Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 306 (2007) 457–468
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Role of the integrin-linked kinase (ILK) in determining neuronal polarity

Wei Guo [a,b], Hui Jiang [a,b], Virginia Gray [c], Shoukat Dedhar [c], Yi Rao [b,d,*]

[a] *Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Graduate School of the Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China*
[b] *National Institute of Biological Sciences, Beijing, 7 Zhongguanchun Life Sciences Park, Beijing 102206, China*
[c] *BC Cancer Research Centre, 675 West 10th Avenue, Vancouver, British Columbia, Canada V5Z 1L3*
[d] *Department of Neurology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Avenue, Ward 10-185 Chicago, IL 60611, USA*

Received for publication 15 November 2006; revised 13 March 2007; accepted 14 March 2007
Available online 20 March 2007

Abstract

The establishment of axon–dendrite polarity in mammalian neurons has recently been shown to involve the kinases Akt and GSK-3β. Here we report the function of the integrin-linked kinase (ILK) in neuronal polarization. ILK distribution is differential: with more of it present in the axonal tips than that in the dendritic tips of a polarized neuron. Inactivation of ILK by chemical inhibitors, a kinase-inactive mutant or siRNAs inhibited axon formation, whereas a kinase hyperactive ILK mutant induced the formation of multiple axons. Biochemical studies indicate that ILK is upstream of Akt and GSK-3β. Manipulations of multiple intracellular components indicate that ILK is functionally upstream of Akt and GSK-3β but downstream of PI3K in neuronal polarity. These results reveal a key role of ILK in the formation of neuronal polarity and suggest a signaling pathway important for neuronal polarity.

Keywords: Integrin-linked kinase; Neuronal polarity; Glycogen synthase kinase-3β; Akt; PI3K; Axon determination

Introduction

Neuronal function relies on its axon–dendrite polarity. Cellular and molecular studies have used cultured pyramidal neurons from the rodent hippocampus as the standard model for studying mechanisms responsible for neuronal polarization (Bradke and Dotti, 2000; Craig and Banker, 1994; Horton and Ehlers, 2003; Wiggin et al., 2005). A hippocampal pyramidal neuron sends out several initial similar neurites. Later stage, one of the neurites is clearly longer than the others and is positive for molecular markers of the axon, whereas the others become the dendrites (Banker and Cowan, 1977, 1979; Dotti and Banker, 1987; Dotti et al., 1988).

At the subcellular level, neurite competition and centrosomes have been implicated in axon–dendrite specification (Banker and Cowan, 1977, 1979; Dotti and Banker, 1987; Dotti et al., 1988; de Anda et al., 2005). Cytoskeleton dynamics is involved in neuronal polarization (Baas et al., 1988; Bradke and Dotti, 1999). At the molecular level, the evolutionarily conserved complex Par3, Par6 and atypical PKC has been found to play a role in neuronal polarization (Nishimura et al., 2004, 2005; Shi et al., 2003), although not in *Drosophila* (Rolls and Doe, 2004). Two small GTPases Rap1b and Cdc42 (Nishimura et al., 2005; Schwamborn and Puschel, 2004; Shi et al., 2003), and the SAD kinases (Kishi et al., 2005) are involved in neuronal polarization. The SAD kinases are the only components that have so far been found to play a role in neuronal polarization in both invertebrates (Crump et al., 2001) and vertebrates (Kishi et al., 2005). The relationship of these intracellular molecules is not clear.

A potential pathway has been suggested by findings of the roles of Akt and the glycogen synthase kinase-3β (GSK-3β) in establishing and maintaining neuronal polarity (Jiang et al., 2005; Shi et al., 2003; Yoshimura et al., 2005). Their downstream components include the collapsin response mediator protein-2 (CRMP-2) (Yoshimura et al., 2005) and adenomatous polyposis coli (APC) (Shi et al., 2004; Zhou et al., 2004), both regulators of MT dynamics. The upstream regulators of Akt and

* Corresponding author. Department of Neurology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Avenue, Ward 10-185 Chicago, IL 60611, USA. Fax: +1 312 503 6089.
E-mail address: y-rao@northwestern.edu (Y. Rao).

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.03.019

GSK-3β include phosphoinositide-3 kinase (PI3K) and the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Jiang et al., 2005; Shi et al., 2003; Yoshimura et al., 2005). However, none of these upstream molecules can directly regulate the activity of Akt and GSK-3β, there is therefore a major gap in this pathway.

PI3K activation leads to Akt phosphorylation at Ser473 and its activation, which can in turn cause GSK-3β phosphorylation at Ser9 and its inactivation (Cross et al., 1995). There are several enzymes implicated as the Ser473 kinase of Akt including PDK1, Akt itself, ILK, DNA-PKcs and mTOR (Balendran et al., 1999; Feng et al., 2004; Persad et al., 2001; Sarbassov et al., 2005; Toker and Newton, 2000). There are also multiple enzymes whose activation can directly or indirectly lead to GSK-3β inactivation, including the MAP kinase (Brady et al., 1998), p70 ribosomal S6 kinase (p70S6K) (Armstrong et al., 2001), protein kinase A (PKA) (Fang et al., 2000), protein kinase C (PKC) (Ballou et al., 2001) and the integrin-linked kinase (ILK). Our previous studies have failed to find evidence for the involvement of the MAP kinases, PKA, or PKC in the formation of neuronal polarity (Jiang et al., 2005). We have now studied the role of ILK because of its potential roles upstream of both Akt and GSK-3β.

ILK was identified as a β1-integrin cytoplasmic domain binding protein (Hannigan et al., 1996). It contains four ankyrin repeats at the amino terminus followed by a pleckstrin homology (PH) domain and a catalytic domain near the carboxyl terminus (Hannigan et al., 1996). It can be activated by phosphatidylinositol-3,4,5-triphosphate (PIP_3) (Delcommenne et al., 1998; Persad et al., 2001) and can function downstream of

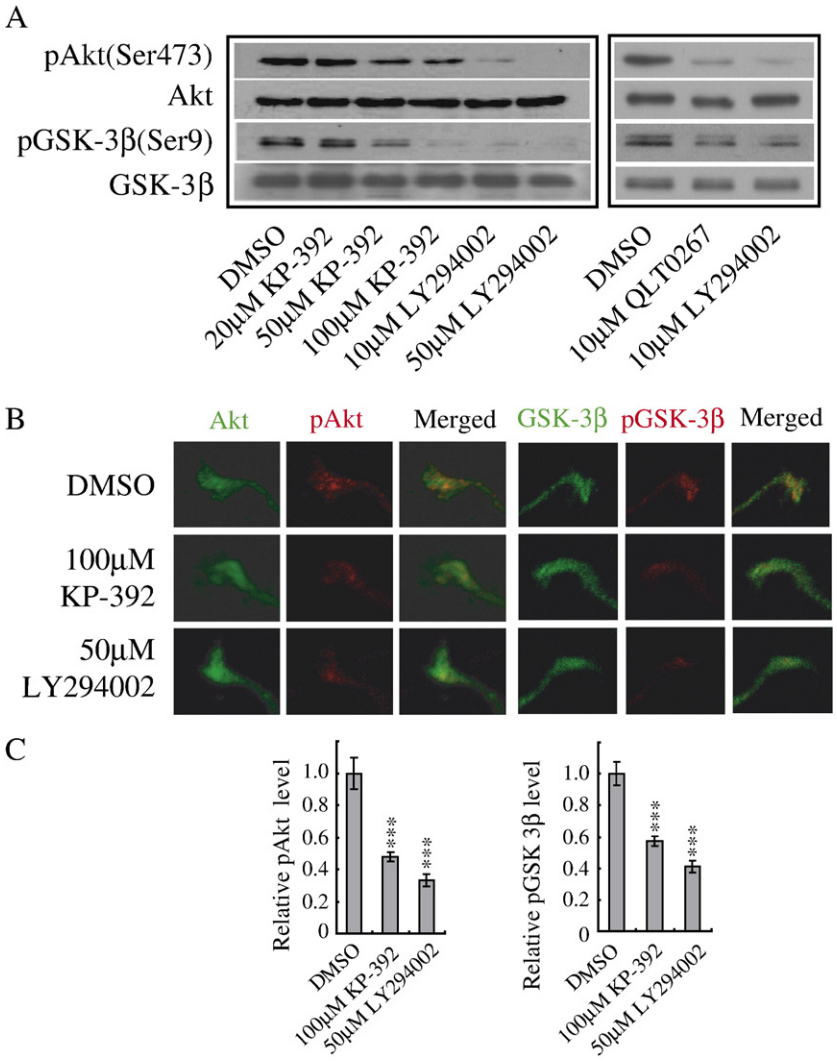

Fig. 1. Effect of ILK inhibition on Akt and GSK-3β phosphorylation in hippocampal neurons. (A) Western analysis of Akt phosphorylation at Ser473 and GSK-3β phosphorylation at Ser9, after treatment with the control vehicle (DMSO), the ILK inhibitor KP-392, QLT0267, and the PI3K inhibitor LY294002. pAkt (Ser473): Akt phosphorylated at Ser473; Akt: total Akt; pGSK-3β (Ser9): GSK-3β phosphorylated at Ser9; GSK-3β: total GSK-3β. (B) Immunostaining of Akt, pAkt, GSK-3β and pGSK-3β in the growth cones after treatment with DMSO, 100 μM KP-392 or 50 μM LY294002. (C) Quantification of pAkt/Akt and pGSK-3β/GSK-3β ratios in growth cones. The ratios in growth cones were relative with that in DMSO treated neurons as 1. pAkt/Akt ratios for DMSO treatment: 1.0±0.09 (n=15), KP-392: 0.48±0.03 (n=15), LY294002: 0.33±0.4 (n=15). pGSK-3β/GSK-3β ratios for DMSO: 1.0±0.07 (n=10), KP-392: 0.57±0.03 (n=10), LY294002: 0.41±0.4 (n=10). Statistical analyses were performed between DMSO and ILK inhibitors. Asterisks indicate statistical significance (Student's t test; ***$p < 0.001$).

growth factor receptors and integrins (Dedhar et al., 1999; Hannigan et al., 2005; Wu and Dedhar, 2001). In vitro biochemical assays and cell biology have shown that ILK could phosphorylate Akt at Ser473 (Delcommenne et al., 1998; Lynch et al., 1999; Persad et al., 2001; Troussard et al., 2003), and GSK-3β at Ser9 (Delcommenne et al., 1998; Lynch et al., 1999; Persad et al., 2001; Troussard et al., 2003).

