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RESEARCH ARTICLE

The Scaffold Protein KATNIP Enhances CILK1 Control of Primary Cilia

Jacob S. Turner,[a]* Ellie A. McCabe,[a]* Kevin W. Kuang,[a] Casey D. Gailey,[a#] David L. Brautigan,[b] Ana Limerick,[a] Elena X. Wang,[a] Zheng Fu[a]

[a]Department of Pharmacology, University of Virginia, Charlottesville, Virginia, USA
[b]Department of Microbiology, Immunology and Cancer Biology, University of Virginia, Charlottesville, Virginia, USA

ABSTRACT The primary cilium functions as a cellular sensory organelle and signaling antenna that detects and transduces extracellular signals. Mutations in the human gene *CILK1* (ciliogenesis associated kinase 1) cause abnormal cilia elongation and faulty Hedgehog signaling, associated with developmental disorders and epilepsy. CILK1 is a protein kinase that requires dual phosphorylation of its TDY motif for activation and its extended C-terminal intrinsically disordered region (IDR) mediates targeting to the basal body and substrate recognition. Proteomics previously identified katanin-interacting protein (KATNIP), also known as KIAA0556, as a CILK1 interacting partner. In this study we discovered that CILK1 colocalizes with KATNIP at the basal body and the CILK1 IDR is sufficient to mediate binding to KATNIP. Deletion analysis of KATNIP shows one of three domains of unknown function (DUF) is required for association with CILK1. KATNIP binding with CILK1 drastically elevated CILK1 protein levels and TDY phosphorylation in cells. This resulted in a profound increase in phosphorylation of known CILK1 substrates and suppression of cilia length. Thus, KATNIP functions as a regulatory subunit of CILK1 that potentiates its actions. This advances our understanding of the molecular basis of control of primary cilia.

KEYWORDS CILK1, KATNIP, kinase, phosphorylation, primary cilia

INTRODUCTION

Most mammalian cells rely on a microtubule-based protrusion on their apical surface, known as the primary cilium, to detect and transmit extracellular signals.[1,2] Primary cilia dysfunction is responsible for a broad range of human disorders known as ciliopathies.[3,4] Proper spatial arrangement of membrane receptors, ion channels, enzymes, and signaling molecules along the surface of the primary cilium is crucial for its sensory and signaling functions. The length of the primary cilium is tightly controlled during tissue development and differentiation, and abnormal length is a key feature of ciliopathies.[5] Human gene *CILK1* (ciliogenesis associate kinase 1), mutated in ciliopathies and epilepsy,[6–9] encodes an evolutionarily conserved protein kinase that serves as a negative regulator of cilia length.[10]

CILK1, formerly known as intestinal cell kinase (ICK),[11] is similar to both mitogen activated protein kinases (MAPKs) and cyclin-dependent kinases (CDKs) in the kinase domain.[12] CILK1 activation requires the phosphorylation of threonine in a MAPK-like TDY motif by cyclin-dependent kinase 20 (CDK20), also known as cell cycle related kinase (CCRK).[13] The phosphorylation of Tyr in the TDY motif is by intramolecular self-phosphorylation.[12] CILK1 differs from classic MAPKs by the presence of a long, unstructured carboxyl-terminal domain (CTD). This intrinsically disordered region (IDR) of

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Address correspondence to Zheng Fu, zf6n@virginia.edu.

*These authors contributed equally.

#Current address: Department of Cell and Developmental Biology, Vanderbilt University, Nashville, Tennessee 37240, USA.

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CILK1 is necessary for ciliary targeting and substrate recognition,[14] presumably by mediating CILK1 interactions with regulatory proteins and substrates.

KATNIP (katanin interacting protein), also known as KIAA0556, is a basal body protein mutated in Joubert syndrome, a ciliopathy.[15] KATNIP was identified as a binding partner for CILK1 from a yeast two-hybrid screen.[15] The only recognizable features of KATNIP are three domains of unknown functions (DUFs). KATNIP binds to microtubules and interacts with ciliary transport proteins and the MT-severing enzyme p60/p80 katanins.[15] Interactions with these proteins have been mapped to different DUFs, implicating that KATNIP acts as a scaffold protein to support multiple ciliary functions.[15] *KATNIP* null fibroblasts exhibit abnormally long cilia, but the molecular basis of this phenotype is yet to be elucidated.[15] Herein, we show that KATNIP colocalizes with CILK1 at the cilia base, binds to the IDR of CILK1 dependent on one DUF domain. The KATNIP-CILK1 association considerably stabilizes CILK1 and enhances its TDY phosphorylation. KATNIP markedly increases CILK1 phosphorylation of substrates and restricts cilia length. These results together demonstrate that KATNIP enhances CILK1 activation and function.

