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Par1b/MARK2 Phosphorylates Kinesin-Like Motor Protein GAKIN/KIF13B To Regulate Axon Formation▽†

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Here we report that Par1b/MARK2 regulates axon formation via phosphorylation of a kinesin superfamily protein GAKIN/KIF13B. Accumulating evidence indicated the importance of the evolutionarily conserved kinase Par1b in the regulation of cell polarity. Using hippocampal neurons in culture, it has been shown that Par1b regulates axon specification, but the underlying mechanism remains uncharacterized. We identify GAKIN/KIF13B as a novel Par1b-binding protein and reveal that GAKIN/KIF13B is a physiological substrate for Par1b, and the phosphorylation sites are conserved from *Drosophila*. In hippocampal neurons, GAKIN/KIF13B accumulates at the distal part of the microtubules in the tips of axons, but not of dendrites. Overexpression of GAKIN/KIF13B by itself can induce the formation of extra axons, which is inhibited by the coexpression of Par1b in a manner dependent on its kinase activity. In contrast, small interfering RNA (siRNA)-mediated knockdown of GAKIN/KIF13B severely retards neurite extension and promotes the axonless phenotype. The extra axon phenotype caused by Par1b siRNA is suppressed by cointroduction of GAKIN/KIF13B siRNA, thus placing the GAKIN/KIF13B function downstream of Par1b. We also find that GAKIN/KIF13B acts downstream of the phosphatidylinositol 3-kinase (PI3K) signaling via Par1b phosphorylation. These results reveal that GAKIN/KIF13B is a key intermediate linking Par1b to the regulation of axon formation.

Cell polarity, which is the basis for generation and function of various types of differentiated cells, is a fundamental phenomenon in cell biology. The *Par* genes were originally discovered in a screen for partitioning defective mutants in *Caenorhabditis elegans* embryos (17). Later, it was shown that the *Par* genes are evolutionarily conserved and play crucial roles in cell polarity (11, 18). A great deal of effort has been focused on clarifying the functional relationships between the Par proteins, and it is now widely accepted that the Par complex, which is composed of Par3/Par6/atypical protein kinase C (aPKC), phosphorylates Par1, causing it to dissociate from the membrane and resulting in the loss of its biological activity (15, 19, 33). Par1 is a Ser/Thr kinase (12), and its mammalian ortholog was originally identified as microtubule affinity-regulating kinase (MARK) (10, 16). Recent studies revealed that Par1b/MARK2 plays a critical role in neuronal polarity in the process of axon specification from multiple candidate neurites using primary cultures of mammalian hippocampal neurons (7, 43). Indeed, Chen et al. indicated that phosphorylation of Par1b significantly occurs at the tips of axons (7), suggesting that localized inactivation of Par1b is important for axon formation. However, the mechanistic details downstream of Par1b in axon formation remain largely uncharacterized.

Guanylate kinase-associated kinesin (GAKIN) (also known as kinesin superfamily 13B [KIF13B]) is a microtubule-based motor protein that was discovered as a novel binding partner for the human ortholog of *Drosophila* tumor suppressor protein Discs large (Dlg) (13). Ectopic expression of GAKIN/KIF13B in MDCK cells induced formation of extremely long protrusions, and the expressed GAKIN/KIF13B proteins localized at the tips of the protrusions (4). GAKIN/KIF13B also associates directly with centaurin-α1 (also known as phosphatydylphosphatidylinositol-3,4,5-triphosphate [PIP3]-binding protein [PIP3BP]), a GTPase-activating protein (GAP) for Arf6 (36). Through the interaction with centaurin-α1/PIP3BP, GAKIN/KIF13B can transport PIP3-containing lipid vesicles on microtubules to their plus ends, and the authors suggested that it plays a role in the intracellular transport of PIP3 and contributes to neuronal polarity (14, 25). Indeed, PIP3 transport to axon tips has been mentioned to occur (3). Collectively, GAKIN/KIF13B appears to be a very important molecule in neuronal polarity, but the functional and regulatory mechanisms of GAKIN/KIF13B remain to be clarified.

We report here the identification of GAKIN/KIF13B as a novel *in vivo* substrate for Par1b and present evidence that GAKIN/KIF13B plays a critical role in axon formation in neurons, which is negatively regulated by Par1b-mediated phosphorylation. Further, GAKIN/KIF13B is regulated downstream of phosphatidylinositol 3-kinase (PI3K) signaling via Par1b-mediated phosphorylation.

MATERIALS AND METHODS

Antibodies and chemicals. Anti-Par1b antibody was raised in New Zealand White rabbits. His-Par1b-CTF (histidine-tagged Par1b [His-Par1b] C-terminal fragment [CTF]) (amino acids 509 to 699) was expressed in *Escherichia coli* JM109 and purified with nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen, Hilden, Germany) and used for immunization. The serum was affinity purified with GST-Par1b-CTF (glutathione *S*-transferase [GST] fused to Par1b-

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CTF) conjugated to CNBr-activated Sepharose 4B beads (Amersham Pharmacia, Piscataway, NJ). For Par1b knockdown experiments, anti-Par1b antibody provided by S. Ohno (Yokohama City University) (33) was used. Two anti-GAKIN antibodies were also raised in rabbits. His-CAP-Gly (histidine-tagged, glycine-rich cytoskeleton-associated protein) (amino acids 1528 to 1826) was expressed in *E. coli* DH5α, and His-GAKIN (full length) was expressed in Sf9 cells with recombinant baculoviruses. These proteins were purified with Ni-NTA agarose beads and used for immunization. The serum was affinity purified with GST-CAP-Gly conjugated to CNBr-activated Sepharose 4B beads. The purified antibody obtained from the His-CAP-Gly-immunized rabbits was used in most experiments. To detect rat endogenous GAKIN by immunoblotting, the other antibody was used. Mouse anti-β-tubulin, mouse anti-MAP2 (MAP2 stands for microtubule-associated protein 2), mouse anti-FLAG (M2), and rabbit anti-FLAG antibodies were from Sigma-Aldrich (St. Louis, MO). Mouse anti-Myc (9E10) and rabbit anti-Myc (A-14) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-Tau-1 (MAB3420) antibody was from Chemicon (Temecula, CA). Rabbit anti-14-3-3β antibody was from Abcam (Cambridge, United Kingdom). Mouse anti-phospho-Ser 14-3-3 binding motif (4E2) and rabbit anti-phospho-Ser 14-3-3 binding motif were from Cell Signaling (Danvers, MA). LY294002 was from Alexis Corporation (San Diego, CA). Myristoylated protein kinase C ζ peptide inhibitor was from Biomol (Plymouth Meeting, PA). KT5720 was from Calbiochem (San Diego, CA).

Constructs and RNA interference (RNAi). The cDNAs encoding two constructs of human Par1b, the wild-type (WT) and kinase-negative (KN) forms, were generated as described previously (34). The deletion constructs of Par1b, Kinase-UBA (amino acids 1 to 329) and Spacer-ELKL (amino acids 329 to 691), were generated by digestion with appropriate restriction enzymes. The cDNA encoding human GAKIN (KIAA-0639) was provided by the Kazusa DNA Research Institute (Kisarazu, Japan). GAKIN mutants (S1381A, S1410A, and AA) were generated with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The nucleotide sequences of the cDNAs were verified by DNA sequencing. The deletion constructs of GAKIN, Motor (amino acids 1 to 481), Stalk (amino acids 807 to 1638), Stalk1 (amino acids 333 to 1113), Stalk2 (amino acids 1113 to 1638), and CAP-Gly (amino acids 1638 to 1826) were generated by digestion with appropriate restriction enzymes. The cDNA fragments were inserted into pCMV-Tag (Stratagene), pGEX (Amersham), pQE (Qiagen), pFastBac1 (Invitrogen, Carlsbad, CA), pTRE2, and pCAGGS (Clontech Laboratories, Palo Alto, CA) plasmid vectors.

Two individual small interfering RNA (siRNA) duplex oligonucleotides against *Par1b*, rat *Par1b*, and rat *GAKIN*/*KIF13B* were purchased from Invitrogen (Carlsbad, CA). The target mRNA sequences are as follows: Par1b #1 siRNA, 5′-CAGATAGTGTCTGCTGTGCAGTACT-3′; Par1b #2 siRNA, 5′-CGGCCTTCAGCTGATCTGACCAATA-3′; rat Par1b #1 siRNA, 5′-GCTGGATACTTTCTGTGGTAGTCCT-3′; GAKIN/KIF13B #1 siRNA, 5′-GGCCATTGAAGTGTATGGACATAAA-3′; GAKIN/KIF13B #2 siRNA, 5′-CCACTGATGGAAGAGTGCATATTGT-3′; and Control siRNA, 5′-GAGTCTCGTGGAATCGAACGTTAAA-3′.

Culture of COS-7, MDCK, and SH-SY5Y cells. COS-7, MDCK, and SH-SY5Y cells were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum. COS-7 cells were seeded in a 60-mm dish, cultured overnight, and transfected with Lipofectamine (Invitrogen). The cells were harvested with lysis buffer (40 mM Tris-HCl at pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100) 24 h after transfection. MDCK and SH-SY5Y cells (approximately 50 to 60% confluent in 60-mm dishes) were transfected with *Par1b* siRNAs or control siRNA (300 pmol for MDCK cells and 450 pmol for SH-SY5Y cells) with Lipofectamine 2000 (Invitrogen) and cultured for 24 h. MDCK Tet-off stable clones expressing FLAG-tagged Par1b (FLAG-Par1b) or green fluorescent protein (GFP) as a control were generated by cotransfection of pTRE2 and pTK-Hyg (Clontech) vectors and selected with hygromycin for 2 weeks. Clones were grown in the presence of 0.5 μg/ml doxycycline and harvested after the removal of doxycycline.

Identification of Par1b-interacting proteins. For identification of Par1b-binding proteins, lysates of MDCK Tet-off stable clones (GFP and FLAG-Par1b) were incubated with anti-FLAG M2 agarose beads (Sigma) for 2 h. The beads were washed five times and suspended in SDS-PAGE sample buffer. The bound proteins were separated by SDS-PAGE and stained with the SilverQuest silver staining kit (Invitrogen). The bands of interest were analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) and tandem mass spectrometry (MS-MS) (4700 proteomics analyzer; Applied Biosystems, Foster City, CA).

