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Kinesin-14 Pkl1 targets γ-tubulin for release from the γ-tubulin ring complex (γ-TuRC)

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Abbreviations: γ-TuSC, γ-tubulin small complex; γ-TuRC, γ-tubulin ring complex; MTOC, microtubule-organizing center; Klp, kinesin-like protein; SPB, spindle pole body; TRIP, targeting and regulatory interference at poles; aa, amino acid(s); SDM, site-directed mutagenesis; NLS, nuclear localization signal; GFP, green fluorescent protein; WCE, whole-cell extract

The γ-tubulin ring complex (γ-TuRC) is a key part of microtubule-organizing centers (MTOCs) that control microtubule polarity, organization and dynamics in eukaryotes. Understanding regulatory mechanisms of γ-TuRC function is of fundamental importance, as this complex is central to many cellular processes, including chromosome segregation, fertility, neural development, T-cell cytotoxicity and respiration. The fission yeast microtubule motor kinesin-14 Pkl1 regulates mitosis by binding to the γ-tubulin small complex (γ-TuSC), a subunit of γ-TuRC. Here we investigate the binding mechanism of Pkl1 to γ-TuSC and its functional consequences using genetics, biochemistry, peptide assays and cell biology approaches in vivo and in vitro. We identify two critical elements in the Tail domain of Pkl1 that mediate γ-TuSC binding and trigger release of γ-tubulin from γ-TuRC. Such action disrupts the MTOC and results in failed mitotic spindle assembly. This study is the first demonstration that a motor protein directly affects the structural composition of the γ-TuRC, and we provide details of this mechanism that may be of broad biological importance.

Introduction

The division and differentiation of eukaryotic cells depend on transformation of the microtubule cytoskeleton spatially and temporally into polarized structures that enable specialized microtubule networks and processes.[1] These transformations require the γ-tubulin ring complex (γ-TuRC). This multi-protein assembly organizes microtubule polarity and directs microtubule growth.[2,3] Further, it provides a scaffold for formation of the typical 13-protofilament microtubule[4,5] and thereby ensures uniform microtubule diameters and dynamic behavior necessary for many cellular processes.

Fission yeast *S. pombe* is a well-established model system to study the microtubule cytoskeleton and the structure and function of γ-TuRC. Similar to centrosomes of human cells, spindle pole bodies (SPBs) in yeast form the primary MTOC that regulates mitosis.[6-9] Structural models of the yeast γ-tubulin small complex (γ-TuSC) and γ-TuRC have been proposed,[5,10] in which seven γ-TuSC, each forming a V shape, assemble into a ring. This assembly positions γ-tubulin molecules into the required template to initiate growth of microtubule ends. In *S. pombe*, the γ-TuSC proteins are γ-tubulin, Alp4 and Alp6.[11] Human γ-tubulin functionally replaces the *S. pombe* protein,[12] indicating that conserved mechanisms may underlie γ-TuSC function. Details of γ-TuSC assembly into γ-TuRC and its regulated activation remain unclear.

Knowledge of how regulators can perturb the γ-TuRC, particularly at the critical interface of growing microtubule ends with γ-TuSC, is needed to understand its mechanism. Kinesin-like motor proteins (Klps) bind tubulin and are known organizers and regulators of microtubules.[13] kinesin-14 Klps are ubiquitous and one of multiple families present in eukaryotes.[14] In addition to a conserved ATP-dependent Motor domain, kinesin-14 members have unique Tail domain elements that provide functional specialization. *S. pombe* kinesin-14 Pkl1 is the first known Klp regulator of γ-TuSC. Genetically, Pkl1 interacts with all proteins of the γ-TuSC in *S. pombe*[15,16] and its Motor domain binds to γ-tubulin.[17] The Pkl1 Tail domain is also required,[18] but its role was previously unclear. Here we show that the Pkl1 Tail is sufficient to regulate γ-TuRC, independent of all other Pkl1 domains in vivo and in vitro. Two unique elements in this domain allow capture of γ-TuRC and disruption of γ-TuSC by removal of γ-tubulin proteins.