RESULTS

CILK1 colocalizes with KATNIP at centrioles. GFP-CILK1 was previously shown to be predominantly localized to the basal body in cells, as visualized by fluorescence microscopy.[14,16] In contrast, GFP-CILK1 kinase domain (residues 1–284) without its extended C-terminal region was diffusely distributed throughout the cell.[14] Thus, the C-terminal domain (residues 285–632), composed entirely of an IDR, is required for localization of CILK1 to the basal body. Our hypothesis was that the CILK1 C-terminus interacted with another protein anchored in the basal body. KATNIP is a basal body protein reported as a CILK1 interacting protein in a proteomic study.[17] Therefore, we used fluorescence microscopy to test whether full length CILK1 colocalizes with KATNIP. We transfected plasmids encoding GFP-CILK1 and Flag-KATNIP into NIH3T3 cells. Centrioles were visualized by staining with anti-γ-tubulin. We observed a predominant overlap of fluorescent GFP-CILK1 (green) and anti-Flag-KATNIP (red) signals with anti-γ-tubulin (magenta) (Fig. 1). This colocalization occurs on both the mother and daughter centrioles, though we observed more intense staining of the mother (Fig. S1). A total of 188 individual cells were examined, in three independent experiments, and the overlap of fluorescence was observed in every one. These results demonstrated CILK1 colocalization with KATNIP at centrioles.

CILK1 IDR interacts with KATNIP. We mapped regions of CILK1 required for interaction with KATNIP by testing portions of the protein for binding by coprecipitation. We co-expressed full-length or a series of truncated GST-CILK1 (Fig. 2A) with full length Flag-KATNIP in HEK293T cells and pulled down GST-CILK1 using Glutathione Sepharose beads. We immunoblotted to quantify the amount of Flag-KATNIP bound to various GST-CILK1 on beads. As a control we immunoblotted for the expression levels in Flag-KATNIP in cell extracts and calculated the ratio of Flag-KATNIP protein in the two fractions. Truncation of only 61 residues from the C-terminus of CILK1 (1-571) reduced Flag-KATNIP binding by 30% compared to the full-length CILK1 (1-632) (Fig. 2B). Further truncation of CILK1 to residue 477 or to residue 377 or to 284 essentially eliminated binding of Flag-KATNIP. The N-terminal kinase domain (1–284) alone did not bind Flag-KATNIP. Indeed, the C-terminal IDR segment of CILK1 (285–632) was sufficient to bind Flag-KATNIP and was as effective as the full-length CILK1 in the pulldown assay (Fig. 2B). These data clearly showed interaction between KATNIP and the C-terminal IDR of CILK1 and that CILK1 IDR is necessary and sufficient for the interaction with KATNIP.

DUF2 is required for KATNIP-CILK1 interaction. Analysis of the KATNIP sequence based on NCBI Conserved Domain Database (CDD) identified three DUFs (domain of unknown functions), as illustrated in Fig. 3A. We hypothesized that one or more of these DUFs would be responsible for binding to the IDR in CILK1. We generated four truncated KATNIP constructs comprised of different DUFs, each with a Flag tag for

