Structural basis for microtubule recognition by the human kinetochore Ska complex

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The ability of kinetochores (KTs) to maintain stable attachments to dynamic microtubule structures (‘straight’ during microtubule polymerization and ‘curved’ during microtubule depolymerization) is an essential requirement for accurate chromosome segregation. Here we show that the kinetochore-associated Ska complex interacts with tubulin monomers via the carboxy-terminal winged-helix domain of Ska1, providing the structural basis for the ability to bind both straight and curved microtubule structures. This contrasts with the Ndc80 complex, which binds straight microtubules by recognizing the dimeric interface of tubulin. The Ska1 microtubule-binding domain interacts with tubulins using multiple contact sites that allow the Ska complex to bind microtubules in multiple modes. Disrupting either the flexibility or the tubulin contact sites of the Ska1 microtubule-binding domain perturbs normal mitotic progression, explaining the critical role of the Ska complex in maintaining a firm grip on dynamic microtubules.
Establishment of physical connections between the chromosomes and the spindle microtubules (MTs) via the kinetochore (KT) is essential for faithfully segregating the duplicated chromosomes to daughter cells. A key property of the functional KT is its ability to maintain attachments to the plus end of MTs, as they undergo cycles of polymerization and depolymerization commonly known as dynamic instability. As MT depolymerization contributes to the force required for driving chromosome segregation, MT-biding factors that can stay attached to and/or track depolymerizing MTs are essential. At the outer KT, a protein interaction network called the KMN network (consisting of the protein KNL1 and the protein complexes Min12 and Ndc80) provides the direct binding site for MTs. Among these components, the Ndc80 complex is the major MT-binding factor, whereas KNL1 also possesses MT-binding ability. The Ndc80 complex is a heterotrimer composed of Ndc80, Nuf2, Spc24 and Spc25. The globular domains of Ndc80 and Nuf2 are connected to the globular domains of Spc24 and Spc25 via a long coiled-coil structure, resulting in an ~60-nm dumbbell-shaped architecture. Although the globular heads of Spc24 and Spc25 mediate the KT association, those of Ndc80 and Nuf2 directly interact with MTs, stabilizing MTs. The Ndc80 complex can track depolymerizing MTs when attached to microtubules and it influences MT dynamics by stabilizing MTs. In budding yeast, a monomeric protein complex called Dam1 can form a ring around MTs and cooperates with the Ndc80 complex in maintaining stable KT attachments to dynamic MTs. However, no obvious structural Dam1 homologue has been identified in metazoans.

Originally discovered in a proteomics screen, the Ska complex is now recognized as a key element required for maintaining stable KT–MT attachments. The ternary Ska complex, composed of Skal, Sk2 and Sk3, localizes to the outer KT in a KMN-dependent manner. There, it is regulated by the Aurora B kinase, much like the Dam1 complex. Cells depleted of the Ska complex fail to maintain stable KT–MT attachments, resulting in chromosome segregation failure followed by cell death. The Ska complex can directly interact with MTs and track depolymerizing MTs in vitro. Accordingly, it has been proposed that the Ska and Ndc80 complexes form an integrated MT-binding assembly. The ability of the Ska complex to track depolymerizing MTs in vitro and its dependency on the KMN for its localization and function suggest that the Ska complex may be a functional equivalent of the Dam/DASH complex in metazoans. Besides stabilizing KT–MT attachments, the Ska complex has also been implicated in silencing the spindle checkpoint.

During MT growth and shrinkage, MTs undergo important conformational changes. Protofilaments adopt a curved conformation during MT depolymerization and a straight conformation during polymerization. Understanding the structural basis for how the Ndc80 and Ska complexes interact with dynamic MTs is indispensable for understanding the mechanistic aspects of KT–MT attachments. Structural characterizations of Ndc80–MT interactions have shown that the Ndc80 complex binds MTs by interacting at the dimeric interface of α- and β-tubulins. This mode of interaction is also thought to influence the plus-end dynamics of MTs. Although no atomic structure of the Dam1 complex is available, electron microscopy studies have provided insight into how multimeric Dam1 complexes assemble into a ring-like structure encircling MTs. Our previous work demonstrated that the Ska complex is a dimer of triple helical bundles formed by Skal, Sk2 and Sk3, resulting in a W-shaped structure with a maximum interatomic distance of ~350 Å. The MT-binding domains (MTBDs) of the Ska complex protrude at the ends of the W-shaped homodimer, suggesting a transversal mode of MT binding at the KT–MT interface. A recently reported nuclear magnetic resonance (NMR) structure of the Caenorhabditis elegans Skal–MTBD revealed the involvement of a winged-helix domain in MT recognition. At this point, information on how the Ska complex interacts with MTs is crucial to understand the role of the Ska complex in potentially coupling MT dynamics and chromosome segregation. By combining X-ray crystallography, crosslinking/mass spectrometry (MS) and biochemistry, we have here characterized the MTBD of the human Ska complex and evaluated its interaction with MTs in vitro and in vivo. We show that the Ska complex, unlike the Ndc80 complex, can bind tubulin monomers in different orientations via its multiple MT contact sites, allowing it to recognize MTs in a conformation-independent manner. These results provide novel structural and functional insights into the role of the Ska complex in maintaining stable attachments to dynamic MTs.