Results

Two novel elements in the kinesin-14 Pkl1 Tail are required for function and spindle pole targeting. It was previously shown that

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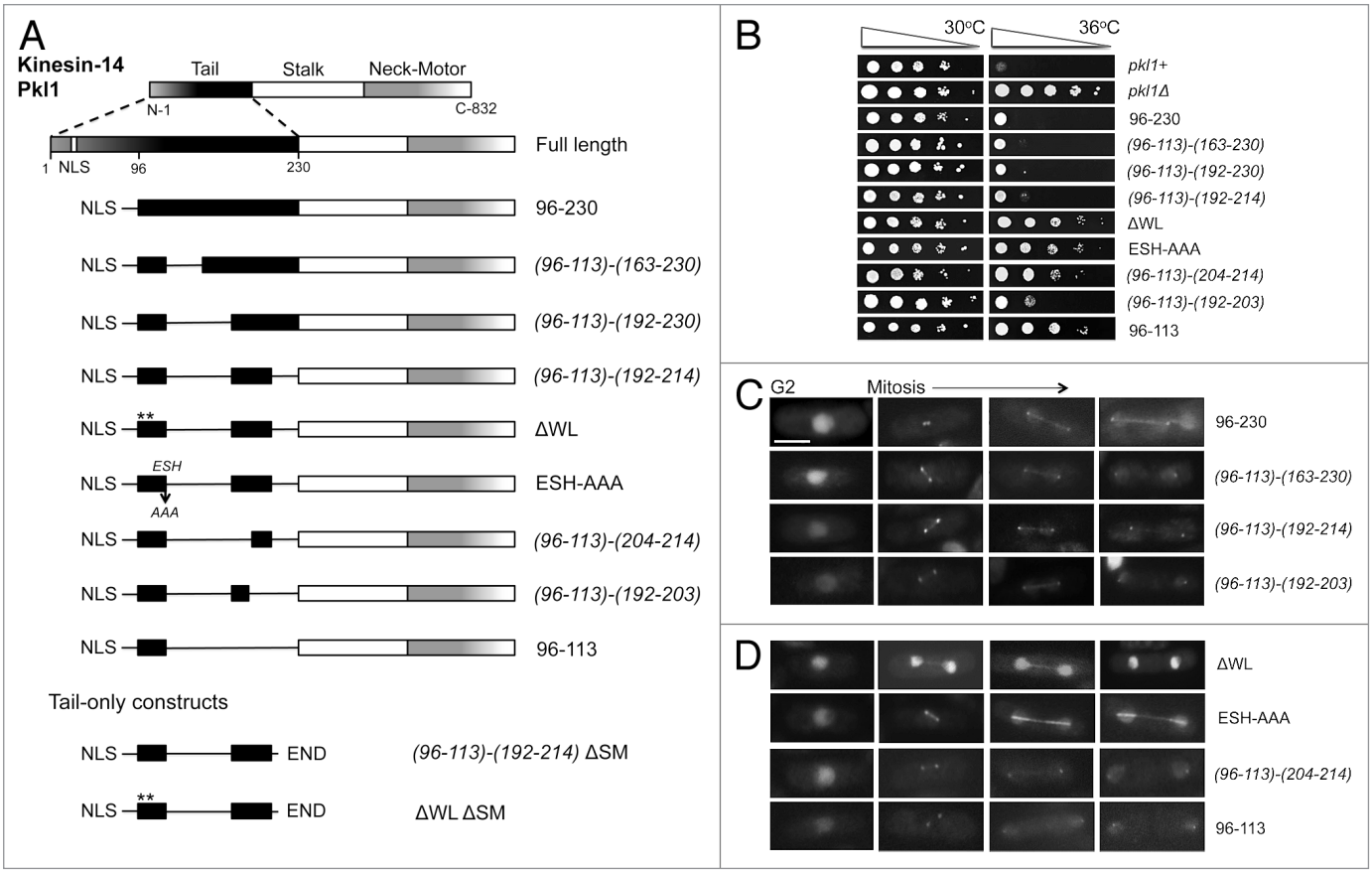

Figure 1. Two domains in kinesin-14 Pkl1 regulate bipolar spindle assembly. (**A**) Diagram of full-length Pkl1 showing domains. Tail constructs are derived from starting construct 96–230 as shown. Amino acid regions 1–95, 114–191 and 204–230 are dispensable for function in our assays. Two Tail-only derivatives were also generated and are expanded on in **Figure 2**. The 10 aa nuclear localization signal (NLS) sequence is deleted in amino acids 1–95 and must be fused to each construct for nuclear entry. (**B**) Representative images (n > 3) of serial dilution growth assays for monitoring Pkl1 function in the presence of altered Tail domain elements. Note, these constructs contain the unaltered Pkl1 Stalk, Neck, and Motor domains. Gradient bars indicate increasing dilutions of cells. Functional Pkl1 inhibits growth at 36°C. (**D and E**) Spindle localization of GFP-Pkl1 fusion proteins with modified Tail regions. Tail derivatives shown in (**D**) are functional, whereas Tail derivatives shown in (**E**) are non-functional based on serial dilution growth assays in (**C**). Therefore, spindle pole targeting is necessary but not sufficient for function. Representative images from n = 200. Scale bar is 5 microns.

