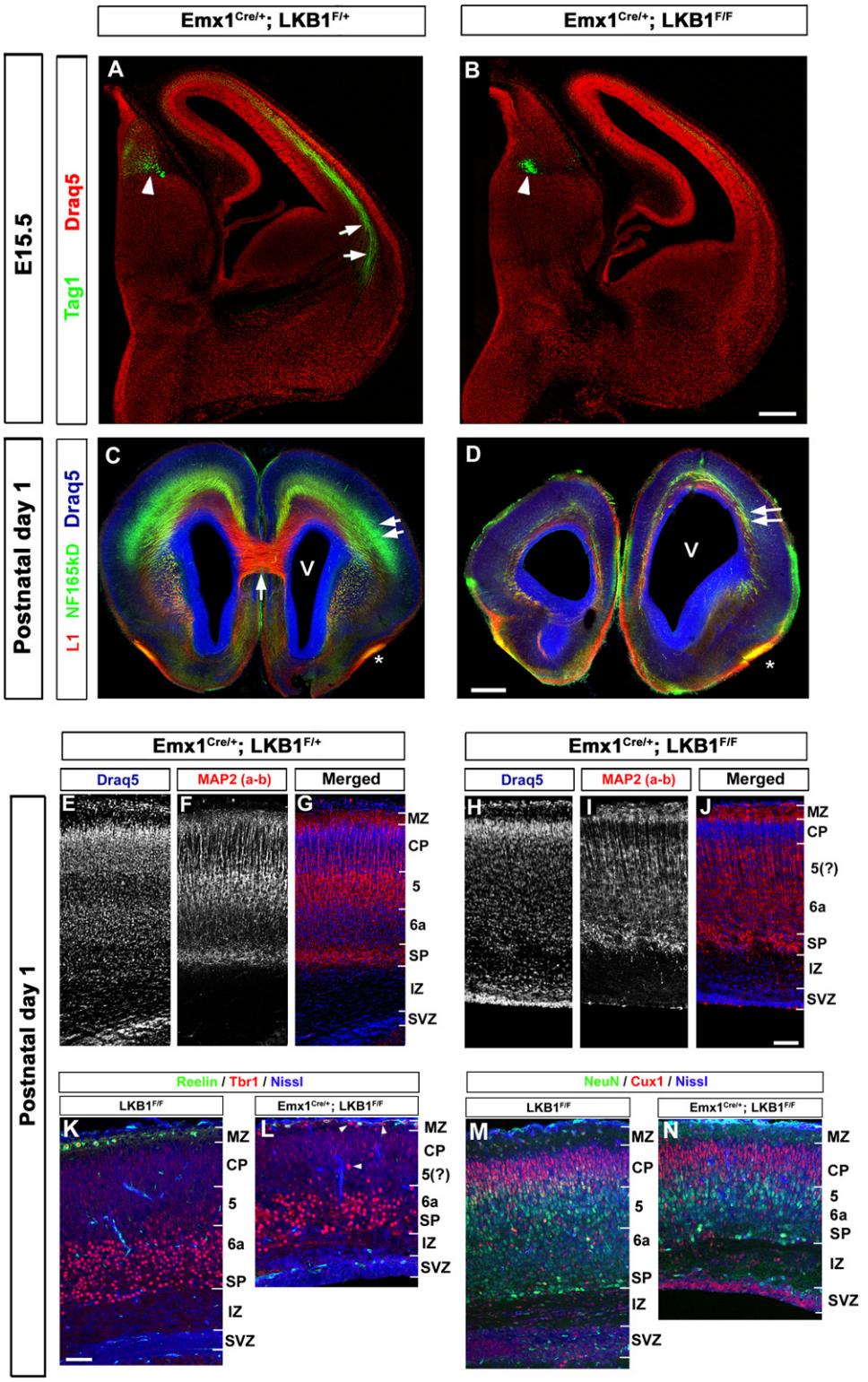

Figure 2. LKB1-Deficient Cortical Neurons Lack Axons In Vivo

(A and B) Coronal sections of E15.5 cortex showing TAG1-positive corticofugal axons (arrows in [A]) growing through the intermediate zone and into the internal capsule in controls ($Emx1^{Cre/+}$;$LKB1^{F/+}$) and their absence in mutants ($Emx1^{Cre/+}$;$LKB1^{F/F}$). Axons are present in the fornix of both control and mutant embryos (arrowheads). The scale bar represents 150 microns.

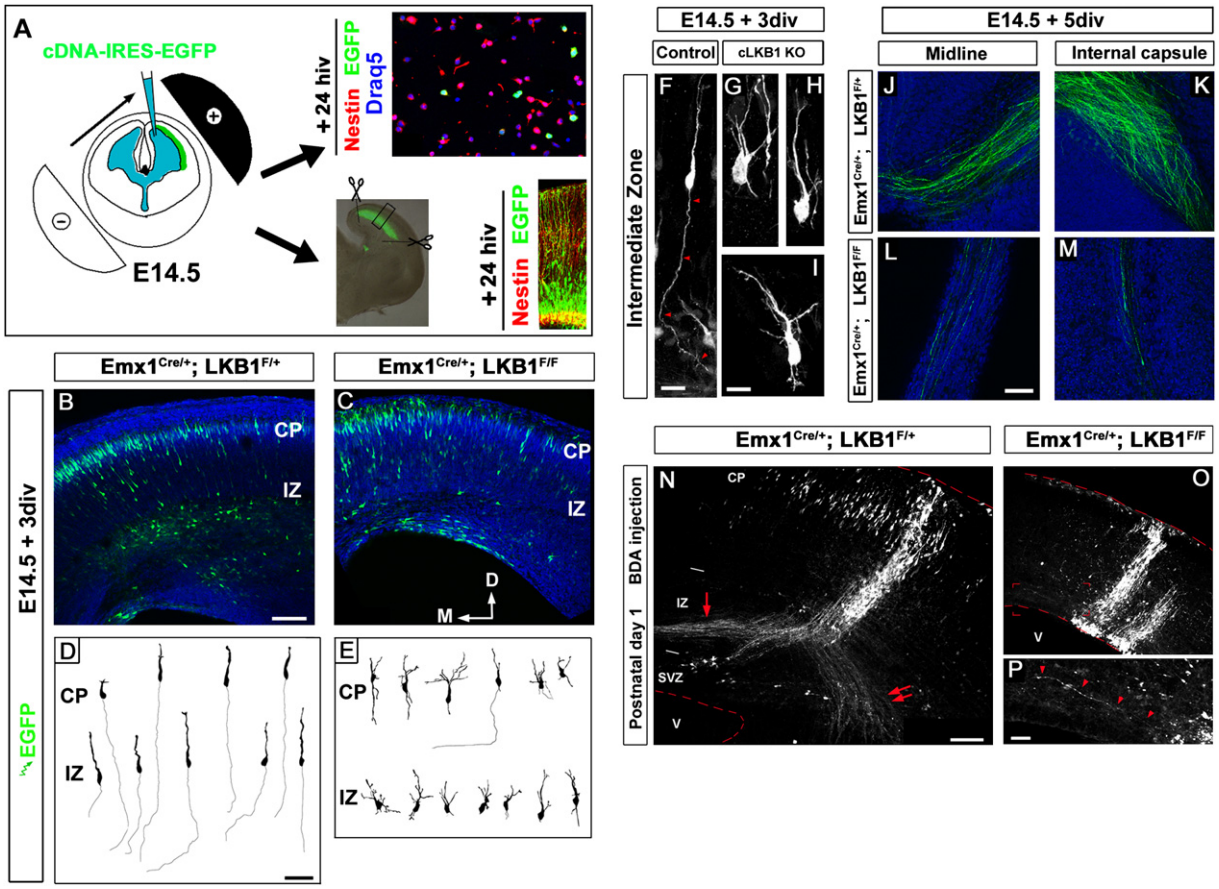

Figure 3. LKB1 Is Required for Axon Initiation

(A) E14.5 ex vivo cortical electroporation targets radial glial progenitors in the dorsal telencephalon as shown by acute dissociation, culture for 24 hr and immunostaining for Nestin, a radial glial marker. After 5–7 days in vitro >95% of EGFP-labeled cells differentiate into TuJ1 positive neurons (data not shown).

(B and C) Organotypic slice cultured for 3 days in vitro reveals radial migration of EGFP-labeled neurons into the IZ and accumulation in the CP in mutants and controls. The scale bar represents 80 microns.

(D–I) Computer-based reconstruction (D and E) and micrographs (F–I) of EGFP-labeled neurons shows absence of axon but supernumerary dendrites in mutants compared to controls. The scale bar represents 50 microns.

(J–M) After 5 days in vitro, EGFP+ neurons extend large number of axons toward the corpus callosum and internal capsule in control slices, but few axons emerge from EGFP-positive neurons in slices from mutants. The scale bar represents 20 microns.

(N–P) Anterograde axon tracing using BDA microinjections labels many callosal (single arrow) and corticofugal (double arrows) axons in controls but few in mutants. (P) is a high-magnification panel of areas boxed in of area boxed in (O). (N and O) The scale bar represents 100 microns. (P) The scale bar represents 20 microns.

DIV, days in vitro; other abbreviations are as in Figure 1.

catalytically inactive LKB1 mutants (LKB1^{D194A} or LKB1^{K78I}; Figure 6A and Figure S7A). Similar results were obtained with SAD-B (data not shown). These results show that LKB1 can phosphorylate SAD kinases. To ask whether this phosphorylation is required for SAD kinase activity, we made use of our previous observation that SAD kinases phosphorylate the microtubule-associated protein Tau at S262 (Kishi et al., 2005). This result was obtained in cell lines that expressed LKB1. In contrast, when we coexpressed SAD-A and Tau in HeLa cells, then probed lysates with a phospho-specific antibody to pTau (S262), no phosphorylation was detectable.

(C and D) L1-positive callosal axons and neurofilament-positive cortical axons are present in control but nearly absent in mutant cortex at birth. The scale bar represents 300 microns.

(E–J) MAP2(a-b) staining reveals a largely preserved dendritic architecture in the mutant cortex. The scale bar represents 40 microns.