Immunofluorescence microscopy. Cells cultured on coverslips were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 20 min followed by 5-min treatment with 0.2% Triton X-100. The cells were blocked with 10% fetal bovine serum in PBS for 1 h, incubated with primary antibodies for 2 h, washed with PBS, and then further incubated with Alexa 488- or Alexa 546-conjugated secondary antibodies (Molecular Probes, Eugene, OR) for 1 h. After the coverslips were washed, they were mounted on slides and observed with a conventional fluorescence microscope or with a confocal laser microscope (FV1000; Olympus, Tokyo, Japan).

Kinase assay. The full-length and various deletion forms of FLAG-tagged GAKIN (FLAG-GAKIN) were transiently expressed in COS-7 cells and purified with anti-FLAG M2 agarose beads. The protein-bound beads were washed five times and used as substrates. The kinase reaction for His-Par1b was performed in 30 μl of kinase buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 2 mM EGTA, 0.5 mM dithiothreitol) containing [γ-^{32}P]ATP at 30°C for 30 min. The reaction mixtures were subjected to SDS-PAGE, immunoblotting, and autoradiography.

Microtubule cosedimentation assay. FLAG-GAKIN and Myc-tagged Par1b (Myc-Par1b) were transiently expressed in COS-7 cells and lysed in PIPES buffer (80 mM PIPES [pH 6.8], 1 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100). After centrifugation at 100,000 × *g* for 30 min at 4°C, tubulin in the supernatant was polymerized and stabilized by the addition of 2 mM GTP, 20 μM Taxol, and 1 mM $MgCl_2$. After incubation for 30 min at 37°C, FLAG-GAKIN was allowed to form complexes with microtubules by the addition of 2 mM adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP), and further incubated for 30 min at 37°C. After centrifugation at 150,000 × *g* for 1 h at 25°C, the supernatant and pellet (containing microtubules and associated proteins) were analyzed by immunoblotting.

Primary culture of hippocampal neurons. Hippocampi were dissected from rats at embryonic day 18 (E18 rats), digested with trypsin, and dissociated by pipetting. The neurons were transfected with plasmid vectors (2 μg) or siRNAs (100 pmol) in each cuvette by electroporation with the rat neuron Nucleofector kit (Amaxa Biosystems, Cologne, Germany) and plated at a density of 30,000 cells per glass coverslip (coated with laminin and poly-D-lysine) in DMEM with 10% fetal bovine serum. After the neurons attached to the substrate, the medium was changed to neurobasal medium with B27 supplement, 0.5 mM glutamine, and antibiotics. For transfection after plating, dissociated neurons were plated at a density of 200,000 cells per glass coverslip. After the neurons were cultured for 48 h, they were transfected with plasmid vectors (2 μg) with Lipofectamine 2000. LY294002 (20 μM), myristoylated protein kinase Cζ (PKCζ) peptide inhibitor (10 μM), and KT5720 (200 nM) were added to the medium 24 h after plating, and the cells were incubated for an additional 24 h.

Statistical analysis. All statistical analyses were done with the two-tailed Student's *t* test. Error bars in the graphs represent standard deviations (SDs).

RESULTS

GAKIN/KIF13B is a novel Par1b-binding protein. We searched for novel Par1b-interacting proteins in MDCK epithelial cells. We generated MDCK clonal cell lines stably expressing FLAG-tagged Par1b or GFP (control) and subjected lysates of these cells to immunoprecipitation with anti-FLAG beads. We identified a 250-kDa protein that specifically coprecipitated with FLAG-Par1b but not with GFP as a control (Fig. 1A). This protein was excised from the gel, and mass spectrometry of the trypsin-digested samples yielded 10 peptides matching the sequence of GAKIN/KIF13B (data not shown).

To examine the association of endogenous Par1b and GAKIN/KIF13B, antibodies against these proteins were raised in rabbits (see Fig. S1A in the supplemental material). Immunoprecipitation and immunoblotting analyses with these antibodies confirmed that endogenous Par1b and GAKIN/KIF13B coprecipitated with each other, though the amount was small (Fig. 1B). Next, we examined where in Par1b and GAKIN/KIF13B the interaction occurs. Because two Par1b deletion constructs, Kinase-UBA (amino acids 1 to 329) and Spacer-ELKL (amino acids 329 to 691), failed to bind GAKIN/KIF13B (data not shown), it appears that nearly the entire length of Par1b is necessary for binding with GAKIN/KIF13B. GAKIN/KIF13B contains a Motor domain, a Stalk region, which contains a FHA domain and binding regions for human Dlg (hDlg) and centaurin-α1/PIP3BP, and a CAP-Gly domain (Fig. 1C). Four deletion constructs (Motor, Stalk1, Stalk2, and

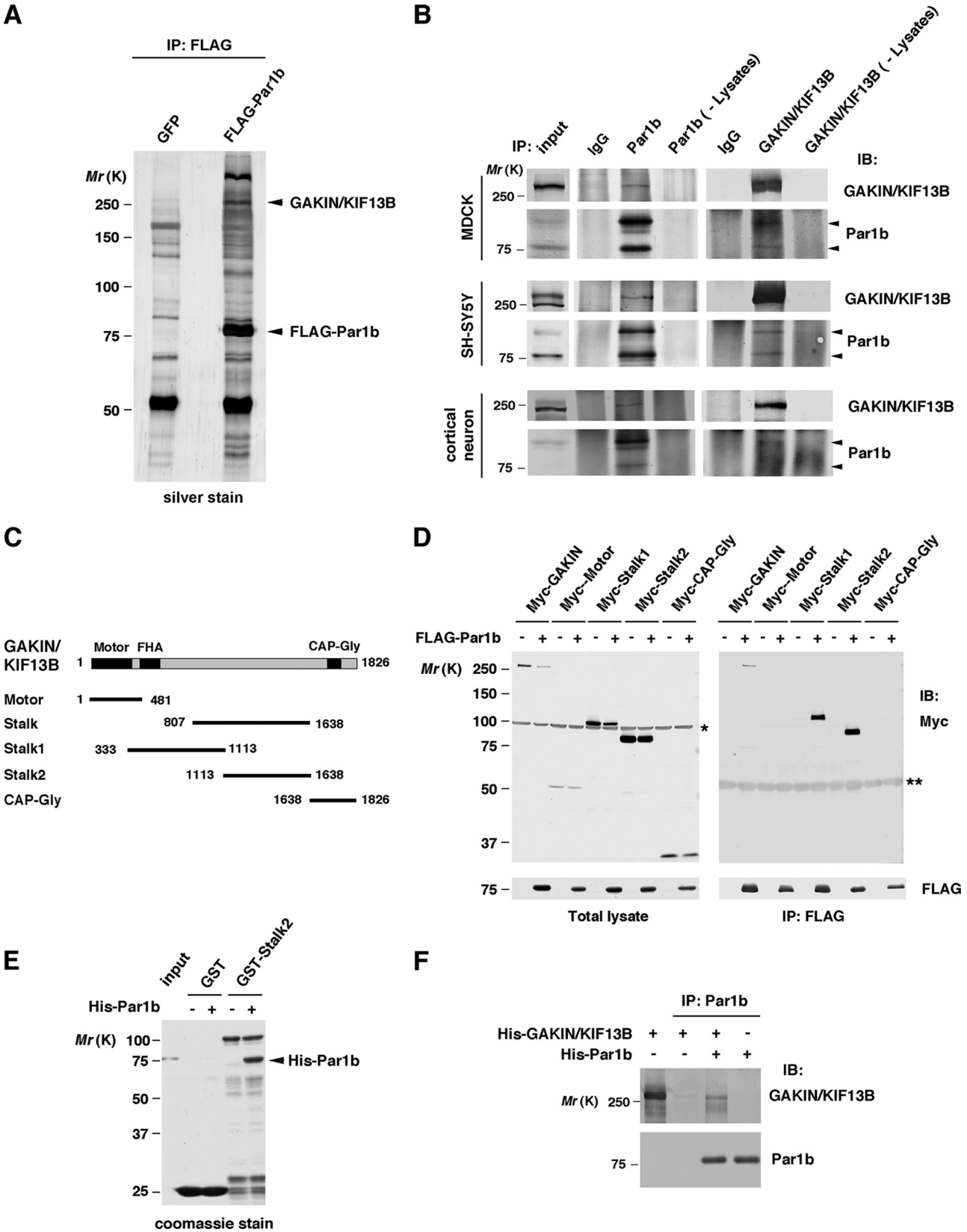

FIG. 1. Binding of Par1b to GAKIN/KIF13B. (A) Anti-FLAG immunoprecipitates (IP) from lysates of MDCK clones stably expressing FLAG-Par1b or GFP as a control were subjected to SDS-PAGE and visualized by silver staining. The positions of FLAG-Par1b and GAKIN/KIF13B are indicated to the right of the gel. The relative molecular weights (in thousands) [*Mr*(K)] are indicated to the left of the gel. (B) Equal amounts of lysates of MDCK, SH-SY5Y cells, or cortical neurons at 2 DIV were subjected to immunoprecipitation (IP) with antibody against Par1b

