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Microtubule Tip Tracking by the Spindle and Kinetochore Protein Ska1 Requires Diverse Tubulin-Interacting Surfaces

Highlights

- The Ska1 complex associates with and oligomerizes soluble tubulin heterodimers
- Mutating specific Ska1 surfaces disrupts tubulin, but not microtubule binding
- The Ska1 complex tracks the plus end of both growing and shrinking microtubules
- Ska1 tubulin binding mutants prevent tip tracking and proper mitotic progression

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In Brief

Monda and Whitney et al. demonstrate that the kinetochore-localized Ska1 complex autonomously tracks with both growing and depolymerizing microtubule ends *in vitro* and that this activity requires multiple microtubule binding surfaces. Ska1 mutants that bind microtubules, but are unable to tip track, result in defective mitotic progression in cells.



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SUMMARY

The macromolecular kinetochore functions to generate interactions between chromosomal DNA and spindle microtubules [1]. To facilitate chromosome movement and segregation, kinetochores must maintain associations with both growing and shrinking microtubule ends. It is critical to define the proteins and their properties that allow kinetochores to associate with dynamic microtubules. The kinetochore-localized human Ska1 complex binds to microtubules and tracks with depolymerizing microtubule ends [2]. We now demonstrate that the Ska1 complex also autonomously tracks with growing microtubule ends in vitro, a key property that would allow this complex to act at kinetochores to mediate persistent associations with dynamic microtubules. To define the basis for Ska1 complex interactions with dynamic microtubules, we investigated the tubulin-binding properties of the Ska1 microtubule binding domain. In addition to binding to the microtubule lattice and dolastatin-induced protofilament-like structures, we demonstrate that the Ska1 microtubule binding domain can associate with soluble tubulin heterodimers and promote assembly of oligomeric ring-like tubulin structures. We generated mutations on distinct surfaces of the Ska1 microtubule binding domain that disrupt binding to soluble tubulin but do not prevent microtubule binding. These mutants display compromised microtubule tracking activity in vitro and result in defective chromosome alignment and mitotic progression in cells using a CRISPR/Cas9-based replacement assay. Our work supports a model in which multiple surfaces of Ska1 interact with diverse tubulin substrates to associate with dynamic microtubule polymers and facilitate optimal chromosome segregation.

RESULTS AND DISCUSSION

The Ska1 Microtubule Binding Domain Oligomerizes Tubulin around Microtubules

We sought to define the properties of the Ska1 microtubule binding domain (MTBD; [2]) that are required for its activities in microtubule tracking and chromosome segregation. Our previous work using electron microscopy (EM) found that the human Ska1 complex binds to microtubules, forming an ill-structured decoration on the microtubule surface [2, 3] (Figure S1A). In contrast, when we incubated the monomeric human or C. elegans Ska1 microtubule binding domain with microtubule polymers, we found that it formed striking spiral structures that curled around the microtubule lattice (Figures 1A and 1B). To assess the nature of these oligomeric structures, we used cryo-EM and image analysis to generate a low-resolution reconstruction of the C. elegans SKA-1 microtubule binding domain bound to microtubules (Figure 1C; see Method Details). These reconstructions revealed prominent density for the microtubule lattice, an outer ring surrounding the microtubule, and additional density connecting the two rings. The outer ring has more density than could easily be accounted for by the Ska1 MTBD, which has a molecular weight of \sim 15 kDa. Instead, this outer ring is consistent with a filament of tubulin based on its structure, conformation, and its diameter of \sim 5 nm (Figure 1C). The density connecting the microtubule and outer ring therefore most likely corresponds to the SKA-1 microtubule binding domain.

This behavior of the Ska1 MTBD is strikingly similar to that observed for the kinesin-13 MCAK, which also induces the formation of a protofilament-like structure encircling microtubules [4–6]. In the case of both Ska1 and MCAK, it is unlikely that such a spiral or ring-like assembly is formed in cells (as occurs for the fungal Dam1 complex, for example [7, 8]). Indeed, the full Ska1 complex did not generate similar spirals around microtubules (Figure S1A), suggesting that this ordered structure is



Figure 1. The Ska1 Microtubule Binding Domain Associates with Soluble Tubulin Dimer

(A) Negative stain transmission electron microscopy image of the human Ska1 microtubule binding domain forming oligomeric assemblies around a microtubule. (B) Cryo-EM image of the *C. elegans* SKA-1 microtubule binding domain forming oligomeric assemblies around microtubules.

(C) 2 views of the cryo-EM reconstruction of the *C. elegans* SKA-1 microtubule binding domain bound to a microtubule showing the presence of the microtubule lattice, an outer oligomeric ring most likely composed of tubulin, and density connecting these rings most likely corresponding to the SKA-1 microtubule binding domain.

(D) Sedimentation assay demonstrating that incubation of soluble tubulin heterodimer with Ska1 MTBD or Ska1 complex results in the formation of large assemblies. Coomassie-stained gel showing supernatant (S) and pellet (P) fractions from the indicated conditions.

(E) Negative stain transmission electron microscopy images of the assemblies formed following incubation of the *C. elegans* SKA-1 MTBD or human Ska1 MTBD with soluble tubulin. Rings formed by the incubation of dolastatin-10 with tubulin are shown as a comparison. Scale bars represent 100 nm. See also Figure S1B. (F) Size exclusion chromatography traces and Coomassie-stained gels of the peak fractions for the indicated samples demonstrating co-elution of the Ska1 MTBD with RB3-SLD-tubulin. Fractions run on the gel are indicated by the orange, blue, and pink bars.

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Figure 2. The Ska1 Microtubule Binding Domain Uses Multiple Distinct Surfaces to Interact with Tubulin

(A) Left: schematic of the human Ska1 complex highlighting the location of the Ska1 microtubule binding domain within the complex, and within the Ska1 protein. Right: structure of the human Ska1 microtubule binding domain (PDB: 4C9Y) [12] showing the locations of the indicated residues targeted for mutational analysis.

(B) Coomassie-stained gels showing the pellet fractions from representative co-sedimentation assays for the binding of the indicated Ska1 microtubule binding domain mutants to either microtubule polymers, dolastatin-induced rings, or soluble tubulin dimer. For microtubules and dolastatin-induced rings, quantifications indicate the percentage of MTBD bound to the substrate for 2 experiments, with the mean ± SD shown. For tubulin, the quantification indicates the percentage of tubulin in the pellet for 7 independent experiments, with the mean ± SD shown. See Figure S2A for the Coomassie-stained gels of the supernatant fractions.

constrained by the positioning of the MTBDs within the intact Ska1 complex. Intriguingly, we note that formation of a tubulin spiral around microtubules by the Ska1 MTBD suggests that the monomeric Ska1 MTBD is able to bind simultaneously to two tubulin subunits—in this case, tubulin subunits present in the microtubule lattice as well as soluble tubulin heterodimers.

Ska1 Binds to Tubulin Dimer to Form a Higher-Order Assembly

We next tested directly whether Ska1 is able to interact with unpolymerized, soluble tubulin heterodimers. We incubated the Ska1 MTBD with an equimolar concentration of GDP-tubulin heterodimers in the absence of added GTP to prevent microtubule polymerization. Surprisingly, this resulted in the formation of an opaque precipitate that pelleted after centrifugation (data not shown), suggesting that oligomeric assemblies were formed. We therefore assessed sedimentation through a glycerol cushion. In agreement with our prior results that the Ska1 microtubule binding domain behaves as a monomer in solution [2], no sedimentation was detected for the Ska1 microtubule binding domain alone (Figure 1D). In contrast, incubation of the Ska1 MTBD with tubulin heterodimer resulted in the formation of an assembly containing both tubulin and the Ska1 MTBD that sedimented by centrifugation (Figure 1D). Interestingly, similar assemblies were also observed following the addition of the full-length human Ska1 complex to soluble tubulin (Figure 1D), indicating that this property is preserved in the physiological complex.