These considerations led us to investigate the involvement of ILK in determining the axon–dendrite polarity and to examine its functional relationship with the Akt/GSK-3β pathway. We report here that ILK distribution is preferential in axonal tips of polarized neurons, that biochemically it is required for phosphorylation of Akt at Ser473 and GSK-3β at Ser9 in hippocampal neurons, and that ILK is functionally upstream of Akt and GSK-3β in determining neuronal polarity. These results demonstrate that ILK is a key component in the signaling pathway involved in mammalian neuronal polarization.

Materials and methods

Materials

Antibodies

Antibodies (and their suppliers) used are: anti-ILK (Upstate and Cell Signaling), anti-Tau-1 (Chemicon), anti-MAP2 (Chemicon), anti-synapsin1 (Chemicon), anti-GSK-3β (Chemicon and Cell Signaling), anti-phospho-GSK-3β (Ser9) (Cell Signaling), anti-Akt (Cell Signaling and New England Biolabs), and anti-phospho-Akt (Ser473) (Cell Signaling).

Chemicals

LY294002 was purchased from Calbiochem and GSK-3β inhibitor SB415286 from Tocris.

KP-392 (also known as KP-SD-1) was identified in a high-throughput kinase assay using highly purified recombinant ILK. It has a high degree of selectivity when tested against 150 protein kinases, and shows greater than 100-fold selectivity over PDK-1, DNA-PK, GSK-3 and PKB isoforms (unpublished data). KP-392 has previously been extensively characterized in terms of inhibiting PKB and GSK-3 phosphorylation in a variety of cell types, including neurons (Mills et al., 2003; Persad et al., 2001; Troussard et al., 2000).

QLT0267 is a second-generation ILK inhibitor, derived from KP-392. It is more potent than KP-392 and inhibits ILK kinase activity at 26 nmol/L in a cell-free assay using highly purified recombinant ILK. It has a similar selectivity profile to KP-392, but inhibits activation of downstream effectors of ILK in cells at between 1 and 10 micromolar concentrations. Among 150 kinases tested under similar conditions, QLT0267 is highly specific, showing ~1000-fold selectivity over kinases including CK2, CSK, DNA-PK, PIM-1, PKB/Akt and PKC, and ~100-fold selectivity over other kinases such as Erk-1, GSK-3β, LCK, PKA, p70S6K, and RSK1 (Troussard et al., 2006; Younes et al., 2005). The effects of QLT0267 are similar to those of dominant-negative ILK mutants and siRNA. Furthermore, constitutively active ILK protects the downstream substrates from inhibition by QLT0267 (Koul et al., 2005; Younes et al., 2005).

DNA constructs

ILK-WT, ILK-S343A and ILK-S343D were generously provided to us by Shoukat Dedhar, GSK-3β-S9A/pCS2 was generously provided to us

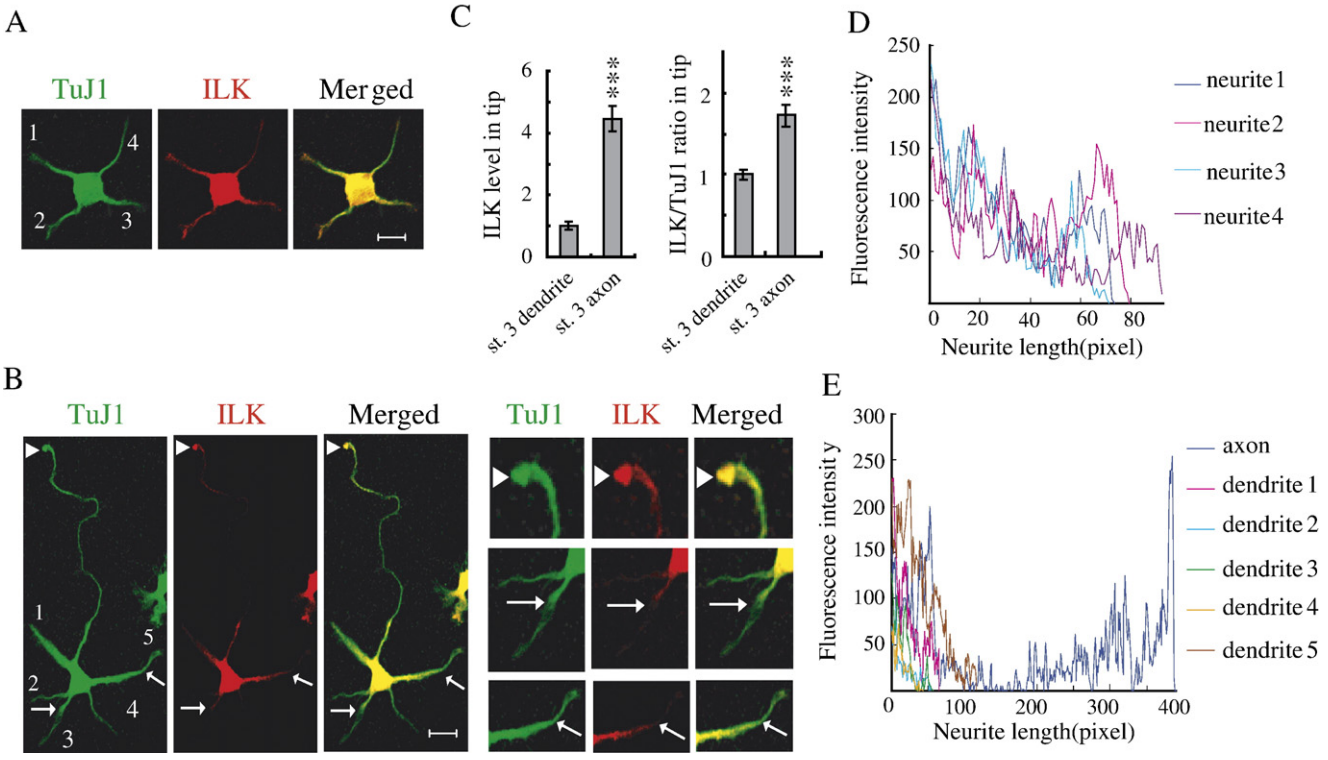

Fig. 2. Distribution of ILK in axonal and dendritic tips of hippocampal neurons. (A and B) Distribution of ILK in st. 2 and st. 3 rat hippocampal neurons. Immunocytochemical staining with an anti-ILK antibody (in red) and the anti-TuJ1 antibody (in green). The small panels on the right part of (B) are higher magnification pictures of the tips of axons and dendrites. The scale bar is 20 μm. Note that, in panel B, ILK staining can be detected in the tip of the axon, whereas ILK is not detected in the tips of the dendrites. The white arrows in panel B marked the ending points of ILK staining while the staining of TuJ1 is detectable distal to these points. (C) Relative ILK levels and ILK/TuJ1 ratios in neurite tips of st. 3 neurons. The average level of ILK in dendrites was normalized to 1.00±0.14 (n=12). The relative level in axons was 4.46±0.40 (n=12). The ILK/TuJ1 ratio in dendrites was normalized to 1.00±0.06 (n=12). The relative ratio in axons was 1.72±0.12 (n=12). Asterisks indicate statistical significance (Student's t test; ***$p<0.001$). (D and E) Fluorescence intensities of anti-ILK immunostaining in the neurites of st. 2 and st. 3 neurons. Fluorescence intensities were measured from the neurites, as shown in panels A and B.

by P. S. Klein. Myr-HA-Akt/pLNCX was a generous gift from W.R. Sellers.

Neuronal culture and transfection

Hippocampal neuronal cultures have been described previously (Jiang et al., 2005). For inhibition of neuronal polarity, the inhibitors were added to the culture media during medium changes. For Western analysis and ILK inhibition in neurite tips, all inhibitors were added 5 h before lysis. Transfection of neurons was carried out immediately after dissociation, using the Amaxa Nucleofector device following the manufacturer's protocol. Plasmids were co-transfected with GFP at a ratio of 3:1. After the indicated time points, neurons were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4 °C for 20 min and processed for immunocytochemistry.

Immunocytochemistry

Fixed neurons were washed with PBS three times for 10 min, and treated with PBS containing 0.1% Triton X-100 for 10 min. Neurons were then blocked by 10% normal goat serum in PBS for 1 h at room temperature. After blocking, neurons were incubated with primary antibodies at 4 °C overnight. After washing with PBS for 6×5 min, secondary antibodies were added and incubated at room temperature for 1 h, washed with PBS washing for 6×5 min before visualization under a fluorescent microscope.

Imaging

Images were acquired with a Zeiss 20× or 40× objective of confocal laser-scanning microscope. The fluorescence levels were based on single confocal planes. Images were collected sequentially (multi-track). The excitation lasers were of 488 nm and 543 nm, and the emission at 505–530 nm and 560–615 nm. For the relative levels of pAkt and pGSK-3β in Fig. 1C, and ILK in Fig. 2C, fluorescence intensities were measured in the entire growth cone or the neurite tip. The ILK fluorescence intensities in Figs. 2D, E are based on line profiles through the neurites. Morphometric measurements were performed using the Metamorph software (Universal Imaging) by setting a threshold of fluorescence intensity and were automatically counted and logged into Excel.

SiRNAs

Knockdown of ILK was achieved by oligo RNA-based siRNAs. The target sequence is 5′-CCC CUG AAG CCC UGC AAA A-3′ for ILK-R. The precise sequences for ILK-R are 5′-CCC CUG AAG CCC UGC AAA ATT-3′ and 5′-UUU UGC AGG GCU UCA GGG GTT-3′ for rat ILK. The control (non-targeting) sequences are 5′-UUC UCC GAA CGU GUC ACG UTT-3′ and 5′-ACG UGA CAC GUU CGG AGA ATT-3′. The relevant sequence of human ILK is 5′-CCC CCG AAG CUC UGC AGA A-3′ and cannot be targeted by the designed RNAi. Neurons were transfected using the Amaxa Nucleofector device. For Western analysis, neurons were harvested 48 h after transfection. For immunostaining, neurons were fixed 5 days after transfection.