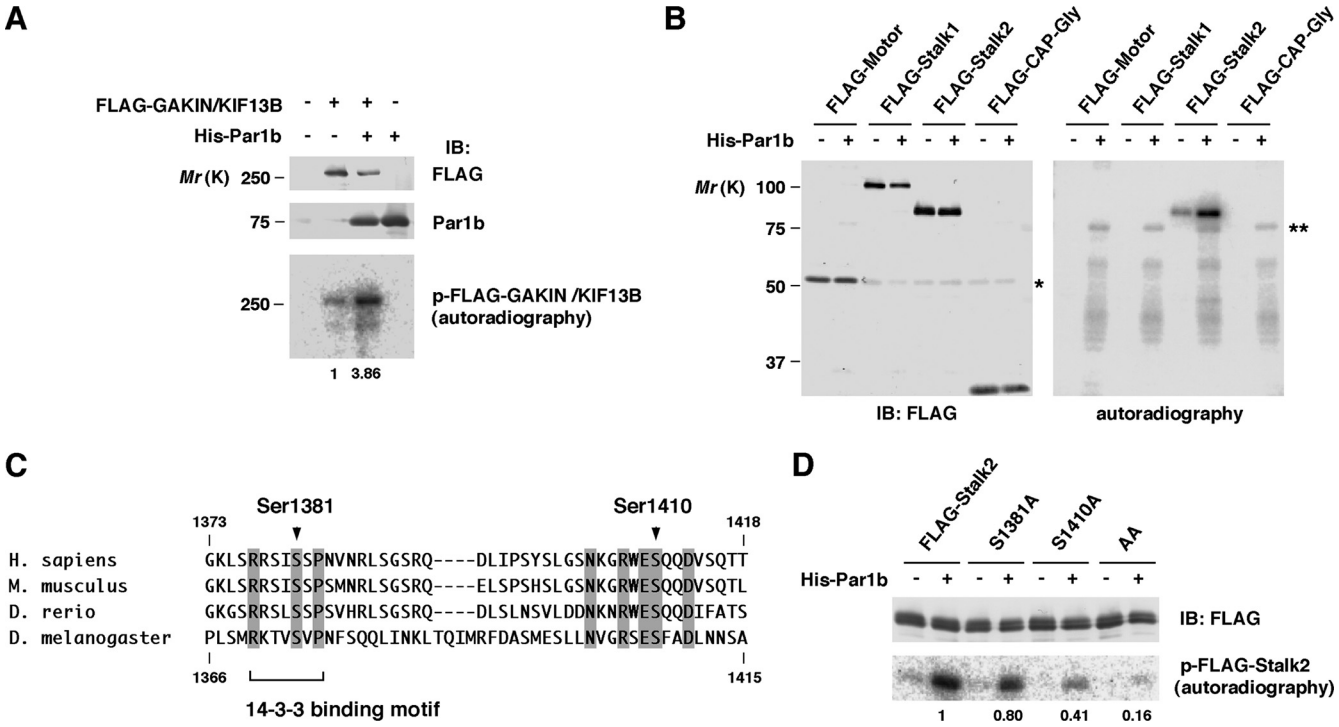

FIG. 2. Phosphorylation of GAKIN/KIF13B by Par1b. (A) FLAG-GAKIN/KIF13B was expressed in COS-7 cells and then immunoprecipitated. The precipitates were subjected to kinase assays for His-Par1b (+). The control experiments (−) were performed with samples that had been obtained by subjecting *E. coli* lysate (not expressing His-Par1b) to the same purification procedure as His-Par1b. Autoradiography represents the phosphorylation level of FLAG-GAKIN/KIF13B. (B) The indicated deletion constructs of GAKIN/KIF13B were expressed and subjected to kinase assays as in panel A. The positions of IgG (*) and autophosphorylated His-Par1b (**) are indicated. (C) Alignment of the amino acid sequence of the region of human GAKIN/KIF13B containing Ser1381 and Ser1410 with the corresponding sequences. The human (*Homo sapiens*), mouse (*Mus musculus*), zebrafish (*Danio rerio*), and fruit fly (*Drosophila melanogaster*) sequences are shown. The evolutionarily conserved amino acids surrounding Ser1381 and Ser1410 are shown on a gray background. The 14-3-3 binding motif is also indicated. (D) The indicated mutagenized constructs of FLAG-Stalk2 were subjected to kinase assays as in panel A.

CAP-Gly) of GAKIN/KIF13B were generated, and each was tested for its ability to interact with FLAG-Par1b. We found that the interaction occurs at Stalk1 and Stalk2 (Fig. 1D), suggesting that at least two independent Par1b-binding sites exist. Furthermore, an *in vitro* binding assay with purified recombinant His-Par1b and GST-Stalk2 revealed a direct interaction between these proteins (Fig. 1E). We could not determine whether GST-Stalk1 binds His-Par1b directly *in vitro* because recombinant GST-Stalk1 was not expressed in sufficient quantities in *E. coli* (data not shown). Finally, we expressed and purified full-length recombinant His-Par1b and His-GAKIN/KIF13B (see Fig. S1B in the supplemental material), and subjected them to an *in vitro* binding assay using anti-Par1b antibody. The results clearly indicated that His-GAKIN/KIF13B binds His-Par1b (Fig. 1F), thus confirming the interaction of full-length proteins.

GAKIN/KIF13B is phosphorylated by Par1b. It was reported that GAKIN/KIF13B is heavily phosphorylated by unidentified kinases (13). Given that the Ser/Thr kinase Par1b associates with GAKIN/KIF13B, we examined whether Par1b could phosphorylate GAKIN/KIF13B. FLAG-GAKIN/KIF13B overexpressed in COS-7 cells was immobilized on beads, and the beads were subjected to His-Par1b kinase assays. As shown in Fig. 2A, His-Par1b phosphorylated FLAG-GAKIN/KIF13B. Next, we examined the phosphorylation sites in GAKIN/KIF13B. To do this, FLAG-tagged deletion constructs of GAKIN/KIF13B were used as substrates for His-Par1b kinase assays (Fig. 1C). Among these deletion con-

or GAKIN/KIF13B. The precipitates were analyzed by immunoblotting (IB) with the indicated antibodies. Immunoprecipitations with control rabbit IgG and without lysates (− Lysates) were also performed as controls. The multiple bands for Par1b represent alternative splicing isoforms (indicated by the black arrowheads to the right of the gels). (C) Domain map of GAKIN/KIF13B and its deletion constructs (the numbers are the positions of the amino acid residues). (D) COS-7 cells were transfected with the indicated constructs, and the lysates were subjected to anti-FLAG immunoprecipitation. The lysates and precipitates were analyzed by immunoblotting with the indicated antibodies. The positions of nonspecific bands (*) and IgG (**) are indicated. (E) Recombinant His-Par1b was incubated with GST-Stalk2 or GST immobilized on beads as a control. Proteins were subjected to SDS-PAGE and visualized by staining with Coomassie brilliant blue. Ten percent of His-Par1b was loaded as input. (F) His-GAKIN/KIF13B and His-Par1b were mixed and immunoprecipitated with anti-Par1b antibody. The precipitates were analyzed by immunoblotting with the indicated antibodies.

structs, only FLAG-Stalk2 was phosphorylated by His-Par1b (Fig. 2B). Furthermore, we also confirmed that purified GST-Stalk2 was phosphorylated by His-Par1b (data not shown), indicating that Par1b phosphorylates GAKIN/KIF13B directly.

When we compared the amino acid sequences of GAKIN/KIF13B in various species, we noticed that two serine residues in the Stalk2 region, Ser1381 and Ser1410, were evolutionarily conserved from *Drosophila melanogaster* to mammals (Fig. 2C). In addition, the sequence surrounding Ser1381 matched the consensus sequence for the 14-3-3 binding motif, RSX pSXP or RXXXpSXP (pS stands for phosphorylated serine) (39, 40). Because several reports have indicated that Par1b phosphorylates the 14-3-3 binding motif in its substrates, including histone deacetylase 7 (HDAC7) and Bazooka/Par3 (5, 8), we focused on these Ser residues as candidate phosphorylation sites. To test this possibility, three mutated constructs of FLAG-Stalk2 in which these Ser residues were substituted for Ala (S1381A, S1410A, and AA [S1381A/S1410A]) were subjected to His-Par1b kinase assays. The phosphorylation signals of S1381A and S1410A were weaker than that of FLAG-Stalk2 and barely detectable for the AA mutation (Fig. 2D), suggesting that Par1b phosphorylates GAKIN/KIF13B at both Ser1381 and Ser1410.

Par1b phosphorylation regulates GAKIN/KIF13B accumulation. Our finding that GAKIN/KIF13B was phosphorylated at Ser1381, which matches the consensus 14-3-3 binding motif, led us to investigate whether the phosphorylation by Par1b could induce 14-3-3 binding with GAKIN/KIF13B. We expressed FLAG-Stalk2 of GAKIN/KIF13B with/without Myc-Par1b and performed immunoprecipitation analyses. We found that the amount of endogenous 14-3-3β coprecipitated with FLAG-Stalk2 increased by the coexpression of Myc-Par1b (Fig. 3A). This augmentation requires Par1b kinase activity because the kinase-negative (KN) mutated form of Par1b did not promote coprecipitation. Furthermore, the S1381A and AA mutants did not interact with 14-3-3β. These results are consistent with the idea that phosphorylation of Ser1381 results in the association with 14-3-3. It should also be noted that a small amount of coprecipitation was observed in the absence of Myc-Par1b coexpression, suggesting that GAKIN/KIF13B is phosphorylated by endogenous Par1b or some other kinase(s).

Next, we investigated the phosphorylation status of GAKIN/KIF13B using an anti-phospho-Ser 14-3-3 binding motif antibody (anti-p-Ser 14-3-3 binding motif antibody) that recognizes the 14-3-3 binding consensus motifs in a phosphorylation-dependent manner (28). As shown in Fig. 3A, the coexpression of Myc-Par1b WT increased the signal intensity at FLAG-Stalk2. In addition, no signal was observed with the mutation in which Ser1381 was replaced with Ala. We also confirmed that part of the endogenous GAKIN/KIF13B in MDCK cells and primary neurons (Fig. 3B) is phosphorylated and associates with 14-3-3β. To examine whether this phosphorylation is dependent on Par1b, two siRNAs against Par1b, Par1b #1 and Par1b #2, were transfected to MDCK or SH-SY5Y cells or hippocampal neurons. These siRNAs reduced the amount of endogenous Par1b compared with control nonsilencing siRNA (Fig. 3C). The phosphorylation signals of GAKIN/KIF13B with anti-p-Ser 14-3-3 binding motif antibody were also decreased, indicating the importance of Par1b in the phosphorylation of Ser1381 of GAKIN/KIF13B *in vivo*.