a contiguous 135 amino acid segment that lacks the first 95 amino acids of the Pkl1 Tail domain retains function.[18] As an initial step, we generated eight small deletions to this 135 aa Tail domain (**Fig. 1A**) and analyzed these constructs for Pkl1 activity in the double mutant strain (*pkl1*Δ, *cut7*-22ts). In *S. pombe* the two Klps encoded by these genes, kinesin-14 Pkl1 and kinesin-5 Cut7, provide antagonistic activities in spindle assembly.[19] The double mutant strain that lacks Pkl1 grows at 36°C, but growth is arrested when Pkl1 activity is restored by addition of functional constructs. Changes to growth are observed by plating an increasing serial dilution of cells (**Fig. 1B**). All constructs shown in **Figure 1A** were expressed with the strong-nmt promoter and integrated into the genome. In summary, amino acids in Tail regions 96–113 and 192–203 are essential for function, whereas amino acids 114–191 and 204–230 can be deleted. The mutagenesis of residues Trp, Leu (WL) or Glu, Ser, His (ESH) within region 96–113 resulted in loss of function, indicating that these amino acids are essential. Genetic analysis reveals that the Pkl1 Tail can be reduced to a 30 aa sequence comprised of regions 96–113 and 192–203 that along with the remaining Klp domains retains full function in our growth assays.

The ability of kinesin-14 Pkl1 to regulate mitotic spindle assembly requires its localization to spindle poles.[18] To monitor whether our altered Pkl1 constructs localize to poles, we inserted the coding sequence of GFP at the N terminus and analyzed these GFP-Pkl1 fusion proteins by time-lapse microscopy. In **Figure 1D**, spindle pole localization appears similar to the starting control GFP-Pkl1-96–230 for functional derivatives that are GFP-Pkl1-(96–113)-(163–230), GFP-Pkl1-(96–113)-(192–214) and GFP-Pkl1-(96–113)-(192–203). The serial growth assays (**Fig. 1C**) indicate that for region 192–214, segment 192–203 is most important, since Pkl1 activity is lost when it is removed. In the derivative GFP-Pkl1-(96–113)-(204–214) that is missing this important region, spindle pole body localization remains. Removal of region 204–214 does not alter function or localization further (**Fig. 1C and E**). This suggests that although region 192–203 is functionally important, 96–113 is the primary γ-TuRC spindle pole-targeting element.

The ESH to AAA mutation in region 96–113 reduces spindle pole localization and enhances Pkl1 signal on microtubule minus ends. This is more readily seen on longer anaphase B spindles (**Fig. 1E**). We expect that Pkl1 localization here is primarily on microtubules of uniform polarity, since few if any anti-parallel microtubule bundles extend to poles at this late stage.[13,20] Some retention to poles is likely, since Pkl1 has low affinity for microtubules,[21] and so we interpret this as ineffectual loading of Pkl1 onto γ-TuRC. Deletion of WL residues in this region appears to remove Pkl1 from poles, though background nuclear staining complicates its analysis here. In the Pkl1 Tail domain, the two sequence elements 96–113 and 192–203 are therefore required for Pkl1 localization to spindle poles and its ability to regulate spindle assembly from this site.

The Pkl1 Tail can function independently from the Motor domain in vivo. We next investigated whether the Tail domain can localize to the γ-TuRC independently from Motor domain binding to γ-tubulin. To this end, we generated two Tail-only constructs that remove the remaining Pkl1 Stalk, Neck and Motor domains, referred to as ΔSM (delete Stalk to Motor; **Figs. 1A and 2**). Whereas a short Pkl1 Tail domain containing contiguous regions (96–113)–(192–214) ΔSM localizes to spindle poles (**Fig. 2A**), the sole deletion of WL residues in 96–113 prevents spindle pole targeting completely (**Fig. 2B**). In serial dilution growth assays, it was found that the Tail-only derivative (96–113)–(192–214) ΔSM expressed in the (*pkl1*Δ, *cut7-22ts*) strain has significant activity to limit growth at the restrictive temperature vs. the nonfunctional Pkl1 Tail derivative ΔWL ΔSM and *pkl1*Δ. These data indicate that two regions of the Pkl1 Tail are alone sufficient to target and regulate the γ-TuRC, and that this activity is enhanced by the additional Pkl1 Stalk, Neck and Motor domains (**Fig. 1C**).