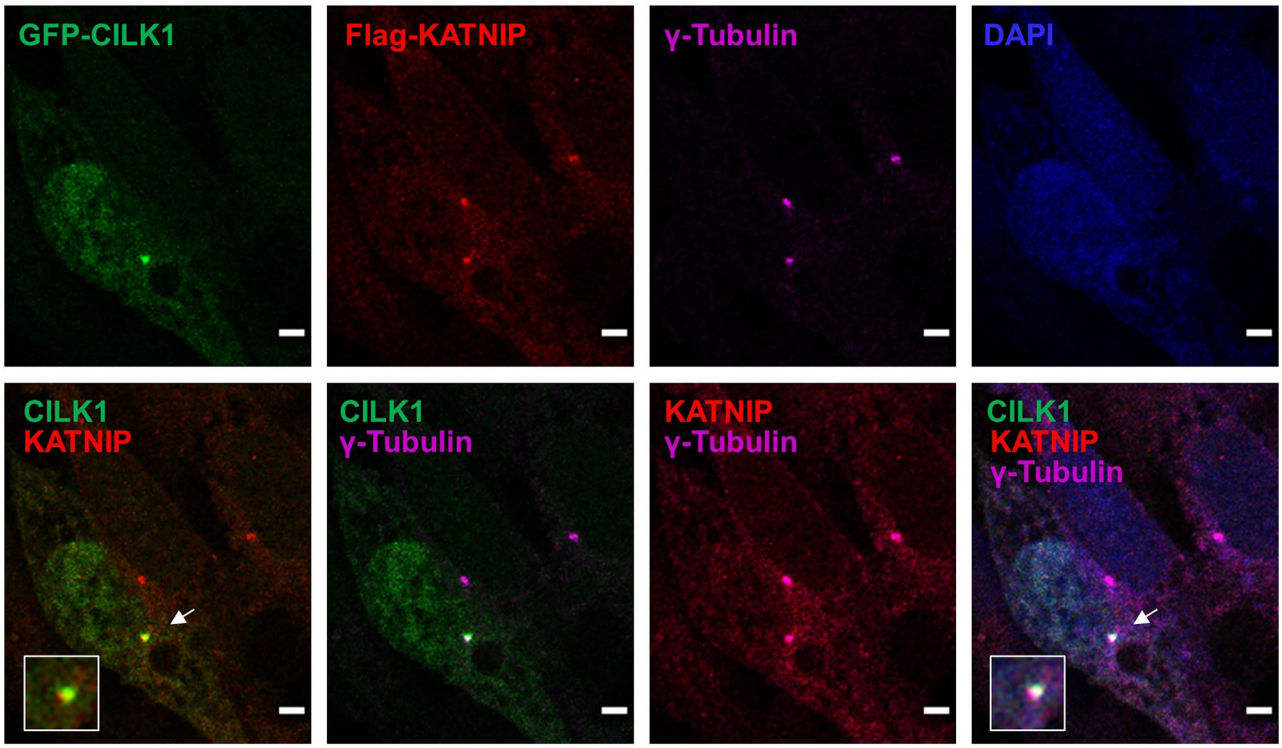

FIG 1 Colocalization of CILK1 and KATNIP at centrioles. NIH-3T3 cells were cotransfected with GFP-CILK1 and Flag-KATNIP and immunostained with primary antibodies recognizing the Flag tag and the centriole marker γ-tubulin followed by Alexa Fluor-conjugated secondary antibodies. The confocal fluorescence images show GFP-CILK1 (green), Flag-KATNIP (red), γ-tubulin (cyan), and nucleus (DAPI, blue). Overlay of GFP-CILK1 and Flag-KATNIP is shown as yellow and overlay of GFP-CILK1, Flag-KATNIP, and γ-tubulin as white. Representative images from 188 cilia examined, three independent experiments. Scale bar, 3 μm.

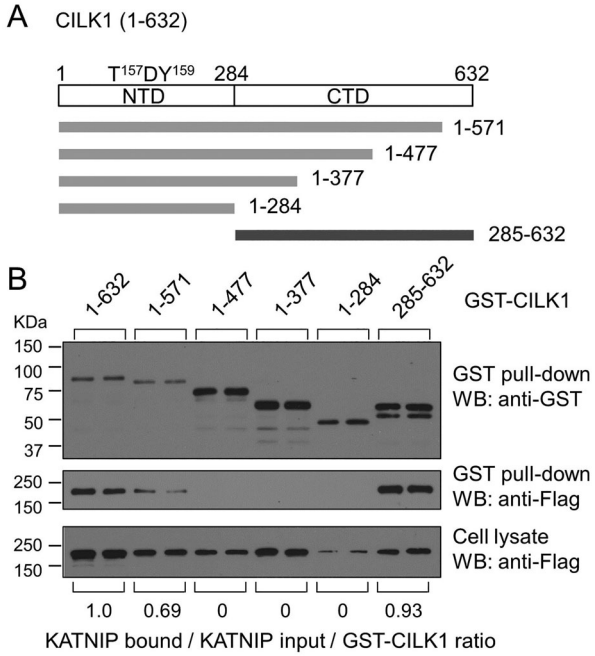

FIG 2 CILK1 IDR is sufficient to mediate binding to KATNIP. (A) Schematic diagrams of human CILK1 domain structures (NTD, N-terminal domain; CTD, C-terminal domain; the TDY motif in the activation loop) and CILK1 truncated constructs used in (B). (B) GST-tagged CILK1 full length or truncated constructs were cotransfected with Flag-KATNIP into HEK293T cells. Flag-KATNIP associated with GST-CILK1 and in whole cell extracts were detected by Western blotting with a Flag antibody. The ratios of Flag-KATNIP associated with GST-CILK1 to the input from cell extracts were indicated. Results with biological replicates were confirmed in two independent experiments.