Western analysis

Cells were lysed in the lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 μg/ml leupeptin). Samples were solubilized with the loading buffer, heated to 100 °C for 5 min, and resolved by SDS–PAGE using 10% acrylamide. Equal amounts of samples were loaded into discontinuous gels (10% acrylamide tested under reducing conditions). Proteins were transferred to PVDF membrane for 1.5 h at 100 V, blocked with 5% BSA for 1 h at room temperature. Membranes were then incubated with the primary antibodies in blocking solutions at 4 °C overnight before detection with HRP-conjugated secondary antibodies. Chemiluminescence was detected with ECL solution.

Results

Requirement of ILK for Akt and GSK-3β phosphorylation in hippocampal neurons

Biochemically, ILK can phosphorylate Akt at Ser473 and GSK-3β at Ser9 in vitro or in several cell types (Delcommenne et al., 1998; Lynch et al., 1999; Persad et al., 2001; Troussard et al., 2003). We examined whether ILK was required for the

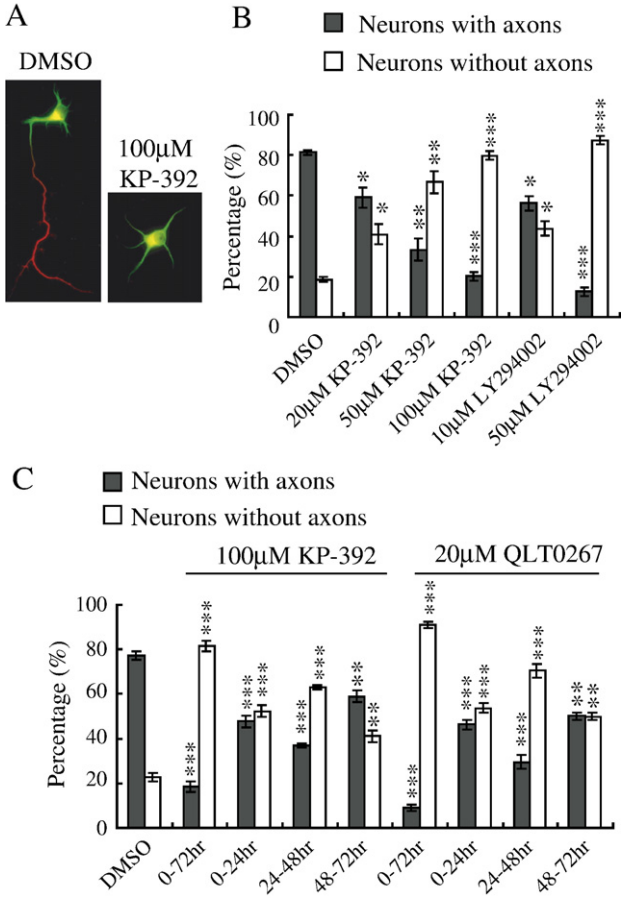

Fig. 3. Loss of neuronal polarity upon treatment with the inhibitors KP-392 and QLT0267. (A) The ILK inhibitor KP-392 inhibited the establishment of neuronal polarity. Neurons were stained with anti-MAP2 (green) and Tau-1 (red). The left panel shows a neuron with normal polarity (with an axon in red and dendrites in green) in the presence of DMSO. The right panel shows a neuron without polarity after treatment with 100 μM KP-392: it has 5 dendrites (staining green for anti-MAP2) but no axons (staining red for Tau-1). (B) Quantification of polarity defects in neurons treated with DMSO, KP-392 or the PI3K inhibitor LY294002. DMSO: 81.4±1.1% polarized neurons (n=202); 20 μM KP-392: 50.0±5.0% (n=193); 50 μM KP-392: 33.4±5.3% (n=198); 100 μM KP-392: 20.2±2.2% (n=182); 10 μM LY294002: 56.2±3.5% (n=119); 50 μM LY294002: 12.6±2.1% (n=155). (C) Quantification of polarity defects in neurons treated with 100 μM KP-392 and 20 μM QLT0267 in different time windows. DMSO: 77.3±1.8% polarized neurons (n=226). 100 μM KP-392: 18.4±2.3% (n=313) from 0 to 72 h; 47.8±2.6% (n=216) from 0 to 24 h; 37.0±0.9% (n=269) from 24 to 48 h; 58.9±2.5% (n=257) from 48 to 72 h. 20 μM QLT0267: 9.0±1.4% (n=273) from 0 to 72 h; 46.2±2.1% (n=274) from 0 to 24 h; 29.5±3.0% (n=228) from 24 to 48 h; 50.1±1.7% (n=220) from 48 to 72 h. Statistical analyses were performed between DMSO and ILK inhibitors. Asterisks indicate statistical significance (Student's t test; *$p<0.05$, **$p<0.01$, ***$p<0.001$).

phosphorylation of Akt and GSK-3β in hippocampal neurons during the developmental stages when neuronal polarity was formed.

Hippocampal neurons were isolated from embryonic day 18 (E18) SD rats. We treated cultured neurons with the vehicle DMSO, or different concentrations of the ILK inhibitor KP-392 (Mills et al., 2003; Persad et al., 2001; Troussard et al., 2000), or another ILK inhibitor QLT0267 (Troussard et al., 2006; Younes et al., 2005), or the PI3K inhibitor LY294002. The specificities of the ILK inhibitors have been demonstrated and discussed in Materials and methods.

Akt phosphorylation at Ser473 and GSK-3β phosphorylation at Ser9 were detected with specific antibodies. LY294002 served as a positive control because we have previously shown that it inhibited Akt and GSK-3β phosphorylation (Jiang et al., 2005) (Fig. 1A). When the extracts from hippocampal neurons were assayed, the ILK inhibitor KP-392 and QLT0267 both reduced phosphorylation of Akt at Ser473 and GSK-3β at Ser9