Results

Skal2–255 is essential for correct mitotic progression. We previously demonstrated that the C-terminal domains of Skal (Skal2–255) and Sk3 (Sk332–412) are essential for the function of the Ska complex. Deletion of Skal2–255 completely abolished the MT-binding ability of the complex, supporting its role in recognizing MTs. Secondary structure predictions suggested that Skal2–255 possesses a globular domain preceded by an unstructured region of 40 amino acids. Proteolysis experiments using trypsin and MS analysis identified a stable fragment of Skal encompassing residues 133–255 (Skal133–255; Fig. 1a). Consistent with our previous work, Skal133–255 showed weak MT binding on its own. The full-length (FL) Ska complex is a dimer, and we asked whether dimerization of Skal133–255 would increase its affinity for MTs. Exploiting the propensity of glutathione S-transferase (GST) to dimerize, we tested the ability of GST-fused Skal133–255 to bind MTs. Although GST–Skal133–255 is not strictly equivalent to the FL Ska1 dimer in the native complex, and thus might bind MTs differently, it clearly interacted with MTs more efficiently than Skal133–255. These observations suggest that Skal133–255 (from now on referred to as MTBD) is the major MT-binding element within the Ska complex, which, on dimerization, can bind MTs more efficiently.

We next evaluated the requirement of the Ska1–MTBD and the loop (Skal133–132, referred to as Skal loop) connecting the amino-terminal helical domain (Skal1–91) for correct mitotic progression. As reported earlier, Skal1 depletion resulted in a significant increase in mitotic timing (Fig. 1c; see Supplementary Fig. S1a for deletion efficiency). A majority of cells showed metaphase-like appearance (Fig. 1d and Supplementary Movie 1), but roughly a third of the cells showed prolonged prometaphase (Fig. S1a for depletion efficiency). A majority of cells showed metaphase-like appearance (Fig. 1d and Supplementary Movie 1), but roughly a third of the cells showed prolonged prometaphase with chromosome congression defects (Supplementary Movie 2), probably reflecting a more complete depletion of the Ska complex. FL mCherry-Ska1 efficiently rescued depletion of Ska1, with chromosome congression defects (Supplementary Movie 2), probably reflecting a more complete depletion of the Ska complex. FL mCherry-Ska1 efficiently rescued depletion of endogenous Ska1 (Supplementary Movie 3) and cells progressed through mitosis comparably with control (GL2-treated) cells (Fig. 1c). Replacement of endogenous Ska1 by Skal1–132 failed to rescue normal progression through mitosis (Fig. 1c): onset of anaphase was delayed and the frequency of apoptosis increased, but most of the cells showed proper chromosome alignment with timings comparable to cells rescued with Skal1 wild type (WT; Fig. 1d, Supplementary Table S1 and Supplementary Movie 4). Moreover, Skal1–132 localized to KT but failed to decorate spindle MTs and showed no bundling activity when compared with Skal1 WT (Supplementary Fig. S1b,c). These observations are
Figure 1 | Characterization of functional determinants of Ska1. (a) Limited proteolysis of the Ska192–255 with trypsin that led to the formation of a stable fragment identified by MS as Ska1133–255. Uncropped scan of the gel is shown in Supplementary Fig. S8a. (b) Top, representative SDS–PAGE of cosedimentation assays comparing the MT-binding activity of Ska1133–255 and GST–Ska1133–255. Bottom, quantification of the MT-binding assays in b (mean ± s.d., n = 4, ***P ≤ 0.001, t-test). (c) Box-and-whisker plot showing the elapsed time (min) between nuclear envelope breakdown (NEBD) and anaphase onset/death for individual cells. The total number of cells (n) from two or more independent experiments is given above each box. Lower and upper whiskers represent 10th and 90th percentiles, respectively. Table summarizing information from the live cell experiments shown below regarding the average time in mitosis (from NEBD until anaphase onset/cell death) and the percentage of cells dying in mitosis. (d) Representative stills from time-lapse video-microscopy experiments illustrating mitotic progression of HeLa S3 cells stably expressing histone H2B-GFP treated as in c. Time in h:min is indicated. T = 0 was defined as the time point at which NEBD became evident. Scale bar, 10 μm. (e) Cartoon representation of the structure of human Ska1–MTBD, which possesses a modified winged-helix domain with an elongated shape. The length of the structure is 50 Å whereas the width is approximately 30 Å. Secondary structure elements are labelled. (f) Sequence alignment of human Ska191–255 showing amino acid conservation between H. sapiens (hs), M. musculus (mm), X. tropicalis (xt) and D. rerio (dr). Secondary structure elements are shown below the aligned sequences. Amino acid conservation is highlighted in grey.