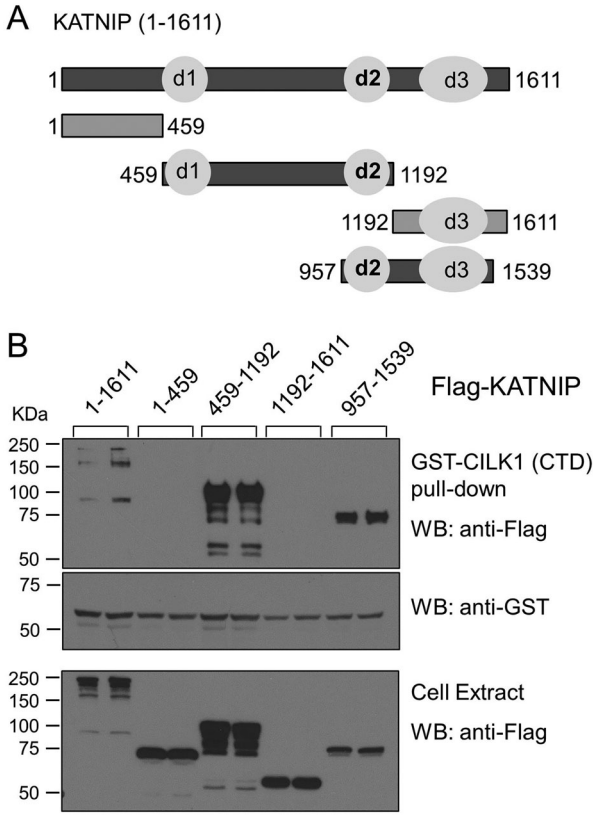

FIG 3 DUF2 of KATNIP is required for interaction with CILK1. (A) Schematic diagrams of KATNIP with three DUFs (domain of unknown functions) and various DUF-deleted constructs used in (B). (B) GST-CILK1 (CTD) was cotransfected with Flag-KATNIP full-length, or deletion constructs into HEK293T cells. Flag-KATNIP associated with GST-CILK1 (CTD) and in cell extracts were detected by a Flag antibody, and GST-CILK1 (CTD) in the pulldown by a GST antibody on Western blots. Results with biological replicates were confirmed in two independent experiments.

identification (Fig. 3A). We individually co-expressed these KATNIP truncated proteins with GST-CILK1-IDR (285-632) in HEK293T cells. Pulldown of protein complexes on GST beads revealed the presence of the full-length KATNIP (1–1611), the fragment of 459–1192 (DUF1 and DUF2), and the fragment of 957-1539 (DUF2 and DUF3), but not the N-terminal fragment of 1-459 (no DUF) or the C-terminal fragment of 1192–1611 (DUF3) (Fig. 3B). All the fragments were abundantly expressed in cells based on anti-Flag immunoblotting of cell extracts (Fig. 3B). These data showed that the protein repeat DUF2 (residues 955–1099) is required for KATNIP interaction with CILK1.

KATNIP stabilizes and activates CILK1. We next examined the effects of KATNIP on CILK1. We transfected cells with either GFP-CILK1 or Flag-KATNIP or both together and immunoblotted the cell extracts with antibodies recognizing CILK1 protein and the phospho-TDY motif of CILK1 (Fig. 4A). When co-expressed with Flag-KATNIP, there was an impressive 5-fold increase in the protein level of GFP-CILK1 (Fig. 4B) and a 2-fold increase in the TDY motif phosphorylation (Fig. 4C), compared to expression without KATNIP. This enhancement of CILK1 protein level and activity by KATNIP was reflected in increased phosphorylation of two endogenous CILK1 substrates, KIF3A and Scythe (Fig. 4A).[13,14,18] We concluded that KATNIP binding stabilized CILK1, probably by reducing its degradation, and enhanced its activation.

A separation of KATNIP actions on CILK1. Since KATNIP fragments, 459–1192 (DUF1 and DUF2) and 957–1539 (DUF2 and DUF3), bind CILK1 (Fig. 3A), we tested if they also enhanced CILK1 activity. We cotransfected either full-length Flag-KATNIP or truncated proteins with GFP-CILK1 into HEK293T cells, pulled down Flag-KATNIP from cell extracts by anti-Flag immunoprecipitation, and immunoblotted for total and phospho-CILK1 associated with Flag-KATNIP. Reinforcing the results in Fig. 3B, full-length and fragments 459–1192 and 957–1539 of KATNIP stably associated with and