Because several proteins, including BAD (Bcl2 antagonist of cell death) (mitochondrion-cytosol) (42) and FKHRL1 (a member of the Forkhead family of transcription factors) (nucleus-cytosol) (6), are known to show altered intracellular localization in response to 14-3-3 binding, we examined whether coexpression of Par1b could affect GAKIN/KIF13B localization in MDCK cells. Consistent with previous observations (4), overexpression of Myc-GAKIN/KIF13B induced the formation of protrusions, and Myc-GAKIN/KIF13B was heavily concentrated at the tips of these protrusions (Fig. 3D and E) (see Fig. S2 in the supplemental material). Interestingly, coexpression of FLAG-Par1b dispersed Myc-GAKIN/KIF13B throughout the cytoplasm and suppressed GAKIN/KIF13B-induced formation of protrusions. In contrast, FLAG-Par1b KN showed no effect. To further confirm that the effect of Par1b depends on phosphorylation of GAKIN/KIF13B, we performed similar experiments with the AA mutant of Myc-GAKIN/KIF13B. When this mutated construct was expressed alone, localization of Myc-GAKIN/KIF13B AA was similar to that of Myc-GAKIN/KIF13B. However, the localization did not change in response to coexpression of FLAG-Par1b. Taken together, these data indicate that Par1b regulates intracellular localization (accumulation) of GAKIN/KIF13B through phosphorylation.

To further examine the molecular mechanism of GAKIN/KIF13B accumulation, we treated MDCK cells expressing Myc-GAKIN/KIF13B with nocodazole, which depolymerizes microtubules. Nocodazole treatment clearly abolished the accumulation of GAKIN/KIF13B, suggesting the importance of microtubules (see Fig. S3A in the supplemental material). Next, we examined the possible effect of Par1b on the interaction of GAKIN/KIF13B with microtubules. A significant amount of FLAG-GAKIN/KIF13B expressed in COS-7 cells was coprecipitated (pellet) with Taxol-stabilized microtubules (see Fig. S3B in the supplemental material). However, when Myc-Par1b was coexpressed, the amount of FLAG-GAKIN/KIF13B in the pellet fraction decreased, further supporting that GAKIN/KIF13B accumulation occurs in a microtubule-dependent manner. It has been reported that an intramolecular interaction in GAKIN/KIF13B inhibits its microtubule binding activity (41). Therefore, we next examined whether phosphorylation by Par1b affects the intramolecular interaction in GAKIN/KIF13B using FLAG-Motor and Myc-Stalk constructs (Fig. 1C). First, we could confirm the intramolecular interaction between FLAG-Motor and Myc-Stalk or Myc-Stalk AA (see Fig. S3C in the supplemental material). However, when Myc-Par1b was coexpressed, the amount of Myc-Stalk coprecipitated with FLAG-Motor showed an increase compared to that of Myc-Stalk AA. Thus, phosphorylation of GAKIN/KIF13B by Par1b augments the intramolecular interaction. Collectively, consistent with the results in Fig. 3D and E, phosphorylation-induced intramolecular interaction of GAKIN/KIF13B should negatively regulate its microtubule binding activity, resulting in the dispersion of GAKIN/KIF13B in the cytoplasm.

Endogenous GAKIN/KIF13B accumulates at axon tips. It has been suggested that GAKIN/KIF13B is involved in neuronal polarity (14). Primary cultures of mammalian hippocampal neurons are used routinely for analyses of cell polarity (3). Therefore, first, we examined localization of endogenous

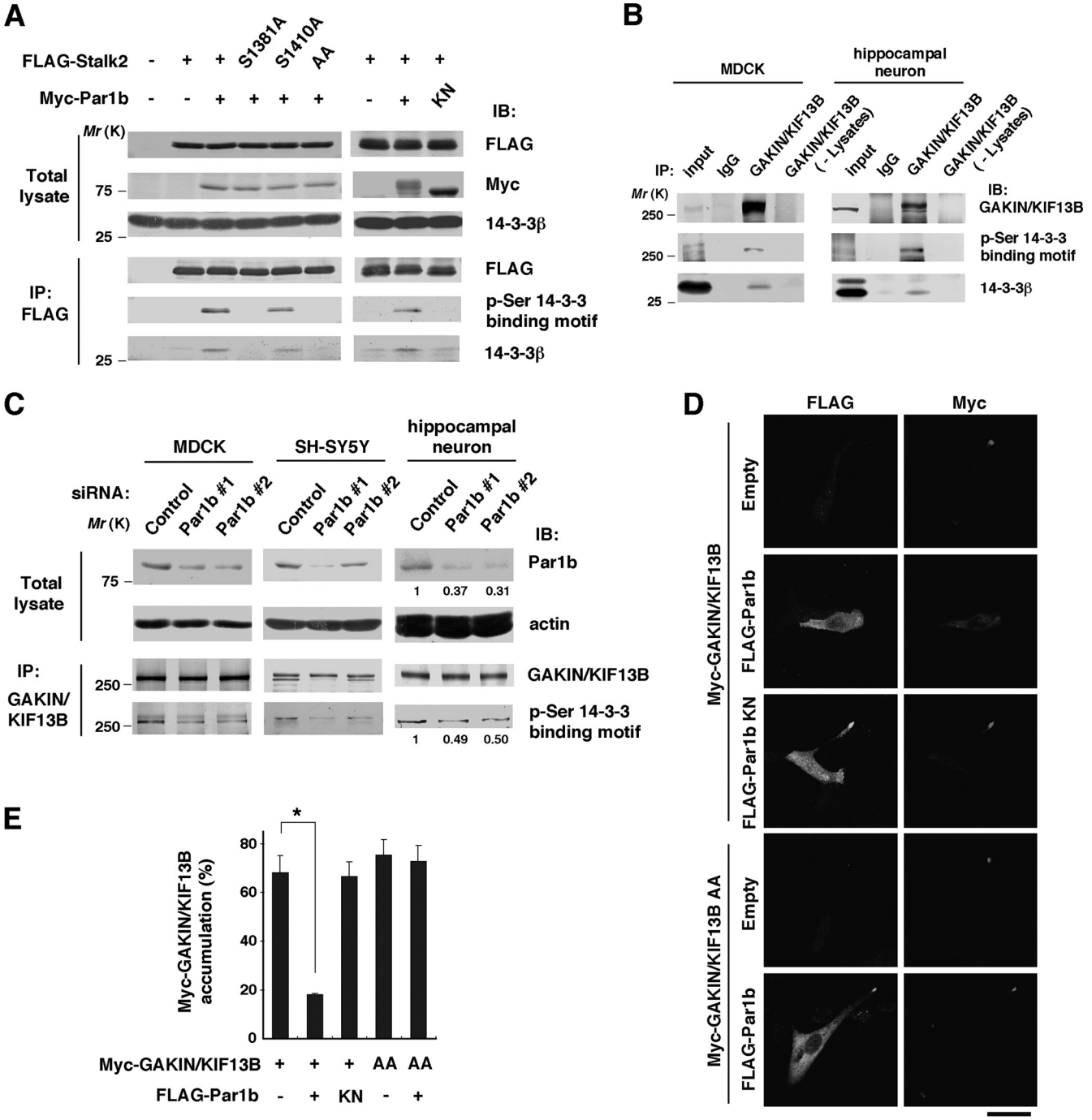

FIG. 3. Par1b phosphorylation induces 14-3-3 binding with GAKIN/KIF13B. (A) COS-7 cells were transfected with the indicated constructs. The lysates and anti-FLAG immunoprecipitates were subjected to immunoblotting with the indicated antibodies. (B) Lysates of MDCK cells or hippocampal neurons at 2 DIV were subjected to immunoprecipitation with antibody against GAKIN/KIF13B. The precipitates were analyzed by immunoblotting with the indicated antibodies. Immunoprecipitations with rabbit IgG and without lysates were performed as controls. (C) MDCK, SH-SY5Y cells, or hippocampal neurons at 2 DIV were transfected with the indicated siRNAs. The lysates and anti-GAKIN/KIF13B immunoprecipitates were subjected to immunoblotting with the indicated antibodies. The numbers below the column indicate the ratio of band intensity normalized to actin (means of three independent experiments). (D) MDCK cells were transfected with the indicated constructs. The cells were cultured for an additional day and stained for FLAG and Myc. Bar, 20 μm. (E) Myc-GAKIN/KIF13B accumulation in MDCK cells. Myc-GAKIN/KIF13B accumulation is defined as large dot-like signals observed exclusively at the tips of protrusions. Three independent experiments were performed, and >60 cells were counted in each experiment. The values show the means plus SDs (error bars). Values that are significantly different ($P < 0.001$) are indicated by an asterisk.