consistent with a previous report31 and show the requirement of the Ska1–MTBD for ensuring stable KT–MT interactions and timely mitotic progression, although this domain seems dispensable for initial chromosome alignment. In contrast, replacement of WT Ska1 by Ska1–MTBD resulted not only in delayed anaphase onset and problems in maintaining a tight metaphase plate (Fig. 1c and Supplementary Movie 5) but also increased the time for initial chromosome alignment.
The structural analysis showed that the Ska1–MTBD is related to are stabilized in an extended conformation, followed by eight of 25.1 and 26.8, respectively (Table 1), and superpose well with helical segments (

This precluded the assessment of the MT-bundling ability of this localization of the Ska1–MTBD, possibly through a nuclear

We next sought to specifically evaluate the functional requirement of the Ska1 loop. Transfection of Ska1Δloop(SGSG), where amino acids 92–132 were replaced by a short linker sequence (GSSG), delayed both chromosome alignment and anaphase onset by twofold (Fig. 1c, Supplementary Table S1 and Supplementary Movie 6). It is to be noted that the Ska1Δloop(SGSG) can bind MTs with comparable efficiency to the WT Ska complex in vitro MT-binding assay (Supplementary Fig. S1d). Interestingly, the bulk of the Ska1Δloop(SGSG) mutant showed no or weak KT localization, suggesting a potential role for the loop in mediating intermolecular interactions required for KT localization (Supplementary Fig. S1b). Together, these results suggest that the flexibility associated with the loop region (and/or intermolecular interactions mediated by it) is required for timely progression through mitosis.

Ska1–MTBD possesses a modified winged-helix motif. To understand the structural basis for the ability of human Ska complex to bind MTs, we obtained crystals of the Ska1–MTBD in two different crystal forms that diffracted X-rays to about 2 Å (Table 1). The structure was determined by single anomalous dispersion (SAD) experiments using crystals obtained from selenomethionine-incorporated samples. Models from crystal form I (space group C222_1) and II (space group P3_2) were refined to 2.1 and 2.3 Å with R factors of 19.6 and 22.5, and Rfree factors of 25.1 and 26.8, respectively (Table 1), and superpose well with an overall root mean square deviation of 0.6 Å. Residues 133–142 are stabilized in an extended conformation, followed by eight α-helical segments (α1–α8) and a C-terminal β-hairpin (Fig. 1e,f). The structural analysis showed that the Ska1–MTBD is related to a winged-helix domain, a domain known for its ability to bind DNA and in mediating protein–protein interactions. The Ska1–MTBD differs from the canonical winged-helix domain by the incorporation of two additional modules (Fig. 1e and Supplementary Fig. S2a).

Human and C. elegans Ska1–MTBDs show structural variations. During the course of this work, the NMR structure of the MTBD of the C. elegans Ska1 was reported (Table S1). The Ska1–MTBD of C. elegans shares 28% sequence identity and 46% sequence similarity with its human counterpart. Structural comparisons show that the overall topology of the human and C. elegans Ska1–MTBDs is the same (structures superpose with an overall root mean square deviation of 3.0 Å). However, in the C. elegans structure, helices α5 and α8, and the β-strands β2 and β3 are in a different orientation relative to the rest of the structure, resulting in noticeable changes in the surface charge distribution of the MTBD (Supplementary Fig. S2b–d). Structure-based sequence alignment reveals that C. elegans amino acid Thr168 acts as a hinge residue between α3 and α5 (Supplementary Fig. S2c). In contrast, the corresponding amino acid in higher vertebrates is proline (182, human numbering), an amino acid that has limited backbone conformational flexibility (Supplementary Fig. S2c).