(K–N) Layers form normally in LKB1 mutant cortex, as revealed by layer-specific markers Tbr 1 (layers 1, 6a, and subplate), reelin (layer 1), and Cux1 (layers 2–4). Few displaced Tbr-1-positive neurons in LKB1-deficient cortex are marked by arrowheads in (L). The scale bar represents 50 microns. Abbreviations are as in Figure 1.

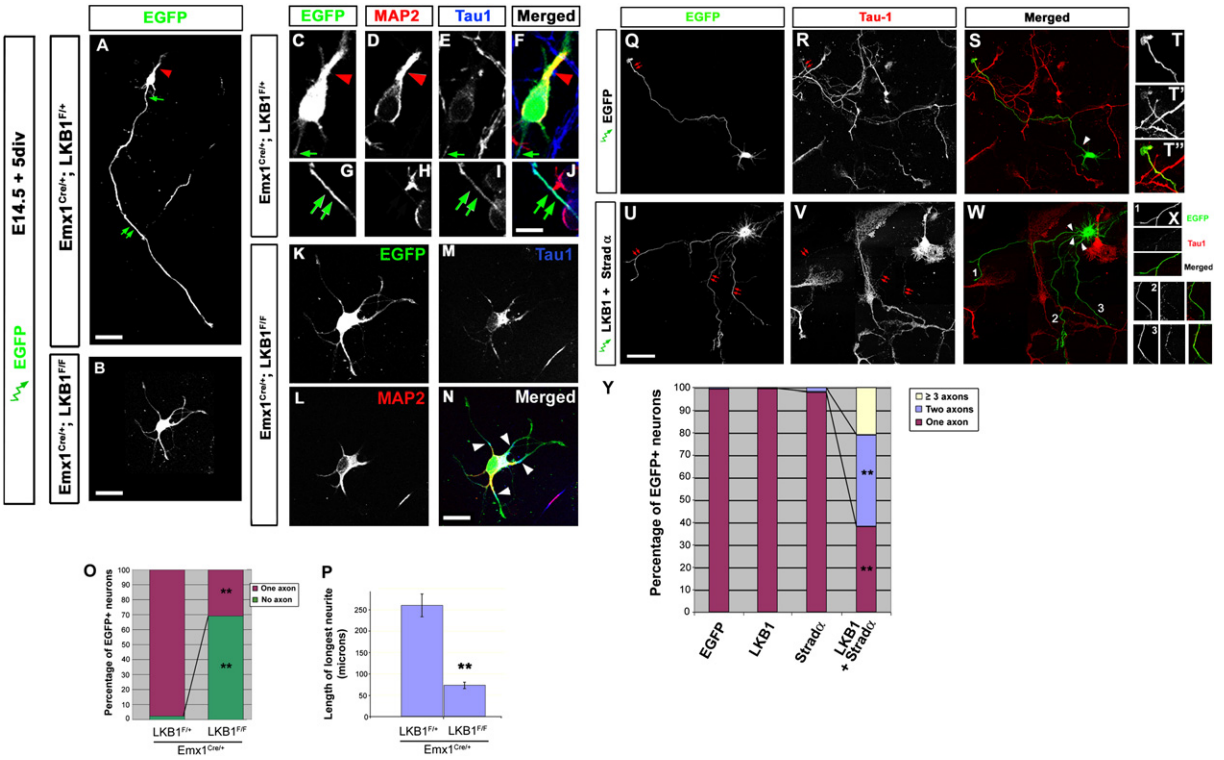

Figure 4. LKB1 Is Necessary and Sufficient for Neuronal Polarization

(A and B) Cortical progenitors from control (A) or LKB1 mutant (B) E14.5 embryos were electroporated with EGFP and maintained in dissociated culture for 5 days in vitro (div). Control neurons display a single, long axon (double arrows in [A]) and short dendrites (arrowhead in [A]), whereas LKB1-deficient cortical neurons do not display any long axon-like process. (A) The scale bar represents 30 microns. (B) The scale bar represents 20 microns.

(C–J) High magnification of the neurons shown in (A) and (B) reveals the MAP2-positive but Tau-1 negative dendrites (arrowhead in [G]–[J]) and a single Tau-1-positive but MAP2 negative axon (G–J) of the control neuron. The scale bar represents 10 microns.

(K–N) In (K)–(N), EGFP+ cortical neurons deficient for LKB1 only form short MAP2 and Tau-1 double-positive neurites (arrowheads in [N]) but no long, Tau1-positive, MAP2-negative axon. The scale bar represents 15 microns.

(O) Percentage of EGFP+ neurons with one or no Tau1-positive axon in neuronal cultures from E14.5 control ($Emx1^{Cre/+}$; $LKB1^{F/+}$; n = 70 from three independent experiments) or mutant embryos ($Emx1^{Cre/+}$; $LKB1^{F/F}$; n = 44 from three independent experiments). **$p < 0.01$, chi-square test.

(P) Length of the longest neurite in E14.5 neuronal cultures from control and mutant embryos. **$p < 0.01$ nonparametric Mann-Whitney test (n as in [O]).

(Q–T'') E14.5 cortical neurons electroporated with EGFP have only one Tau1-positive axon (double arrows; shown at higher magnification T–T'') after 7 div. The scale bar represents 40 microns.

(U–X) Simultaneous overexpression of LKB1 and Stradα induces supernumerary Tau1-positive axons (arrowheads in [W]). (X1)–(X3) shows high-magnification views of axons numbered 1–3 in (W). Arrowheads in (S) and (W) indicate the axon initiation site. The scale bar represents 40 microns.

(Y) Percentage of neurons with one (purple), two (blue), or more than two (yellow) >100 μm long Tau1-positive axon in conditions shown in (A)–(P). Each treatment is quantified from three independent experiments (EGFP n = 136 neurons; LKB1 n = 171; Stradα n = 103; LKB1+Stradα n = 110). *$p < 0.05$ and **$p < 0.01$, chi-square test comparison with EGFP.

Coexpression of LKB1, however, resulted in robust tau phosphorylation (Figure 6A). Tau phosphorylation resulted from LKB1-dependent phosphorylation of SAD-A rather than a direct effect of LKB1 because no phosphorylation was observed when SAD-A was omitted or mutated to a form that cannot be phosphorylated by LKB1 ($SAD\text{-}A^{T175A}$; Figure 6A and Figure S7B). Thus, LKB1 is an activator of SAD kinases.

To determine whether LKB1 is the endogenous activator of SAD kinases in the developing cortex, we probed lysates from control and $Emx1^{Cre/+}$;$LKB1^{F/F}$ cortex with anti-SAD-A, anti-SAD-B, and the pSAD (T-al) antibody. Levels of SAD-A and SAD-B showed no major or consistent difference between mutants and controls (n = 8), but levels of pSAD (T-al) were dramatically decreased in the mutant cortex (Figure 6B). As noted above, low levels of LKB1 persist in the mutants (Figure S2G), and may account for the residual pSAD (T-al). We cannot, however, rule out the possibility that other kinases in cortex are capable of phosphorylating SAD kinases. Nonetheless, these results demonstrate that LKB1 is the major activator of SAD kinases in neonatal cortex.

LKB1 can phosphorylate the activation loop of 14 related kinases in nonneural cells but many of these can be phosphorylated and activated by other kinases as well (Alessi et al., 2006). Is LKB1 the major activator of all of these kinases in brain? To test this possibility, we used a phospho-specific antibody to the activation loop

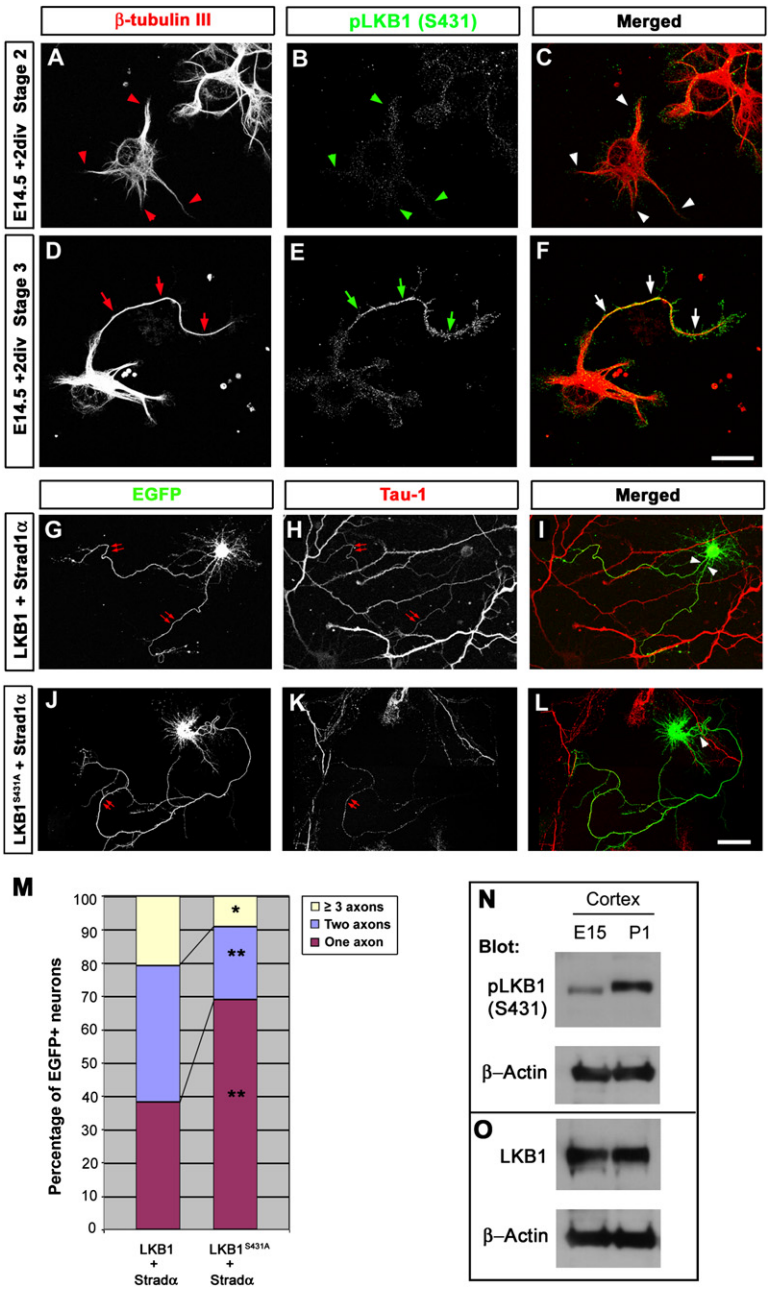

Figure 5. Axonal Localization and Axon Specification Activity of pLKB1-S431

(A–F) E14.5 cortical neurons cultured for 2 days and stained with antibodies to β-tubulin III (TuJ1) and pLKB1(S431). Stage 2 neurons, with multiple short neurites of equal length, have low levels of pLKB1(S431) in all neurites (A–C). Stage 3 neurons, in which one neurite has acquired axonal characteristics, show systematic enrichment of pLKB1(S431) in the axon (arrows in [D]–[F]). The scale bar represents 15 microns.