To define the nature of the Ska1-tubulin assembly, we next used negative stain transmission EM to visualize the *C. elegans* and human Ska1 MTBD (Figures 1E and S1B) or the human Ska1 complex (Figure S1B) following incubation with soluble tubulin heterodimer. We observed striking ring-like assemblies reminiscent of the ring-like protofilament structures induced by the incubation of tubulin with the compound dolasta-tin-10 (Figures 1E and S1B; also see [2]). Although tubulin ring formation is not a common property of microtubule binding proteins, we note that similar assemblies have been observed following addition of the CAP-Gly domains of p150^{Glued} or CLIP-170 to soluble tubulin [9], or following the addition of the motor domain of the kinesin-8 Kif18A to stabilized microtubules [10].

Because incubation of tubulin with the Ska1 MTBD results in large oligomeric assemblies, to directly test its binding to soluble tubulin we utilized the stathmin-like domain (SLD) of RB3, which associates with two tubulin heterodimers and maintains tubulin in an unpolymerized state [11]. We found that both the Ska1 MTBD (Figure 1F) and the Ska1 complex (Figure S1C) associated with the RB3-tubulin complex based on altered migration in size exclusion chromatography. Together, these results demonstrate that Ska1 associates with tubulin heterodimers in addition to its previously defined interaction with polymeric tubulin substrates.

Generation of Separation of Function Mutations for Microtubule/Tubulin Binding by Targeting Distinct Surfaces on the Ska1 Microtubule Binding Domain

To specifically probe the contributions of Ska1 microtubule tip tracking to chromosome segregation, it is critical to identify separation of function mutants that maintain the ability to bind microtubules. The Ska1 MTBD forms a winged-helix structure and has multiple distinct surfaces with positively charged residues that contribute to microtubule binding (Figure 2A; [2, 12]). Prior work suggested that these distinct surfaces allow Ska1 to bind to microtubules in diverse orientations [12]. Although several models could explain Ska1-induced tubulin oligomerization, we hypothesized that this reflects Ska1 binding to multiple tubulin heterodimers in diverse orientations. Because it seems unlikely that microtubule binding would require multiple competent binding surfaces simultaneously, we sought to identify Ska1 mutants that bound to microtubules but did not induce tubulin oligomerization. To do this, we generated a series of mutants targeting distinct surfaces of the Ska1 MTBD with single or double amino acid substitutions in positively charged, surface-exposed residues known to contribute to microtubule interactions [2, 12]. As compared to a four-residue mutant (K183A K184A K223A K226A) previously shown to severely compromise microtubule binding (Figures 2B and S2A; [12]), these mutants largely retained the ability to associate with microtubule polymers (Figures 2B and S2A). These Ska1 mutants also bound to dolastatininduced protofilament-like tubulin rings, albeit with some reductions in apparent binding activity (Figures 2B and S2A). Significantly, the majority of these mutations prevented formation of larger assemblies with tubulin heterodimer (Figures 2B and S2A) and also compromised binding to the RB3-tubulin complex (Figures 2C and S2B). We additionally analyzed the K183A K184A mutant in the context of the full Ska1 complex. Similar to the K183A K184A mutant MTBD, binding to microtubules and dolastatin rings was maintained for the K183A K184A Ska1 complex, but tubulin assembly and binding to the RB3tubulin complex was compromised (Figures S2C and S2D). Thus, multiple distinct surfaces on Ska1 are required for promoting assembly of soluble tubulin heterodimers, and this activity is separable from the binding of Ska1 to the cylindrical microtubule lattice (Figure 2D).

The Ska1 Complex Tracks with Both Depolymerizing and Elongating Microtubule Plus Ends

We next sought to test the behavior of the Ska1 complex in the presence of dynamic microtubules. We demonstrated previously that the Ska1 complex is able to track with the depolymerizing microtubule end *in vitro* following induced depolymerization by ablation of a stabilizing microtubule cap [2]. To test Ska1 interactions with dynamically growing and shrinking microtubules, here we utilized GMPCPP-stabilized microtubule seeds attached to a coverslip and altered the concentration of soluble tubulin to promote either microtubule depolymerization or elongation

(D) Table summarizing the binding data from this figure.

⁽C) Coomassie-stained gels of the size exclusion chromatography fractions for binding of the Ska1 MTBD and mutants to an RB3-SLD-tubulin complex. The gel for wild-type MTBD is duplicated from Figure 1F. RB3-SLD-tubulin peak fractions (see Figure 1F) are shown for each of the Ska1 MTBD mutants. Quantifications indicate the amount of Ska1 MTBD co-eluting with RB3-SLD relative to wild-type for 2 experiments, with the mean ± SD shown. All mutants show reduced or eliminated binding to RB3-SLD-tubulin. See also Figure S2B.



Figure 3. In Vitro Analysis of Ska1 Interactions with Dynamic Microtubules

(A) Schematic of the in vitro total internal reflection fluorescence (TIRF) assay to visualize Ska1 complex associations with dynamic microtubules.

(B) Representative kymographs of GFP-Ska1 complexes on elongating and depolymerizing microtubules, as visualized with GFP fluorescence. Also see Movie S1. The vertical scale bar represents 30 s, and the horizontal scale bar represents $2 \mu m$; microtubule plus ends point to the right. Enlarged images are shown for the green, yellow, and blue boxed regions. The kymograph on the right of the depolymerization panel is an equivalently enlarged region from a distinct microtubule. All enlargements shown were chosen to highlight Ska1 complex diffusion near the elongating and depolymerizing ends, their encounter with ends, and brief tracking. For the enlargements, the vertical scale bar represents 3 s, and the horizontal scale bar represents 2 μm .

(C) Representative kymographs of the indicated GFP-Ska1 complexes on polymerizing microtubules. Tracking by wild-type Ska1 appears as an almost continuous bright line because at this timescale, individual complexes cannot be discriminated. Microtubule plus ends point to the right. Percentage of microtubules (mean \pm SD) that display Ska1 complex tip-tracking is shown below each kymograph. Data are based on \geq 38 microtubules for each protein.

(legend continued on next page)

(Figure 3A; see Method Details). We observed binding and diffusion of GFP-labeled wild-type Ska1 complex on the GDP-containing microtubule lattice (Figure 3B), as well as an enrichment of diffusing Ska1 complexes on the guanosine-5'-[(α,β) -methyleno]triphosphate (GMPCPP)-containing microtubule seed (Figures S3A and S3B). Consistent with our prior work [2], when microtubule depolymerization was induced by the removal of soluble tubulin, the Ska1 complex showed an enrichment at the shortening microtubule ends. At low Ska1 complex concentration, we observed that individual molecules associated with and tracked the microtubule plus end only briefly, indicating that this tracking is not highly processive (Figure 3B). Additionally, although work from others has not detected plus-end tracking of elongating microtubules by the Ska1 complex [13], we observed a notably increased brightness for GFP-Ska1 complex fluorescence at growing microtubule ends (Figures 3B and S3C; Movie S1) indicative of plus-end tracking with microtubule polymerization.