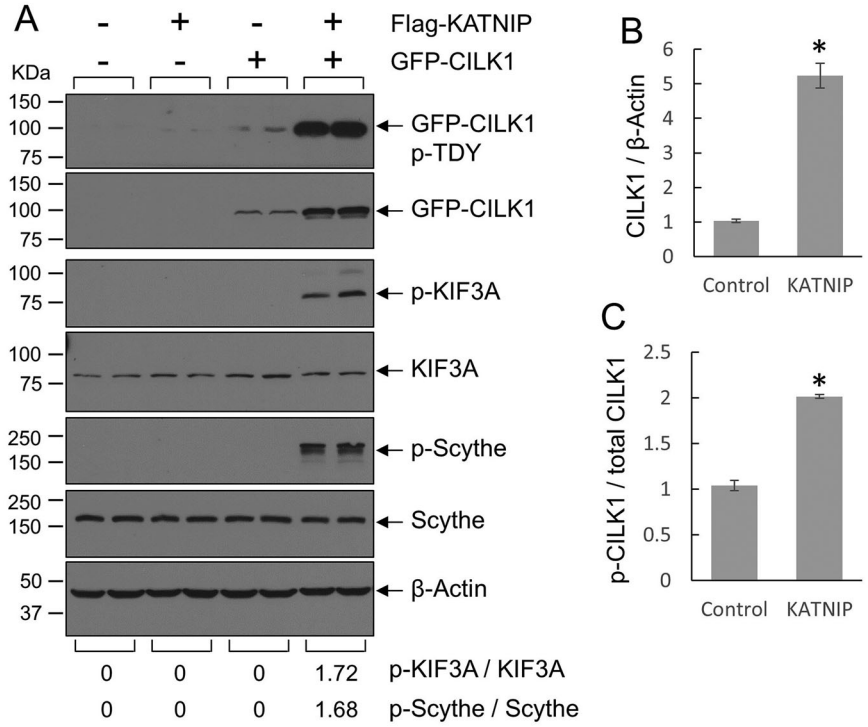

FIG 4 KATNIP stabilization and activation of CILK1. (A) HEK293T cells were transfected with either Flag-KATNIP or GFP-CILK1 or both. Total and phospho-signals of CILK1 and its substrates, KIF3A and Scythe, were detected by Western blotting with antibodies as indicated. β-Actin was blotted as the loading control. The ratios of the phospho-signals to the total signals of endogenous KIF3A and Scythe were indicated. Results with biological replicates were confirmed in two independent experiments. (B) Quantification of GFP-CILK1 against the loading control β-Actin signals, shown as mean ± SD, two-tailed Student's *t* test, $^*P < 0.05$. (C) Quantification of phospho-TDY against total GFP-CILK1 signals, shown as mean ± SD, two-tailed Student's *t* test, $^*P < 0.05$.

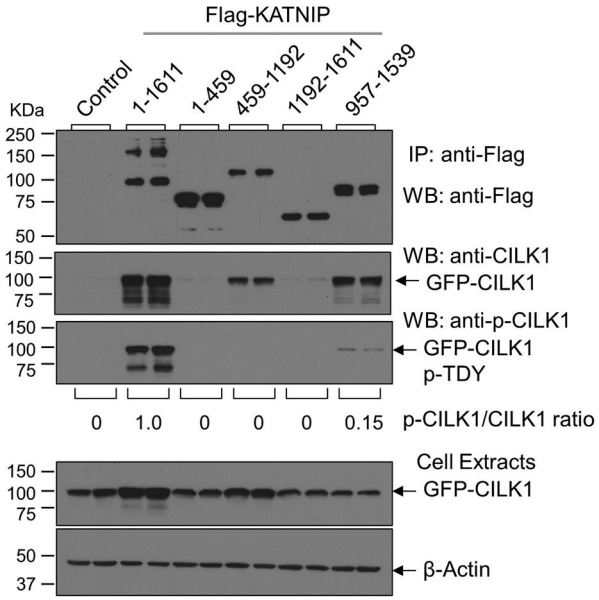

FIG 5 KATNIP binding via DUF2 is not sufficient for activation of CILK1. GFP-CILK1 was cotransfected with Flag-KATNIP full-length or deletion constructs into HEK293T cells. Anti-Flag immunoprecipitation samples were blotted for Flag-KATNIP, total and phospho-TDY signals of GFP-CILK1. Cell extracts were blotted for the total input of GFP-CILK1 and the loading control β-Actin. Results with biological replicates were confirmed in two independent experiments.

coprecipitated CILK1 (Fig. 5). However, we only observed enhanced TDY phosphorylation in CILK1 associated with the full-length KATNIP. In contrast, the phospho-TDY signal in CILK1 was barely detected in complexes with KATNIP fragments (Fig. 5). The results showed that KATNIP fragments 459–1192 and 957–1539 bound CILK1 but did

not produce an increase in kinase activity. This suggests that KATNIP binding via DUF2 is required for binding but not sufficient for activation of CILK1.

KATNIP enhances CILK1 restriction of cilia length. CILK1 negatively regulates cilia length, dependent on its kinase activity.[19,20] To evaluate the effect of KATNIP on CILK1 control of the primary cilium in living cells, we transfected separate plates of NIH-3T3 cells with (1) GFP-CILK1 alone or (2) Flag-KATNIP alone or (3) GFP-CILK1 in combination with Flag-KATNIP. We used Arl13B and γ-tubulin antibodies to stain the cilia body and cilia base, respectively. In confocal microscopic images of cells transfected with GFP-CILK1 alone or in combination with Flag-KATNIP, we measured cilia length in GFP-positive and GFP-negative cells (Fig. 6A). GFP-CILK1 and Flag-KATNIP colocalized with γ-tubulin at the base (Fig. S2), consistent with the results in Fig. 1. In confocal images of cells transfected with Flag-KATNIP alone, we identified Flag-KATNIP-positive cells by Flag antibody fluorescence as illustrated in Fig. 1 and Fig. S2 and measured cilia length in Flag-positive and Flag-negative cells. The expression of GFP-CILK1 alone caused a modest decrease in cilia length. In contrast, there was not a significant difference in cilia length in Flag-KATNIP positive cells compared to Flag-KATNIP negative cells (Fig. 6C).