(G–L) E14.5 cortical neurons coelectroporated with $LKB1^{S431A}$ and Stradα show a reduced probability of having multiple axons emerging from the cell body of neurons when compared to neurons coelectroporated with wild-type LKB1 and Stradα. Arrows indicate axon in (G)–(L); arrowheads indicate soma in (I) and (L). The scale bar represents 25 microns.

(M) Percentage of neurons with one (purple), two (blue), or more than two (yellow) >100 μm long Tau1-positive axons in conditions shown in (G)–(L). Each treatment is quantified from three independent experiments (LKB1 + Stradα, n = 110; $LKB1^{S431A}$ + Stradα, n = 119). *$p < 0.05$ and **$p < 0.01$, chi-square test comparison.

(N and O) Western blot analysis of total LKB1 (R) and pLKB1 (S431) (Q) in the developing cortex at E15 and P1. Blots were reprobed with anti-β-actin to assess equal loading.

of AMP-activated kinase, pAMPK (T172), which is activated by LKB1 in muscle and liver (Sakamoto et al., 2005; Shaw et al., 2005). In cortex, in contrast, levels of pAMPK (T172) were not detectably affected by loss of LKB1 (Figure 6B). This result emphasizes the selective association of LKB1 and SAD kinases in the developing brain.

We also used pSAD (T-al) antibodies to localize phosphorylated SAD kinases in the brain. Whereas SAD kinases are present throughout the cortex in both dendrites and axons of cortical neurons (Kishi et al., 2005), pSAD (T-al) is concentrated in cortical axons at E15.5 (Figure S8). Staining is nearly absent from $Emx1^{Cre/+}$;$LKB1^{F/F}$ cortex, reflecting both lack of SAD activation and loss of axons (Figures 6C–6F). Likewise, levels of pTau (S262), which are greatly decreased in SAD-A/B mutant brain (Kishi et al., 2005), are decreased in Emx1Cre/+;$LKB1^{F/F}$ cortex (data not shown).

Taken together, these results suggest that LKB1 function in cortical development depends on its ability to activate SAD kinases. To further test this idea, we designed short hairpin RNAs that selectively and efficiently knock down SAD-A and SAD-B protein levels in heterologous cells (Figure 6G). We expressed these shRNAs along with LKB1 and Stradα in dissociated cortical neurons

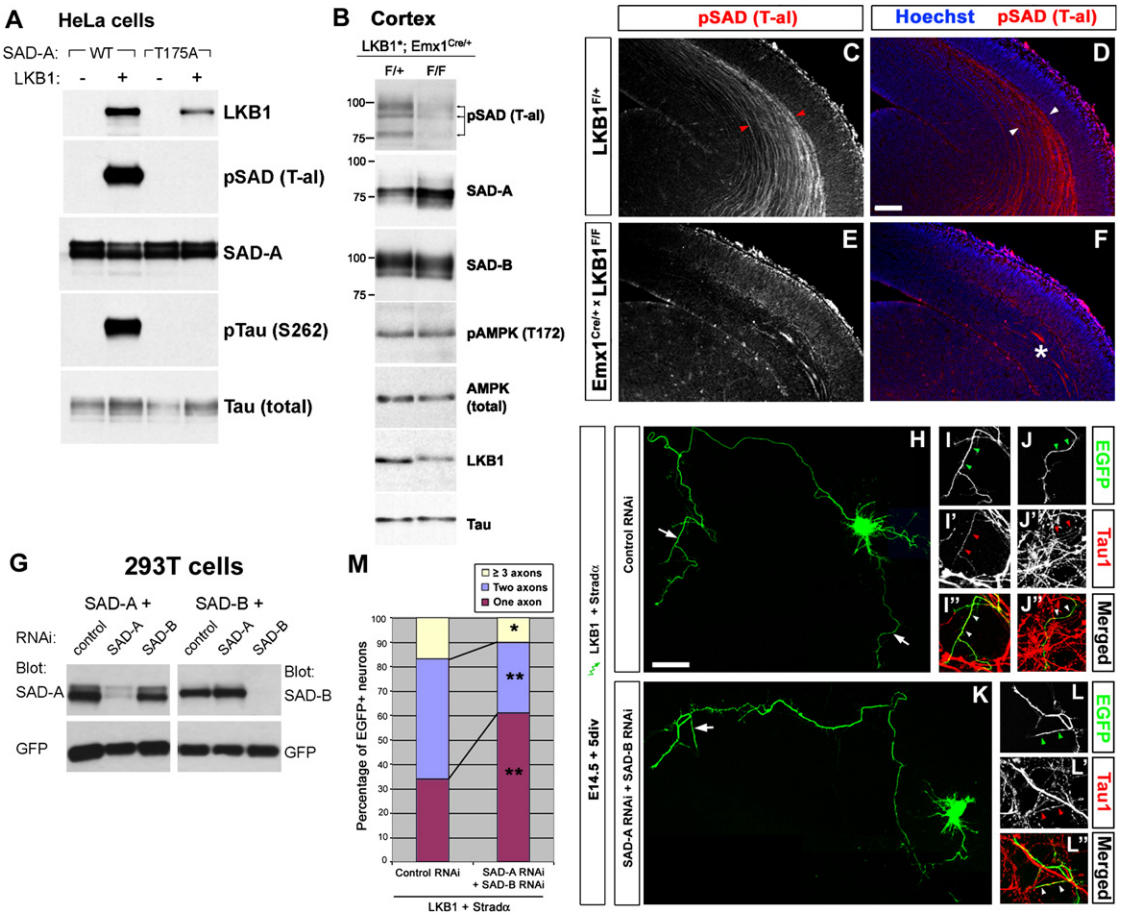

Figure 6. LKB1 Function in Axon Specification Requires SAD Kinases

(A) Cotransfection of LKB1 and wild-type SAD-A in HeLa cells results in strong phosphorylation of SAD-A on T175 (lanes 1 and 2). Mutation of SAD-A T175 to A eliminates phosphorylation (lanes 3 and 4). Phosphorylation of SAD-A at T175 by LKB1 activates its kinase activity as assessed by robust phosphorylation of Tau on S262.

(B) Reduced phosphorylation of SAD A/B in the cortex of LKB1 knockout ($Emx1^{Cre/+}$; $LKB1^{F/F}$) compared to control mice ($Emx1^{Cre}$; $LKB1^{F/+}$) at birth. Levels of SAD-A, SAD-B, and Tau (~70 kDa, isoform shown, but others are also unaffected) are not altered in the mutant. Levels of AMPK and of pAMPK (T172) are also not affected by deletion of LKB1. The samples shown are from nonconsecutive lanes from the same gel.

(C–F) Marked decrease of pSAD (T-al) in the cortex of LKB1 mutant (E and F) compared to control mice (C and D) at E15.5. The arrowheads in (C) and (D) and the asterisk in (F) mark the location of the intermediate zone. The scale bar represents 125 microns.

(G) Validation of the specificity of short hairpin sequences directed at SAD-A and SAD-B kinases. 293T cells were cotransfected with SAD-A or SAD-B cDNAs together with control RNAi or RNAi targeting SAD-A or SAD-B. Effective and selective knock-down of SAD-A and SAD-B kinases is achieved. Immunoblot for GFP serves as a loading control.

(H–L'') Cotransfection of LKB1 and Stradα with control shRNA (H–J'') in E14.5 cortical neurons results in multiple Tau1 positive axons, whereas shRNA's targeting SAD-A and SAD-B attenuate the ability of LKB1 over-activation to induce multiple axons (K–L''). (I)–(J'') and (L)–(L'') show details of the GFP and Tau1 staining in regions marked by arrows in (H)–(K), respectively. The scale bar represents 25 microns.

(M) Percentage of neurons with one (purple), two (blue) or more than two (yellow) >100 μm long Tau1-positive axons in conditions shown in H-L''. Each condition is quantified from 3 independent experiments (LKB1+Stradα+Control RNAi n = 65; LKB1+Stradα+SAD-A/B RNAi n = 69). *$p < 0.05$ and **$p < 0.01$, chi-square test comparison.

using the ex vivo electroporation method described above. The ability of overexpressed LKB1 and Stradα to induce supernumerary axons was unaffected by control shRNA (Figures 6H–6J''; compare to Figure 5P), but was decreased 2-fold by a combination of shRNAs targeting SAD-A and SAD-B (Figures 6K and 6L'' and 6M). We conclude that SAD-A/B kinases act downstream of LKB1 to promote axon specification during neuronal polarization.