Diverse plus tip tracking proteins associate with elongating microtubules in cells, but most of these represent "hitchhiker" molecules that associate with microtubule ends through the end-binding (EB) family of proteins, rather than acting as "autonomous tip trackers" [14]. Although the Ska1 complex has been proposed to interact with EB1 [15], our results indicate that the Ska1 complex is capable of EB1-independent tracking of polymerizing microtubules in vitro. The ability of the Ska1 complex to bind to soluble tubulin and track elongating microtubule plus ends is reminiscent of the autonomously tracking microtubule polymerase XMAP215 [16]. However, unlike XMAP215, the addition of Ska1 complex did not significantly change the microtubule polymerization rate (Figure S3D). Imaging at a high frame rate revealed that the enrichment of Ska1 complex fluorescence at the dynamic microtubule end persisted for an average of ~ 1 s on growing microtubules, similar to the tracking behavior detected on shrinking microtubules (Figure S3E). Thus, the Ska1 complex is an autonomous microtubule tip tracking protein with the ability to remain associated with both polymerizing and depolymerizing microtubule plus ends. This property has not been reported for other human kinetochore-localized microtubule-associated proteins [17], highlighting Ska1 as an important player for kinetochore tracking of microtubule ends.

Ska1 Mutants Defective in Soluble Tubulin Binding Show Reduced Microtubule End-Tracking Behavior In Vitro

We next tested mutant Ska1 complexes containing a subset of the mutations characterized above that prevented MTBDinduced tubulin oligomerization and represent each of the distinct surfaces on the Ska1 MTBD (K183A K184A, K223A K226A, R155A, and R236A; Figure 2A). Based on fluorescent decoration along the entire microtubule polymer, each complex displayed similar binding to the microtubule lattice (Figure S3F). However, some mutants required a 2- to 3-fold increased concentration of GFP-Ska1 complex to achieve a similar level of microtubule decoration (see Method Details), indicating a slightly reduced affinity for microtubules, in agreement with the co-sedimentation assays described above.

Strikingly, we found that all four Ska1 mutant complexes failed to track with the plus end of elongating microtubules (Figure 3C), despite associating with the microtubule lattice. As we observed for the wild-type Ska1 complex, the microtubule polymerization rate in the presence of the mutant complexes was unchanged (Figure S3D). These mutants additionally displayed defects in associating with depolymerizing microtubules. Three of the mutant Ska1 complexes tested (K223A K226A, R155A, and R236A) failed to exhibit tip tracking as evidenced by the lack of enhanced brightness at the shortening microtubule ends (Figure 3D; Movie S2). The Ska1 K183A K184A mutant complex did display increased GFP-intensity at the depolymerizing microtubule end relative to the adjacent microtubule lattice. However, the tracking for this mutant appeared less robust and persistent relative to the wild-type Ska1 complex as judged by the brightness and continuity of the tracking signals (Figure 3D). These data indicate that multiple, distinct, positively charged tubulin interaction surfaces on the Ska1 microtubule binding domain contribute to robust association with dynamic microtubule ends.

Multi-faceted Ska1-Tubulin Binding Contributes to Proper Chromosome Segregation

Prior work has examined the cellular consequences of Ska1 mutants with severely compromised microtubule binding activity [2, 12]. In contrast, the mutations characterized here disrupt soluble tubulin interactions and microtubule tracking without eliminating binding to the microtubule lattice. To test the effects of these precise mutations on kinetochore function and chromosome segregation, we next utilized an inducible CRISPR/Cas9based replacement strategy in human HeLa cells [18]. Consistent with prior RNAi-based studies [2, 3, 12, 19-23], induction of the Cas9 nuclease in cells constitutively expressing a guide RNA (sgRNA) targeting Ska1 resulted in severe defects in chromosome alignment and mitotic progression that could be rescued by expression of a guide resistant version of GFP-Ska1 (Figures 4A-4D; [18]). In agreement with our prior work [2], we found that the microtubule binding domain of Ska1 was required to fully rescue the loss of endogenous Ska1, as knockout of Ska1 in cells expressing GFP-Ska1 ∆MTBD (lacking residues 132-255) resulted in a mitotic delay and chromosome alignment defects (Figures 4A-4D).

We next tested the consequences of expressing the four Ska1 mutants analyzed in the microtubule tip-tracking assay (K183A K184A, K223A K226A, R155A, and R236A; Figure S4A). In contrast to Ska1 Δ MTBD, which does not localize to spindle microtubules, all four mutants displayed detectable localization to microtubules, in addition to their kinetochore localization (Figure 4B). Interestingly, none of these mutants were able to fully compensate for the loss of endogenous Ska1, as evidenced by increased chromosome alignment defects (Figure 4C) and a mitotic delay (Figures 4D and S4B). However, these defects

⁽D) Representative kymographs of the indicated GFP-Ska1 complexes on depolymerizing microtubules. Also see Movie S2. Microtubule plus end points to the right. Percentage of microtubules (mean \pm SD) that display Ska1 complex tip-tracking is shown below each kymograph. Data are based on \geq 29 microtubules for each protein. ** for the K183A K184A mutant indicates that this mutant shows a similar percentage of microtubules that display tracking, but the tracking signal is less robust relative to the wild-type Ska1 complex. The vertical scale bar represents 20 s, and the horizontal scale bar represents 1 μ m. See also Figure S3.

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Figure 4. Ska1 Microtubule Binding Domain Mutants Disrupt Proper Mitotic Progression

(A) Immunofluorescence images of microtubules (DM1A), DNA (Hoechst), kinetochores (ACA), and GFP-Ska1 in the indicated conditions. The Ska1 inducible knockout (iKO) was induced with doxycycline for 4 days prior to imaging. The fluorescence is not scaled identically in each panel due to acquisition on different days. The scale bar represents 10 μ m.

(B) Fluorescent images of live cells stably expressing the indicated GFP-Ska1 constructs. Images are provided for a qualitative comparison of localization, but the GFP fluorescence is not scaled identically in each panel due to acquisition on different days. The scale bar represents 10 μ m.

(C) Quantification of mitotic phenotypes in control cells (uninduced Ska1 iKO), Ska1 iKO cells, and cells expressing the indicated GFP-tagged Ska1 constructs in the iKO background 4 days following induction of CRISPR-Cas9. n = 100 cells/condition.

(D) Quantification of mitotic duration (chromosome condensation to anaphase onset; mean \pm SD) in control cells and the indicated GFP-Ska1 cell lines 4 days following induction of CRISPR/Cas9. Control (n = 22), Ska1 iKO (n = 24), wild-type (n = 18), Δ MTBD (n = 10), K183A K184A (n = 9), K223A K226A (n = 37), R155A (n = 34), and R236A (n = 17). The mutants are statistically different from the wild-type rescue condition and the Ska1 Δ MTBD mutant based on unpaired t tests (see Figure S4B). The asterisk for the Ska1 iKO indicates that most cells did not exit mitosis during the imaging such that these data under-represent the mitotic duration.

were generally not as severe as those observed for Ska1 Δ MTBD (Figures 4C, 4D, and S4B). Based on the ability of these mutants to localize to kinetochores (Figure 4B) and associate with micro-tubules *in vitro* (Figures 2B and S3F) and in cells (Figure 4D), it is tempting to speculate that the observed mitotic defects are the result of a reduced ability of these mutants to track dynamic microtubules.

Intriguingly, we note that we were able to isolate a viable stable cell line expressing Ska1 Δ MTBD but apparently lacking endogenous Ska1 (Figure S4C; see Method Details). When compared to Ska1 Δ MTBD-expressing cells 4 days after induction of the Ska1 knockout, this stable cell line displayed similar defects in chromosome alignment and mitotic progression (Figures S4D and S4E), suggesting that the viability of the Ska1 Δ MTBD stable replacement cell line is not due to long-term adaptation to the loss of endogenous Ska1. Therefore, although the Ska1 microtubule binding domain is not essential for viability in cell culture, it is required for efficient chromosome alignment and timely cell division, both of which critically depend on optimal kinetochoremicrotubule interactions.