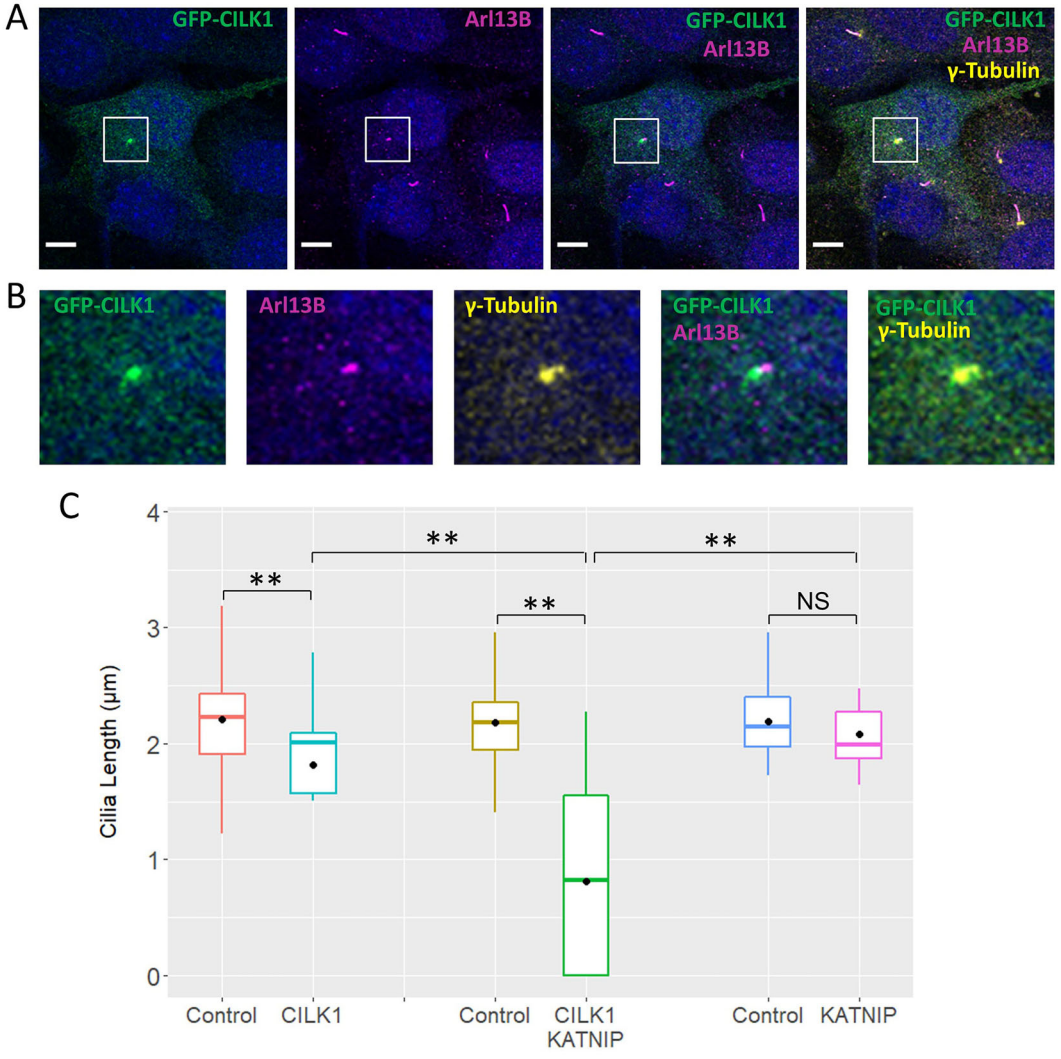

FIG 6 KATNIP enhances CILK1 reduction of cilia length. (A) NIH3T3 cells were cotransfected with GFP-CILK1 and Flag-KATNIP and stained for cilia body and base by Arl13B and γ-tubulin antibodies, respectively. Scale bar, 5 μm. (B) Magnified images of the region highlighted in square in (A), showing both single and double channels of fluorescence. (C) A box plot showing the distribution of numerical values of cilia length in GFP-CILK1-negative (control, n = 98) and GFP-CILK1-positive cells (n = 35), Flag-KATNIP-negative (control, n = 40) and Flag-KATNIP-positive cells (n = 42), median with interquartile range box and min/max whiskers, two independent experiments. One-way ANOVA followed by post hoc Tukey HSD test was used to assess the significance of differences between pairs of group means. The significance level was at *P* values of 0.05 (*) and 0.01 (**). NS = not significant at both *P* values.