Pkl1 directly triggers release of γ-tubulin from the γ-tubulin ring complex. In the Pkl1 Tail, the two critical elements are separated by 77 amino acids, which may suggest separable activities to target γ-TuRC and negatively regulate its function (**Fig. 3**). To test this hypothesis, we synthesized three Pkl1 Tail peptides containing 6xHis tags (**Fig. 3A**) referred to as P-γTRC (region 96–113), P-γTRC-SDM (site-directed mutagenesis; region 96–113 with the mutations WL to AA and ESH to GSG) and P-γtBD (mutated region 96–113 plus 192–203). We analyzed these peptides directly on purified γ-TuRC isolated by fast protein liquid chromatography (FPLC) as previously described[3,11,16] (**Fig. 3B**). Peptides are retained on paramagnetic cobalt beads with strong affinity for poly-histidine tagged proteins (Invitrogen). The isolated γ-TuRC fraction is first precleared against empty beads before addition to the peptide-bead mixture for 1–2 h at 4°C. The fraction supernatant is then removed and the peptide-bead-protein complexes washed 3×. Bound proteins are eluted with 300 mM imidazole and analyzed by silver staining and western (**Fig. 3D**). P-γTRC binds γ-TuRC on beads, and the multiprotein complex is retained through all washes. The negative control peptide, P-γTRC-SDM, is unable to bind and retain γ-TuRC, consistent with our in vivo analyses. P-γtBD, unlike P-γTRC, does not retain γ-TuRC and instead pulls γ-tubulin from the complex. Not all γ-tubulin is removed from the γ-TuRC in these experiments, as seen in pre-elution washes (**Fig. 3E**). This peptide data establishes two important mechanistic details. First, the Pkl1 Tail is the primary functional element in the kinesin-14/MTOC mechanism, with two subcomponents for targeting and γ-TuRC disruption. We refer to these contiguous elements as a TRIP domain, for targeting and regulatory interference at poles. Second, whatever functions the Motor domain provides in vivo are not necessary to regulate γ-TuRC in vitro.

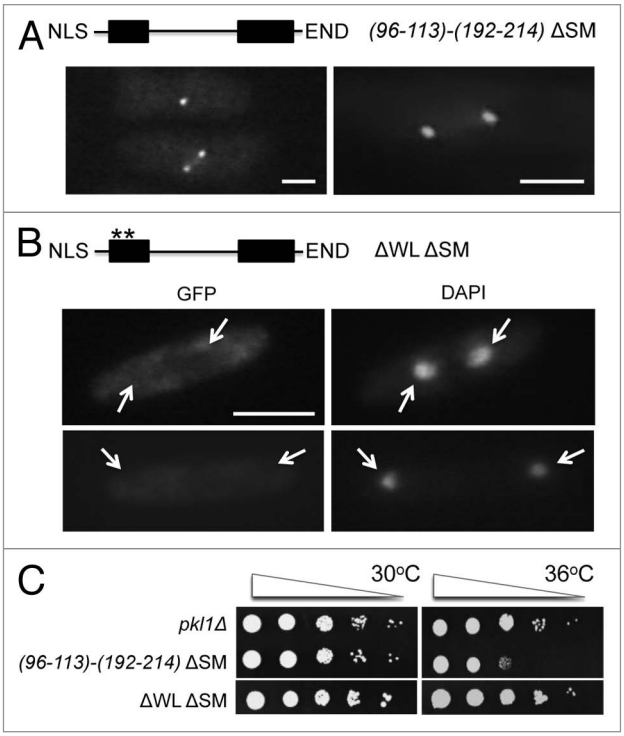

Figure 2. The Pkl1 Tail alone retains sufficient targeting and regulation in vivo. Critical Pkl1 Tail elements were analyzed separately from its other domains by generating Tail-only derivatives (ΔSM: delete Stalk-Motor). (**A**) Tail-only construct (96–113)-(192–214) ΔSM retains spindle pole targeting. Scale bar is 2 microns in both images. (**B**) Tail-only construct ΔWL is absent from poles. Scale bar is 5 microns. Asterisks mark the position of deleted residues. (**C**) Tail-only construct (96–113)-(192–214) ΔSM is sufficient for Pkl1 function with respect to *pkl1*Δ and nonfunctional ΔWL ΔSM in serial dilution growth assays (n > 3). Gradient bars indicate increasing dilutions of cells. Growth inhibition at 36°C indicates functional activity.

Discussion

In this study, we expand our earlier findings in fission yeast on a novel mitotic mechanism, that is kinesin-14 Klp regulation of γ-TuRC for spindle assembly. Here we identify two critical Tail elements in kinesin-14 Pkl1 that enable targeting to γ-TuRC and regulation by removal of γ-tubulin from γ-TuSC in vitro. This is the first report describing how γ-TuRC function is disabled and provides details of how this mechanism is accomplished by a kinesin-14. We refer to the critical Tail elements as a two-component TRIP domain, for targeting and regulatory interference at poles and use this domain to identify other kinesin-14 family members