Together, our data indicate that multiple surfaces on the Ska1 microtubule binding domain are required for associations with soluble tubulin, bi-directional microtubule tip tracking activity *in vitro*, and optimal mitotic progression in cells.

Ska1 Uses Multiple Distinct Surfaces to Interact with Microtubules

Our work demonstrates that the Ska1 complex is able to associate with and autonomously track both depolymerizing ([2]; Figure 3B) and polymerizing (Figure 3B) microtubules. This contrasts with other key components of the kinetochore-microtubule interface, such as the Ndc80 complex, which at a singlemolecule level fails to associate with dynamic microtubule tips [2, 24]. Defining the mechanistic basis for the tip-tracking ability of the Ska1 complex and its contribution to the kinetochoremicrotubule interface are important goals. Importantly, our work reveals several properties of the Ska1 microtubule binding domain that may contribute to its tip-tracking activity and interactions with microtubule ends at mitotic kinetochores. First, we find that Ska1 is able to interact with diverse tubulin substrates, including the cylindrical microtubule lattice, protofilament-like tubulin structures, soluble tubulin heterodimer, and an RB3-tubulin complex (Figure 2). Second, we identify an involvement of diverse surfaces of the Ska1 MTBD in tracking with dynamic microtubules in vitro (Figure 3), suggesting the potential for heterogeneity in the conformation with which Ska1 binds microtubules, consistent with prior crosslinking analyses [12]. The absence of a specific conformation by which the Ska1 MTBD interacts with the microtubule further contrasts it with the highly stereotypical binding displayed by the Ndc80 complex ([25, 26]; also see [12]).

The tubulin-binding properties that we have identified here most likely facilitate the association of Ska1 with dynamic microtubule ends, a key requirement at kinetochores [17]. For example, we speculate that instead of "hopping" between specific binding sites on the tubulin subunits within the microtubule lattice in a manner that is susceptible to the loss of an interaction, the Ska1 microtubule binding domain could "roll" by simultaneously or sequentially engaging multiple surfaces of the Ska1 MTBD and tubulin to maintain their association. This mode of motility is consistent with the lack of a specific or regular microtubule-binding orientation for the Ska1 complex as observed by EM (Figure S1A; [2, 3, 26]). We note that a structurally unrelated microtubule-binding protein KLP10-A, a kinesin-13 family member, has also been suggested to diffuse along the microtubule lattice by engaging multiple, distinct microtubule-binding surfaces [27]. Finally, our cellular analysis suggests that Ska1-mediated tip tracking most likely contributes to proper cell division. Thus, the features and properties of the Ska1 microtubule binding domain defined here contribute to its roles at the kinetochore-microtubule interface. In addition, although the Ska1 MTBD is necessary and sufficient for Ska1 complex microtubule binding and spindle localization [2], we note that recent work has also suggested a contribution to microtubule interactions from Ska3 [28]. Ultimately, these activities must be regulated [13, 29, 30] and integrated with other players at the kinetochore, including the Ndc80 complex [2, 31, 32], to ensure faithful chromosome segregation during every cell division.

STAR*METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two movies and can be found with this article online at https://doi.org/10.1016/j.cub.2017.10.018.

AUTHOR CONTRIBUTIONS

Conceptualization, I.M.C., J.K.M., E.W.-K., and E.L.G.; Methodology, I.P.W., J.K.M., E.V.T., E.L.G., E.W.-K., and I.M.C.; Investigation, I.P.W., J.K.M., E.V.T., E.W.-K., and I.M.C.; Writing – Original Draft, I.M.C. and J.K.M.; Writing – Review & Editing, I.M.C., J.K.M., I.P.W., E.V.T., E.W.-K., and E.L.G.; Funding Acquisition, I.M.C. and E.L.G., E.W.-K., and R.A.M.; Supervision, I.M.C., E.L.G., and R.A.M.

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STAR*METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Tubulin (clone DM1A)	Sigma-Aldrich	Cat# T9026; RRID: AB_477593
Human polyclonal anti-Centromere	Antibodies Incorporated	Cat# 15-234-0001; RRID: AB_2687472
Rabbit polyclonal anti-Ska1	Cheeseman lab [3]	N/A
Goat anti-rabbit IgG-HRP	GE Healthcare	Cat# RPN4301; lot: 9721201
Sheep anti-mouse IgG-HRP	GE Healthcare	Cat# NA931V; lot: 9729340
anti-Digoxigenin-AP (anti-DIG), Fab fragments from sheep	Roche Applied Science	Cat# 11093274910
Bacterial and Virus Strains		
E. coli: One Shot Mach1 T1 Phage-Resistant Chemically Competent E. coli	Thermo Fisher Scientific	Cat# C862003
E. coli: BL21(DE3) Competent E. Coli	Cheeseman lab	N/A
Chemicals, Peptides, and Recombinant Proteins		
Hoechst-33342	Sigma Aldrich	Cat# B2261
Urea	Millipore Sigma	Cat# 1084870500; CAS: 57-13-6
Complete EDTA-free protease inhibitor cocktail	Sigma Aldrich	Cat# 4693159001
Proteinase K	New England Biolabs	Cat# P8107
PreScission protease (pGEX3C-PreScission)	Cheeseman lab	N/A
TEV protease (pRK793)	Cheeseman lab	N/A
GMPCPP	Jena Bioscience	Cat# NU-405
Tubulin protein (> 99% pure): porcine brain	Cytoskeleton	Cat# T240
Dolastatin-10	Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute	NSC# 376128
Paclitaxel	Sigma Aldrich	Cat# T7402
400-mesh C-flat grids	Electron Microscopy Sciences	Cat# CF-2/2-4C-50
Doxycycline	Sigma Aldrich	Cat# D9891
Bovine tubulin	Purified as described in [33]	N/A
DIG-tubulin	Bovine tubulin labeled with Digoxigenin (Invitrogen) as described in [34]	Cat# A-2952
Hilyte647-tubulin	Bovine tubulin labeled with Hilyte647 (Anaspec) as described in [34]	Cat# 81256
Pluronic F127	Sigma-Aldrich	Cat# P2443
Mg-GTP	Sigma-Aldrich	Cat# A9187
GMPCPP	Jena Bioscience	Cat# NU405-S
Catalase	Sigma-Aldrich	Cat# C-40
Glucose oxidase	Sigma-Aldrich	Cat# G-2133
Glucose	Sigma-Aldrich	Cat# G-8270
BSA	Sigma-Aldrich	Cat# A7638
DTT	Invitrogen	Cat# 15508-13
Methyl cellulose	MP Biomedicals	Cat# 155492