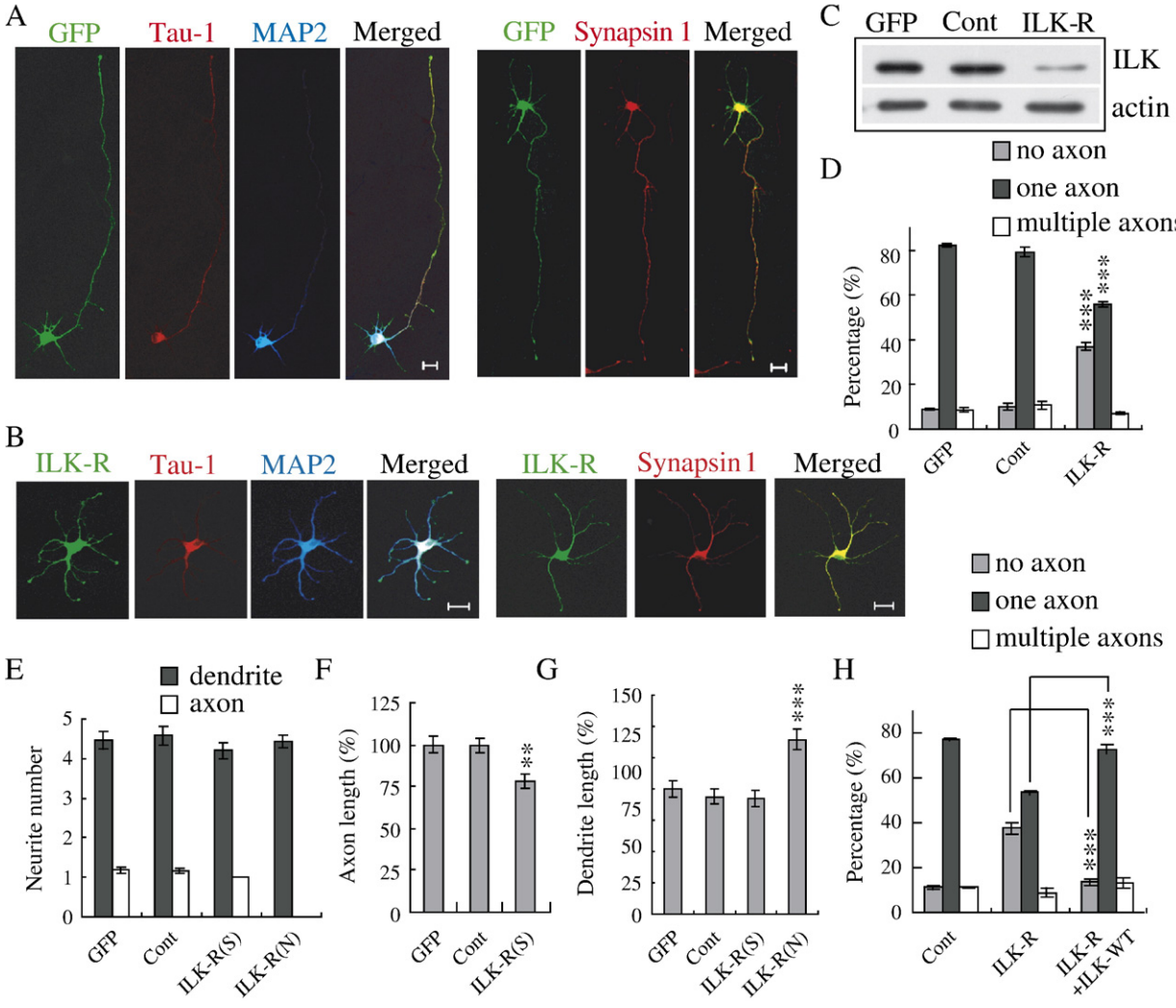

Fig. 4. Inhibition of Neuronal Polarity by an ILK siRNA. (A) Neurons transfected with GFP alone showing normal localization of the dendritic marker MAP2 in the soma, the entire length of dendrites, and the proximal part of the axon, whereas the axonal markers Tau-1 and synapsin 1 in the soma and the distal part of the axon. (B) Neurons cotransfected with GFP and the ILK siRNA (ILK-R) were stained with dendritic and axonal markers. The axon was lost from neurons transfected with ILK-R. (C) Western analysis showing reduction of ILK protein expression in hippocampal neurons after ILK-R siRNA treatment. (D) Quantification of polarity defects. Cont: control siRNA. Neurons with no axon: GFP 9.1±0.41% (n=203), Cont 10.1±1.6% (n=205), ILK-R 36.9±1.8% (n=175). Neurons with a single axon: GFP 82.3±0.93%; Cont 79.2±2.1%, ILK-R 55.9±1.2%. Neurons with multiple axons: GFP 8.7±1.1%, Cont 10.7±1.8%, ILK-R 7.2±0.73%. (E) Number of neurites per neuron. Numbers of dendrites in neurons transfected with ILK-R were counted in two categories: (S) being neurons with single axons while (N) being neurons with no axons. GFP (n=47): 1.18±0.08 axons and 4.47±0.22 dendrites. Cont (n=47): 1.17±0.06 axons and 4.58±0.23 dendrites. ILK-R(S) (n=47): 1 axons and 4.2±0.20 dendrites. ILK-R(N) (n=47): 0 axons and 4.44±0.16 dendrites. (F) The average axon length of GFP-transfected neurons was normalized as 100%. GFP (n=65): 100±5.4%. Cont (n=66): 99±4.6%. ILK-R(S) (n=67): 77±4.2%. (G) The average dendrite length of GFP-transfected neurons was normalized as 100%. GFP (n=104): 100±6.3%. Cont (n=120): 94±5.6%. ILK-R(S) (n=85): 92±6.6%. ILK-R(N) (n=95): 139±8.1%. Statistical analyses were performed between Cont and ILK-R. (H) ILK-R effect was rescued by a human wild type ILK cDNA. Quantification of polarity defects. Neurons with no axon: Cont 11.3±0.80% (n=285), ILK-R 37.6±2.4% (n=289), ILK-R+ILK-WT 13.7±1.4% (n=290). Neurons with a single axon: Cont 77.4±0.77%, ILK-R 53.7±1.0%, ILK-R+ILK-WT 73.1±2.1%. Neurons with multiple axons: Cont 11.4±0.36%, ILK-R 8.7±1.9%, ILK-R+ILK-WT 13.2±2.3%. Asterisks indicate statistical significance (Student's t test; $^{**}p<0.01$, $^{***}p<0.001$).

(Fig. 1A and Supplementary Fig. 1). Because the functionally important difference of activity is that in the tips of neurites (Jiang et al., 2005; Shi et al., 2003), we also examined the distribution of phospho-Akt and phospho-GSK-3β in the neurite tips of polarized stage (st.) 3 neurons. Both the ILK inhibitor KP-392 and the PI3K inhibitor LY294002 decreased the phosphorylation of both Akt and GSK-3β (Figs. 1B, C).

Distribution of ILK in the neurites of hippocampal neurons

To investigate the significance of ILK in neuronal polarity, we determined the distribution of ILK in hippocampal neurons during the developmental stages relevant to neuronal polarity. Hippocampal neurons from E18 rats were cultured and stained with a monoclonal antibody specifically recognizing ILK (Mills et al., 2003) and the TuJ1 antibody recognizing the neuronal-specific class III β-tubulin.

ILK was found in the tips of all neurites in st. 2 non-polarized neurons (Figs. 2A, D and Supplementary Fig. 2). However, there is more ILK present in axonal tips than that in dendritic tips in st. 3 polarized neurons (Figs. 2B, E and Supplementary Fig. 2). The relative ILK level and the ILK/TuJ1 ratio in the axons were both significantly higher than those in dendrites of the polarized st. 3 neurons (Fig. 2C).

Inhibition of neuronal polarity by an ILK pharmacological inhibitor, siRNA and a kinase inactive ILK mutant

To study the functional role of ILK, we used three different ways to inhibit ILK: KP-392 and QLT0267 (two specific pharmacological inhibitors), ILK-S343A (a kinase inactive ILK mutant) and small interfering RNA (siRNA) (Persad et al., 2001). The PI3K inhibitor LY294002 is known to inhibit neuronal polarization (Shi et al., 2003) and was used as a positive control. The polarity of neurons were judged both by the length of longest neurite being 2 times longer than other neurites and the localization of molecular markers revealed by immunostaining with specific antibodies. Tau-1 stained the distal part of axons whereas the anti-MAP2 antibody stained the entire length of dendrites and the proximal part of axons (as well as the soma). Synapsin1, which is a presynaptic protein, is also a marker for axons.

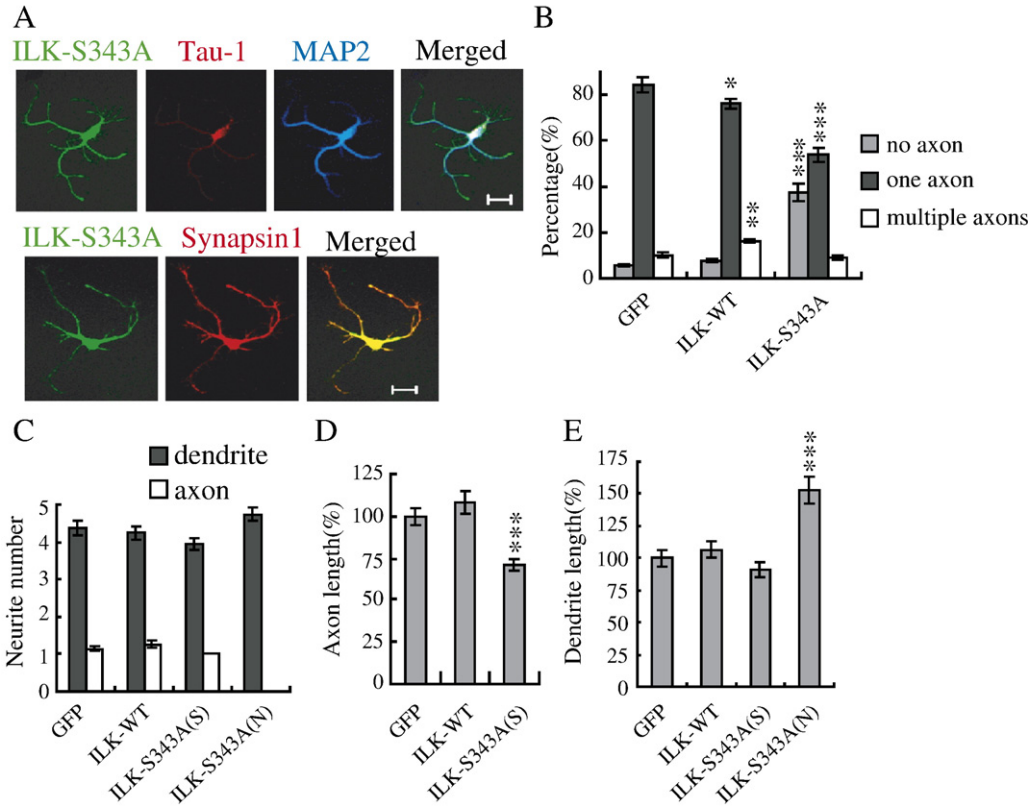

Fig. 5. Inhibition of axon formation by a kinase inactive ILK mutant. (A) Neurons transfected with ILK-S343A and GFP were stained with dendritic and axonal markers. ILK-S343A transfection caused the loss of the axon. (B) Quantification of polarity defects. Neurons with no axon: GFP 5.5±0.51% (n=344), ILK-WT 7.7±0.77% (n=252), ILK-S343A 37.3±3.9% (n=198). Neurons with a single axon: GFP 84.5±1.6%, ILK-WT 76.0±1.0%, ILK-S343A 53.8±3.1%. Neurons with multiple axons: GFP 10.0±1.2%, ILK-WT 16.3±0.40%, ILK-S343A 8.9±0.93%. Statistical analyses were performed between GFP and ILK-WT or ILK-S343A. (C) Number of neurites per neuron. The numbers of dendrites of neurons cotransfected with ILK-S343A and GFP were counted in two categories: (S) being neurons with single axons while (N) being neurons with no axons. GFP (n=44): 1.16±0.08 axons and 4.36±0.20 dendrites. ILK-WT (n=45): 1.27±0.09 axons and 4.24±0.18 dendrites. ILK-S343A(S) (n=38): 1 axons and 3.95±0.17 dendrites. ILK-S343A(N) (n=42): 0 axons and 4.74±0.17 dendrites. (D) The average axon length of GFP-transfected neurons was normalized as 100%. GFP (n=60): 100±5.2%. ILK-WT (n=64): 109±6.6%. ILK-S343A(S) (n=62): 71±3.5%. (E) The average dendrite length of GFP-transfected neurons was normalized as 100%. GFP (n=149): 100±5.0%. ILK-WT (n=120): 106±6.1%. ILK-S343A(S) (n=91): 91±5.8%. ILK-S343A(N) (n=91): 152±10%. Asterisks indicate statistical significance (Student's t test; *p<0.05, **p<0.01, ***p<0.001).

Cultured hippocampal neurons were treated with DMSO, KP-392 or LY294002 for 3 days. KP-392 increased the number of neurons without polarity in a dose dependent manner: axon formation was inhibited by KP-392 (Figs. 3A, B), as did LY2 94002. We also used another ILK inhibitor QLT0267 which is more potent than KP-392 and highly selective for ILK (Koul et al., 2005; Younes et al., 2005). We found that QLT0267 also inhibited axon formation. There were only 46.3±3.2% polarized neurons (n=266) treated with 10 μM QLT0267 relatively to 78.7±1.5% polarized neuron (n=260) treated with DMSO. To determine whether continuous treatment for 3 days are required to see the effect, we treated the hippocampal neurons with the inhibitors in different time windows and found they affected axon formation and the establishment of neuronal polarity with 24-h treatment in any of the first 3 days (Fig. 3C).

We designed a siRNA ILK-R for ILK. It was co-transfected into rat hippocampal neurons with a cDNA encoding GFP. Western analysis showed that ILK-R could decrease the level of ILK efficiently (Fig. 4C). Neurons transfected with siRNAs and GFP were cultured for 5 days and stained with axonal and dendritic markers. To exclude the possibilities that ILK down-regulation reduced neuronal development or axonal growth, we analyzed the transfected neurons at day 5, when they were more mature than those at 3 days. At this stage synapsin1 is an additional axon marker, although tau-1 could mark axons early at day 2.