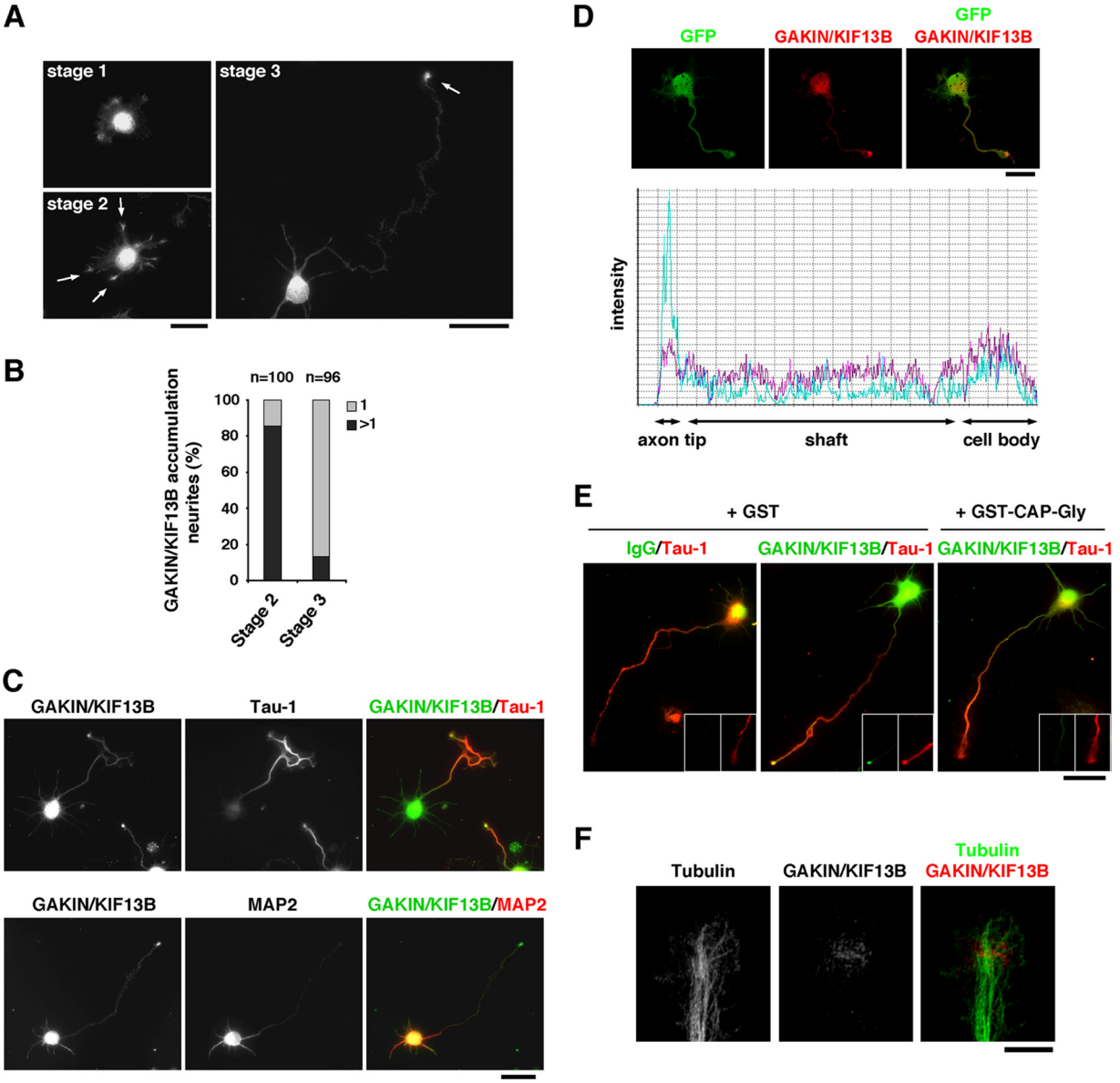

FIG. 4. Accumulation of GAKIN/KIF13B at axon tips. (A) Representative GAKIN/KIF13B-stained images of stage 1, stage 2, and stage 3 hippocampal neurons from E18 rats are indicated. The accumulation of GAKIN/KIF13B at the tips of neurites is indicated by the small white arrows. Bars, 20 μm. (B) Percentage of hippocampal neurons that have a single neurite (gray) or two or more neurites (black) containing GAKIN/KIF13B accumulation at stage 2 ($n = 100$) or stage 3 ($n = 96$). GAKIN/KIF13B accumulation is defined here as a level greater than 4 times the shaft intensity level. (C) Neurons at 3 DIV were stained for GAKIN/KIF13B and Tau-1 or MAP2. Merged images are also presented (GAKIN/KIF13B shown in green; Tau-1 and MAP2 shown in red). Bar, 20 μm. (D) (Top) Neurons were transfected with GFP and then stained for GAKIN/KIF13B at 3 DIV (green, GFP; red, GAKIN/KIF13B). Bar, 20 μm. (Bottom) The fluorescence intensity in the axon tip, shaft, and cell body was measured and is shown in the graph plot using line scan imaging (purple, GFP; blue, GAKIN/KIF13B). (E) Anti-GAKIN/KIF13B antibody was preincubated with GST (control) or GST-CAP-Gly immobilized on beads for 2 h. Neurons at 3 DIV were stained with absorbed anti-GAKIN/KIF13B antibody or IgG (control) (green) together with anti-Tau-1 antibody (red). Green and red signals at the axon tips are also shown separately. Bar, 20 μm. (F) Neurons at 3 DIV were stained for β-tubulin and GAKIN/KIF13B, and the growth cone area is magnified and indicated. A merged image is also shown (GAKIN/KIF13B [red]; β-tubulin [green]). Bar, 5 μm.

GAKIN/KIF13B in primary cultures of hippocampal neurons from E18 rats. Neurons attach to the culture dishes and form lamellipodia (stage 1) and extend several short processes of similar lengths (stage 2). Then, a single neurite elongates rapidly to become an axon (stage 3) (9). Staining with anti-GAKIN/KIF13B antibody showed positive signals in the soma and neurites, but significant accumulation was observed at the tips of several or all neurites in the majority of stage 2 neurons (Fig. 4A and B). In contrast, at stage 3, such accumulation was observed at the tip of the longest neurite (probably an axon) but had mostly disappeared from the other short neurites, which would likely become dendrites. To confirm whether accumulation of GAKIN/KIF13B during stage 3 occurs at axon tips, neurons were double stained for GAKIN/KIF13B and

axonal marker Tau-1 or dendritic marker MAP2. Tau-1 staining revealed that the long neurites positive for GAKIN/KIF13B were axons, and MAP2 staining showed that GAKIN/KIF13B did not accumulate at dendrite tips (Fig. 4C). To quantify these localization data, we performed line scan analyses by expressing GFP as a volume marker and compared the signal intensity with that of anti-GAKIN/KIF13B. The results indicated that the GAKIN/KIF13B signal is stronger than the GFP signal at the axon tip (Fig. 4D) (see Fig. S4 in the supplemental material).

To eliminate the possibility of nonspecific staining, the anti-GAKIN/KIF13B antibody was first absorbed with plenty of the GST fusion form of immunogen (GST-CAP-Gly) or GST immobilized on beads (control) and then used for staining. As shown in Fig. 4E, the anti-GAKIN/KIF13B antibody pretreated with GST as a control showed a positive signal at the axon tip, but the signal disappeared after incubation with GST-CAP-Gly. However, it should be noted that the signal in the soma might be nonspecific because it still remained regardless of immunogen absorption. Immunoblotting analysis using lysates of hippocampal neurons showed that anti-GAKIN/KIF13B antibody monospecifically recognized an approximately 250-kDa band (see Fig. S1C in the supplemental material), consistent with the electrophoretic mobility of GAKIN/KIF13B. To confirm further the specificity of this antibody, immunoprecipitation of neuron lysates was performed. Immunoblotting of the precipitates revealed specific signal of approximately 250 kDa. No other nonspecific signal was detected (see Fig. S1D in the supplemental material).

Because GAKIN/KIF13B is a kinesin 3 family plus-end-directed microtubule motor protein, we performed double staining of GAKIN/KIF13B and microtubules (anti-β-tubulin antibody) in the growth cones of axons. We found that GAKIN/KIF13B accumulated in a spot-like manner at the central area of the growth cone, where microtubules are concentrated, and several microtubules further extend their distal ends over this central area (Fig. 4F).

Ectopic expression of GAKIN/KIF13B induces extra axon formation. Next, we examined the effect of GAKIN/KIF13B expression in hippocampal neurons. To visualize the morphology of neurons, we cotransfected GFP (Fig. 5A [the coexpression rate was greater than 90%]). Transfection with Myc-GAKIN/KIF13B decreased the rate of polarized neurons, of which the longest process was at least twice as long as the other processes (Fig. 5B). Further, a greater number of neurons expressing Myc-GAKIN/KIF13B possessed two or more axons positive for Tau-1 staining (Fig. 5A). The rate of such neurons was only 5.6% in the control experiment, but GAKIN/KIF13B expression significantly increased the rate to 35.6% (Fig. 5C). When FLAG-Par1b was coexpressed, the rate of neurons containing two or more Tau-1-positive neurites was significantly reduced. In addition, the AA mutation of GAKIN/KIF13B lacking the Par1b phosphorylation sites was resistant against Par1b, suggesting that Par1b negatively regulates GAKIN/KIF13B via phosphorylation, consistent with the previous reports indicating the crucial importance of Par1b in neuronal polarity (7, 43).

A very important finding obtained from the expression analyses is the formation of two or more axons, because this point has never been referred to in the previous study (14). To confirm this finding, we performed expression experiments again by transfecting high-density plated neurons with lipofection at 2 days *in vitro* (DIV) culture, according to the method employed in the previous study. Myc-GAKIN/KIF13B induced the formation of additional long Tau-1-positive axons at 3.5 DIV (Fig. 5D) and was localized at the tips of all axons, similar to endogenous GAKIN/KIF13B (Fig. 5E). To further confirm the axon-forming ability of GAKIN/KIF13B, we also stained 7 DIV neurons expressing GAKIN/KIF13B with an antibody for growth-associated protein 43 (GAP43), another axonal marker. The results indicated that the long additional neurites induced by GAKIN/KIF13B were also positive for GAP43 (see Fig. S5 in the supplemental material). The phenotype of neurons expressing Myc-GAKIN/KIF13B AA was more pronounced than that of neurons expressing Myc-GAKIN/KIF13B, with many bifurcating axon branches (Fig. 5D). Collectively, these results confirm extra axon formation by GAKIN/KIF13B expression, suggesting the instructive role of GAKIN/KIF13B in axon formation.

GAKIN/KIF13B functions downstream of Par1b in axon formation. Loss-of-function analyses of GAKIN/KIF13B have never been performed, and thus, the importance of endogenous GAKIN/KIF13B in axon formation remains to be determined. We performed knockdown analyses by transfecting neurons with siRNAs for GAKIN/KIF13B, together with GFP construct to visualize the morphology. Transfections with two siRNAs for GAKIN/KIF13B, GAKIN/KIF13B #1 and GAKIN/KIF13B #2, reduced the amount of endogenous GAKIN/KIF13B from the levels in control siRNA-transfected neurons (Fig. 6A). We confirmed a significant reduction of the GAKIN/KIF13B signal at the neurite tips at 2 DIV culture (see Fig. S4B in the supplemental material). At 3 DIV, GAKIN/KIF13B knockdown severely retarded the extension of long neurites (Fig. 6B). We then stained neurons with Tau-1 antibody and examined the length of neurites, which revealed that GAKIN/KIF13B knockdown significantly increased the number of neurons lacking a Tau-1-positive axon (Fig. 6C and D). Moreover, expression of Myc-GAKIN/KIF13B, which is of human origin and resistant against GAKIN/KIF13B #2 siRNA because of a three-base-pair mismatch, could rescue the axonless phenotype (Fig. 6D). These results are quite consistent with those of the overexpression analyses (Fig. 5), together supporting a positive role of GAKIN/KIF13B in axon formation.