Ska interacts with MTs using a multipartite mode of binding. The basic nature of the Ska1–MTBD (predicted pI = 9.2) led us to hypothesize that the Ska complex recognizes MTs through electrostatic interactions. Analysis of the electrostatic surface potential revealed the existence of contiguous positively charged patches all over the Ska1–MTBD surface (Fig. 2a), suggesting the potential involvement of multiple MT contact sites. Of the 23 Lys (K)/Arg (R) residues that are present in the Ska1–MTBD, 14 are clearly exposed to solvent (Fig. 2a). To identify the critical residues required for MT binding, K/R residues that cluster on the surface were mutated to Ala (A) in the context of the FL human Ska complex. Before subjecting the mutants to MT cosedimentation assays, we analysed their size-exclusion chromatography profiles to rule out the influence of the mutations on the overall structure of the complex. All the mutants behaved identically to the WT Ska complex, suggesting that mutations do not affect the proper folding of the Ska complex (Supplementary Fig. S3c). Although mutations at K170/177 and K135/206

### Table 1 | Data collection, phasing and refinement statistics.

|                                | Crystal form 1         | Crystal form 2         |
|--------------------------------|------------------------|------------------------|
| **Data collection**            |                        |                        |
| Space group                    | C222_1                 | P3_2                   |
| **Cell dimensions**            |                        |                        |
| a, b, c (Å)                    | 39.01, 161.58, 104.48  | 47.18, 47.18, 116.50  |
| α, β, γ (°)                    | 90, 90, 90             | 90, 90, 120            |
| **Wavelength**                 | 0.98                   | 1.54i                  |
| **Resolution (Å)**             | 63.9–2.0 (2.12–2.01)   | 58.3–2.3 (2.27–2.25)   |
| **R颃**                        | 7.8 (45.5)             | 4.8 (30.8)             |
| **Root mean square deviation** |                        |                        |
| Bond lengths (Å)               | 0.008                  | 0.008                  |
| Bond angles (°)                | 1.09                   | 1.12                   |

Values in parentheses are for highest-resolution shell.
showed no major effects on MT binding, mutations at R155/236/245, a region reported recently to be critical for MT binding, together with two new regions identified in this study, K183/203/206 and K217/223/226/227, all showed significant reductions in MT binding (Kd = 20 ± 4.5, 17.1 ± 5.5 and 14.5 ± 2.8 μM, respectively, versus WT = 2.9 ± 0.6 μM), pointing to the existence of multiple MT interaction sites (Fig. 2b). Confirming this notion, a combination of R236/245A with R155A resulted in stronger reduction in MT binding than R236/245A alone (Fig. 2b). These results provide clear evidence that the human Ska complex binds MTs through a multipartite binding mode of Ska1–MTBD.

Multipartite MT binding is required for Ska complex function. We next evaluated the functional significance of the positively charged clusters of the Ska1–MTBD using small interfering RNA (siRNA) rescue assays with the above K or R to A mutants. In line with in vitro results, cells transfected with K170/177A and K183/184/203/206A showed partial rescue compared to WT (Fig. 2a), whereas cells transfected with K183/184/203/206/217/223/226/227A, K183/184/203/206/217/223/226/227/R236/245A, and (7+2) K183/184/203/206/217/223/226/227/R236/245A showed almost complete rescue compared to WT (Fig. 2b). These results provide clear evidence that the human Ska complex binds MTs through a multipartite binding mode of Ska1–MTBD.
proteins that interact with MTs by recognizing acidic tails of crosslinked residues on the three-dimensional structures of Ska1–showed crosslinks with tubulin monomers. Mapping of the and function, two (K183/184/203/206 and K216/217/223/226) three K/R clusters that we identified to be crucial for MT binding highlighting the specificity of the interaction (Fig. 4a,b). Further-
analysed on its own or in the context of the Ska complex, identical crosslinks with MTs, regardless of whether it was involved the MTBD of Ska1. The Ska1–MTBD made almost crosslinks observed for the FL Ska complex bound to MTs (Supplementary Fig. S5f). Consistent with our biochemical and functional analyses, most of the crosslinks observed for the FL Ska complex bound to MTs involved the MTBD of Ska1. The Ska1–MTBD made almost identical crosslinks with MTs, regardless of whether it was analysed on its own or in the context of the Ska complex, highlighting the specificity of the interaction (Fig. 4a,b). Furthermore, Sk2, which has been shown not to have any MT-binding activity, did not produce any crosslinked peptides with tubulin, confirming the specificity of the crosslinking reaction. Among the three K/R clusters that we identified to be crucial for MT binding and function, two (K183/184/203/206 and K216/217/223/226) showed crosslinks with tubulin monomers. Mapping of the crosslinked residues on the three-dimensional structures of Ska1–MTBD and MTs showed that these clusters contact globular/ folded regions of tubulin monomers (unlike most MT-binding proteins that interact with MTs by recognizing acidic tails of tubulins) mainly at two helices: H3 and H4 of β-tubulin and H3 and H12 of α-tubulin (Fig. 4c). Interestingly, intermolecular contacts of Sk1 with H4 of β-tubulin and H12 of α-tubulin seem to be sequence specific, as these tubulin residues are unique to α- and β-isomers (results not shown).