ILK-R could reduce the number of neurons with axons and increase the number of neurons without axons (Figs. 4A, B for staining and D for statistics). The number of dendrites was not affected by ILK siRNAs (Fig. 4E). In neurons whose axons were still present (ILK-R (S) in Fig. 4F), axon length was reduced. Only in neurons without axons (ILK-R (N) in Fig. 4G), the length of dendrites was increased. Because the siRNA ILK-R used here only targets the rat, but not the human, we used a cDNA expressing the wild type human ILK cDNA to examine whether the effect caused by siRNA could be rescued and found that the human ILK cDNA indeed reversed the phenotype of siRNA (Fig. 4H). Taken together, these results indicate that ILK siRNAs could inhibit axon formation in two manners: either completely eliminating axons, or reducing axon length. The effect on dendrite length was conditional upon

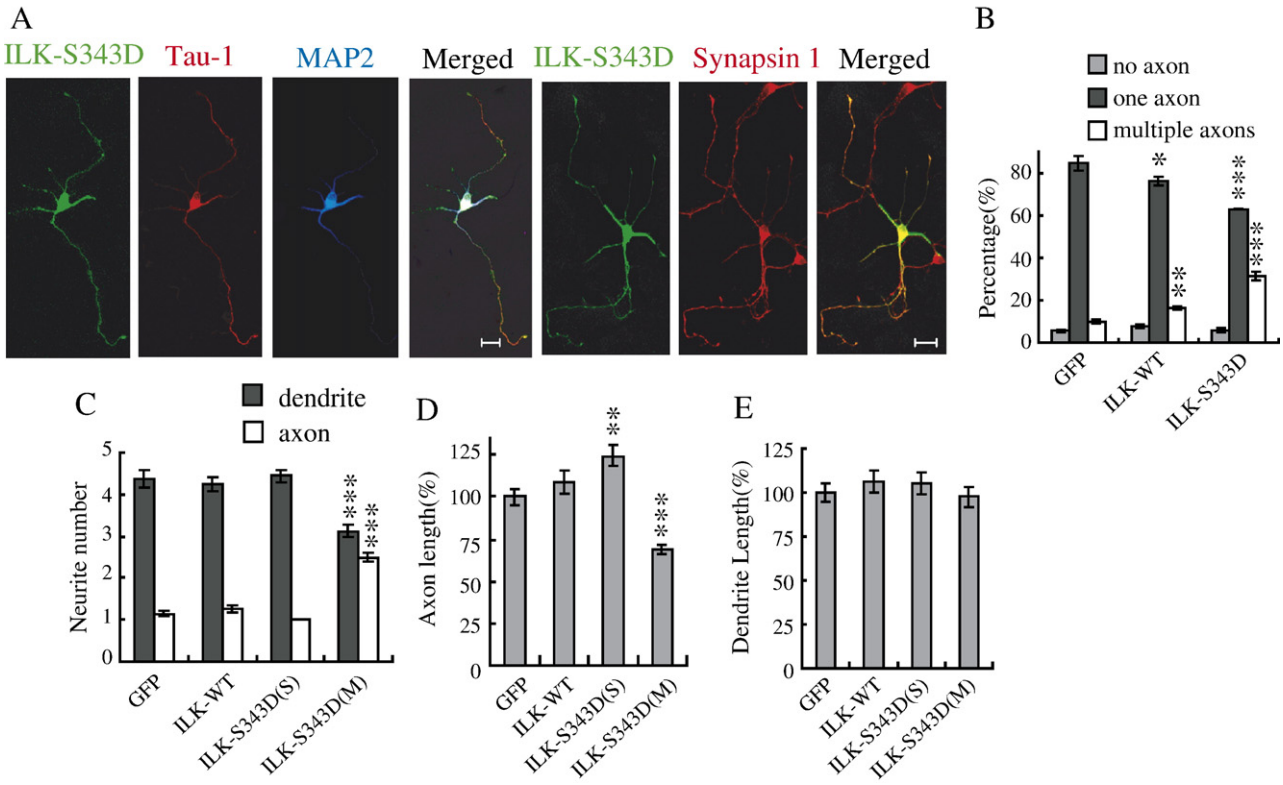

Fig. 6. Formation of multiple axons in neurons transfected with a hyperactive ILK mutant. (A) Neurons transfected with ILK-S343D were stained with dendritic and axonal markers. ILK-S343D transfected neurons had multiple axons. (B) Quantification of polarity defects. Neurons with no axon: GFP 5.5±0.51% (n=344), ILK-WT 7.7±0.77% (n=252), ILK-S343D 5.8±0.37% (n=312). Neurons with a single axon: GFP 84.5±1.6%, ILK-WT 76.0±1.0%, ILK-S343D 62.8±0.32%. Neurons with multiple axons: GFP 10.0±.2%, ILK-WT 16.3±0.40%, ILK-S343D 31.2±0.32%. (C) Neurite numbers per neuron. Numbers of dendrites of neurons co-transfected with GFP and ILK-S343D were counted in two categories: (S) was neurons with single axons while (M) was neurons with multiple axons. GFP (n=44): 1.16±0.08 axons and 4.36±0.20 dendrites. ILK-WT (n=45): 1.27±0.09 axons and 4.24±0.18 dendrites. ILK-S343D(S) (n=60): 1 axon and 4.45±0.14 dendrites. ILK-S343D(M) (n=54): 2.49±0.11 axons and 3.13±0.15 dendrites. (D) The average axon length of GFP-transfected neurons was normalized as 100%. GFP (n=60): 100±5.2%. ILK-WT (n=64): 109±6.6%. ILK-S343D(S) (n=61): 125±6.5%. ILK-S343D(M) (n=77): 69±2.8%. (E) The average dendrite length of GFP-transfected neurons was normalized as 100%. GFP (n=149): 100±5.0%. ILK-WT (n=120): 106±6.1%. ILK-S343D(S) (n=110): 105±6.0%. ILK-S343D(M) (n=88): 98±5.8%. Statistical analyses were performed between GFP and ILK-WT or ILK-S343D. Asterisks indicate statistical significance (Student's t test; *$p<0.05$, **$p<0.01$, ***$p<0.001$).

elimination of axons, which is most likely due to a redistribution of materials that would have been used for axons into the existing dendrites, as we have previously observed in several situations with axon elimination (Jiang et al., 2005). All phenotypes of ILK siRNAs were similar to the GSK-3β mutant defective in phosphorylation, consistent with a role for ILK being upstream of GSK-3β.

Transfection with a kinase inactive ILK ILK-S343A also increased the number of neurons without axons (Figs. 5A, B). ILK-S343A did not affect the dendrite number (Fig. 5C). Similar to ILK RNAi treatment, ILK-S343A decreased axon length in neurons retaining a single axon (ILK-S343A(S) in Fig. 5D). The dendrites were significantly longer only after the axon was eliminated by ILK-S343A (ILK-S343A(N) in Fig. 5E). Wild type ILK had a small, but significant, effect on neuronal polarity (Figs. 5B–E), which is qualitatively similar to that of the hyperactive ILK-S343D, as will be discussed below.