In addition, we performed double-knockdown experiments of Par1b and GAKIN/KIF13B to show the *in vivo* functional relationship between these proteins. As reported previously (7), transfection of rat Par1b #1 induced the multiple axon phenotype containing two or more axons 6.4% in control neurons and 36.2% in Par1b knockdown neurons (Fig. 6E and F). However, cotransfection of GAKIN/KIF13B #1 with rat Par1b #1 clearly suppressed the multiple axon phenotype (8.5%). Rather, these double-knockdown neurons tended to become axonless, like GAKIN/KIF13B knockdown neurons. Thus, GAKIN/KIF13B knockdown is dominant against Par1b knockdown, placing the function of GAKIN/KIF13B downstream of Par1b in axon formation.

We have shown that Par1b promotes phosphorylation of GAKIN/KIF13B and association with 14-3-3β (Fig. 2 and 3), suggesting that 14-3-3 is also important for axon formation. We first examined the localization of endogenous 14-3-3β in hippocampal neurons at 3 DIV by immunostaining (see Fig. S6A

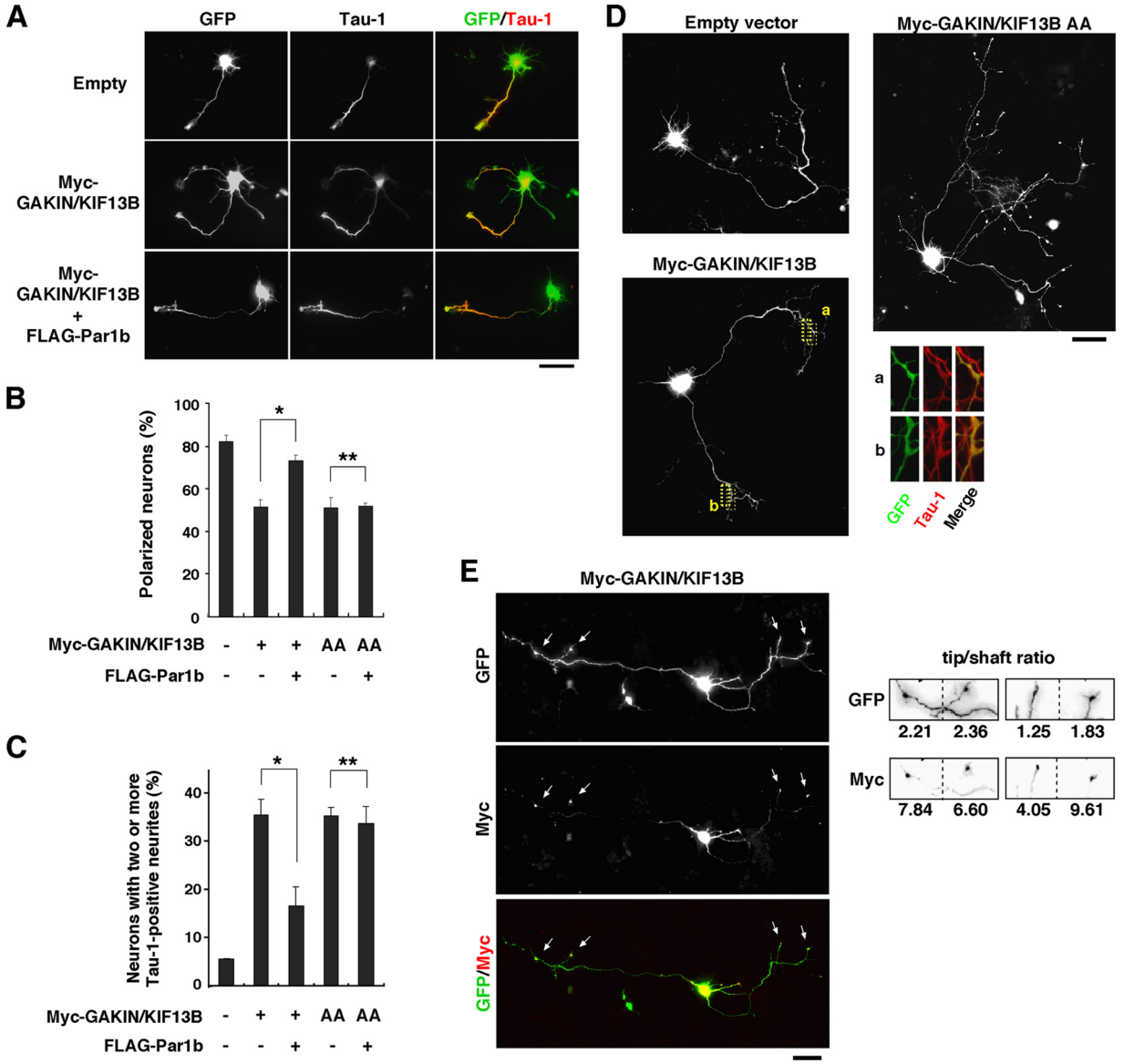

FIG. 5. GAKIN/KIF13B plays a positive role in axon formation. (A) Hippocampal neurons from E18 rats were transfected with empty vector, Myc-GAKIN/KIF13B, or Myc-GAKIN/KIF13B plus FLAG-Par1b together with GFP and then stained for Tau-1 at 3 DIV. Merged images are also presented (GFP shown in green and Tau-1 in red). Bar, 20 μm. (B) Neurons were transfected with the indicated constructs, and the percentage of polarized neurons was determined at 3 DIV. Three independent experiments were performed, and >80 cells were counted in each experiment. The values in panels B and C are means plus SDs (error bars). Values that are significantly different are indicated as follows: *, $P < 0.01$; **, $P = 0.33$. (C) Percentage of neurons that have two or more axons positive for Tau-1. Three independent experiments were performed, and >80 cells were counted in each experiment. Values that are significantly different are indicated as follows: *, $P < 0.01$; **, $P = 0.55$. (D) Neurons were transfected with empty vector, Myc-GAKIN/KIF13B, or Myc-GAKIN/KIF13B AA together with GFP at 48 h after plating by lipofection and observed for GFP fluorescence at 3.5 DIV. Magnified views of the two areas surrounded by yellow dotted lines (a and b) in neurons transfected with Myc-GAKIN/KIF13B and labeled by GFP fluorescence (green), Tau-1 staining (red), and both GFP fluorescence and Tau-1 staining (merged images) are shown. Bar, 20 μm. (E) Neurons were transfected with Myc-GAKIN/KIF13B together with GFP as in panel D. A merged image is also shown (green, GFP; red, Myc). Arrows indicate the tips of the axon-like neurites are indicated by the small white arrows. Bar, 20 μm. The tip/shaft ratios of the GFP and Myc signal intensities are shown to the right of the photomicrographs.

in the supplemental material). 14-3-3β was detected within the soma and proximal neurites, but not in the distal part of axons. Next, we examined the effect of the dominant-negative 14-3-3 construct (DN-14-3-3) (29) on axon formation. Expression of DN-14-3-3 increased the multiple axon phenotype containing two or more axons, as does GAKIN/KIF13B (see Fig. S6B in the supplemental material). Collectively, 14-3-3 appears to functionally inhibit the GAKIN/KIF13B function in axon formation.

PI3K functions upstream of GAKIN/KIF13B via Par1b phosphorylation. To further explore the regulatory mechanism of GAKIN/KIF13B in axon formation, we examined GAKIN/KIF13B accumulation in stage 2 neurons prior to axon specification. First, we ectopically expressed FLAG-Par1b, with