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Cell Lines		
Human: HeLa, female Cervix Adenocarcinoma Cells	Cheeseman lab	N/A
Human: HeLa, Inducible Cas9, Ska1 sgRNA (cKM250.3)	Cheeseman lab [18]	N/A
Human: HeLa, Inducible Cas9, Ska1 sgRNA, GFP-Ska1 CRISPR/Cas9 hardened (clW13.28)	This paper	N/A
Human: HeLa, Inducible Cas9, Ska1 sgRNA, GFP- Ska1 ∆MTBD CRISPR/Cas9 hardened (clW14.23)	This paper	N/A
Human: HeLa, Inducible Cas9, Ska1 sgRNA, GFP- Ska1 K183A K184A CRISPR/Cas9 hardened (clW19.1)	This paper	N/A
Human: HeLa, Inducible Cas9, Ska1 sgRNA, GFP- Ska1 K223A K226A CRISPR/Cas9 hardened (cIW20.F)	This paper	N/A
Human: HeLa, Inducible Cas9, Ska1 sgRNA, GFP-Ska1 R236A CRISPR/Cas9 hardened (clW18.I)	This paper	N/A
Human: HeLa, Inducible Cas9, Ska1 sgRNA, GFP-Ska1 R155A CRISPR/Cas9 hardened (cIW22.A)	This paper	N/A
Human: HeLa, Induced Cas9, Ska1 sgRNA, GFP-Ska1 ΔMTBD CRISPR/Cas9 hardened (Stable endogenous knockout) (clW14.23v2)	This paper	N/A
Oligonucleotides		
Forward primer for Ska1 genomic amplification: 5'- gtacggtaccgtggttagaaac-3'	This paper	N/A
Forward primer for Ska1 genomic amplification: 5'- gattacctgggcccatttctt-3'	This paper	N/A
Reverse primer for Ska1 genomic amplification: 5'-cgttatgcaaaaatctaaaattacctaag-3'	This paper	N/A
Reverse primer for Ska1 genomic amplification: 5'-cattaaggaacgcatgactgt-3'	This paper	N/A
Recombinant DNA		
pET3aTr-His-Precission-human Ska1 MTBD (pJS249)	Cheeseman lab [2]	N/A
pET3aTr-His-Precission-human Ska1 MTBD K183A K184A K203A K206A (pIC400)	This paper	N/A
pET3aTr-His-Precission-human Ska1 MTBD K183A K184A (pIW28)	This paper	N/A
pET3aTr-His-Precission-human Ska1 MTBD K203A K206A (pIW39)	This paper	N/A
pET3aTr-His-Precission-human Ska1 MTBD K223A K226A (pDK616)	This paper	N/A
pET3aTr-His-Precission-human Ska1 MTBD R236A (pIW40)	This paper	N/A
pET3aTr-His-Precission-human Ska1 MTBD R155A (pJS278)	This paper	N/A
pET3aTr-His-Precission-human Ska1 MTBD R245A (pJS281)	This paper	N/A
pET3aTr-C.elegans Ska1 MTBD-His (pJS263)	Cheeseman lab [2]	N/A
pST39-His-GFP-human Ska1; human Ska2 (pJS183)	Cheeseman lab [2]	N/A
pST39-His-GFP-human Ska1 K183A K184A; human Ska2 (pIW87)	This paper	N/A
pST39-His-GFP-human Ska1 K223A K226A; human Ska2 (pIW90)	This paper	N/A
pST39-His-GFP-human Ska1 R236A; human Ska2 (pIW84)	This paper	N/A

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CellPress

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
pST39-His-GFP-human Ska1 R155A; human Ska2 (pIW95)	This paper	N/A
pGEX-6P-1-human Ska3 (pCB4)	Cheeseman lab [3]	N/A
pST39-His-TEV-human Ska1; human Ska2 (pJS182)	This paper	N/A
pST39-His-TEV-human Ska1 K183A K184A; human Ska2 (pIW86)	This paper	N/A
pBAT4-RB3-SLD (pJM160)	This paper	N/A
pBABEblast-GFP-human Ska1 CRISPR/Cas9 hardened (pIW69)	This paper	N/A
pBABEblast-GFP-human Ska1 CRISPR/Cas9 hardened K183A K184A (pIW85)	This paper	N/A
pBABEblast-GFP-human Ska1 CRISPR/Cas9 hardened K223A K226A (pIW88)	This paper	N/A
pBABEblast-GFP-human Ska1 CRISPR/Cas9 hardened R236A (pIW82)	This paper	N/A
pBABEblast-GFP-human Ska1 CRISPR/Cas9 hardened R155A (pIW93)	This paper	N/A
pBABEblast-GFP-human Ska1 ∆MTBD CRISPR/Cas9 hardened (pIW70)	This paper	N/A
Software and Algorithms		
Photoshop CS5.1, version 12.1	Adobe	http://www.adobe.com
Image Studio Lite Version 5.2.5	LI-COR	https://www.licor.com
GraphPad Prism 7, Version 7.0c	GraphPad software	https://www.graphpad.com
UCSF Chimera	Molecular graphic images were produced using the Chimera package from Computer Graphics Laboratory, University of California, San Francisco.	https://www.cgl.ucsf.edu/chimera/
Metamorph	Molecular Devices	https://www.moleculardevices.com
Other		
Clarity Western ECL Substrate	Bio-Rad	Cat# 1705060
TOPO TA Cloning Kit For Sequencing	Invitrogen	Cat# 450030
Ni-NTA agarose	QIAGEN	Cat# 30230
Glutathione agarose	Sigma Aldrich	Cat# G4510
HiLoad 16/600 Superdex 200 pg	GE Healthcare	Cat# 28989335
NAP-5 column	GE Healthcare	Cat# 17-0853-01
Vivaspin 3 kDa MWCO spin concentrator	GE Healthcare	Cat# 28-9323-58
Vivaspin 10 kDa MWCO spin concentrator	GE Healthcare	Cat# 28-9323-60
Vivaspin 50 kDa MWCO spin concentrator	GE Healthcare	Cat# 28-9323-62
AcquaStain	Bulldog Bio	Cat# AS001000
Superdex 200 Increase 3.2/300	GE Healthcare	Cat# 28990946
Nitrocellulose, Amersham Protran 0.2 NC	GE Healthcare	Cat# 10600001

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Iain Cheeseman (icheese@wi.mit.edu). Plasmids are also available through Addgene (http://www.addgene.org/lain_Cheeseman/).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human tissue culture cell lines used in this study are listed in the Key Resources Table. All HeLa (female cervix adenocarcinoma cells, not authenticated) lines used in this study were cultured in Dulbecco's modified Eagle medium supplemented with 10% tetracycline-free fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM L-glutamine at 37°C with 5% CO₂.

All recombinant protein expression was performed in BL21 (DE3) E. coli.

METHOD DETAILS

Molecular Biology

E. coli expression vectors for human Ska1 MTBD, *C. elegans* SKA-1 MTBD, and the polycistronic vector containing human GFP-Ska1 and human Ska2 were described in [2]. Human Ska3 was described in [3]. Human His-TEV-Ska1 and human Ska2 was cloned into pST39. The stathmin-like domain (SLD) of RB3 was generated by cloning into pBAT4 the cDNA encoding residues 49-189 with an additional alanine at its N terminus and, at its C terminus, a GGSG linker followed by a 6xHis tag. GFP-tagged Ska1 and Ska1 ΔMTBD for expression in human cells were described in [2] and here mutated by site-directed mutagenesis to harden against CRISPR-Cas9 by introducing the maximal number of basepair changes within the sequence recognized by the guide RNA while maintaining the amino acid sequence. Mutations in human Ska1 MTBD and human GFP-Ska1 complex were introduced by site-directed mutagenesis.

Protein Purification

All protein expression was done in BL21(DE3) *E. coli*. For purification of ceSKA-1 MTBD, human Ska1 MTBD and variants, expression was induced using 0.1 mM IPTG overnight at 18°C. Cells were lysed and sonicated in 50 mM potassium phosphate (KPi), 300 mM NaCl, 10 mM imidazole, 0.1% TWEEN 20, pH 8.0 (His lysis buffer). Cleared supernatant was incubated with Ni-NTA agarose (QIAGEN) for 1 hr at 4°C, washed three times with 50 mM KPi, 500 mM NaCl, 40 mM imidazole, 0.1% TWEEN 20, 5 mM beta-mer-captoethanol (βME), pH 8.0 (His wash buffer), followed by elution with 50 mM KPi, 500 mM NaCl, 250 mM imidazole, 5 mM βME, pH 7.0 (His elution buffer). The eluted protein was supplemented with PreScission protease at 4°C overnight to remove the 6x-His tag, and purified on a Superdex 200 16/600 column in 20 mM HEPES, 150 mM KCl, 1 mM DTT, pH 7.0 (Gel filtration buffer). Gel filtration fractions were concentrated in a Vivaspin 3 kDa MWCO spin concentrator (GE Healthcare).