DISCUSSION

A Kinase Pathway Required for Cortical Neuron Polarization

LKB1 is a tumor-suppressor mutated in more than 80% of patients presenting the cancer predisposition syndrome called Peutz-Jeghers Syndrome (Alessi et al., 2006). Activation of LKB1 is sufficient to polarize mammalian

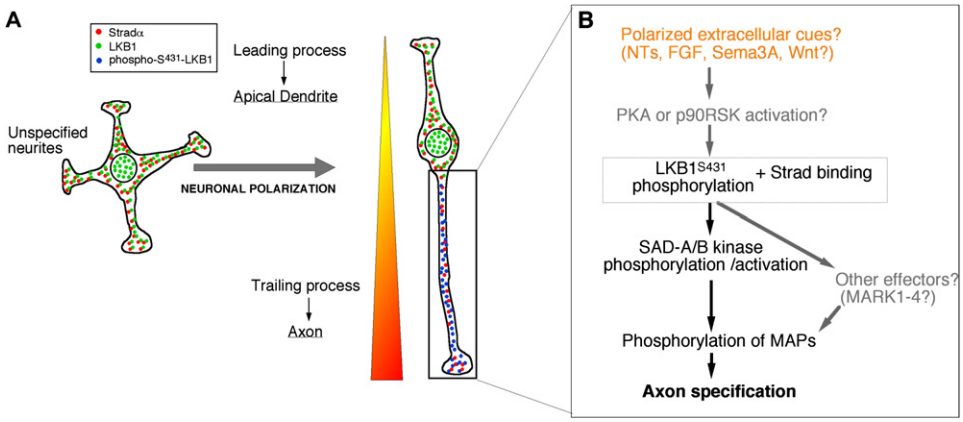

Figure 7. Model of the Kinase Pathway Identified in This Study

We propose that specific extracellular signals control axonal specification and polarization in cortical neurons by activating LKB1 and SAD kinases in the neurite becoming the axon. See Discussion for details.

epithelial cells in the absence of cell-cell contact (Baas et al., 2004). LKB1 is viewed as a master regulator of cell polarity because it is the only protein so far shown to have this activity. The *C. elegans* and *Drosophila* orthologs of *LKB1* (*Par4* and *dLKB1*, respectively) are also regulators of embryonic polarity, controlling anterior-posterior axis formation in both species and epithelial cell polarity in flies (Martin and St Johnston, 2003; Watts et al., 2000). Previous studies had not examined the role of LKB1 or its orthologs in neuronal polarity. The severe and specific defects we documented in LKB1 conditional knockout mice demonstrate a unique and nonredundant role for LKB1 in polarization of cortical neurons.

Several biological activities mediated by LKB1 require the phosphorylation of S431. For example, oocyte polarity defects seen in dLKB1 null mutant flies are effectively rescued by reintroduction of wild-type dLKB1 but not by dLKB1 bearing a single point mutation at the equivalent site (S535) (Martin and St Johnston, 2003). We provide several lines of evidence suggesting that phosphorylation of this site is also important for neuronal polarization. This site is a substrate for PKA and p90RSK (Sapkota et al., 2001), which are both activated in response to many extracellular cues in multiple cell types. Specifically, BDNF has been shown to induce phosphorylation of LKB1 on Serine 431 in a p90RSK-dependent manner (Arthur et al., 2004). Moreover, Shelly et al. (2007) provide evidence that induction of axon differentiation by local presentation of brain-derived neurotrophic factor (BDNF) requires PKA-dependent LKB1 phosphorylation on S431. Taken together with our data, these results support the idea that LKB1 phosphorylation at this site serves as an intracellular sensor of extracellular polarizing signals in vivo (see Figure 7).

In contrast to Shelly et al. (2007), we did not observe asymmetric localization of total LKB1 protein in cultured cortical neurons. This difference might be due to the nature of the systems used. Our cultures consisted of electroporated cortical neuronal progenitors undergoing polarization for the first time in vitro, whereas, the hippocampal neurons cultured by Shelly et al. (2007) had already undergone polarization in vivo before dissociation. Most importantly, both studies provide strong evidence that local activation of LKB1 by phosphorylation on S431 is critical for axon specification.

How are effects of LKB1 on neuronal polarity mediated? Mice lacking LKB1 show defects in neuronal polarization similar to those demonstrated previously in SAD-A/B double mutants (Kishi et al., 2005). Moreover, SAD-A and SAD-B are inactive both in cultured cells and in developing cortex in the absence of LKB1. Thus, LKB1 regulates cortical neuronal polarization at least in part by activating SAD-A and SAD-B.

SAD kinases phosphorylate the microtubule-associated protein Tau on S262 in a KXGS motif present in the tubulin binding domain (Kishi et al., 2005). The KXGS motif is also found in other microtubule-associated proteins including MAP2, MAP4 and Doublecortin (Ebneth et al., 1999; Schaar et al., 2004). SAD kinases appear likely to phosphorylate at least some of these proteins (B.N.L. and J.S. unpublished). Microtubule binding proteins such as Tau and doublecortin have been implicated in axon specification and neuronal polarization (Caceres and Kosik, 1990; Deuel et al., 2006; Kempf et al., 1996; Koizumi et al., 2006). Thus, one way in which SAD kinases may affect neuronal polarization is by regulating cytoskeletal dynamics prior to or coincident with the specification of the axon. Current studies are aimed at identifying the preferred sites of SAD kinase phosphorylation and determining the identity of SAD kinase substrates involved in neuronal polarization.

Multiple Molecular Mechanisms Controlling Neuronal Polarity

Many signaling molecules have been implicated in polarization of cultured mammalian neurons. They include

glycogen synthase kinase 3β (GSK3β), phosphotidylinositol 3′ (PI3) kinase, atypical protein kinase C (aPKC), protein kinase B (AKT), the phosphatase PTEN, the tubulin binding protein CRMP-2, the small GTPases Rac1 and cdc42, their regulators DOCK 7, Rap1b, and Tiam-1, and vertebrate orthologs of *C. elegans* Par1, Par3 and Par6 (Chen et al., 2006; Da Silva et al., 2005; Inagaki et al., 2001; Jiang et al., 2005; Menager et al., 2004; Schwamborn and Puschel, 2004; Shi et al., 2003, 2004; Yoshimura et al., 2005, 2006; Yu et al., 2000). These proteins may act in distinct neuronal populations or at distinct times of development. It is also possible, however, that several of these proteins interact with or function via the PKA/p90RSK-LKB1-SAD-A/B pathway that we have described.

Particularly interesting in this regard are the Par proteins (Par1-6) initially identified as regulators of embryonic polarity in nematodes (Kemphues et al., 1988). As noted above, LKB1 is the vertebrate ortholog of Par-4 and SAD is related to Par-1. All of the *C. elegans* Par proteins act as parts of a single pathway, and there is increasing evidence that their vertebrate orthologs form complexes and act together (Wiggin et al., 2005). Recently, a complex containing Par3 and Par6 has been implicated in control of hippocampal neuron polarity (Shi et al., 2003). Other components of this complex include cdc42 and aPKC, both of which have also been shown to affect axon initiation in vitro (Chen et al., 2006; Schwamborn and Puschel, 2004). A rodent ortholog of Par-1, MARK2, has also been implicated in neuronal polarization (Biernat et al., 2002; Chen et al., 2006). In a model derived from studies of *C. elegans* embryos and mammalian epithelial cells, Par4/LKB1 phosphorylates Par-1/MARK, which phosphorylates Par-3 on two distincts Serine residues creating a binding site for Par-5/14-3-3, which modulates the activity of the Par3/Par6/aPKC complex (Benton and St Johnston, 2003). In neurons, a similar cascade might be important for axon specification, with SAD-A/B acting along with or in place of MARKs (see Figure 7).

LKB1 has several substrates other than SAD-A/B and MARKs. Phosphorylation of one of them, AMPKα, is not affected in LKB1-deficient cortex at birth, but might be affected at later stages. LKB1 activates AMPK in muscle and liver, and conditional mutants lacking LKB1 in these organs exhibit altered metabolism and function (Sakamoto et al., 2005; Shaw et al., 2005). Similarly, AMPK and other substrates of LKB1 might regulate functional properties of adult neurons. Interestingly, SAD-B has recently been implicated in synaptic function (Inoue et al., 2006).

Neuronal Polarization In Vitro versus In Vivo

LKB1 can be phosphorylated on S431 in response to a polarized distribution of extracellular cues or spontaneously in the apparent absence of such cues. In the latter case, local anisotropies in the substrate might exist or small stochastic differences among neurites might be detected and amplified, leading to one neurite becoming an axon (Arimura and Kaibuchi, 2005). Thus, axonal specification could result from either directed (in vivo) or stochastic (in vitro) local phosphorylation of LKB1 and subsequent local activation of SAD kinases (Figure 7). Subsequently, either repression of S431 phosphorylation or active dephosphorylation by ubiquitous phosphatases might repress axonal differentiation or promote dendritic differentiation in other processes. Later still, LKB1 and/or SAD-A/B might be activated in dendrites; indeed, our previous study provided evidence for SAD-dependent changes in dendritic cytoskeleton (Kishi et al., 2005).

In vivo, the initial step of axon/dendrite polarization most likely results from the ability of unpolarized neuron progenitors to polarize intracellular components in response to asymmetrically distributed extracellular cues. In the cerebral cortex, it is now clear that polarization occurs during radial migration, before neurons reach their final position in the CP. The leading process becomes the apical dendrite and the trailing process becomes the axon (Figure 7) (Hand et al., 2005; Hatanaka et al., 2004; Hatanaka and Murakami, 2002; Noctor et al., 2004; Sidman and Rakic, 1973; Solecki et al., 2004). It is therefore important to identify such cues. In *C.elegans,* a local gradient of Netrin dictates the pole of the neuron from which an axon emerges (Adler et al., 2006). In the developing mammalian cortex, a gradient of Semaphorin3A within the CP act as a chemorepulsive cue to bias polarized outgrowth of axons toward the intermediate zone (Polleux et al., 1998). Such extracellular cues might locally activate the kinase signaling cascade identified in this study, regulating key cytoskeletal components and leading to polarized differentiation of axons and dendrites.

EXPERIMENTAL PROCEDURES

Animals

Mice were used according to protocols approved by the Institutional Animal Care and Use Committees at the University of North Carolina-Chapel Hill and Harvard University, and in accordance with NIH guidelines. Time-pregnant females were maintained in a 12 hr light/dark cycle and obtained by overnight breeding with males of the same strain. Noon following breeding is considered as E0.5.

Histochemistry and In Situ Hybridization

In situ hybridization was carried out using digoxigenin-labeled riboprobes as described previously (Dufour et al., 2003). Probes were generated from the following mouse cDNA clones purchased from Open Biosystems (Huntsville, AL): LKB1 (Genbank: BF233256, IMAGE: 4159322). Strad-α (BC058517, IMAGE:5717255). Strad-β (BI220161, IMAGE:5098489), MO25-α (BU509982, IMAGE:6504639).