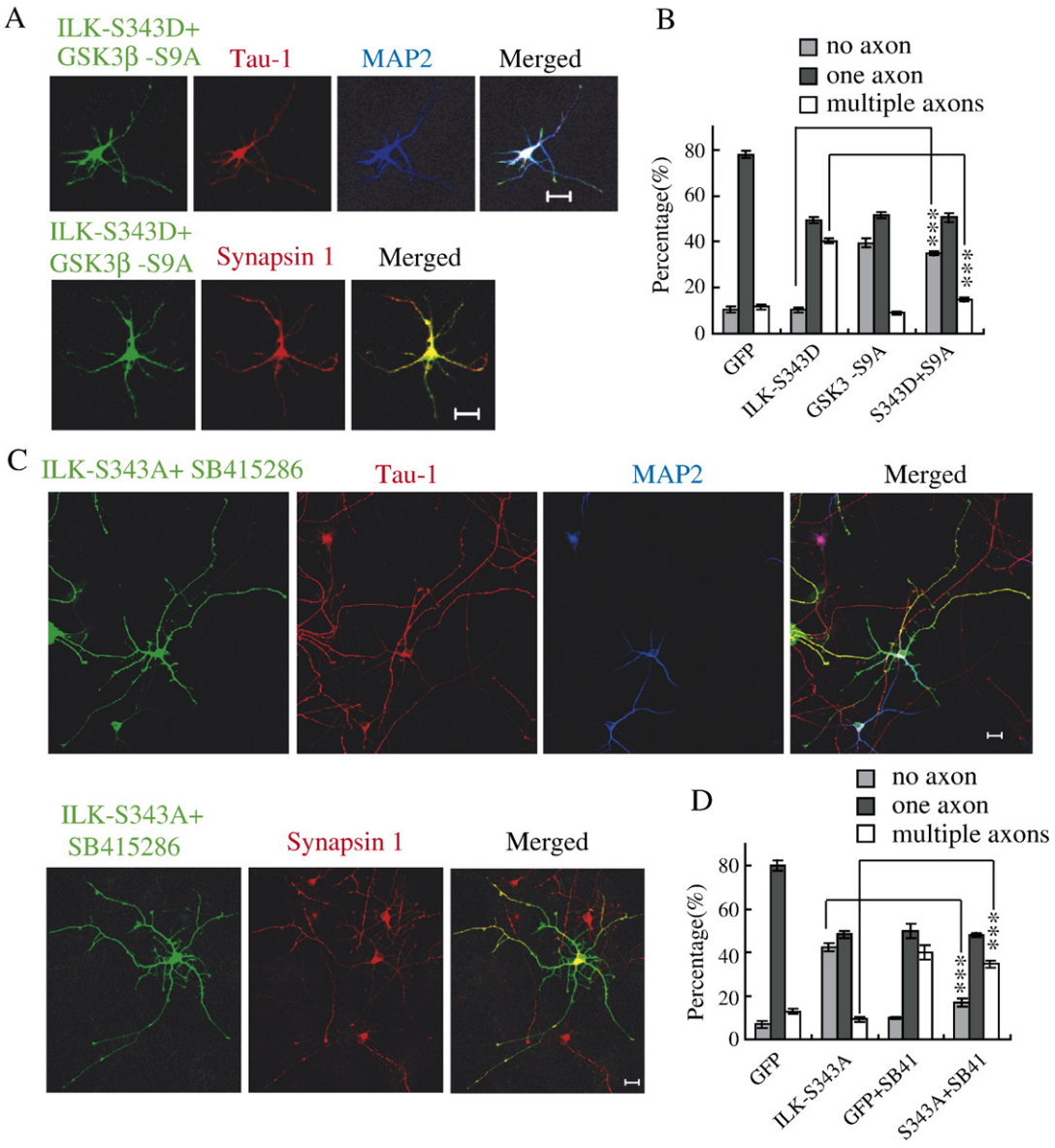

Fig. 7. Functional relationship of ILK with GSK-3β in the formation of neuronal polarity. (A) Neurons cotransfected with ILK-S343D and GSK3β-S9A had no axon. Neurons were stained with dendritic and axonal markers. (B) Quantification of polarity defects in neurons cotransfected with ILK-S343D and GSK3β-S9A. Neurons with no axon: GFP 10.4±1.2% (n=222), ILK-S343D 10.4±1.0% (n=228), GSK3β-S9A 39.4±2.0% (n=213), ILK-S343D+GSK3β-S9A 34.8±1.8% (n=249). Neurons with a single axon: GFP 78.0±1.6%, ILK-S343D 49.3±1.1%, GSK3β-S9A 51.6±1.4%, ILK-S343D+GSK3β-S9A 50.5±1.8%. Neurons with multiple axons: GFP 11.7±1.1%, ILK-S343D 40.3±1.1%, GSK3β-S9A 9.0±0.64%, ILK-S343D+GSK3β-S9A 14.7±0.92%. (C) Neurons transfected with ILK-S343A in the present of GSK3β inhibitor SB415286 had multiple axons. Neurons were stained with dendritic and axonal markers. (D) Quantification of polarity defects in neurons transfected with ILK-S343A in the present of GSK3β inhibitor SB415286. Neurons with no axon: GFP 6.9±1.6% (n=234), ILK-S343A 42.4±1.8% (n=209), GFP+SB41 10.0±0.6% (n=171), ILK-S343A+SB41 17.2±1.8% (n=223). Neurons with a single axon: GFP 80.1±2.2%, ILK-S343A 48.4±1.5%, GFP+SB41 50.0±3.3%, ILK-S343A+SB41 48.1±0.8%. Neurons with multiple axons: GFP 12.9±1.1%, ILK-S343A 9.2±1.0%, GFP+SB41 40.1±3.1%, ILK-S343A+SB41 34.8±1.7%. Asterisks indicate statistical significance (Student's t test; ***$p < 0.001$).

Taken together, inhibition by the chemical inhibitors, siRNAs and the kinase inactive mutant (ILK-S343A) results in the similar phenotype of axon elimination, indicating that ILK is required for the formation of the axon, and suggesting the possibility that ILK could be involved in the establishment of neuronal polarity.

Induction of multiple axons by a kinase hyperactive ILK mutant

To investigate the effect of ILK activation, we used ILK-S343D, a kinase hyperactive ILK mutant (Persad et al., 2001). ILK-S343D increased the number of neurons with multiple axons, but decreased the number of neurons with a single axon (Figs. 6A, B). The total number of neurites per neuron was not significantly changed, indicating that the formation of multiple axons was at the expense of dendrites (Fig. 6C). In neurons transfected with ILK-S343D that still had a single axon, the axons were longer than those transfected with GFP alone (ILK-S343D(S) in Fig. 6D). The length of axons was shorter when multiple axons formed (ILK-S343D(M) in Fig. 6D). The length of dendrite was not affected by ILK-S343D transfection (Fig. 6E). These results indicate that ILK activation causes the formation of multiple axons and promote the growth of single axons. These results are similar to GSK-3β inactivation (Jiang et al., 2005). Akt activation also causes the formation of multiple axons, but it also increases the total number of neurites (Jiang et al., 2005). ILK-S343D cannot mimic the effect of Akt activation on the total number of neurites.

Functional relationship of ILK to GSK-3β, Akt, and PI3K

To determine the functional relationship between ILK and another molecule in the formation of neuronal polarity, we manipulated the activities of both ILK and the other molecule and examined whether the phenotype of manipulating one molecule (X) predominated over the phenotype of manipulating

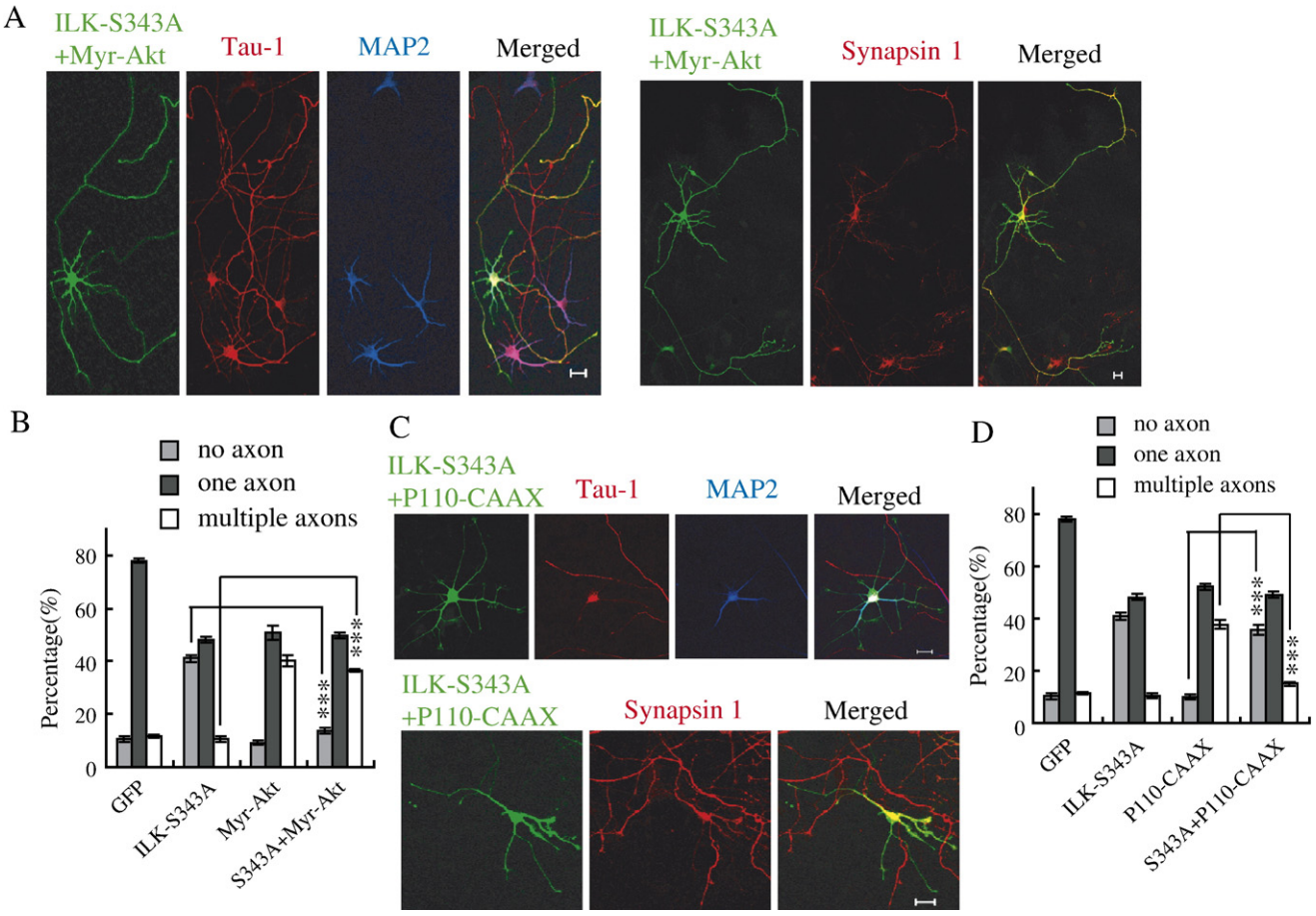

Fig. 8. Functional relationship of ILK with Akt and PI3K in the formation of neuronal polarity. (A) Neurons co-transfected with ILK-S343A and Myr-Akt had multiple axons. Neurons were stained with dendritic and axonal markers. (B) Quantification of polarity defects in neurons cotransfected with ILK-S343A and Myr-Akt. Neurons with no axon: GFP 10.5±1.2% (n=242), ILK-S343A 41.1±1.4% (n=227), Myr-Akt 9.2±0.67% (n=232), ILK-S343A+Myr-Akt 13.9±1.0% (n=247). Neurons with a single axon: GFP 78.1±0.76%, ILK-S343A 48.3±1.1%, Myr-Akt 50.8±2.6%, ILK-S343A+Myr-Akt 49.7±0.96%. Neurons with multiple axons: GFP 11.5±0.52%, ILK-S343A 10.6±0.95%, Myr-Akt 40.0±2.1%, ILK-S343A+Myr-Akt 36.4±0.43%. (C) Neurons cotransfected with ILK-S343A and P110-CAAX had no axon. Neurons were stained with dendritic and axonal markers. (D) Quantification of polarity defects in neurons cotransfected with ILK-S343A and P110-CAAX. Neurons with no axon: GFP 10.5±1.2% (n=242), ILK-S343A 41.1±1.4% (n=227), P110-CAAX 9.9±0.94% (n=218), ILK-S343A+P110-CAAX 35.7±1.7% (n=258). Neurons with a single axon: GFP 78.1±0.76%, ILK-S343A 48.3±1.1%, P110-CAAX 52.2±1.1%, ILK-S343A+P110-CAAX 49.2±1.2%. Neurons with multiple axons: GFP 11.5±0.52%, ILK-S343A 10.6±0.95%, P110-CAAX 37.8±1.7%, ILK-S343A+P110-CAAX 15.1±0.76%. Asterisks indicate statistical significance (Student's t test; ***$p < 0.001$).

another molecule (Y). If phenotypic predomination occurs, it would support an epistatic relationship between X and Y, with X being downstream of Y.

Because the phenotype of ILK activation is opposite to GSK-3β activation, the relationship of ILK and GSK-3β was determined by two experiments: one by increasing the activities of both ILK and GSK-3β, the other by decreasing the activities of both ILK and GSK-3β. As shown previously, the constitutively active ILK-S343D increased the percentage of neurons with multiple axons and decreased the percentage of neurons with a single axon. A constitutively active GSK-3β mutant GSK3β-S9A increased the percentage of neurons with no axon and decreased the percentage of neurons with a single axon (Jiang et al., 2005). When both the constitutively active ILK (ILK-S343D) and the constitutively active GSK-3β (GSK-3β-S9A) were introduced into hippocampal neurons, the predominant phenotype was that of GSK-3β-S9A (Figs. 7A, B). The GSK-3β inhibitor SB415286 induced multiple axons (Jiang et al., 2005), whereas the kinase inactive ILK mutant ILK-S343A inhibited axon formation. When both ILK and GSK-3β were inhibited (by the introduction of ILK-S343A and SB415286) in hippocampal neurons, the phenotype was that of the GSK-3β inhibitor SB415286 (Figs. 7C, D). Thus, in both types of experiments, the effects of manipulating GSK-3β activity predominate over those of ILK manipulation, indicating that that ILK acts upstream of GSK-3β.