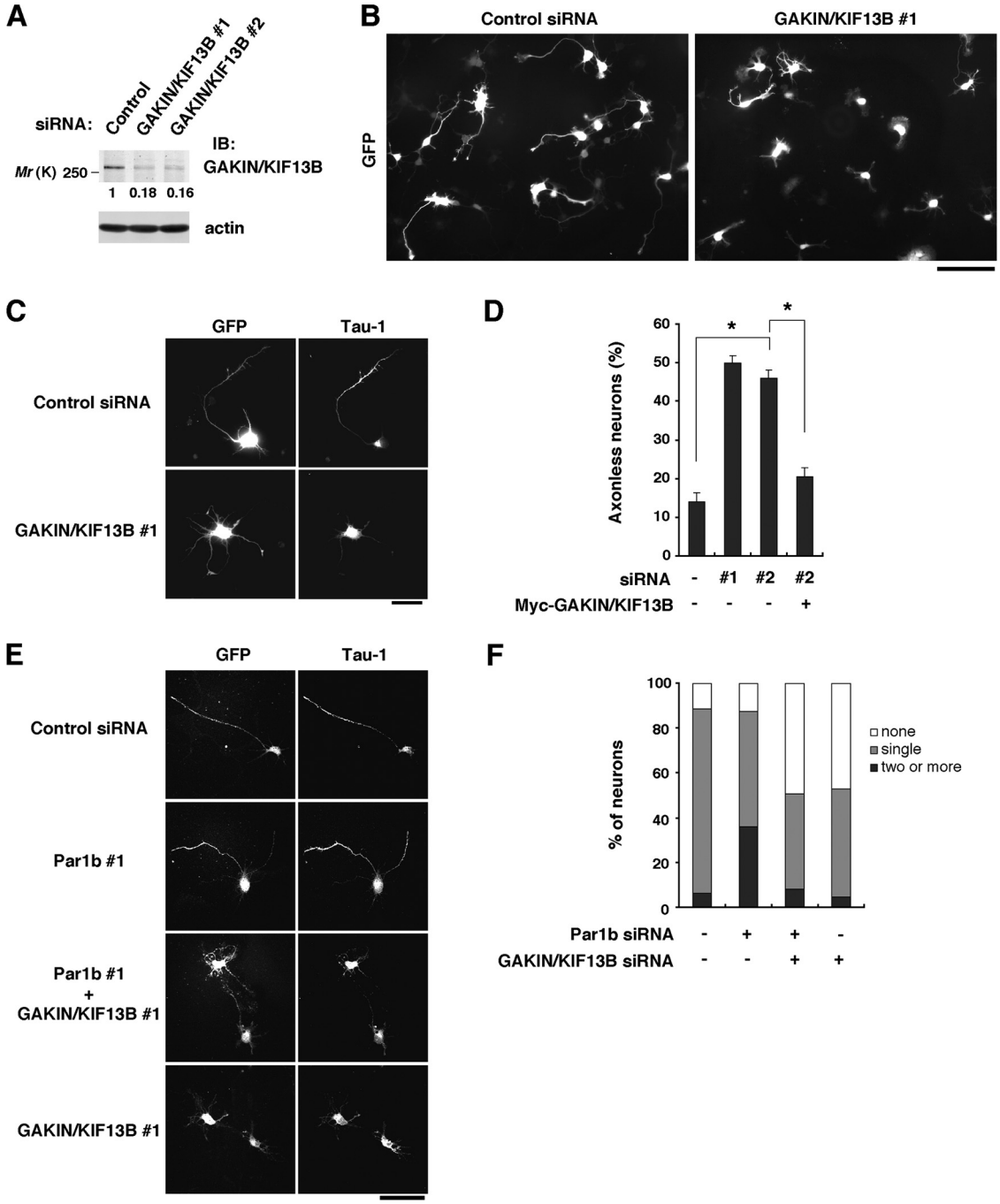

FIG. 6. GAKIN/KIF13B functions downstream of Par1b in axon specification. (A) Hippocampal neurons from E18 rats were transfected with the indicated siRNAs and GFP by electroporation. Lysates of the neurons at 2 DIV were then subjected to immunoblotting with anti-GAKIN/KIF13B antibody. The numbers below the top blot indicate the ratios of band intensity normalized to actin. (B) Neurons transfected as in panel A were visualized with GFP at 3 DIV. Bar, 50 μm. (C) Neurons transfected as in panel A were stained for Tau-1 at 3 DIV. Bar, 20 μm. (D) Percentage of axonless neurons at 3 DIV. Three independent experiments were performed, and >80 cells were counted in each experiment. The values show the means plus SDs (error bars). Values that are significantly different ($P < 0.001$) are indicated by an asterisk. The axons were determined here to be neurites positive for Tau-1 staining and longer than 100 μm. (E) Neurons transfected with the indicated siRNAs were stained for Tau-1 at 3 DIV. Bar, 50 μm. (F) Percentage of neurons with none, one (single), or two or more axons at 3 DIV. Three independent experiments were performed, and >80 cells were counted in each experiment.

GFP to visualize cell morphology and examined the possible effect on GAKIN/KIF13B accumulation. As shown in Fig. 7A, FLAG-Par1b clearly disturbed GAKIN/KIF13B accumulation at the tips of neurites, whereas FLAG-Par1b KN did not. This effect of Par1b on GAKIN/KIF13B accumulation appears to be analogous to the results in MDCK cells (Fig. 3D and E). Because Par1b has been reported to have a negative effect on axon formation (7, 43), our results suggest that it does so

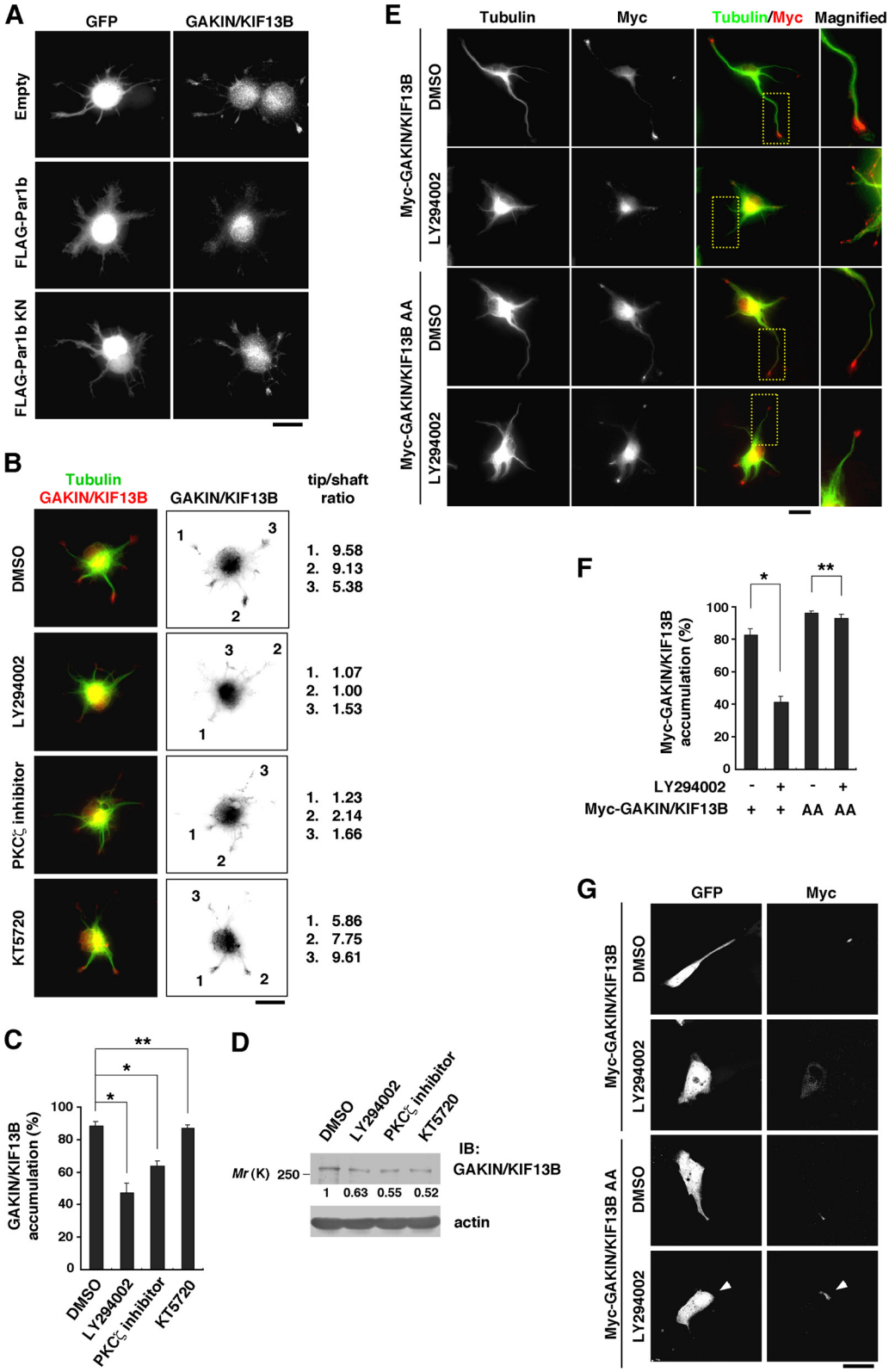

FIG. 7. PI3K regulates GAKIN/KIF13B localization. (A) Hippocampal neurons from E18 rats were transfected with the indicated constructs together with GFP and then stained for GAKIN/KIF13B at 2 DIV. Bar, 10 μm. (B) Neurons at 1 DIV were treated with LY294002 (20 μM), PKCζ inhibitor (10 μM), KT5720 (200 nM), or DMSO (control) for an additional 24 h and stained for β-tubulin and GAKIN/KIF13B (β-tubulin [green]; GAKIN/KIF13B [red]). The tip/shaft ratios of the GAKIN/KIF13B signal intensities are also shown. Bar, 10 μm. (C) Percentage of neurites with GAKIN/KIF13B accumulation at the tip of the neurite at 2 DIV. Three independent experiments were performed, and >80 cells were counted in each experiment. The values show the means plus SDs (error bars). Values that are significantly different are indicated as follows: *, $P < 0.001$; **, $P = 0.50$. (D) Lysates of

through phosphorylation and prevention of accumulation of GAKIN/KIF13B at the tips of growing neurites.

Recent studies revealed that Par1b is phosphorylated at Thr595 by aPKC, such as PKCζ (15, 19, 33). This phosphorylation induces dissociation of Par1b from the membrane, and this is believed to negatively regulate Par1b function. PKCζ can be activated directly by 3-phosphoinositide-dependent protein kinase 1 (PDK1) in a phosphatidylinositol 3-kinase (PI3K) activity-dependent manner (21, 24), which is crucial for axon specification (31). Therefore, we tested the effects of a PI3K inhibitor (LY294002) and a PKCζ inhibitor on GAKIN/KIF13B localization at stage 2 before polarization. We also tested the effect of the PKA inhibitor KT5720. Control dimethyl sulfoxide (DMSO) and KT5720 had no effect on GAKIN/KIF13B accumulation at the neurite tips, whereas LY294002 and the PKCζ inhibitor both disturbed GAKIN/KIF13B localization (Fig. 7B and C). The amount of GAKIN/KIF13B in neurons treated with LY294002 or the PKCζ inhibitor did not differ from that in KT5720-treated neurons (Fig. 7D).

To examine whether the effect of LY294002 on GAKIN/KIF13B accumulation occurs via Par1b, we transfected neurons with rat Par1b siRNAs and then subjected them to LY294002 treatment. The results indicated that the accumulation of GAKIN/KIF13B at the neurite tips was restored to a small but significant extent by Par1b knockdown (see Fig. S7A and B in the supplemental material). Next, we examined the effect of LY294002 on Myc-GAKIN/KIF13B AA. Similar to endogenous GAKIN/KIF13B, Myc-GAKIN/KIF13B also showed significant accumulation at the tips of neurites, and LY294002 prevented accumulation, resulting in smaller Myc-GAKIN/KIF13B signals at the ends of microtubules (Fig. 7E and F). In contrast, accumulation of Myc-GAKIN/KIF13B AA was not inhibited by LY294002. Collectively, these findings suggest that the PI3K signaling may act upstream of Par1b to regulate the accumulation of GAKIN/KIF13B in neurons.

We performed similar experiments in MDCK cells. As shown in Fig. 7G, LY294002 abolished both the accumulation of Myc-GAKIN/KIF13B and the formation of protrusions, which is very similar to our observation in Par1b-coexpressing cells (Fig. 3D). As expected, Myc-GAKIN/KIF13B AA was resistant to dispersion by LY294002. These results are consistent with the idea that GAKIN/KIF13B accumulation is regulated by Par1b-dependent phosphorylation under the control of PI3K signaling. We also noticed that Myc-GAKIN/KIF13B AA in the LY294002-treated MDCK cells showed clear accumulation but did not induce protrusions and stayed at the cortical region of the cell, suggesting that the PI3K signaling not only regulates GAKIN/KIF13B accumulation but also is required for elongation of protrusions.