Cultured neurons and brain sections were immunostained by previously described methods (Polleux and Ghosh, 2002). The following antibodies were used: chicken anti-GFP (Upstate), mouse anti-Tuj1 (β-III tubulin) (Sigma), rabbit anti-pLKB1 (S431) antibodies (Cell Signaling, Santa Cruz), rabbit anti-Tbr1 (gift from Robert Hevner), anti-Cux1 (gift of Dr. Christopher Walsh, Harvard), rabbit anti-total-LKB1 (Upstate), mouse anti-nestin (BD Bioscience), mouse anti-neurofilament 165 (2H3, Developmental Studies Hybridoma Bank), rat anti-L1 (Chemicon), mouse anti-MAP2 (a/b isoforms) Clone AP20 (Sigma), mouse mAb anti-Tau-1 (Chemicon), rabbit anti-activated caspase 3 (Cell Signaling), rabbit anti-phospho-Histone H3 (Cell Signaling), mouse anti-TAG1 (4D7; Developmental Studies Hybridoma Bank), mouse anti-Reelin (Calbiochem), mouse anti-NeuN (Chemicon),

Neurotrace fluorescent Nissl stain (Invitrogen) and the DNA labeling compound Draq5 (Axxora). All images were captured using a LEICA TCS SL confocal microscope.

Constructs

The LKB1-K78I mutant was generously provided by Dr. Lewis Cantley via the Addgene plasmid repository (Addgene plasmid 8591, http://www.addgene.org). All cDNAs were subcloned into a pCIG2 vector (Hand et al., 2005), which contains a (cDNA)-IRES-EGFP under the control of a CMV-enhancer/chicken β-actin promoter. tdTomato was expressed from the plasmid pCIT (gift of Dr. Tom Maynard; UNC Chapel Hill) which utilizes the tdTomato cDNA provided by Dr. Roger Tsien. miR-based shRNAs targeting the coding regions of SAD-A and –B were designed and inserted into vectors using procedures detailed in Supplementary Methods.

Electroporation and Primary Cultures

Mouse cortical progenitors were electroporated ex vivo at embryonic day (E) 14.5 and subsequently cultured as dissociated cells or slices as previously described (Hand et al., 2005; Polleux and Ghosh, 2002) (see Supplementary Methods for details).

Analysis of SAD-A/B Phosphorylation

To generate a pSAD (T-al) antibody, the peptide sequence N-KGDSLLE(pT)SCGSPHY-COOH was synthesized and conjugated to KLH for immunization of New Zealand white rabbits. Terminal bleeds were passed over a column containing the nonphosphorylated version of the immunogenic peptide followed by affinity purification using a column containing the phosphorylated peptide (Covance Research Products, Denver, PA). ELISA and immunoblotting confirmed that the purified antibody lacked reactivity toward the nonphosphorylated peptide as well as the nonphosphorylated SAD-A/B proteins. See Supplementary Methods for more details.

SDS-PAGE immunoblotting using the following antibodies: anti-phospho-SAD-A/B (pSAD (T-al); 1:5000), anti-SAD-A (hybridoma supernatant 1:5), anti-SAD-B (crude serum 1:5000) (Kishi et al., 2005), anti-LKB1 (1:1000, Upstate), anti-AMPK and anti-pAMPK (T172) (1:1000, Cell Signaling), anti-pTau (Ser262) and anti-Tau antibodies were from Stressgen (Ann Arbor, MI).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and eight figures and can be found with this article online at http://www.cell.com/cgi/content/full/129/3/549/DC1/.

ACKNOWLEDGMENTS

We thank Drs. Kevin Jones and Ron DePinho for providing the $Emx1^{Cre}$ and the $LKB1^{Flox}$ mouse lines, respectively; Roger Tsien, Steve Elledge, Robert Hevner, and Christopher A. Walsh for providing reagents; Yongqin Wu for excellent technical assistance; and Eldon Peters, Rocky Cheung, and Hannah Bishop for help making constructs. This project was funded by a Pew Scholar Award in Biomedical Sciences (FP), grants from the NIH-NINDS (to F.P. and J.R.S.), and the NINDS Institutional Center Core Grant to Support Neuroscience Research (P30 NS45892-01). B.N.L. is a fellow of the Damon Runyon Cancer Research Foundation (DRG-1914-06).

Received: October 31, 2006
Revised: January 8, 2007
Accepted: March 5, 2007
Published: May 3, 2007

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Cell, Volume *129*

Supplemental Data

LKB1 and SAD Kinases Define a Pathway Required for the Polarization of Cortical Neurons

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Supplemental Experimental Procedures

Generation and Validation of SAD-A and –B shRNA constructs

miR-based shRNA targeting the coding regions of SAD-A and –B were designed using the shRNA Retriever function on the RNAi Central website (Cold Spring Harbor Laboratories, http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA). Hairpins were inserted into the backbone vectors pSM2C and pPRIME-CMV-GFP (kind gifts of Steve Elledge, Harvard Medical School, Boston, MA; (Stegmeier et al., 2005) using previously described methods (Paddison et al., 2004).

Validation of shRNA construct specificity was done by transfecting 293T or HeLa cells with pcDNA-SAD-A or –B and the RNAi plasmid of interest at a 1:4 ratio. Immunoblotting was performed using SAD-A and –B antibodies to determine the extent of knockdown (see **Fig. 7G** and **Suppl. Fig. 5**). The validated targeting sequences are shown below with the numbers in parentheses indicating the nucleotide coordinates of the respective cDNAs with 1 representing the adenosine of the start codon. The control RNAi is directed against the firefly luciferase coding sequence and has been described in (Stegmeier et al., 2005).

SAD-A (194-214) 5'-AGCGAGAGATTGCCATCTTGA-3'

SAD-B (468-488) 5'-GCCAGAGAACCTGCTGTTGGA-3'

Immunoblotting using pSAD (T-al) antibody

To analyze SAD kinase phosphorylation in vitro, HeLa and 293T cells (ATCC) were cultured in DMEM with 10% (v/v) fetal calf serum, 2mM L-glutamine and penicillin/streptomycin. Transfections were performed in 6 well dishes using FuGene 6 (Roche, Indianapolis, IN) according to the manufacturer's specifications. DNA constructs (0.5 micrograms of each) used for co-transfection were: pcDNA-SAD-A-WT or -T175A (constructed by site-directed mutagenesis and

sequence verified), GFP-4RTau (Kishi et al., 2005) and wild-type or mutant LKB1 vectors described above. Forty-eight hours after transfection, lysates were prepared and analyzed. Anti-pTau (Ser262) and anti-Tau antibodies were from Stressgen (Ann Arbor, MI).

To analyze SAD kinase phosphorylation in vivo, brains of newborn pups were dissected and the right hemisphere of the cerebral cortex was removed and immediately flash frozen in liquid nitrogen. Cortices were homogenized in a denaturing buffer consisting of 100 mM Tris-HCl, 10 mM magnesium acetate, 6 M urea, 2% (w/v) SDS, Complete EDTA-free protease inhibitors (Roche, Indianapolis, IN), phosphatase inhibitor cocktails (Calbiochem) and 25 U/ml Benzonase (Novagen) to reduce lysate viscosity. Lysates were boiled for 5 minutes and protein concentrations were analyzed by BCA assay (Pierce). Equivalent amounts of lysate (20 micrograms) were analyzed by SDS-PAGE (7% resolving gels) and immunoblotting using the following antibodies: anti-phospho-SAD-A/B (pSAD (T-al); 1:5000), anti-SAD-A (hybridoma supernatant 1:5), anti-SAD-B (crude serum 1:5000) (Kishi et al., 2005), anti-LKB1 (1:1000, Upstate), anti-AMPK and anti-pAMPK (T172) (1:1000, Cell Signaling).

Biotinylated Dextran Amine Injections

Injections were carried out as described previously ((Chang et al., 2000); AWP and FP, data not shown). A solution of 10% Biotinylated Dextran Amine (BDA-MW 3000; Molecular Probes) and 0.5% Fast Green in 0.1M PBS (pH 7.4) was microinjected into the cortex. Brains were incubated in oxygenated (95/5% O_2/CO_2) artificial cerebrospinal fluid (5mM KCl, 2mM MgCl, 10mM D-Glucose, 1.25mM NaH_2PO_4, 2mM $CaCl_2$, 125mM NaCl, 25mM $NaHCO_3$) at 37^o C for 5 hours followed by immersion in 4% PFA. Brains were embedded in 3% low gelling point agarose and vibratome sectioned coronally in 100 m intervals. Sections were permeabilized and blocked with 5% goat serum, 3% BSA, 3% Triton, 0.01%, NaN_3 in PBS and probed with 10 g/mL AlexaFluor 546 conjugated Streptavidin (Molecular Probes).

Electroporation and Primary Cultures

Mouse cortical progenitors were electroporated *ex vivo* at embryonic day (E) 14.5 or E15.5 as described (Hand et al., 2005). Briefly, DNA constructs were injected into the lateral ventricle of each embryo and electroporated using an ECM 830 electroporator (BTX) with four 100 ms pulses separated by 100 ms intervals at 40 V. Following electroporation, cerebral cortices were dissected and enzymatically dissociated as described previously (Polleux and Ghosh, 2002). $5x10^4$ cells were plated onto glass coverslips coated with poly-L-lysine and laminin and cultured in serum-free media (Neurobasal containing both B27 and N2 supplements, L-Glutamine and

Penicillin/Streptomycin) and fixed in 4% paraformaldehyde for immunohistochemistry. For slice cultures, embryonic brains were electroporated and dissected as described above. The brains were then embedded in 3% low temperature gelling agarose and 250 μm-thick vibratome sections were cut using a LEICA VT100S vibratome and placed on poly-L-lysine/laminin coated transwell inserts and cultured organotypically using an air interface protocol (Polleux and Ghosh, 2002).