Co-expression of Flag-KATNIP and GFP-CILK1 together resulted in a statistically significant reduction in cilia length, compared with the control GFP-negative cells that are either GFP/Flag double negative or GFP-negative/Flag-positive (Fig. 6C). These results demonstrated that KATNIP enhanced the suppressive effect of CILK1 on cilia length in living cells.

DISCUSSION

In this study, we discovered that the basal body protein KATNIP acts as a regulatory subunit of kinase CILK1 to restrict its intracellular localization and amplify its negative control of cilia length. One striking observation was that KATNIP binding to the C-terminal IDR of CILK1 stabilized CILK1 protein, resulting in a 5-fold increase in the protein levels in cells. Interactions between enzyme subunits are known to provide stabilization of holoenzymes by protecting individual subunits from proteolytic degradation, as demonstrated with cyclic AMP-dependent protein kinase.[21] The effects are consistent with the current view that binding partners stabilize intrinsically disordered proteins (IDP) by inducing a disorder-to-order transition of unstructured regions.[22] Based on deletion mapping of KATNIP interactions, we propose that the DUF2 domain induces this transition in CILK1 IDR and the complex accumulates in cells.

In addition to stabilization of CILK1 protein, KATNIP enhanced TDY phosphorylation of CILK1. Binding between CILK1 and KATNIP appears to be necessary but not sufficient for activation because certain fragments of KATNIP are bound CILK1 without an increase in its activity. One possible mechanism is that KATNIP may recruit both CILK1 and its upstream activating kinase, CDK20/CCRK (cyclin-dependent kinase 20/cell cycle related kinase), to the same protein complex. We envision that DUF2 binds to CILK1 while other DUFs in KATNIP may associate with CDK20 to promote phosphorylation of the threonine in the TDY motif of CILK1. Alternatively, CILK1 could exist in an autoinhibited state with the IDR blocking the active site and KATNIP binding would displace the IDR to afford accessibility of CDK20 to the activation loop of CILK1, without formation of a ternary complex. These alternative possible mechanisms for KATNIP activation of CILK1 require further study.

CILK1 and MAK are related human genes from a common ancestor that has an evolutionarily conserved role as a negative regulator of cilia and flagella length.[23–25] MAK also has a TDY motif in its activation loop and a long IDR at the C-terminus, but the IDR sequence is divergent from that of CILK1.[10] KATNIP was identified as a binding partner for both CILK1 and MAK in a cilia interactome study.[17] In Fig. 3 and Fig. S3, when CILK1 and MAK were co-expressed with KATNIP in cells both showed increased TDY phosphorylation, suggesting that CILK1 and MAK may both be regulated by KATNIP.

KATNIP was named as a Katanin-interacting protein based on yeast two-hybrid interactions. The dimeric katanins A1/B1 are known to sever microtubules.[26] In addition to the results presented here, we have preliminary evidence that KATNIP forms a complex with katanin and promotes CILK1-dependent phosphorylation of katanin B1 (not shown). Our hypothesis is that KATNIP promotes activation of CILK1, as shown here, and also assembles a complex of CILK1 with its substrate katanin to activate microtubule severing that reduces the length of cilia. Our future studies will test this hypothesis.

MATERIALS AND METHODS

Reagents. Glutathione Sepharose 4B (17-0756-01) and Gammabind Plus Sepharose® beads (17-0886-01) were from GE Healthcare (Chicago, IL, USA). Anti-FLAG tag affinity beads (ab270704) were from Abcam (Cambridge, MA, USA). pEBG-GST-CILK1 encoding full length and truncated constructs of GST-CILK1 and pEGFP-CILK1 encoding GFP-CILK1 were described previously.[12] pCMV6-Myc-Flag-CILK1 (RC213609), described previously,[27] and pCMV6-Myc-Flag-KATNIP (RC220412) were from Origene (Rockville, MD, USA). KATNIP truncated constructs were cloned into pCMV6-Myc-Flag vector at Custom DNA Constructs LLC (Mount Sinai, NY, USA).