To study the functional relationship between ILK and Akt, we used the kinase inactive ILK mutant S343A and the constitutively active Akt mutant myr-Akt, derived by Akt fusion with the myristoylation signal of Src (Ramaswamy et al., 1999). It was not possible to do the complementary experiments of inhibiting Akt and activating ILK because Akt inhibition caused neuronal death (Jiang et al., 2005), precluding studies of Akt inhibition on neuronal polarity. As shown previously (Jiang et al., 2005), myr-Akt increased both the number of axons per neuron and the number of dendrites per neuron. Here we found that myr-Akt phenotype predominated over that of ILK-S343A in increasing axons and dendrites (Figs. 8A, B). These results are consistent with the idea of Akt being downstream of ILK.

To determine the relationship between ILK and PI3K, we used P110-CAAX, a constitutively active form of the catalytic subunit of PI3K (Hu et al., 1995). P110-CAAX alone induced multiple axons formation, similar to the hyperactive ILK mutant S343D. When P110-CAAX was introduced together with the kinase inactive ILK mutant ILK-S343A, the phenotype of the latter predominated over that of P110-CAAX: the percentage of neurons with multiple axons decreased while that with no axon increased (Figs. 8C, D). These results indicate that ILK is downstream of PI3K in determining neuronal polarity.

Discussion

Our results have led to two conclusions. First, ILK plays an important role in determining the axon–dendrite polarity of hippocampal pyramidal neurons. Second, ILK functions downstream of the PI3K but upstream of Akt and GSK-3β. ILK is therefore an important link in the signaling pathway involved in the formation of neuronal polarity (Fig. 9).

Support for the first conclusion came from our studies of ILK distribution and function. ILK is present in all neurites at st. 2 but preferentially present in the tips of the axons at st. 3. Inhibition of ILK activity by three different methods (a pharmacological inhibitor, a kinase inactive construct, and siRNAs) all result in inhibition of the axon formation but did not affect dendrite formation. A kinase hyperactive ILK induces the formation of multiple axons with concomitant reduction of dendrites.

Support for the second conclusion came from biochemical and functional studies of the relationship of ILK with other signaling molecules. The distribution of ILK reported here is similar to those of phosphorylated Akt and phosphorylated GSK-3β in hippocampal pyramidal neurons (Jiang et al., 2005; Shi et al., 2003; Yoshimura et al., 2005). Inhibition of ILK activity reduced phosphorylation of Akt and GSK-3β. The phenotype of manipulating ILK activity can be overridden by that of Akt and GSK-3β, indicating that ILK is functionally upstream of Akt and GSK-3β. On the other hand, manipulating ILK activity can override the phenotype of changing PI3K activity, indicating that ILK is downstream of the PI3K.

It is presently unclear whether ILK directly regulates only Akt and then indirectly regulates GSK-3β, or that it directly regulates both Akt and GSK-3β. Biochemically, both possibilities remain plausible because phosphorylation of both Akt and GSK-3β were reduced in hippocampal neurons after ILK inhibition. In several cell types, ILK inhibitor reduced Ser473 phosphorylation of Akt and Ser9 phosphorylation of GSK-3β (Mills et al., 2003; Persad et al., 2001; Zhou et al., 2004).

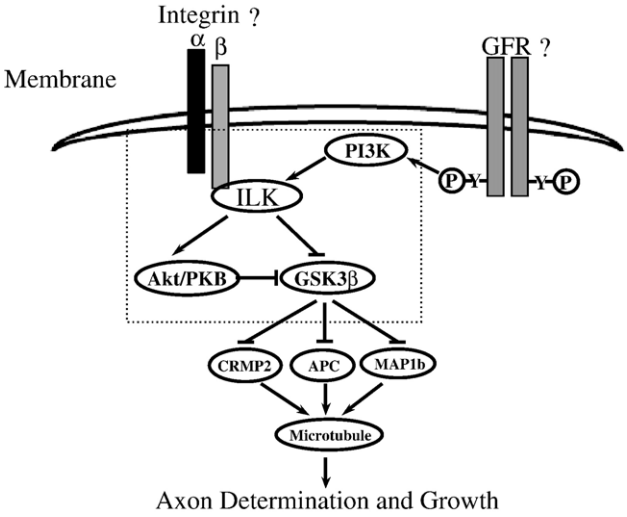

Fig. 9. A model for ILK signaling in neuronal polarity. AKT/GSK-3β pathway is thought to regulate neuronal polarity through CRMP2, APC and MAP1b. ILK functions downstream of the PI3K but upstream of Akt and GSK-3β in determining neuronal polarity. It is not known whether extracellular ligands can function through transmembrane receptors such as integrins or tyrosine kinase receptors in neuronal polarization.

Purified ILK protein can phosphorylate Akt at Ser473 and GSK-3β directly in vitro (Delcommenne et al., 1998; Persad et al., 2001). ILK or its hyperactive mutant increased Akt phosphorylation. The dominant negative ILK and ILK siRNAs reduced Akt and GSK-3β phosphorylation (Delcommenne et al., 1998; Lynch et al., 1999; Persad et al., 2001; Troussard et al., 2003). Conditional knockout of ILK in vitro decreased phosphorylation of Akt and GSK-3β in mouse macrophages (Troussard et al., 2003). Deletion of ILK inhibited Akt phosphorylation stimulated by PDGF but not that by insulin (Sakai et al., 2003). Western analysis of brain extracts showed that phosphorylation of Akt and GSK-3β were not affected in the forebrain of ILK knockout mice (Niewmierzycka et al., 2005), although Akt and GSK-3β phosphorylation in the tips of axons and dendrites have not been examined.

ILK is associated with other molecules, some of which are implicated in neuronal polarization. Its ankyrin domains bind to a number of adaptor and signalling molecules, such as PINCH and the ILK-associated phosphatase (ILKAP) (Leung-Hagesteijn et al., 2001; Tu et al., 1999). PINCH can interact with Nck-2, an adaptor protein containing three Src homology (SH)3 domains and one SH2 domain, which could be potentially linked to phosphorylated tyrosine of growth factor receptors (Tu et al., 1998). Because a specific inhibitor for growth factor receptor tyrosine kinase (RTK), AG1478, prevented neurons from becoming polarized (Shi et al., 2003), it raises the possibility of a potential linkage between RTK and ILK in the determination of neuronal polarity.

ILK is also linked to other proteins ranging from the transmembrane integrins to the cytoplasmic α-parvin (ILKBP), β-parvin (affixin), paxillin (Nikolopoulos and Turner, 2001; Tu et al., 2001; Wu and Dedhar, 2001; Yamaji et al., 2001) and α-PIX (Rosenberger et al., 2003), a guanine nucleotide exchange factor for Rac1 and Cdc42, which are key regulators of actin dynamics. ILK can also directly phosphorylate myosin light chain (MLC) or inactivate the myosin phosphatase target subunit, leading to further stimulation of MLC phosphorylation (Deng et al., 2001; Muranyi et al., 2002), which is also implicated in multiple axons formation (Kim and Chang, 2004). Our finding of a role for ILK in neuronal polarity makes it interesting to delineate the roles and interactions among the ILK interacting proteins in the neuronal polarity.

Acknowledgments

We acknowledge support by the 863 program of Ministry of Science and Technology and by the Beijing Municipal Government; YR acknowledges support by the NIH. SD was supported by the Canadian Institutes for Health Research (CIHR) and the National Cancer Institute of Canada (NCIC). KP-392 and QLT0267 were provided by Quadra Logic Technology Inc (QLT Inc), Vancouver, BC Canada.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.03.019.

References

Armstrong, J.L., Bonavaud, S.M., Toole, B.J., Yeaman, S.J., 2001. Regulation of glycogen synthesis by amino acids in cultured human muscle cells. J. Biol. Chem. 276 (2), 952–956.

Baas, P.W., Deitch, J.S., Black, M.M., Banker, G.A., 1988. Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. Proc. Natl. Acad. Sci. U. S. A. 85 (21), 8335–8339.

Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C.P., Alessi, D.R., 1999. PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. Curr. Biol. 9 (8), 393–404.

Ballou, L.M., Tian, P.Y., Lin, H.Y., Jiang, Y.P., Lin, R.Z., 2001. Dual regulation of glycogen synthase kinase-3beta by the alpha1A-adrenergic receptor. J. Biol. Chem. 276 (44), 40910–40916.

Banker, G.A., Cowan, W.M., 1977. Rat hippocampal neurons in dispersed cell culture. Brain Res. 126 (3), 397–42.

Banker, G.A., Cowan, W.M., 1979. Further observations on hippocampal neurons in dispersed cell culture. J. Comp. Neurol. 187 (3), 469–493.

Bradke, F., Dotti, C.G., 1999. The role of local actin instability in axon formation. Science 283 (5409), 1931–1934.

Bradke, F., Dotti, C.G., 2000. Establishment of neuronal polarity: lessons from cultured hippocampal neurons. Curr. Opin. Neurobiol. 10 (5), 574–581.

Brady, M.J., Bourbonais, F.J., Saltiel, A.R., 1998. The activation of glycogen synthase by insulin switches from kinase inhibition to phosphatase activation during adipogenesis in 3T3-L1 cells. J. Biol. Chem. 273 (23), 14063–14066.

Craig, A.M., Banker, G., 1994. Neuronal polarity. Annu. Rev. Neurosci. 17, 267–310.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378 (6559), 785–789.

Crump, J.G., Zhen, M., Jin, Y., Bargmann, C.I., 2001. The SAD-1 kinase regulates presynaptic vesicle clustering and axon termination. Neuron 29 (1), 115–129.

de Anda, F.C., Pollarolo, G., Da Silva, J.S., Camoletto, P.G., Feiguin, F., Dotti, C.G., 2005. Centrosome localization determines neuronal polarity. Nature 436 (7051), 704–708.