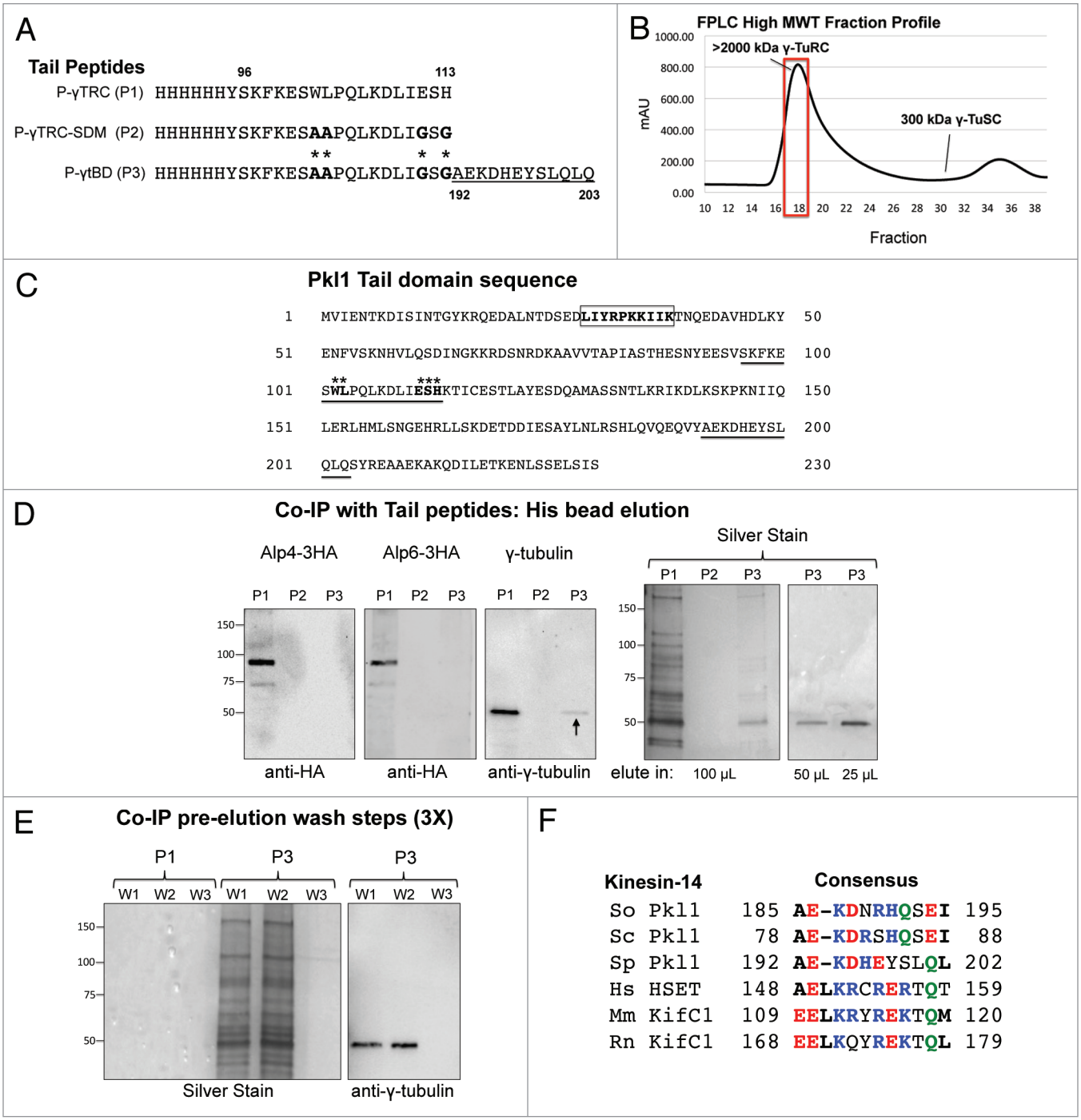

Figure 3. Novel kinesin-14 Tail elements for γ-TuRC binding and γ-tubulin release. Peptide assays. (**A**) Sequences of three Pkl1 Tail peptides (P1, P2, P3) used to analyze the mechanism of γ-TuRC regulation by Pkl1. Starred and bolded residues in P2 and P3 are mutations shown here to disrupt the function of Pkl1. (**B**) Profile of γ-TuRC isolation on Superose 6. MWTs of γ-TuRC and γ-TuSC are indicated along with two MWT markers (2,000 kDa Blue Dextran, 443 kDa Apoferritin). High MWT fraction 17, indicated in red, was used in subsequent assays. (**C**) Native Pkl1 Tail domain sequence. A box indicates the nuclear localization signal (NLS).[18] Underlined sequences correspond to Tail peptides. Bolded amino acids marked with asterisks disrupt function when mutagenized. (**D**) Co-immunoprecipitation (Co-IP) of γ-TuRC occurs with P1, as indicated by westerns of γ-TuSC subunit components Alp4, Alp6 and γ-tubulin. P2 is unable to interact with γ-TuRC as expected. P3 binds to γ-tubulin. Silver stains of the His bead elution indicates γ-TuRC is initially bound but comes off in pre-elution washes shown in (**E**). (**F**) Comparison of the unique P3 sequence (aa 192–203) that contains the γ-TuRC severing activity with similar sequences found in mammalian kinesin-14 Klps in human, mouse and rat. Charged residues are indicated in red (acidic) or (blue) basic. Conserved hydrophobic residues are in black. Conserved glutamine is in green. A cluster of mixed charged residues appears to be important for this function and may help to distinguish kinesin-14 Klp subfamily members.

like human HSET that may share a similar mechanism as kinesin-14 Pkl1.