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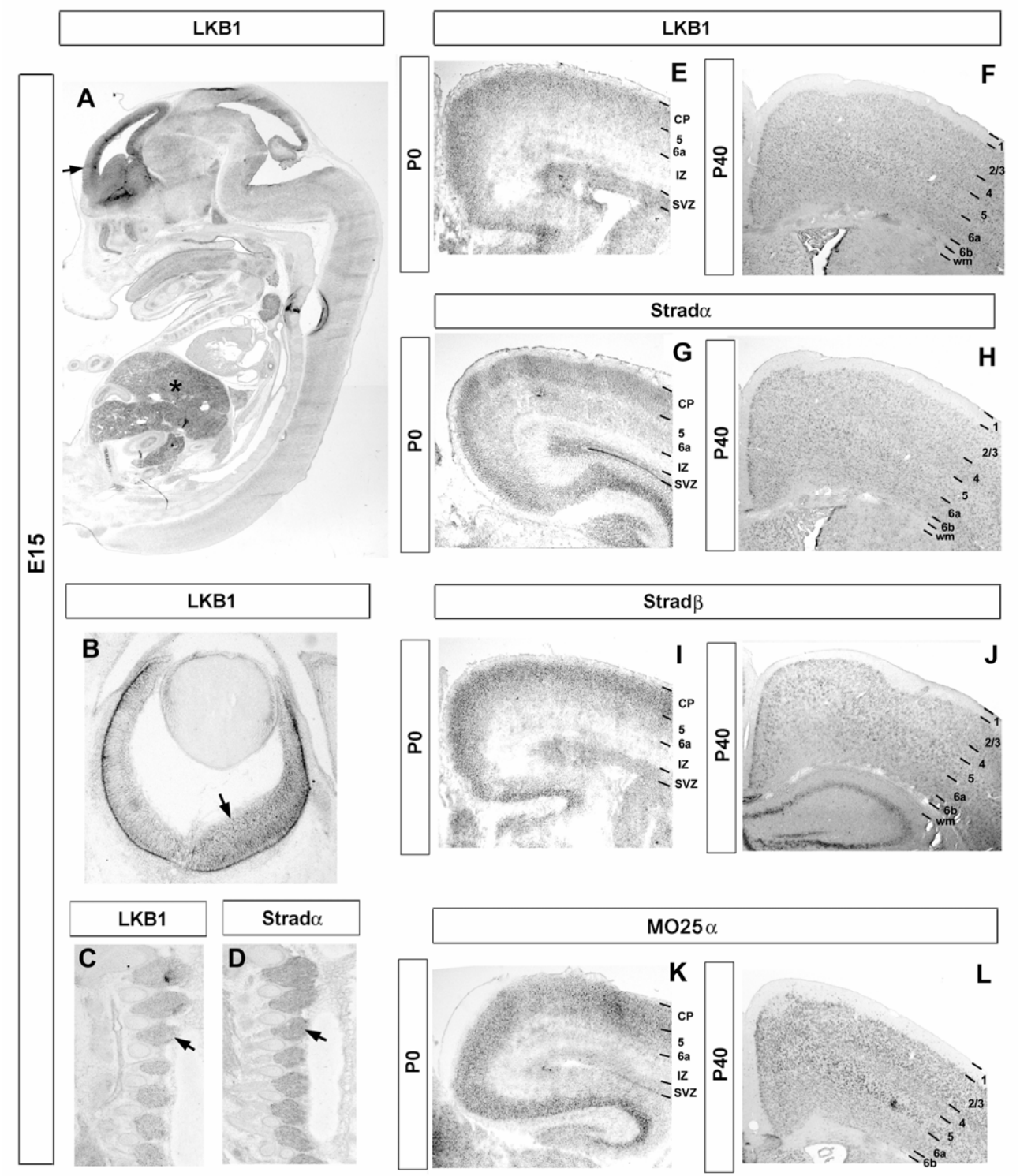
Supplemental Figures



Suppl. Figure 1
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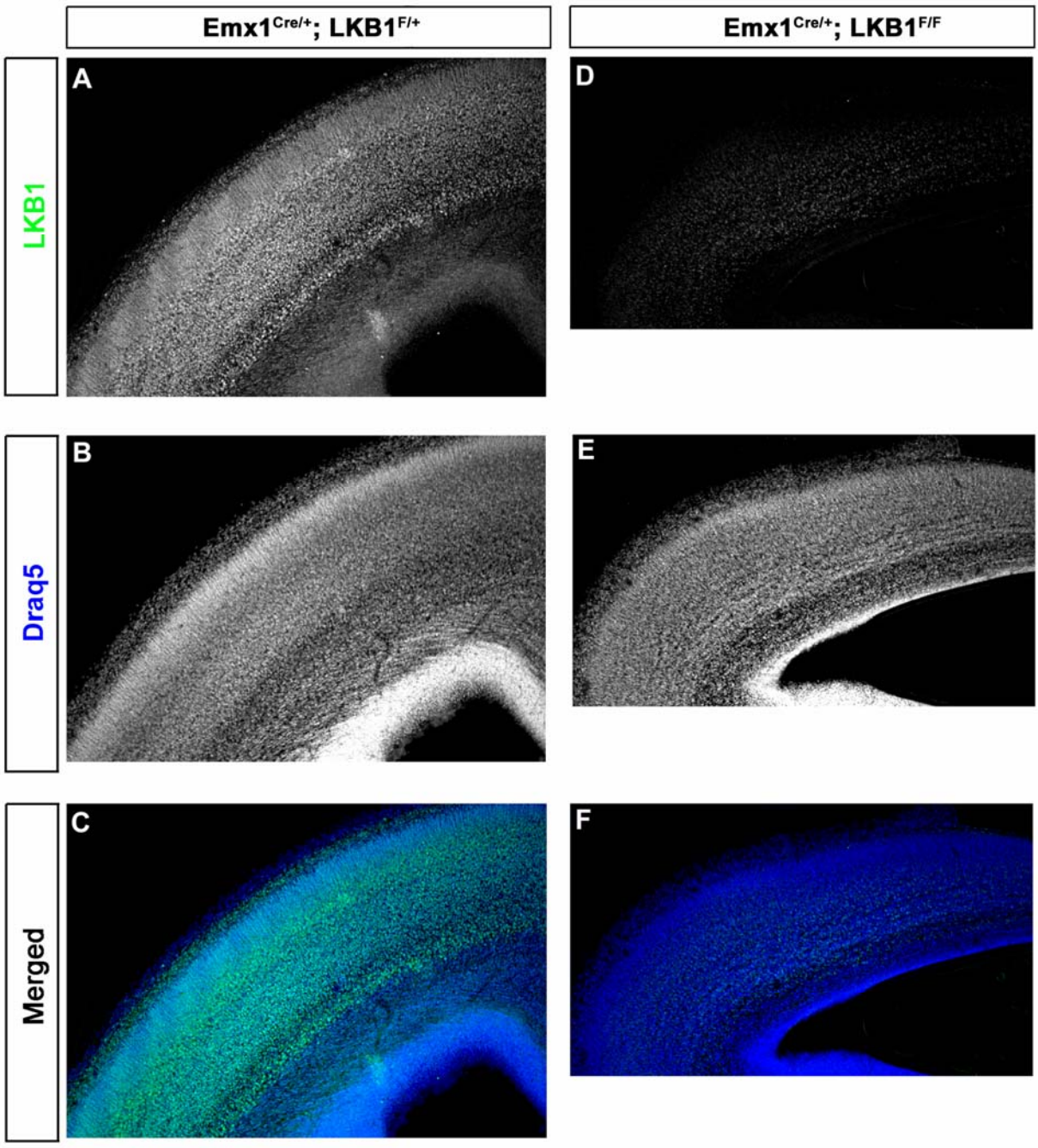
Figure S1. Expression *LKB1* mRNA and its co-activators *Strad*α, *Strad*β and *MO25*α during brain development.

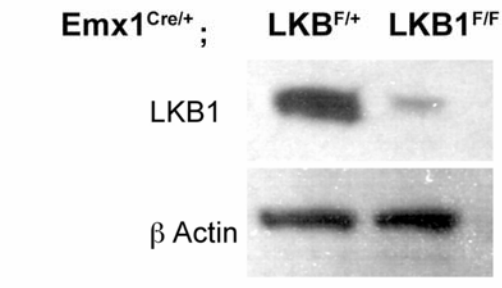
(A) In situ hybridization for *LKB1* on a saggital section of E15.5 mouse embryos reveals its enrichment in the forebrain (arrow) and the liver (star).

(B-D) *LKB1* mRNA is expressed in neural retina (arrow in B) and the dorsal root ganglia (arrows in C) at E15.5 where it is co-expressed with its co-activator Stradα (D).

(E-L) At later stages (P0 and P40) mRNA expression for *LKB1* (E-F) and its co-activators *Strad*α (G-H) *Strad*β (I-J) and *MO25*α (K-L) is maintained in the developing cortex.

Abbreviations: CP, cortical plate; IZ, intermediate zone; SVZ, sub-ventricular zone; wm, white matter.





Suppl. Figure 2
Barnes et al.

Figure S2. Reduction of LKB1 protein expression in the cortex-specific LKB1 conditional knockout mouse.

(A-C) Expression of LKB1 protein in all cortical layers of a post-natal day 1 (P1) mouse (A) as detected by a rabbit polyclonal anti-mouse antibody raised against the C-terminal region of mouse LKB1. Cytoarchitecture is revealed using counterstaining with the nucleic acid dye Draq5 (B).

(D-F) Significant reduction of LKB1 expression in the cortex of a littermate $Emx1^{Cre/+}$; $LKB1^{F/F}$ knockout mouse at P1 (D). Residual staining in D may reflect the fact that $Emx1^{Cre}$ drives expression of Cre-recombinase only in dorsal telencephalic progenitors giving rise to cortical pyramidal neurons but not in ventral telencephalic progenitors giving rise to GABAergic neurons, which comprise 20-25% of all cortical neurons (Gorsky et al. 2002).

(G) Western blot analysis showing reduction of LKB1 expression in the cortex of P1 conditional knockout mouse ($Emx1^{Cre/+}$;$LKB1^{F/F}$) compared to control ($Emx1^{Cre/+}$; $LKB1^{F/+}$) littermate. The blot was re-probed for β-actin as a loading control.