To rule out the possibility that the crosslinking peptides observed are due to nonspecific interactions with free tubulin monomers, we pelleted MTs crosslinked to Ska1–MTBD before MS analysis and compared the results with those obtained from non-pelleted samples (Fig. 4). Analysis of both samples by SDS–PAGE showed identical crosslinked products (Supplementary Fig. S5g). Furthermore, the contact sites observed were almost identical in both pelleted and non-pelleted samples (Supplementary Fig. S5h), attesting to the specificity of the interactions.

In this analysis, we did not detect crosslinks involving the R155/236/245 cluster. This, as well as the fact that we did not detect acidic tails of tubulin monomers, may be due to the following technical reasons: first, EDC, the crosslinking reagent used in this study, does not crosslink arginines; second, peptides derived from the acidic tails of tubulins may have escaped detection by MS possibly because of the presence of posttransla-
tional modifications (notably polyglutamylation) and/or the lack of tryptic cleavage sites, which would result in large peptide fragments that cannot be detected in crosslinked/MS analysis. To overcome the former limitation, we have mutated R155/236/245 to lysine residues in the context of both Ska1–MTBD and FL Ska1, and then tested these mutants in crosslinking/MS experiments (Supplementary Fig. S5c,d). Indeed, the R155/236/245K mutant did reveal interactions between K155 and K245 of Sk1 with H3 of α- and β-tubulin, respectively (Fig. 4c).

Sk1 interacts with tubulin monomers at multiple sites. Having established a multipartite mode of MT binding by the Ska com-
plex, we next aimed at identifying the structural features of MTs that are recognized by the Ska complex. For this purpose, we crosslinked the Ska1–MTBD/Ska complex with MTs, using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). This reagent crosslinks K (and less favourably S, T, Y) to E or D. Analysis of the crosslinked products in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) showed a predomi-
ant band that migrated at the expected molecular weight for one Ska1–MTBD/Ska1 crosslinked to an α-β-tubulin monomer (marked by asterisks in Supplementary Fig. S5a,b).

MS analysis of the crosslinked products allowed us to pinpoint the residues involved in intermolecular recognition between the Ska complex and tubulins (Fig. 4 and Supplementary Fig. S5e). The overall sequence coverage for Ska1/Ska1–MTBD and tubulin monomers was almost complete, except for the flexible C-terminal tails of tubulin (Supplementary Fig. S5f). Consistent with our biochemical and functional analyses, most of the crosslinks observed for the FL Ska complex bound to MTs involved the MTBD of Sk1. The Ska1–MTBD made almost identical crosslinks with MTs, regardless of whether it was analysed on its own or in the context of the Ska complex, highlighting the specificity of the interaction (Fig. 4a,b). Furthermore, Sk2, which has been shown not to have any MT-binding activity, did not produce any crosslinked peptides with tubulin, confirming the specificity of the crosslinking reaction. Among the three K/R clusters that we identified to be crucial for MT binding and function, two (K183/184/203/206 and K216/217/223/226) showed crosslinks with tubulin monomers. Mapping of the crosslinked residues on the three-dimensional structures of Ska1–MTBD and MTs showed that these clusters contact globular/ folded regions of tubulin monomers (unlike most MT-binding proteins that interact with MTs by recognizing acidic tails of tubulins) mainly at two helices: H3 and H4 of β-tubulin and H3 and H12 of α-tubulin (Fig. 4c). Interestingly, intermolecular contacts of Sk1 with H4 of β-tubulin and H12 of α-tubulin seem to be sequence specific, as these tubulin residues are unique to α- and β-isomers (results not shown).