The integration of the TRIP domain with a Klp is logical in that it provides a means to link regulation of γ-TuRC to spindle functions controlled by other motor proteins. In eukaryotes, kinesin-14 and kinesin-5 families are ubiquitous and provide opposing activities in spindle assembly. In *S. pombe*, kinesin-5 also localizes to spindle poles.[22,23] We propose a new model that may apply, in which kinesin-5 association at or near γ-TuRC prevents Pkl1 from binding. This novel competitive relationship is distinct from that used by other microtubule-based kinesin-14 and kinesin-5 members.[24] In most model eukaryotes, two to three kinesin-14 family members are present. An alignment of kinesin-14 sequences from fission yeast and mammals identified those subfamily members predicted to operate at MTOCs (**Fig. 3F**). In human, mouse and rat, the mixed-charge cluster shown follows the sequence WDLK immediately upstream, similar to the TRIP domain. This domain is absent from Drosophila Ncd, and our cross-species studies previously revealed that human HSET but not Ncd rescues Pkl1 function in fission yeast.[18] The TRIP domain and its mechanism to regulate γ-TuRC may therefore be of broad biological significance and useful in distinguishing kinesin-14 roles in vivo.

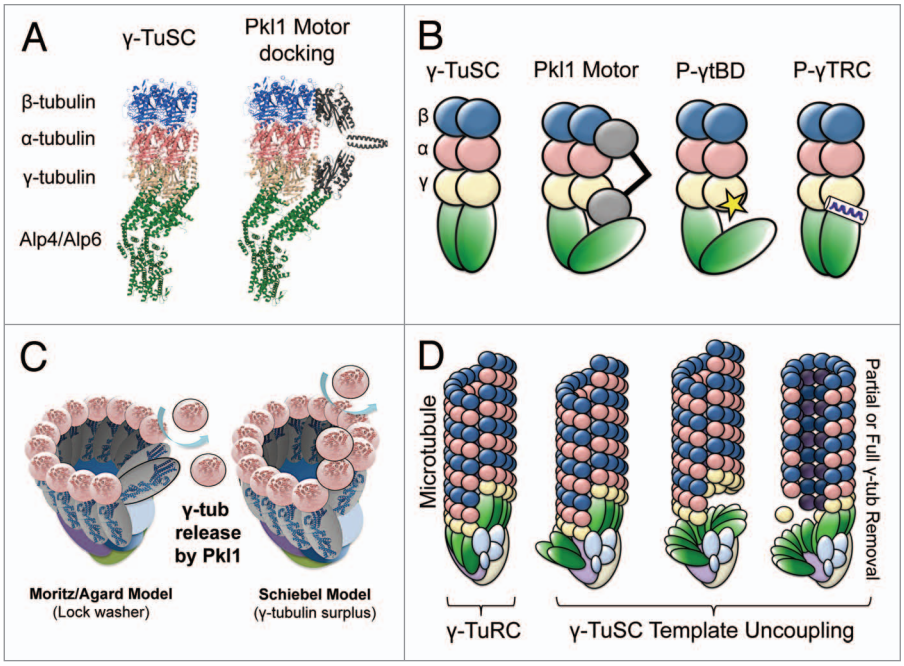

Figure 4. Model for the mechanism of *S. pombe* kinesin-14 Pkl1 regulation of γ-TuRC. Pkl1 associates with the γ-TuRC in vivo to regulate bipolar spindle assembly through Tail and Motor domain interactions. The Pkl1 Motor domain (**A**) binds to γ-tubulin at helix 11, directly adjacent to proposed binding sites of γ-TuSC proteins to γ-tubulin (Alp4 and Alp6 in fission yeast). These adjacent sites may partially overlap. If so, Pkl1 binding could destabilize Alp4/Alp6 interactions with γ-tubulin, priming the system for severing by the Tail domain. This is consistent with enhanced kinesin-14 activity in the presence of the Motor domain in vivo. These concepts are illustrated in (**B**). We propose that two elements of the Pkl1 Tail contribute to this mechanism in different ways, providing γ-TuRC binding and γ-tubulin severing activities. The expected outcome of Motor and Tail activities is to remove γ-tubulin from γ-TuSC and result in conformational inactivation of Alp4/Alp6 as shown in (**C**). The consequence of these actions on microtubules is expected to result in capped microtubule minus ends (**D**). However, full removal of γ-tubulin from microtubule minus ends as well as γ-TuRC may alternatively result in partial or full microtubule depolymerization. The ability of the γ-TuRC to recover from kinesin-14 action remains unclear.