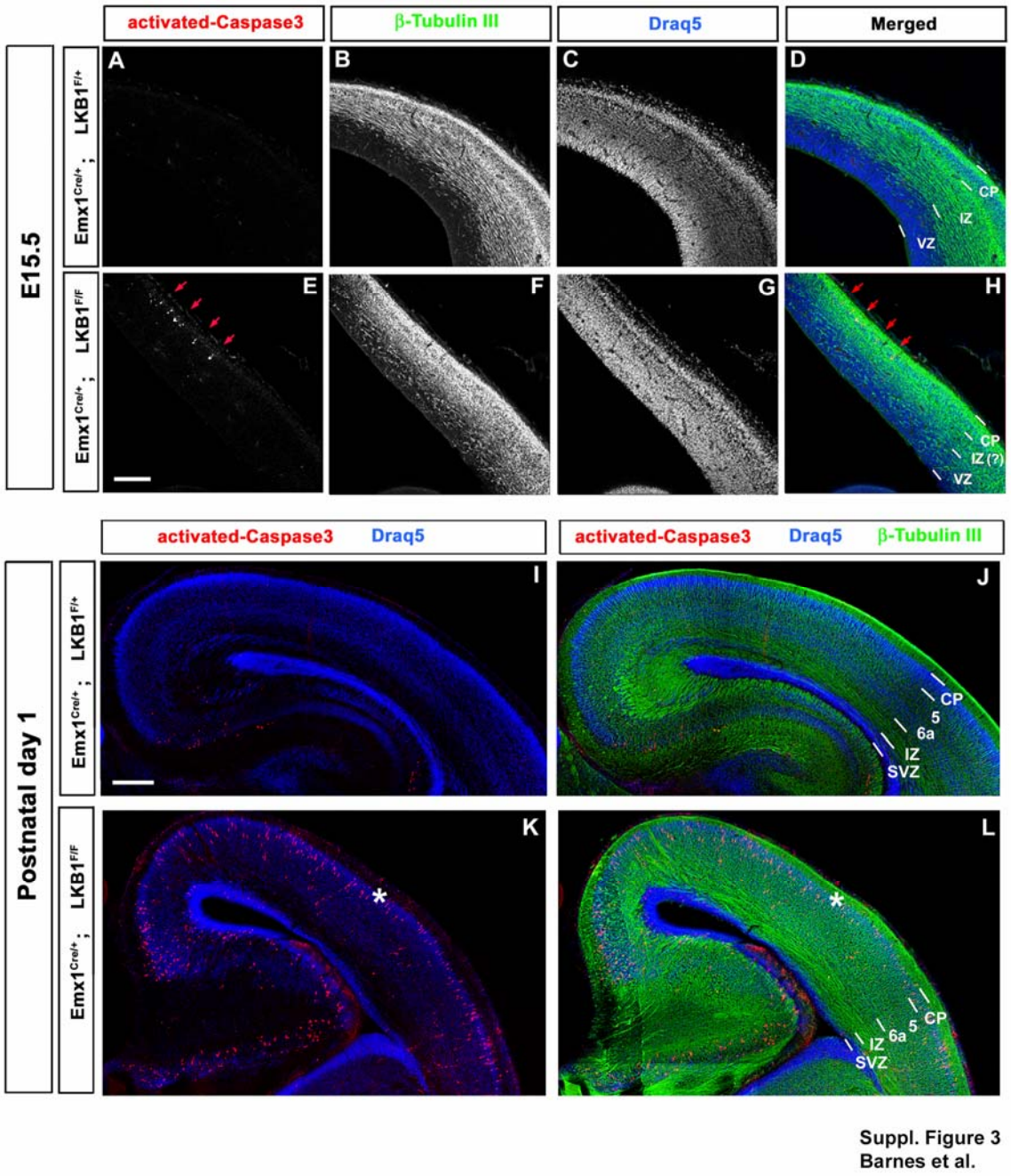


Figure S3. Caspase3-activation in the cortex of conditional LKB1 knockout mice.

(A-H) Sections from E15.5 control and mutant embryos show increased number of Tuj1-positive neurons with high levels of caspase 3 activation (arrows in E and H).

(I-L) At birth (P1) a large number of neurons express high levels of activated-caspase3 in the cortex and hippocampus of LKB1 mutants (K-L). Few neurons express high levels of activated-caspase3 in controls at this stage (I-J). Most cells undergoing apoptosis are located in regions containing post-mitotic neurons expressing β-Tubulin III (star in K-L).

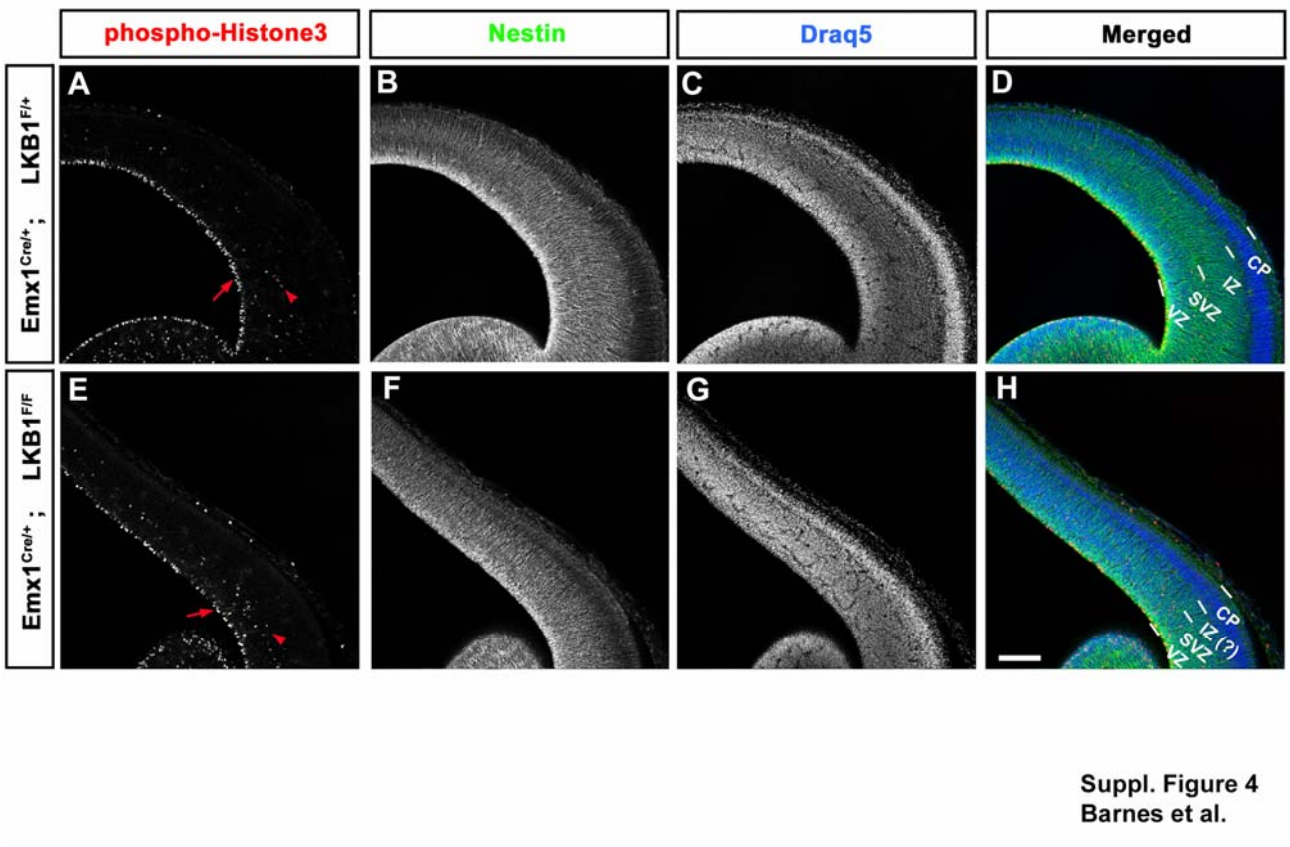


Figure S4. Proliferation of cortical progenitors in the conditional LKB1 knockout embryos at E15.5.

(A-H) Phospho-Histone H3 and Nestin staining in the conditional LKB1 knockout at E15.5 (E-H) reveals no major difference in the number and position of radial glial neural progenitors undergoing mitosis at ventricular (arrows in A and E) and abventricular positions (arrowhead in A and E) when compared to control littermate embryos (A-D).

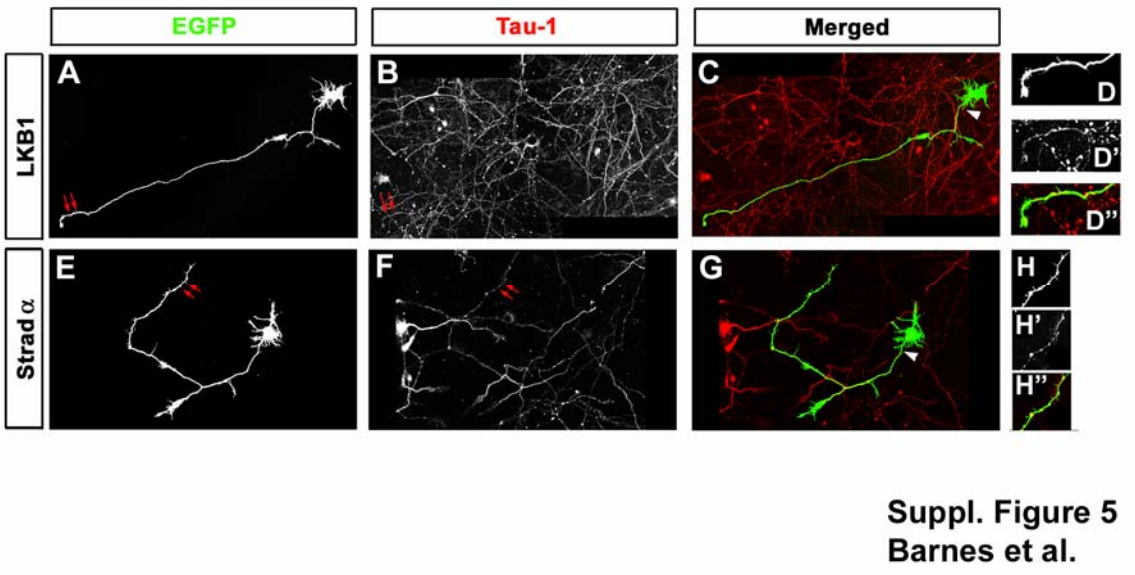


Figure S5. Over-expression of LKB1 or Strada alone is not sufficient to induce multiple axons in cortical neurons.