For purification of untagged Ska1 complex (used in Figures 1D, S1C, S2C, and S1D), all cultures were induced using 0.4 mM IPTG for 4 hr at 37°C. His-Ska1 complexed with Ska2 was purified by the same protocol used for Ska1 MTBD. The eluted protein was supplemented with TEV protease at 4°C overnight to remove the 6x-His tag, and purified on a Superdex 200 16/600 column in Gel filtration buffer. Gel filtration fractions were concentrated in a Vivaspin 10 kDa MWCO spin concentrator (GE Healthcare). Bacteria expressing GST-Ska3 were lysed and sonicated in PBS, 250 mM NaCl, 0.1% TWEEN 20 (GST lysis buffer). Cleared supernatant was incubated with glutathione agarose (Sigma Aldrich) for 1 hr at 4°C, washed three times with GST lysis buffer supplemented with 1 mM DTT (GST wash buffer), and eluted with 50 mM Tris, 75 mM KCl, 10 mM reduced glutathione, pH 8.0 (GST elution buffer). The eluted protein was supplemented with PreScission protease at 4°C overnight to remove the GST tag, and purified on a Superdex 200 16/600 column in Gel filtration buffer. Ska3 containing fractions were passed back over glutathione agarose, concentrated in a Vivaspin 10 kDa MWCO spin concentrator (GE Healthcare), and mixed equimolar with purified Ska1-Ska2, incubated on ice for 30 min and the resulting complex was purified on a Superdex 200 16/600 column in Gel filtration fractions were concentrator (GE Healthcare), and mixed equimolar with purified Ska1-Ska2, incubated on ice for 30 min and the resulting complex was purified on a Superdex 200 16/600 column in Gel filtration fractions were concentrator (GE Healthcare), and mixed equimolar with purified Ska1-Ska2, incubated on ice for 30 min and the resulting complex was purified on a Superdex 200 16/600 column in Gel filtration fractions were concentrated in a Vivaspin 50 kDa MWCO spin concentrator (GE Healthcare).

For purification of His-Ska1 complex (used in Figures S1A and S1B) and His-GFP-Ska1 complex and variants (used in Figures 3 and S3), all cultures were induced using 0.1 mM IPTG overnight at 18°C. His Ska1-Ska2 and His-GFP-Ska1-Ska2 were purified with the same protocol used for Ska1 MTBD, without removal of the 6x-His tag. Bacteria expressing GST-Ska3 was lysed, sonicated, bound to beads and washed identically to the Ska3 purification above. Eluted His-Ska1-Ska2 or His-GFP-Ska1-Ska2 was then added to the glutathione bound GST-Ska3 and incubated at 4°C for 1 hr. The bound complex was washed with GST wash buffer and eluted by cleaving GST-Ska3 off of the beads with PreScission protease at 4°C overnight. The eluted complex was purified on a Superdex 200 16/600 column in gel filtration buffer and concentrated in a Vivaspin 50 kDa MWCO spin concentrator (GE Healthcare).

RB3-SLD was induced using 0.4 mM IPTG for 4 hr at 37°C and purified by the same His-tagged purification strategy used for Ska1 MTBD described above. The Ni-NTA eluate was desalted on a NAP-5 column (GE Healthcare) into BRB80 and concentrated in a Vivaspin 3 kDa MWCO spin concentrator (GE Healthcare).

Co-sedimentation Assays

GMPCPP-stabilized microtubules were generated by incubating 25 μ M tubulin dimer (Cytoskeleton) with 500 μ M GMPCPP (Jena Bioscience) at 37°C for 1 hr. Dolastatin-10-induced rings were generated by incubating 25 μ M tubulin dimer in BRB80 (80 mM PIPES, pH 6.8, 4 mM MgCl, and 1 mM EGTA) and 100 μ M dolastatin-10 (provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute) for 1 hr at room temperature. Microtubules and rings were purified by pelleting through a cushion of BRB80 with 40% glycerol for 10 min at 80000 rpm at room temperature and resuspended in BRB80. The concentration of microtubules was determined by measuring the absorbance at 280 nm after depolymerizing an aliquot of the microtubules in BRB80 + 10 mM CaCl₂ on ice for 15 min. The concentration of dolastatin rings was determined by Coomassie staining relative to a standard curve of tubulin on the same gel. For the tubulin assembly assays, soluble tubulin heterodimer was pre-cleared at 90000 rpm before mixing with the test protein. Co-sedimentation assays were

conducted as described previously [2, 35]. Briefly, 10 µM Ska1 MTBD was mixed with 10 µM of the appropriate tubulin substrate at room temperature and sedimented through a 40% glycerol cushion in BRB80 for 10 min at 80000 rpm. Coomassie gels are stained with AcquaStain (Bulldog Bio). Scanned gels were converted to grayscale in Adobe Photoshop and band intensities were quantified in Image Studio Lite. The graphs, generated in GraphPad Prism, display the percentage of Ska1 MTBD or tubulin pelleted, as determined by dividing the intensity of the pellet sample by the total intensity of the supernatant and pellet for each MTBD construct.

Size Exclusion Chromatography Assays

RB3-SLD and tubulin heterodimer were mixed at a 1:2 molar ratio and incubated on ice for 1 hr. For the right panel of Figure S1C, ~30% less tubulin was incubated with RB3-SLD to limit the concentration of fully saturated tubulin-bound RB3-SLD. In all experiments, Ska1 MTBD or Ska1 complex was then added in an approximately equimolar ratio to RB3-SLD, with the final salt concentration of the reaction being 85 mM KCl. The samples were incubated on ice for an additional 30 min, spun at 13,000 rpm for 10 min, and loaded on a Superdex 200 3.2/300 column on an Akta Micro in 20 mM HEPES, 75 mM KCl, 1 mM DTT, pH 7.0. Peak elution fractions were analyzed on polyacrylamide gels stained with AcquaStain. Scanned gels were converted to grayscale in Adobe Photoshop and band intensities were quantified in Image Studio Lite. For each Ska1 MTBD construct, the intensity of the Ska1 MTBD was measured for each of the 4 peak RB3-SLD-tubulin fractions (the fractions displayed in Figure 2C). The sum of these intensities was divided by the sum of the RB3-SLD intensities in these lanes to correct for differences in RB3-SLD-tubulin concentrations in each experiment. The graphs display these RB3-SLD normalized intensities as percentages relative to wild-type Ska1 MTBD.

Electron Microscopy

Bovine brain microtubules were prepared by polymerizing 5 mg/mL bovine brain tubulin (Cytoskeleton, Inc.) in polymerization buffer (80 mM PIPES, pH 6.8, 1 mM EGTA, 4 mM MgCl₂, 2 mM GTP, 12% dimethyl sulfoxide) for 30 min at 36°C. Paclitaxel was added at 250 µM before further incubation of 30 min at 36°C. The polymerized microtubules were then incubated at room temperature for several hours before use. The *C. elegans* SKA-1 MTBD (13 µM) was mixed with 1 µM microtubules in BRB80 and after a 10 min incubation the sample were adsorbed to glow-discharged 400-mesh C-flat grids (Electron Microscopy Sciences, Hatfield, PA) containing 2.0-µm holes separated by 2.0-µm spacing. The cryosamples were prepared using a manual plunger. Images were acquired using a K2 direct detector (Gatan, Pleasanton, CA) and the Tecnai F-20 microscope (FEI, Hillsboro, OR). Selected segments from 45 images were processed using a single-particle approach and the iterative helical real-space reconstruction procedure [36] with multimodel projection matching of microtubules with various numbers of protofilaments [37]. The rings around the microtubules were not stacked in an ordered manner (Figure 1B), preventing us from constructing a high-resolution map. However, we were able to obtain a low-resolution reconstruction from selected segments of microtubules belonging to the 13 protofilament family. UCSF Chimera [38] was used to display the 3-D reconstruction.