We propose the first model for regulation of γ-TuRC by kinesin-14 that includes Motor binding to γ-tubulin and γ-TuSC disruption by the TRIP domain through γ-tubulin removal. We previously demonstrated that the Pkl1 Motor domain binds to γ-tubulin helix 11.[17] Despite the ability of the TRIP domain to act independently on γ-TuRC, it is clear that in vivo the Motor domain enhances this mechanism. This docking site of the Pkl1 Motor on γ-tubulin may partially overlap with sites used by γ-TuSC. The C-terminal domains of the GCP2/Alp4/Spc97 and GCP3/Alp6/Spc98 families of γ-TuSC proteins are proximate to γ-tubulin helix 11.[10,25] One model is that the Pkl1 Motor domain, when bound to γ-tubulin, interferes with the C terminus of γ-TuSC subunits of γ-TuRC, thus priming the system for more efficient γ-tubulin removal by TRIP (**Fig. 4A and B**).

How the γ-TuSC self-assembles and integrates with additional components to form the γ-TuRC is unknown. Also unclear is how the γ-TuRC can be regulated to control microtubule growth, dynamics and organization. In our peptide assays, Pkl1 removes a subset of γ-tubulin from the γ-TuRC (**Fig. 3E**). A recent study of the budding yeast γ-TuRC indicates that additional γ-tubulin molecules are present along with the seven γ-TuSC subunits and are required for activating the complex[26] (**Fig. 4C**). Kinesin-14 Pkl1 might sever such additional γ-tubulins from the structure, preventing an active γ-TuRC. Alternatively, Pkl1 may sever γ-tubulin from γ-TuSC subunits, perturbing or entirely disrupting the γ-TuRC structure (**Fig. 4D**). We favor a mechanism in which γ-tubulin remains attached to microtubule minus ends, consistent with a cap[27] and with in vitro studies that indicate free minus ends depolymerize more slowly than plus ends[28] shown by TEM to be structurally distinct.[29] As well, the γ-tubulin internal structural domain may not allow rotation to accommodate protofilament bending, as occurs in β-tubulin for depolymerization.[30,31] How γ-tubulin removal by Pkl1 is regulated and the rate at which γ-TuSC and γ-TuRC can recover remains unclear.

The novel mechanism of γ-TuRC regulation by kinesin-14 in *S. pombe* determined by parallel in vivo and in vitro analysis provides the first model for understanding how γ-TuRC can be perturbed to influence microtubules required for spindle assembly. For future experiments, we are developing a high-resolution, dynamic in vitro system for capture and analysis of a single γ-TuRC to determine which γ-tubulins are accessible, where removal is initiated, the critical number of γ-tubulins removed to inactivate the γ-TuRC, the rate of γ-TuRC recovery and TRIP

domain recycling, and to monitor changes to protofilament and microtubule attachment at γ-TuSC of γ-TuRC following TRIP action. This coupled with in vivo studies to determine if mutants can be isolated that are resistant to the action of Pkl1 will provide added details. We expect the mechanistic information defined here to have a fundamental impact on the microtubule cytoskeleton field, revealing new roles for conserved Klp families in spindle assembly while also uncovering details of how structural changes in the γ-TuRC can be generated by motor proteins.

Materials and Methods

Yeast strains, media and growth conditions. *Schizosaccharomyces pombe* media, that is YES or PMAUH, supplemented with 75 μg/ml histidine or uracil and 100 μg/ml adenine, along with standard procedures for genetic manipulation are as described.[32] Yeast transformations used the EZ-YEAST Transformation Kit (MP Biochemicals, cat. 2100-201). For growth assays cells were obtained from logarithmic cultures at 26°C shaking in 10 mls media in baffled flasks, then counted by hemocytometer, equalized and spotted with controls at an initial concentration of 1–3 × 10^6 cells/ml with 1/10 serial dilutions. Triplicate plates were grown at 25°C, 30°C and 36°C. Spotted cells were grown 2–5 d, longer times needed for 25°C. Assays were done at least two times, but usually up to 6× when compared with different constructs. The temperature sensitive (ts) strain 183 (*h*$^+$ *ura4-D18 leu1-32 his3-D1 pkl1*Δ*::his3*$^+$ *cut7-22*^{ts}) lacking *pkl1* was transformed to leu$^+$ prototrophy with integrated *pkl1* derivatives in pRep90x[33] or as GFP-fusion proteins in pRep81[34] (eGFP, Life Technologies). As controls, we used the starting construct 96–230 (+NLS) that has wild type Pkl1 functional activity or the same construct made non-functional by removal of the NLS.[18]