Dedhar, S., Williams, B., Hannigan, G., 1999. Integrin-linked kinase (ILK): a regulator of integrin and growth-factor signalling. Trends Cell Biol. 9 (8), 319–323.

Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., Dedhar, S., 1998. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc. Natl. Acad. Sci. U. S. A. 95 (19), 11211–11216.

Deng, J.T., Van Lierop, J.E., Sutherland, C., Walsh, M.P., 2001. Ca^{2+}-independent smooth muscle contraction. A novel function for integrin-linked kinase. J. Biol. Chem. 276 (19), 16365–16373.

Dotti, C.G., Banker, G.A., 1987. Experimentally induced alteration in the polarity of developing neurons. Nature 330 (6145), 254–256.

Dotti, C.G., Sullivan, C.A., Banker, G.A., 1988. The establishment of polarity by hippocampal neurons in culture. J. Neurosci. 8 (4), 1454–1468.

Fang, X., Yu, S.X., Lu, Y., Bast Jr., R.C., Woodgett, J.R., Mills, G.B., 2000. Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. Proc. Natl. Acad. Sci. U. S. A. 97 (22), 11960–11965.

Feng, J., Park, J., Cron, P., Hess, D., Hemmings, B.A., 2004. Identification of a PKB/Akt hydrophobic motif Ser-473 kinase as DNA-dependent protein kinase. J. Biol. Chem. 279 (39), 41189–41196.

Hannigan, G.E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M.G., Radeva, G., Filmus, J., Bell, J.C., Dedhar, S., 1996. Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. Nature 379 (6560), 91–96.

Hannigan, G., Troussard, A.A., Dedhar, S., 2005. Integrin-linked kinase: a cancer therapeutic target unique among its ILK. Nat. Rev., Cancer 5 (1), 51–63.

Horton, A.C., Ehlers, M.D., 2003. Neuronal polarity and trafficking. Neuron 40 (2), 277–295.

Hu, Q., Klippel, A., Muslin, A.J., Fantl, W.J., Williams, L.T., 1995. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. Science 268 (5207), 100–102.

Jiang, H., Guo, W., Liang, X., Rao, Y., 2005. Both the establishment and the maintenance of neuronal polarity require active mechanisms: critical roles of GSK-3beta and its upstream regulators. Cell 120 (1), 123–135.

Kim, Y., Chang, S., 2004. Modulation of actomyosin contractility by myosin light chain phosphorylation/dephosphorylation through Rho GTPases signaling specifies axon formation in neurons. Biochem. Biophys. Res. Commun. 318 (2), 579–587.

Kishi, M., Pan, Y.A., Crump, J.G., Sanes, J.R., 2005. Mammalian SAD kinases are required for neuronal polarization. Science 307 (5711), 929–932.

Koul, D., Shen, R., Bergh, S., Lu, Y., de Groot, J.F., Liu, T.J., Mills, G.B., Yung, W.K.A., 2005. Targeting integrin-linked kinase inhibits Akt signaling pathways and decreases tumor progression of human glioblastoma. Mol. Cancer Ther. 4 (11), 1681–1688.

Leung-Hagesteijn, C., Mahendra, A., Naruszewicz, I., Hannigan, G.E., 2001. Modulation of integrin signal transduction by ILKAP, a protein phosphatase 2C associating with the integrin-linked kinase, ILK1. EMBO J. 20 (9), 2160–2170.

Lynch, D.K., Ellis, C.A., Edwards, P.A., Hiles, I.D., 1999. Integrin-linked kinase regulates phosphorylation of serine 473 of protein kinase B by an indirect mechanism. Oncogene 18 (56), 8024–8032.

Mills, J., Digicaylioglu, M., Legg, A.T., Young, C.E., Young, S.S., Barr, A.M., Fletcher, L., O'Connor, T.P., Dedhar, S., 2003. Role of integrin-linked kinase in nerve growth factor-stimulated neurite outgrowth. J. Neurosci. 23 (5), 1638–1648.

Muranyi, A., MacDonald, J.A., Deng, J.T., Wilson, D.P., Haystead, T.A., Walsh, M.P., Erdodi, F., Kiss, E., Wu, Y., Hartshorne, D.J., 2002. Phosphorylation of the myosin phosphatase target subunit by integrin-linked kinase. Biochem. J. 366 (Pt 1), 211–216.

Niewmierzycka, A., Mills, J., St-Arnaud, R., Dedhar, S., Reichardt, L.F., 2005. Integrin-linked kinase deletion from mouse cortex results in cortical lamination defects resembling cobblestone lissencephaly. J. Neurosci. 25 (30), 7022–7031.

Nikolopoulos, S.N., Turner, C.E., 2001. Integrin-linked kinase (ILK) binding to paxillin LD1 motif regulates ILK localization to focal adhesions. J. Biol. Chem. 276 (26), 23499–23505.

Nishimura, T., Kato, K., Yamaguchi, T., Fukata, Y., Ohno, S., Kaibuchi, K., 2004. Role of the PAR-3–KIF3 complex in the establishment of neuronal polarity. Nat. Cell Biol. 6 (4), 328–334.

Nishimura, T., Yamaguchi, T., Kato, K., Yoshizawa, M., Nabeshima, Y., Ohno, S., Hoshino, M., Kaibuchi, K., 2005. PAR-6–PAR-3 mediates Cdc42-induced Rac activation through the Rac GEFs STEF/Tiam1. Nat. Cell Biol. 7 (3), 270–277.

Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J.T., Leung, D., Yan, J., Sanghera, J., Walsh, M.P., Dedhar, S., 2001. Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. J. Biol. Chem. 276 (29), 27462–27469.

Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D.B., Perera, S., Roberts, T.M., Sellers, W.R., 1999. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. U. S. A. 96 (5), 2110–2115.

Rolls, M.M., Doe, C.Q., 2004. Baz, Par-6 and aPKC are not required for axon or dendrite specification in *Drosophila*. Nat. Neurosci. 7 (12), 1293–1295.

Rosenberger, G., Jantke, I., Gal, A., Kutsche, K., 2003. Interaction of alphaPIX (ARHGEF6) with beta-parvin (PARVB) suggests an involvement of alphaPIX in integrin-mediated signaling. Hum. Mol. Genet. 12 (2), 155–167.

Sakai, T., Li, S., Docheva, D., Grashoff, C., Sakai, K., Kostka, G., Braun, A., Pfeifer, A., Yurchenco, P.D., Fassler, R., 2003. Integrin-linked kinase (ILK) is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation. Genes Dev. 17 (7), 926–940.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., Sabatini, D.M., 2005. Phosphorylation and regulation of Akt/PKB by the rictor–mTOR complex. Science 307 (5712), 1098–1101.

Schwamborn, J.C., Puschel, A.W., 2004. The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. Nat. Neurosci. 7 (9), 923–929.

Shi, S.H., Jan, L.Y., Jan, Y.N., 2003. Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. Cell 112 (1), 63–75.

Shi, S.H., Cheng, T., Jan, L.Y., Jan, Y.N., 2004. APC and GSK-3beta are involved in mPar3 targeting to the nascent axon and establishment of neuronal polarity. Curr. Biol. 14 (22), 2025–2032.

Toker, A., Newton, A.C., 2000. Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. J. Biol. Chem. 275 (12), 8271–8274.

Troussard, A.A., Costello, P., Yoganathan, T.N., Kumagai, S., Roskelley, C.D., Dedhar, S., 2000. The integrin linked kinase (ILK) induces an invasive phenotype via AP-1 transcription factor-dependent upregulation of matrix metalloproteinase 9 (MMP-9). Oncogene 19 (48), 5444–5452.

Troussard, A.A., Mawji, N.M., Ong, C., Mui, A., St-Arnaud, R., Dedhar, S., 2003. Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. J. Biol. Chem. 278 (25), 22374–22378.

Troussard, A.A., McDonald, P.C., Wederell, E.D., Mawji, N.M., Filipenko, N.R., Gelmon, K.A., Kucab, J.E., Dunn, S.E., Emerman, J.T., Bally, M.B., Dedhar, S., 2006. Preferential dependence of breast cancer cells versus normal cells on integrin-linked kinase for protein kinase B/Akt activation and cell survival. Cancer Res. 66 (1), 393–403 (10.1158/0008-5472. CAN-05-2304).

Tu, Y., Li, F., Wu, C., 1998. Nck-2, a novel Src Homology2/3-containing adaptor protein that interacts with the LIM-only protein PINCH and components of growth factor receptor kinase-signaling pathways. Mol. Biol. Cell 9 (12), 3367–3382.

Tu, Y., Li, F., Goicoechea, S., Wu, C., 1999. The LIM-Only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. Mol. Cell. Biol. 19 (3), 2425–2434.

Tu, Y., Huang, Y., Zhang, Y., Hua, Y., Wu, C., 2001. A new focal adhesion protein that interacts with integrin-linked kinase and regulates cell adhesion and spreading. J. Cell Biol. 153 (3), 585–598.

Wiggin, G.R., Fawcett, J.P., Pawson, T., 2005. Polarity proteins in axon specification and synaptogenesis. Dev. Cell 8 (6), 803–816.

Wu, C., Dedhar, S., 2001. Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. J. Cell Biol. 155 (4), 505–510.

Yamaji, S., Suzuki, A., Sugiyama, Y., Koide, Y., Yoshida, M., Kanamori, H., Mohri, H., Ohno, S., Ishigatsubo, Y., 2001. A novel integrin-linked kinase-binding protein, affixin, is involved in the early stage of cell–substrate interaction. J. Cell Biol. 153 (6), 1251–1264.

Yoshimura, T., Kawano, Y., Arimura, N., Kawabata, S., Kikuchi, A., Kaibuchi, K., 2005. GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. Cell 120 (1), 137–149.

Younes, M.N., Kim, S., Yigitbasi, O.G., Mandal, M., Jasser, S.A., Dakak Yazici, Y., Schiff, B.A., El-Naggar, A., Bekele, B.N., Mills, G.B., Myers, J.N., 2005. Integrin-linked kinase is a potential therapeutic target for anaplastic thyroid cancer. Mol. Cancer Ther. 4 (8), 1146–1156.

Zhou, F.Q., Zhou, J., Dedhar, S., Wu, Y.H., Snider, W.D., 2004. NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC. Neuron 42 (6), 897–912.