To obtain ring-like structures with unpolymerized tubulin, bovine or porcine brain tubulin were mixed in equimolar ratios (1:1 with 1-20 μ M of each component) with *C. elegans* Ska1 MTBD, human Ska1 MTBD or human Ska1 complex in BRB80 and incubated for 5-10 min. The sample was then adsorbed for 1 min onto carbon coated glow-discharged grids before blotting and negatively stained with 1% uranyl acetate. Images were acquired on a FEI Tecnai T12 Transmission Electron Microscope (TEM), using a 4K X 4K Teitz F416 camera.

Cell Culture and Cell Line Generation

Generation of the Ska1 CRISPR/Cas9-based inducible knockout cell line was described in [18]. Cells expressing guide resistant GFP-Ska1 constructs were generated by retroviral infection of the Ska1 inducible knockout cell line and sorted for clonal cell lines. Although the mutants are more highly expressed than endogenous Ska1, they display roughly similar expression levels to each other (see Figure S4A). To induce the knockout, cells were dosed with 1 μ g/mL doxycycline at 0, 24 and 48 hr and assayed at 96 hr (4 days). To generate the stable replacement Ska1 Δ MTBD cell line, single cell sorting was performed on the fourth day and clonal populations were screened by fluorescence microscopy to verify proper localization of the transgene, western blot to verify loss of the endogenous protein, and genomic sequencing to confirm the presence of Cas9 induced frameshift mutations.

For western blotting, cell pellets were incubated on ice for 25 min in urea lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% NP-40, 0.1% SDS, 6.5 M urea, pH 7.5 supplemented with 1 mM PMSF, and Complete EDTA-free protease inhibitor cocktail (Roche). Following size separation on a 12% SDS-PAGE gel, samples were semi-dry transferred to nitrocellulose and blocked for 30 min in 5% skim milk power in TBS + 0.1% Tween-20 (Blocking buffer). Primary and secondary antibodies were diluted in blocking buffer. The Ska1 antibody was described in [3] and used at 1 µg/mL. DM1A (Sigma Aldrich) was used at 1:5,000. HRP conjugated secondary antibodies (GE Healthcare) were used at 1:10,000. All antibody incubations were performed at room temperature for 1 hr. Clarity (Bio-Rad) was used as the ECL substrate.

To assess the genomic locus in the stable Ska1 ΔMTBD replacement cell line, genomic DNA was extracted by lysing the cell pellet in 100 mM Tris, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, pH 8.0, supplemented with Proteinase K (New England Biolabs) at a final concentration of 0.2 mg/mL at 55°C overnight. DNA was precipitated with isopropanol, washed with 75% ethanol and resuspended with TE buffer. PCR primers were designed to bind within the introns surrounding the Ska1 sgRNA target site (5'- gtacggtaccgtggttagaaac-3' and 5'-cgttatgcaaaaatctaaaattacctaag-3' to amplify a 402 nucleotide region centered on the on the sgRNA target site or 5'-gattacctgggcccatttctt-3' and 5'-cattaaggaacgcatgactgt-3' to amplify an 806 nucleotide region centered on the sgRNA target site). The PCR products were run on an agarose gel for gel purification. For both sets of primers, the only detectable product was a single

band of the expected size. Gel purified PCR products were cloned into pCR4-TOPO using the TOPO TA Cloning Kit For Sequencing (Invitrogen). Following transformation of the TOPO reaction product, plasmid DNA was isolated from single bacterial colonies by miniprep and the region of the plasmid containing the insert was sequenced by Sanger sequencing. A total of 23 independent plasmids were sequenced from 2 different preparations of the genomic DNA, each of which displayed the same deletion spanning nucleotides 9-16 of exon 1, resulting in a frameshift and a protein product predicted to terminate after the addition of 7 new amino acids. The absence of any wild-type sequences or in-frame deletions/insertions supports the western blot data indicating a lack of detectable endogenous Ska1 protein.

Fluorescence Microscopy in Cells

For immunofluorescence, cells were fixed with 3.7% formaldehyde in PHEM buffer. Microtubules were visualized using 1:3,000 DM1A (Sigma Aldrich) and centromeres were visualized using 1:300 anti-centromere antibody (ACA). DNA was visualized with 100 ng/mL Hoechst-33342 (Sigma Aldrich). Images were acquired on a DeltaVision Core deconvolution microscope (Applied Precision/GE Healthsciences) equipped with a CoolSnap HQ2 CCD camera with a 100x 1.40 NA Olympus U-PlanApo objective. Images were deconvolved and maximally projected. For time-lapse microscopy, the media was changed to CO_2 -independent media prior to imaging and imaging was performed at 37°C. 3 *Z*-sections were acquired with 5 μ m spacing using fluorescent light at the lowest level usable for data collection. Images were collected at 5-min intervals for 12 hr using a using a 40x, 1.35-NA U-PlanApo objective (Olympus).

In Vitro Microtubule Motility Assays

Tubulin was purified and labeled as in [33, 34]. Microtubule seeds were prepared from a mixture containing 7 parts of unlabeled tubulin and 3 parts of DIG-labeled tubulin supplemented with 1 mM GMPCPP (Jena Bioscience). Flow chambers were prepared as in [39] using silanized coverslips and double stick tape, then anti-DIG antibodies (Roche Applied Science) were adsorbed to the coverslip and the surface was blocked with 1% Pluronic F127. Dynamic microtubules were polymerized from these seeds by adding 5 μ M tubulin mixture containing 10 parts of unlabeled tubulin and 1 part of Hilyte647-labeled tubulin supplemented with 1 mM Mg-GTP. To test tip tracking on elongating microtubules, wild-type GFP-Ska1 was introduced at 10-25 nM and molecular motions were observed at 32°C in imaging buffer, which in addition to soluble tubulin and Mg-GTP contained 80 mM K-PIPES (pH 6.9), 1 mM EGTA, 4 mM MgCl₂, 4 mg/mL BSA, 10 mM DTT, 6 mg/mL glucose, 80 μ g/mL catalase, 0.1 mg/mL glucose oxidase. To examine tracking of shortening microtubule ends, disassembly was induced by removing soluble tubulin with imaging buffer containing GFP-Ska1 complex. Bi-directional tracking was also observed on microtubules undergoing spontaneous dynamic instability in the presence of soluble tubulin (Figure S3C). Some experiments were done in the presence of 0.5% of methyl cellulose to reduce microtubule thermal motions and improve resolution of single molecule motions. This modification did not affect the tip-tracking by Ska1, so results with and without methyl cellulose were combined. Experiments with wild-type and mutant Ska1 complexes were done analogously by using 10-30 nM R155A, 10-30 nM R236A, 35-70 nM K223A K236A, and 35-85 nM K183A K184A. The number of independent experiments for each Ska1 complex mutant was ≥ 2 .