Ska and Ndc80 complexes recognize different features of MTs. Structural characterizations of Ndc80–MT interactions revealed that the Ndc80 complex binds MTs by recognizing the dimeric interface of α- and β-tubulins. This mode of MT binding by the Ndc80 complex makes the interaction sensitive to the conformation of MT protofilaments. Indeed, the Ndc80 complex preferentially binds straight MTs over curved MT protofilaments (vinblastine spirals; Fig. 5a and Supplementary Fig. S6b), in line with previous reports. Our crosslinking/MS and cosedimentation data presented here show that the Ska complex, in contrast to the Ndc80 complex, interacts with MTs by recognizing the regions of tubulin monomers whose accessibility is not perturbed when MTs adopt different conformations. Thus, the Ska complex can bind straight and curved MT protofilaments indiscriminately using the same...
Figure 3 | MT recognition of Ska1 via its multipartite mode of MT binding is a functional requirement for the Ska complex. (a) Box-and-whisker plot showing the elapsed time (min) between nuclear envelope breakdown (NEBD) and anaphase onset/death for individual cells. The total number of cells (n) from two or more independent experiments is given above each box. Lower and upper whiskers represent 10th and 90th percentiles, respectively. Table summarizing information from the live cell experiments shown below regarding the average time in mitosis (from NEBD until anaphase onset/cell death) and the percentage of cells dying in mitosis. (b) Representative stills from time-lapse video-microscopy experiments illustrating mitotic progression of HeLa S3 cells stably expressing histone H2B-GFP. Scale bar, 10 μm.
Figure 4 | Ska1 interacts with MTs by recognizing the globular regions of tubulin monomers in multiple orientations. (a,b) Linkage map showing the sequence position of all the crosslinked residue pairs between (a) Ska1–MTBD (b) Ska complex and α1B and β2B tubulin, where 6 μM human Ska complex/Ska1–MTBD was incubated with 10 μM of MTs in the crosslinking reactions. Crosslinked products were resolved in SDS–PAGE followed by MS analysis. Red and green boxes above the β-tubulin show regions of tubulin involved in longitudinal and lateral contacts, respectively. Crosslinks observed between Ska1 R155/236/245K mutant and MTs are shown in grey. (c) Cartoon representation of tubulin dimer where residues involved in crosslinking with Ska1 residues are highlighted in stick representation. Grey and green lines denote crosslinks observed between K/R clusters of Ska1 and Glu/Asp/Tyr/Thr clusters of β-tubulin and α-tubulin, respectively. The region where Ndc80 interacts with tubulin as reported in Alushin et al.33 is shown in yellow. Important Ska1 residues involved in MT binding are colour coded as in Figs 2 and 3.
contact sites (see $K_d$ values for straight MTs in Fig. 2b and corresponding values for vinblastine spirals in Fig. 5b). In future, it will be interesting to explore the possibility that the presence of the Ndc80 complex (or other Ska-binding partners) could induce some of the binding sites to discriminate between different MT structures.

MT-interacting proteins often interact with MTs by recognizing the acidic tails of tubulin, called ‘E-hooks’. Biochemical and structural characterizations of Ndc80–MT interactions carried out in different laboratories have also highlighted the important contribution of the acidic tail of tubulin in the overall recognition of MTs by the Ndc80 complex13,33. Our crosslinking MS analysis does not provide insight into the possible role of acidic tubulin tails in Ska complex binding, owing to the technical reasons discussed in the previous section. However, as the Ska complex makes multiple contacts with the structured regions of tubulins, we hypothesized that the acidic tail of tubulin may not significantly contribute to the overall recognition of MTs. To evaluate this hypothesis, we tested the ability of the Ska complex to bind subtilisin-treated MTs (where E-hooks are removed by subtilisin treatment) in cosedimentation assays. As expected, the Ndc80 complex showed reduced binding to subtilisin MTs. Interestingly, the Ska complex did not show any noticeable reduction in its ability to bind subtilisin MTs, in line with the view that the critical contacts involve the structured regions of tubulin monomers rather than the acidic tails (Fig. 5c and Supplementary Fig. S6c).