Site-directed mutagenesis, deletion constructs and bioinformatics. Site-directed changes or small deletions of the Tail domain of the *pkl1* gene were done by high-fidelity PCR with mutagenic oligonucleotides that also contained sites for cloning (Integrated DNA Technologies; Roche High Fidelity PCR Master, cat. 2140314). All constructs were sequenced (Northwoods DNA, Inc.; Center for Functional Genomics, University at Albany). For obtaining sequences and sequence analysis we used the following databases and resources: NCBI GenBank and Blastp, Welcome Trust Sanger Institute, the Broad Institute and ExPASy PROSITE. Accession numbers are *Schizosaccharomyces pombe* SpPkl1 (Sanger Center, SPAC_3A11.14c); Pkl1 homologs in *Schizosaccharomyces cryophilus* (Scy) and *Schizosaccharomyces octosporus* (So, Broad Institute SPOG_02038.3 and SPOG_00465.5, respectively); *Homo sapiens* HsHSET (KifC1), *Mus musculus* MmKifC1 and *Rattus norvegicus* RnKifC1 (NP_002254.2, GB_BAA19676.1, and NP_001005878.1, respectively).

Fluorescence microscopy. Live time-lapse images of kinesin-14-GFP fusion proteins were acquired at room temperature on a Zeiss AxioObserver Z1 inverted microscope with Colibri LED illumination, 100× oil 1.45 NA PlanFLUAR or 63× Plan-Apochromat 1.4 NA oil DIC objectives, and Hamamatsu ORCA ER CCD camera using Zeiss AxiovisionRel 4.8 acquisition software. Single plane 1–10 min time-lapse image series at 2–30 sec intervals, or 20-image 0.2-micron Z-stack images were acquired. Representative images are based on n = 200. Images were compiled using Adobe Photoshop (Adobe Systems Inc.) and Microsoft PowerPoint (Microsoft Corp.) software. Images shown in **Figures 1 and 2** are representative of n > 200 per phenotype.

Fast protein liquid chromatography (FPLC) and γ-TuRC isolation. Whole-cell extracts were made by bead beating (Mini-Beadbeater-16: Biospec, cat. 607) in Buffer A as described[11] (50 mM Na_2PO_4, 20% glycerol, pH 7.2, WCE buffer supplemented with 1 mM PMSF and 5 mM Phenanthroline). Triplicate centrifugations at 17,000 × g with times ranging from 1–30 min were used to clarify cell extracts. WCEs were then diluted to 30 mg/ml and ran over a Superose 6 10/300 GL column (GE HealthSciences, cat. 17-5172-01), where half-milliliter fractions were collected and immediately kept on ice. Peak fractions were determined using absorbance units in addition to FPLC analysis of molecular weight standards Blue Dextran (2,000 kDa), Thyroglobulin (669 kDa), Apoferritin (443 kDa) and Bovine Serum Albumin (66 kDa).

Peptide co-immunoprecipitation, western analysis and silver staining. Peptides with 6xHis tags (GenScript) correspond to key elements in the Pkl1 Tail region. Whole-cell extracts generated in phosphate buffer and size fractionated on Superose 6 were used. 100–150 μg of each peptide was conjugated to 25 μL of resuspended cobalt Dynabeads using His affinity (Invitrogen, cat. 10103D) in binding/wash buffer (50 mM NaH_2PO_4, 50 mM NaCl, 0.01% Tween-20, 1 mM imidazole) at 4°C for 30 min. During this time, FPLC fraction 17 (500 μL) was pre-cleared using blank beads under the same conditions. Each peptide-bead mixture was then added to 100 μL of pre-cleared fraction 17 and incubated for 1–2 h at 4°C. After incubation, retained beads were washed 3× with binding/wash buffer before protein complexes were eluted with elution buffer (50 mM NaH_2PO_4, 50 mM NaCl, 0.01% Tween-20, 300 mM imidazole). Both the final elutions and pre-elution wash steps were analyzed by western and silver staining. Antibodies for western include primary rabbit anti-HA epitope tag 1:10,000 (Rockland, cat. 600-401-384), primary mouse monoclonal anti-γ-tubulin clone GTU-88 1:500 (Sigma-Aldrich, cat. T6557), secondary goat-anti-rabbit IgG horseradish peroxidase conjugate 1:10,000 (HRP, Novagen, cat. 71045) and secondary goat-anti-mouse IgG HRP conjugate 1:10,000 (Millipore, cat. AQ132P) with BioRad molecular weight standards. Silver stains were done with a Pierce Silver Stain kit according to the manufacturer's instructions (Pierce, cat. 0024612).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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