(A-H) E14.5 cortical neurons electroporated with LKB1 (A-D) or Stradα (E-H) alone present only one Tau1 positive axons (double arrowhead). Panels in E-E'' and H-H'' represent high magnification of Tau1-positive axons shown in A-C and E-G respectively.

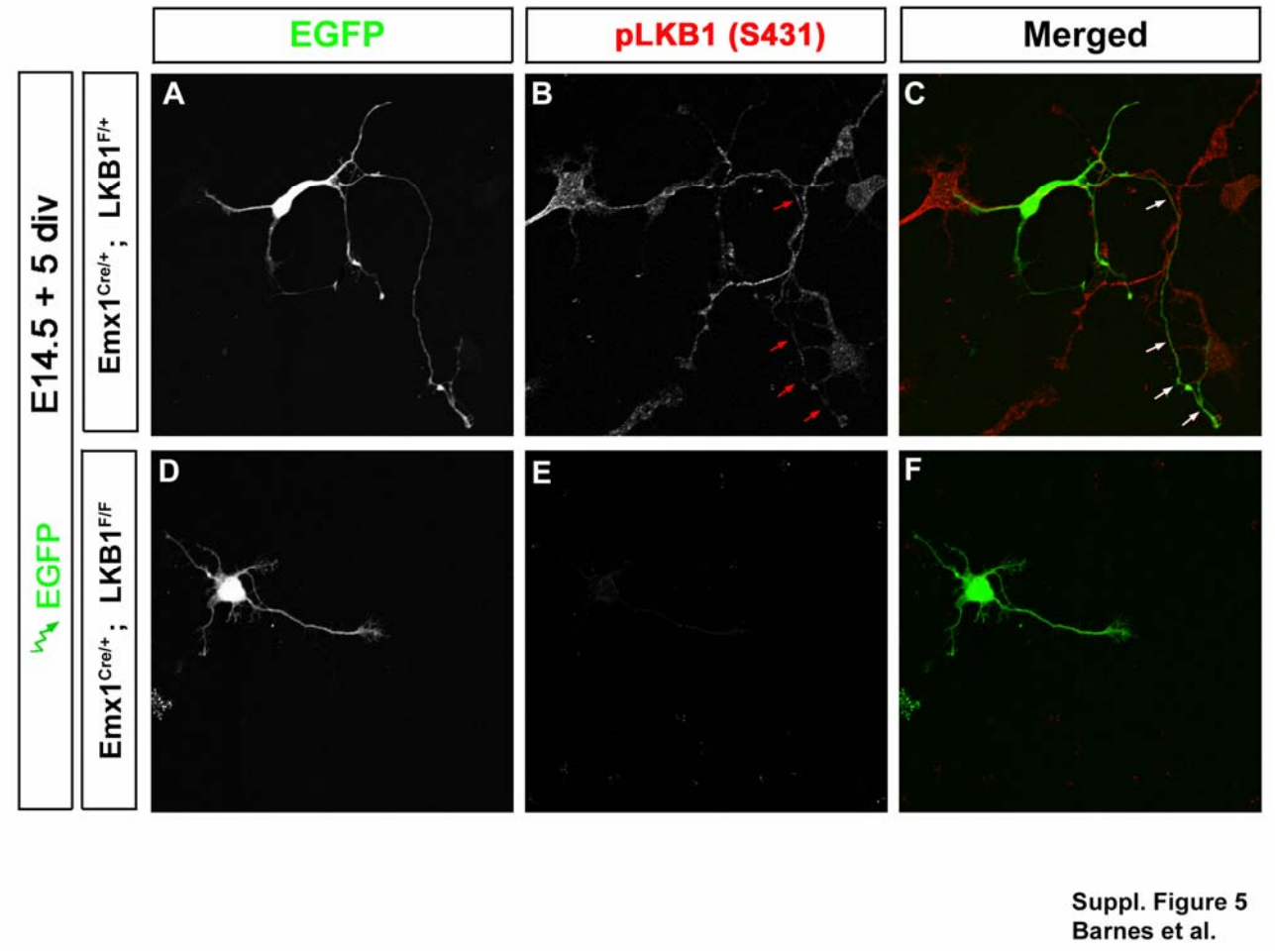


Figure S6. Specificity of the antiserum detecting LKB1 phosphorylated on Serine 431.

(A-C) Control ($Emx1^{Cre/+}$; $LKB1^{F/+}$) E14.5 cortical progenitors electroporated with EGFP and cultured for 5 days in vitro where stained using the antibody recognizing pLKB1 (S431). Anti-pLKB1 (S431) is enriched in axons of cortical neurons (arrows in B-C).

(D-F) The anti-pLKB1 (S431) antibody does not detectably stain neurons cultured from $Emx1^{Cre/+}$; $LKB1^{F/F}$ cortex.

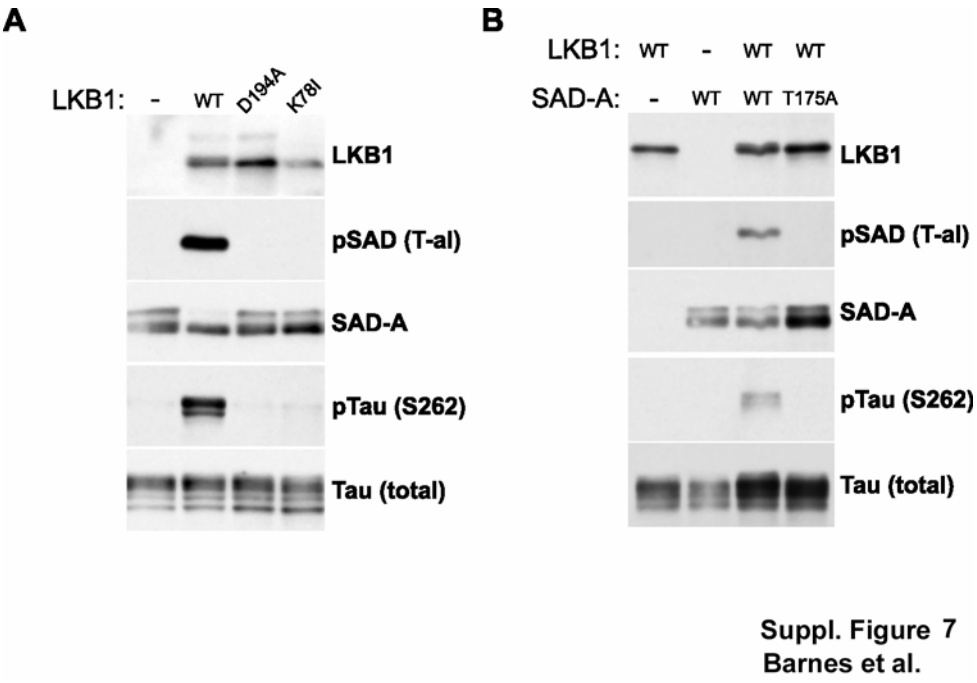


Figure S7. LKB1 kinase activity is required for SAD kinase phosphorylation and activation.

Lysates of HeLa cells co-transfected with the indicated constructs were analyzed by immunoblotting with the antibodies shown.

(A) Expression of SAD-A and wild type LKB1 yields robust SAD-A phosphorylation and activation of SAD-A kinase activity. The kinase-deficient mutants D194A and K78I are unable to phosphorylate and activate SAD-A.

(B) Tau phosphorylation at S262 requires phosphorylation of SAD-A on the activation loop threonine by LKB1. LKB1 does not phosphorylate Tau.

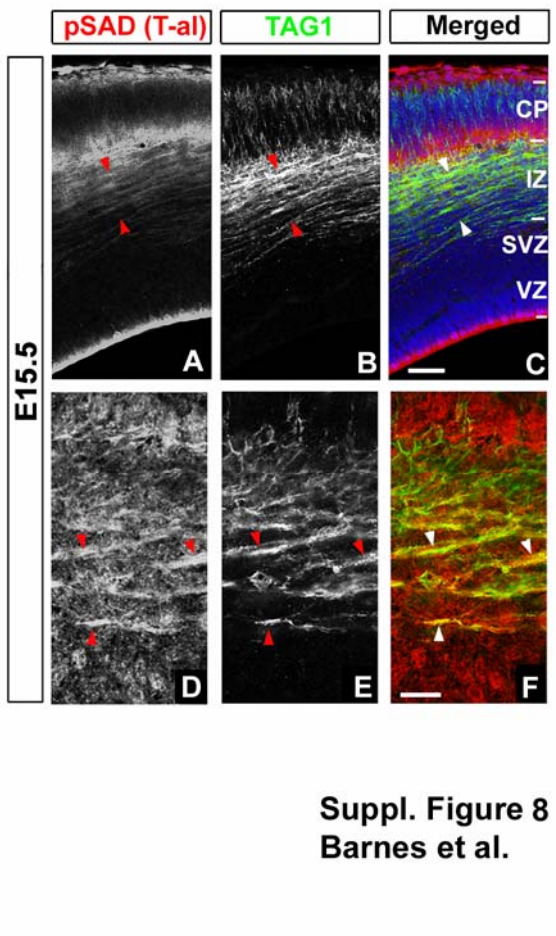


Figure S8. Activated SAD A/B kinases are enriched in corticofugal axons *in vivo*.
(A-F) pSAD (T-al) immunoreactivity is enriched in TAG1-positive corticofugal axons of control cortex at E15.5. Panels D-F show high magnification panels of regions marked by arrowheads in A-C. Arrowheads in D-F emphasize co-localization of pSAD (T-al) and TAG1.