DISCUSSION

Here we have shown that GAKIN/KIF13B plays a critical role in axon formation of hippocampal neurons, which is regulated by Par1b-mediated phosphorylation. The first mammalian counterpart of Par1 discovered was MARK, a kinase that destabilizes microtubules (10, 16). The effect of Par1 on microtubules has generally been attributed to its ability to phosphorylate several MAPs, including Tau and MAP2. Whether such MAPs are involved directly in regulation of polarity is unknown. In this regard, it should be noted that accumulation of GAKIN/KIF13B in the longest neurite and determination of polarity occur at the same time (at the transition from stage 2 to 3 [Fig. 4A and B]). Furthermore, overexpression of GAKIN/KIF13B could partially induce extra axon formation (Fig. 5), and knockdown analyses supported the involvement of GAKIN/KIF13B in axon specification (Fig. 6C and D). Taken together with the previous report (14), GAKIN/KIF13B appears to be an important regulator of neuronal polarity.

In addition, GAKIN/KIF13B is the mammalian ortholog of *Drosophila* Khc-73, which has been shown to play a crucial role in microtubule-induced Pins/Gαi cortical polarity (32). The two phosphorylation sites in GAKIN/KIF13B are also conserved in Khc-73 (Fig. 2C), suggesting that GAKIN might be an important player for cell polarity, of which activity is regulated by phosphorylation. However, in the regulation of Pins/Gαi cortical polarity, Khc-73 functions in a manner independent of Par proteins, and thus, the regulatory mechanism of GAKIN/KIF13B by Par1b in this study cannot be simply applicable to the understanding of the *Drosophila* Pins/Gαi cortical polarity. This suggests an interesting possibility that some unidentified kinase(s) might phosphorylate and regulate Khc-73.

To further understand the role of Par1b in axon specification, it is very important to clarify the spatiotemporal activation/inactivation pattern of Par1b. Chen et al. (7) utilized anti-phospho-Par1b antibody to detect inactivated Par1b, because it is generally thought that Par1b phosphorylation by aPKC leads to its functional inactivation. The authors indicated that phosphorylated Par1b accumulates at the tips of axons, suggesting that Par1b is inactivated in a single neurite, probably by the function of the PI3K and Par complex, and this neurite would then become an axon. Therefore, Par1b inactivation in one neurite appears to be a key step for the specification of axon from multiple candidate neurites. Because Par1b inhibits the

neurons at 2 DIV treated as in panel B were subjected to immunoblotting with anti-GAKIN/KIF13B antibody. The numbers below the top blot indicate the ratios of band intensity normalized to actin. (E) Neurons were transfected with Myc-GAKIN/KIF13B and Myc-GAKIN/KIF13B AA, plated, and cultured for 24 h. LY294002 or DMSO was added, and the cells were incubated for 24 h. Neurons at 2 DIV were stained for β-tubulin and Myc. Merged images (green, β-tubulin; red, Myc) are also presented. The areas outlined by yellow dotted lines in the Tubulin/Myc column are magnified. Bar, 10 μm. (F) Percentage of neurons with accumulation of Myc-GAKIN/KIF13B or Myc-GAKIN/KIF13B AA at the tip of the neurite at 2 DIV in the presence or absence of LY294002. Three independent experiments were performed, and >80 cells were counted in each experiment. The values show the means plus SDs (error bars). Values that are significantly different are indicated as follows: *, $P < 0.001$; **, $P = 0.09$. (G) MDCK cells were transfected with Myc-GAKIN/KIF13B or Myc-GAKIN/KIF13B AA together with GFP. LY294002 or DMSO was added, and the cells were incubated for 20 h. The cells were stained for Myc. Bar, 20 μm. The accumulation of Myc-GAKIN/KIF13B AA in cells treated with LY294002 is indicated by white arrowheads.

accumulation of GAKIN/KIF13B at the tips of neurites (Fig. 7A), GAKIN/KIF13B accumulation should be allowed to occur in a single Par1b-inactivated neurite and stimulate it to become an axon.

Both endogenous and overexpressed GAKIN/KIF13B tend to accumulate at the tips of protrusions, but how such accumulation occurs is unknown. We found that a GAKIN/KIF13B mutant lacking the N-terminal Motor domain partly localized at the centrosome (data not shown), suggesting that GAKIN/KIF13B first localizes at the centrosome and then moves on microtubules to the tips of neurites or axons. When GAKIN/KIF13B reaches the distal ends of microtubules, it would become docked and accumulate there. The results shown in Fig. S3B in the supplemental material indicate that the Par1b-mediated phosphorylation inhibits the interaction of GAKIN/KIF13B with microtubules. This would be important for Par1b to inhibit GAKIN accumulation, because phosphorylated GAKIN/KIF13B loses the microtubule-binding ability and cannot move on microtubules.

In vitro kinase assays indicated that Par1b phosphorylates GAKIN/KIF13B at Ser1381 and Ser1410 (Fig. 2D). Ser1381 matches the consensus for the 14-3-3 binding motif, and indeed, 14-3-3 binding with GAKIN was confirmed by coimmunoprecipitation analyses (Fig. 3B). Monomeric 14-3-3 directly binds one phosphorylated Ser in its target protein, but it functions as a dimer through simultaneous binding to another phosphorylation site, which promotes stable association with the target (40). Ser1410 might be the secondary phosphorylation site for 14-3-3, because the S1410A mutation did not abolish but slightly reduced the amount of coimmunoprecipitation (Fig. 3A). The results shown in Fig. S6 in the supplemental material suggest the negative role of 14-3-3 in axon formation, but the amount of endogenous 14-3-3β appeared to be very small or absent at the tips of not only axons but also of minor neurites. When GAKIN/KIF13B is phosphorylated, it should associate with 14-3-3 and dissociate from the microtubules. Therefore, GAKIN/KIF13B is not able to accumulate at the tips of minor neurites by utilizing the microtubules, which might explain the reason why 14-3-3 inhibits the elongation of minor neurites even though it does not exist at the tips.

It remains unresolved how GAKIN/KIF13B accumulation contributes to axon formation. GAKIN/KIF13B is a motor protein that can transport PIP3-containing lipid vesicles on microtubules through an interaction with centaurin-α1/PIP3BP (14). Because PIP3 is known to accumulate at axon tips and plays an essential role in axon specification (23, 30, 31), GAKIN/KIF13B might contribute to axon formation through regulation of PIP3 accumulation at axon tips. On the other hand, our experimental results with the PI3K inhibitor placed PIP3 upstream of GAKIN/KIF13B (Fig. 7). Therefore, GAKIN/KIF13B can be placed both upstream and downstream of PIP3.

One possible scenario is that GAKIN/KIF13B might participate in a positive-feedback pathway that spatially regulates PIP3 (Fig. 8). A similar feedback regulatory mechanism of PIP3 was first discovered in chemoattractant- or PIP3-stimulated neutrophils (37, 38). In this feedback loop, PI3K and small GTPases in the Rho family, including Rho, Rac, and Cdc42, play crucial roles. In the axon specification process, the Par complex mediates Cdc42-induced Rac activation (26), and this mechanism is thought to play a role in positive-feedback regulation of PIP3, because activated Rac can bind PI3K (35). Such feedback mechanisms should play essential roles in polarity formation, especially spontaneous polarity formation (2) as is observed in the axon specification from several candidate neurites.

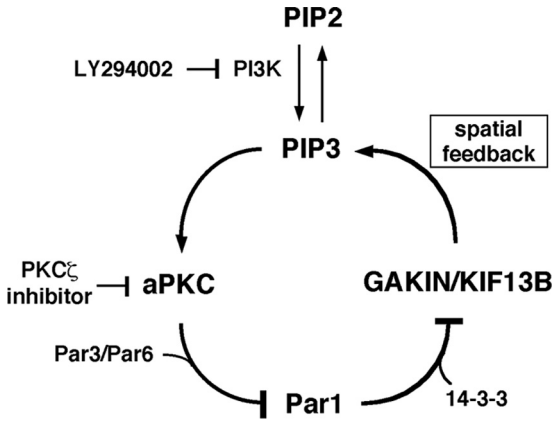

FIG. 8. Schematic diagram of a model for positive-feedback signaling mediated by Par1/GAKIN/KIF13B. PIP3 is generated from PIP2 by PI3K and activates aPKC via PDK1. Activated aPKC forms the complex with Par3/Par6 and phosphorylates Par1. Phosphorylated Par1 is excluded from the membrane and functionally inactivated. Unphosphorylated Par1 phosphorylates GAKIN/KIF13B and inhibits microtubule-dependent accumulation of GAKIN/KIF13B cooperatively with 14-3-3. Unphosphorylated (14-3-3-free) GAKIN/KIF13B accumulates at the protrusion tips with PIP3-containing vesicles. Such a spatial positive-feedback mechanism may assemble PIP3 at specific sites in cells to regulate cell polarity.

GAKIN/KIF13B localization is dependent not only on PIP3 but also on the integrity of microtubules because nocodazole treatment abolishes GAKIN/KIF13B localization (see Fig. S3A in the supplemental material). Such behavior is similar to those of cytoplasmic linker protein (CLIP)-associated proteins (CLASPs). CLASPs are microtubule plus-end-tracking proteins (+TIPs) that can interact with other +TIPs, such as CLIPs and end-binding protein 1 (EB1) (1). CLASPs also localize at the distal ends of microtubules in the leading edge of serum-stimulated motile fibroblasts, which was inhibited by the treatment with PI3K inhibitors. It was later reported that CLASPs associate directly with LL5β, a pleckstrin homology (PH) domain-containing protein that can bind to PIP3 (27) and accumulate at the cortical areas (20). In addition, LL5β is localized around focal adhesions where the cell adheres to the extracellular matrix through integrins. It would be interesting to examine the importance of GAKIN/KIF13B in cellular processes, such as motility and adhesion, and clarify the functional relationship between GAKIN/KIF13B and other PIP3-regulated +TIPs.

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