Antibodies. KIF3A phospho-Thr672 and Scythe phospho-Thr1080 rabbit polyclonal antibodies were generated at GenScript (Piscataway, NJ, USA) and described previously.[13,14] CILK1-phospho-TDY antibody was generated in rabbits against keyhole limpet hemocyanin-coupled phospho-CILK1 peptide RSKPPY[pT]D[pY]VSTR at Open Biosystems, Inc. (Huntsville, AL, USA) and described previously.[27]

Phosphopeptide-specific antibodies were affinity-purified through a positive selection over phosphopeptide antigens followed by negative selections over nonphosphopeptide antigens. GST-tag (B-14) mouse monoclonal (sc-138) and HA-tag (12CA5) mouse monoclonal (sc-57592) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). KIF3A (D7G3) rabbit monoclonal (#8507), HA-tag (C29F4) rabbit monoclonal (#3724), DYKDDDDDK Tag (D6W5B) Rabbit monoclonal (#14793) antibodies were from Cell Signaling Technology (Danvers, MA, USA). Arl13B rabbit polyclonal antibody (17711-1-AP) and Gamma-tubulin mouse monoclonal antibody (66320-1-Ig) were from Proteintech (Rosemont, IL, USA). Goat anti-rabbit IgG (Alexa Fluor 488) antibody (ab150081) and goat anti-mouse IgG (Alexa Fluor 594) antibody (ab150120) were from Abcam (Cambridge, MA, USA).

Cell culture and transfection. HEK293T and NIH-3T3 cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, 10% fetal bovine serum (for HEK293T) or 10% newborn calf serum (for NIH-3T3), and penicillin-streptomycin. HEK293T cells were transfected using a calcium phosphate protocol as described previously.[28] NIH-3T3 cells were transfected using lipofectamine (Invitrogen) following the manufacturer's instruction.

GST pulldown, immunoprecipitation, and immunoblotting. Cells were lysed after 48 h of transfection in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 2 mM EGTA, complete protease inhibitors (Roche), 10 mM sodium orthovanadate, 5 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM β–glycerophosphate, and 1 μM microcystin LR). Cell lysate was cleared by microcentrifugation. GST-CILK1 proteins were pulled down from cell extract using Glutathione Sepharose 4B beads (GE Healthcare) following the manufacturer's instructions. Flag-KATNIP proteins were immunoprecipitated from cell lysate using Flag antibody and captured on Gammabind Sepharose beads (GE Healthcare).

Cell extracts or Sepharose beads were boiled for 5 min in an equal volume of 2x Laemmli sample buffer and resolved by SDS-PAGE. Samples were transferred to a PVDF membrane and blocked for one hour in 5% dry milk before primary antibody incubation in TBS containing 0.1% Tween-20 and 5% bovine serum albumin (BSA) for 90 min at RT or overnight at 4 °C. This was followed by multiple rinses and one-hour incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Chemiluminescence signals were developed using Millipore Immobilon ECL reagents.

Immunofluorescence. NIH-3T3 cells grown on coverslips were fixed by 4% paraformaldehyde (PFA) in PBS, rinsed in PBS, and then permeabilized by 0.2% Triton X-100 in PBS. After one hour in blocking buffer (3% goat serum, 0.2% Triton X-100 in PBS), cells were incubated with primary antibodies at 4 °C overnight followed by rinsing in PBS and one hour incubation with Alexa Fluor-conjugated secondary antibodies. After multiple rinses, slides were mounted in antifade reagent containing DAPI (4′,6-diamidino-2-phenylindole) for imaging via a confocal Laser Scanning Microscopy 700 from ZEISS (Chester, VA, USA) at the UVA Advanced Microscopy Facility.

Cilia length measurement. The Zen 2009 program was used with a confocal Laser Scanning Microscope 700 from ZEISS (Chester, VA, USA) to collect z stacks at 0.5 μm intervals to incorporate the full axoneme based on cilia marker Arl13b staining. All cilia were then measured in ImageJ via a standardized method based on the Pythagorean Theorem in which cilia length was based on the equation $L2 = z2 + c2$, in which "c" is the longest flat length measured of the z slices and "z" is the number of z slices in which the measured cilia were present multiplied by the z stack interval (0.5 μm).

Statistical analysis. Quantified experimental data were analyzed by the two-tailed Student's *t* test to compare the means of two groups or one-way ANOVA and post-hoc Tukey HSD test to assess the significance of differences between pairs of group means. For the *t* test, *P* values < 0.05 were considered as significant. For the Tukey test, the significance level at *P* values of 0.05 and 0.01 was used for evaluation.

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AUTHOR CONTRIBUTIONS

Z.F., D.L.B., J.S.T., and E.A.M. designed research; J.S.T., E.A.M., K.W.K., C.D.G., A.L., E.X.W., and Z.F. performed research; J.S.T., E.A.M., C.D.G., A.L., and Z.F. analyzed data; Z.F. and D.L.B. wrote the paper; J.S.T., E.A.M., K.W.K., C.D.G., A.L., and E.X.W. reviewed and edited the paper.

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ORCID

Zheng Fu http://orcid.org/0000-0003-1238-6695

DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the findings is included within the article or its supplementary materials and is also available here: https://doi.org/10.6084/m9.figshare.23650281.v1

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