Microscopy for the *in vitro* assays was conducted as described in [40]. We used a 488 nm diode laser (Coherent) as a light source to excite GFP fluorescence, and a CUBE 640 nm diode laser (Coherent) for Hilyte647. Imaging was carried out in TIRF mode with 30 ms exposure and rapid switching between 488-nm and 640-nm lasers via acousto-optical tunable filter (AA OPTO-ELECTRONIC). To analyze tracking, the microtubule visible via Hilyte647 fluorescence was fitted with a straight line using Metamorph software (Molecular Devices). Because the position of the dynamic microtubule can change due to thermal rotations, the width of the line was varied to include microtubule images from all frames in the time series. The microtubule-representing line was then transferred onto the GFP image, and the kymograph was plotted along this line. The kymographs with a visually detectable increase in GFP-intensity at the end of microtubule relative to the lattice were identified as tip-tracking events (Figures 3C, 3D, and S3D). The mean and SD of the fraction of tip tracking events were calculated in assumption of binomial probability distribution with n equal to the total number of microtubules collected from $n \ge 2$ independent trials. The intensity of GFP signal associated with the microtubule end was determined using ImageJ by calculating mean pixel intensity along the straight line (2 px width) drawn along the microtubule end in a kymograph. The lattice and background intensities were calculated analogously by using the 2 px wide lines on the microtubule lattice and in the microtubule lattice area of the same kymograph. GFP brightness of the microtubule (Figure S3E) was determined by calculating mean pixel intensity of the entire microtubule lattice area on the kymograph, and subtracting the mean intensity of the similar background area.

QUANTIFICATION AND STATISTICAL ANALYSIS

All quantification methods are described in the Method Details. Statistical parameters are described in the Figure legends.

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Supplemental Information

Microtubule Tip Tracking by the Spindle

and Kinetochore Protein Ska1 Requires

Diverse Tubulin-Interacting Surfaces

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Supplemental Figure 1. Electron microscopy analysis of the Ska1 microtubule binding domain. Related to Figure 1. A. Negative stain transmission electron microscopy images of microtubules alone or with the full human Ska1 complex. Scale bar, 100 nm. B. Additional negative stain transmission electron microscopy images showing tubulin alone, or the assemblies formed following incubation of the *C. elegans* Ska1 MTBD, human Ska1 MTBD, or human Ska1 complex with soluble tubulin. Scale bars, 100 nm. **C**. Size exclusion chromatography traces and Coomassie-stained gels for the indicated samples showing binding of the human Ska1 complex to the RB3-SLD-tubulin complex. For the trace on the left, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. For the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. For the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. For the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. For the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. Solve the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. For the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. For the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. Solve the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. For the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. Solve the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. Solve the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. For the trace on the right, RB3-SLD and tubulin were mixed at a 1:2 molar ratio. Although there is no shift in the elution volume of the Ska1 complex in the presence of the RB3-SLD-tubulin complex, analysis of the peak fractions by SDS-PAGE clearly demonstrates RB3-SLD co-elutes with the Ska1 complex. Ska3 and tubulin migrate similarly on SDS-PAGE and therefore cannot be distinguished from each other. Fractions run on the gels are indicated by the colored bars below the traces.



Δ



Supernatants (from binding experiments in Fig. 2B)

Ska1 K183A K184A complex

31.

22

14



RB3-SLD-Tubulin

Ska1 K183A K184A

RB3-SLD

Ska2

Β

Supplemental Figure 2. Analysis of Ska1 MTBD binding to an RB3-SLD-tubulin complex by size exclusion chromatography. Related to Figure 2. A. Coomassie-stained gels of the corresponding supernatant fractions for the co-sedimentation assays shown in Figure 2B. B. Coomassie-stained gels of the size exclusion chromatography fractions for binding of the Ska1 MTBD and mutants to an RB3-SLD-tubulin complex. The gels in the left column are reproduced from Figure 2C, and wild type is additionally reproduced from Figure 1F, but here, as in Figure 1F, show the fractions from all three peak regions of interest. The right column shows the gels of the equivalent peak fractions from an experimental replicate of each binding experiment. C. Coomassie-stained gels showing the supernatant (S) and pellet (P) fractions from co-sedimentation assays for the binding of wild type or K183A K184A Ska1 complex to either microtubule polymers or dolastatin-induced rings (left gel), or soluble tubulin dimer (right gel). The gel on the right additionally has the supernatant and pellet fractions for wild type Ska1 sedimented in the absence of any tubulin substrate. D. Coomassie-stained gel for the size exclusion chromatography fractions analyzing K183A K184A Ska1 complex with RB3-SLD-tubulin complex. Ska1 complex and RB3-SLD-tubulin elute as partially overlapping, but distinct peaks (as labeled beneath the gel) indicating that, unlike wild type Ska1 complex, this mutant complex does not robustly associate with RB3-SLD-tubulin.



Supplemental Figure 3. Analysis of Ska1 complex microtubule binding and tracking behavior. Related to Figure 3. A. Representative fluorescent images of GFP-Ska1 complexes (green) decorating depolymerizing microtubules. "GDP-lattice" is a microtubule polymerized with GTP and Hilyte647labeled tubulin (red). Asterisks mark the GMPCPP-seed containing DIG-labeled, but not fluorescent, tubulin. Ska1 decoration is strongest at the seed and microtubule plus end, while individual wall-binding Ska1 complexes are also detected on the GDP-lattice. Scale bar, 5 µm. **B**. Representative kymographs showing a depolymerizing microtubule with low Ska1 decoration (10 nM soluble concentration). Under these conditions, Ska1 complex diffusion occurs on the seed with a constrain at the seed borders, while Ska1 tracking of the depolymerizing microtubule end is not visible. Vertical scale bar is 10 s, horizontal is 3 µm. In the enlarged images (yellow and blue boxes), the vertical scale bar is 3 s, horizontal is 3 µm. C. Representative kymograph showing the association of GFP-Ska1 complexes on a single microtubule (processed from Movie S1) as it undergoes both elongation and then spontaneous depolymerization in the presence of soluble tubulin, as visualized with GFP fluorescence. Microtubule plus ends point to the right. **D**. Graph showing individual microtubule polymerization rates with or without GFP-Ska1 complexes with the mean +/- SEM for each complex. Data are based on \geq 37 microtubules for each complex. E. Quantification of the duration of observed tip tracking events for GFP-Ska1 complex on elongating or depolymerizing microtubules. F. GFP intensity along the GDPmicrotubule lattice for the indicated GFP-Ska1 constructs on elongating or depolymerizing microtubules. Data are based on ≥29 microtubules for each Ska1 construct. The whiskers box plot shows the median, the 25th/75th percentile, and minimum/maximum range. The difference in wall decoration between each pair is not significant (p=0.1346, one-way ANOVA test), confirming that differences in tip tracking behavior were not due to variability in the microtubule wall affinity.



Supplemental Figure 4. Cellular analysis of Ska1 microtubule binding domain mutants. Related to Figure 4. **A.** Western blot of the indicated cell lines before induction with doxycycline showing expression levels of each of the Ska1 constructs. **B.** Table showing the p values for the differences in mitotic timing from a statistical comparison of the indicated conditions based on unpaired t tests (based on the experiments in Figure 4C). Green values indicate statistical significance (p<0.05). **C.** Western blot demonstrating the absence of detectable endogenous Ska1 in the stable Δ MTBD replacement cell line. Uninduced parental Ska1 iKO cells were analyzed in a dilution series to determine the threshold where Ska1 can no longer be detected. Asterisks denote non-specific bands. **D.** Quantification of the mitotic defects in the stable Δ MTBD replacement cell line. The uninduced (Control), Ska1 iKO and Δ MTBD 4-day data are reproduced from Figure 4C for comparison. n=100 cells/condition **E.** Quantification of mitotic duration (chromosome condensation to anaphase onset; mean +/- standard deviation) in the stable Δ MTBD replacement cell line (n=15). The uninduced (Control), Ska1 iKO and Δ MTBD 4-day data are reproduced from Figure 4D.