Aurora B sites lie within the MT-binding K/R clusters. The role of Aurora B kinase in correcting erroneous KT–MT attachments by phosphorylating components of the KMN network, notably the Ndc80 complex and KNL1, is well established11,34,43,44. Recent work also showed that Aurora B negatively regulates the KT localization of the Ska complex, possibly influencing interactions with the KMN network30. As noted elsewhere31, all four Aurora B consensus sites (T157, S185, T205 and S242) are located within the Ska1–MTBD (Fig. 6a). Interestingly, two of...
these sites (S185 and T205) are located within the K/R cluster K183/184/203/206 that we have identified as being important for the MT-binding activity of the Ska complex (Fig. 6a). This suggested a direct involvement of S185 and T205 in the Aurora B-mediated phosphoregulation of Ska–MT interactions. To test this possibility, we made phosphomimicking mutants of Ska1 (S185D and S185/T205D) and tested them in MT-binding assays. Although S185D did not show any noticeable reduction in its ability to bind MTs, S185/T205D showed a drastic reduction (Fig. 6b and Supplementary Fig. S7). However, in line with our previous study30, pre-incubation of the Ska complex with Aurora B did not reduce its MT-binding ability, although the Ndc80 complex analysed for control responded as expected (Fig. 6c). To explain these apparently contradictory findings, we considered the possibility that efficient phosphorylation of Ska1 by Aurora B might require prior conformational rearrangements within the MTBD, which might occur either on MT binding or in the context of other KT-associated proteins. To test the former possibility, we first allowed the Ska complex to bind MTs before incubating the MT-bound Ska complex with Aurora B and evaluating the consequences in MT-binding assays. This experiment revealed a small but statistically significant reduction in the Ska–MT interaction in response to Aurora B (Fig. 6d). In line with the above model, normal mode analysis of the MD simulated structures of Ska1–MTBD shows the presence of an intrinsic flexibility associated with the structural element that harbours this MT-binding site and Aurora B consensus sites (Supplementary Movie 10).
S242, are also close to the R cluster R155/236/245, but insertion of phosphomimicking mutations did not show any influence on MT binding (Supplementary Fig. S7). It is interesting that combination of a phosphomimetic mutation at S242D did not abolish MT binding in combination with T157D (this study), although it has been previously shown that combined mutations at S242D and S185D strongly reduced MT binding. This observation, together with our observation that the S185D/T205D combination also drastically diminishes MT binding, demonstrates that Aurora B phosphorylation of Ska1–MTBD can negatively regulate Ska–MT interactions via multiple phosphorylation events.

Discussion

MT-binding activity at the KT is a prime requirement for driving accurate chromosome segregation. Although the Ndc80 complex is considered to be the major contributor to MT binding by the KT, other factors such as the Dam1 complex in budding yeast and the Ska complex in vertebrates are required to efficiently couple MT binding at the KT with chromosome segregation. The ability of the Dam1 complex to form rings around MTs suggested that Dam1-like proteins might work as force couplers that harness the function of the Dam1 complex to form rings around MTs suggested that chromosomes, such as different dynamic properties. In organisms with ‘holocentric’ spindle MTs in vertebrates versus nematodes may exhibit no apparent dynamic oscillations at metaphase. In contrast, in ‘monocentric’ mammalian cells, chromosomes are attached to sites along the chromosome arms, resulting in chromosomes with negatively charged nature of these molecules, and, second, that negatively charged nature of these molecules, and, second, that MT-binding activity at the KT is a prime requirement for driving accurate chromosome segregation. Although the Ndc80 complex is considered to be the major contributor to MT binding by the KT, other factors such as the Dam1 complex in budding yeast and the Ska complex in vertebrates are required to efficiently couple MT binding at the KT with chromosome segregation. The ability of the Dam1 complex to form rings around MTs suggested that Dam1-like proteins might work as force couplers that harness the force associated with MT depolymerization with chromosome movement. However, the proposed functional homologue of Dam1 in humans, the Ska complex, forms a flexible W-shaped structure of triple helical bundles with a maximum interatomic distance of 35 nm (ref. 36). With MTBDs symmetrically positioned at both ends of the dimer, the Ska complex thus appears to exert its function via a different mechanism.

Here we have used biochemical and high-resolution structural analysis to show that the human Ska complex interacts with MTs through the C-terminal domain of Ska1, which forms a variant form of winged-helix domain. This domain has previously been seen as a DNA-binding module in transcription regulators but also as a protein interaction module in a small number of proteins with diverse functions. The Ska complex provides the first instance where this module is used as a MTBD. However, considering that, first, both DNA molecules and MTs are often recognized through electrostatic interactions exploiting the negatively charged nature of these molecules, and, second, that MT-based diffusion motility and DNA-based diffusion shows striking similarities with comparable diffusion coefficients, the use of a winged-helix domain for the Ska1–MTBD may not be surprising. It will be interesting to see whether the Dam1 complex also possesses winged-helix domains in its MT-binding components.

Structural comparisons of human and C. elegans Ska1–MTBDs show conformational variations in the regions that we demonstrate here to be critical for MT binding. This adds to species-specific differences in the overall composition and architecture of the Ska complexes, in that Ska1 and Ska3 associate in a 2:1 complex in C. elegans, whereas the human Ska complex is made of Ska1, Ska2 and Ska3 in a 2:2:2 ratio. It is tempting to suggest that the attachment of chromosomes to spindle MTs in vertebrates versus nematodes may exhibit different dynamic properties. In organisms with ‘holocentric’ chromosomes, such as C. elegans, MTs are attached at multiple sites along the chromosome arms, resulting in chromosomes with no apparent dynamic oscillations at metaphase. In contrast, in ‘monocentric’ mammalian cells, chromosomes are attached to MTs at a discrete site and metaphase-aligned chromosomes show pronounced dynamics.

Using MT-binding assays in combination with siRNA-based rescue assays, we demonstrate here the involvement of at least three tubulin contact sites within Ska1–MTBD for MT recognition. Remarkably, the tubulin contact sites are dispersed across the surface of the Ska1–MTBD and distances between different contact sites range between 15 and 30 Å. Disruption of even one of the tubulin contact sites is enough to perturb normal mitotic progression, suggesting the requirement of all contact sites for efficient function. Crosslinking/MS analysis revealed important molecular details of these Ska–MT interactions, in particular the novel ability of the Ska–MTBD to bind to tubulin monomers. This data together with those from quantitative MT cosedimentation assays demonstrated that tubulin contacts of the Ska1–MTBD can bind straight and curved MTs without apparent preference.

Identifying and characterizing the unique properties of the Ndc80 and Ska complexes is crucial for understanding how these complexes complement each other in providing an integrated interface for efficient MT binding and MT-driven motility. The Ndc80 complex binds MTs through the interaction of the Ndc80-CH (Calponin Homology) domain (called the ‘toe’ at the tubulin dimeric interface (called the ‘toe print’) in a way that favours interactions with the straight conformation of MT protofilaments. Our results show that the Ska complex interacts with the structured regions of tubulin monomers, mainly at helices H3 and H4 of β-tubulin and H12 of γ-tubulin, whose accessibility is not perturbed on MTs assuming different conformations. This feature gives the Ska complex the ability to bind both straight and curved protofilaments with equal efficiency (Fig. 7). One of the sites (Glu110, 156 and 162 in H3) through which β-tubulin makes contact with the Ska1–MTBD is particularly intriguing, as this site is close to the GTP-binding site and also near the regions involved in lateral contacts between adjacent MT protofilaments. Furthermore, this site is recognized by EB1 and has been suggested to be important for EB1’s end-tracking activity and for stabilizing growing MTs. In this context, it would be interesting to know whether the Ska complex can also influence MT dynamics.

One of the intriguing observations made in our study concerns the ability of the Ska complex to bind MTs in multiple different orientations. This combines with the fact that the Ska1–MTBDs are loosely connected to the W-shaped triple helical structure through a 40 amino acid loop, thus providing additional flexibility that may be important to allow the KTs to track disassembling MTs (Fig. 7). This is in stark contrast to the Ndc80 complex, where CH domains of Ndc80 are connected to a rigid helical bundle. We further envisage that the presence of this loop in Ska1 is likely to be critical for efficient MT tracking by the Ska complex and possibly for mediating protein–protein interactions with other KT components. In support of this view, we found that deletion of the Ska1 loop delays mitotic progression of dividing cells.

In summary, our results indicate that the function of the Ska complex is conferred by its ability to interact with regions of tubulin monomers whose accessibility is not affected by different MT structures. These interactions involve multiparticle binding sites and allow MT binding in multiple orientations (Fig. 7). Future structural and functional studies on whether and how Ska–MT interactions are modulated by the presence of other MT-binding proteins, notably the Ndc80 complex, will advance our understanding of the molecular underpinnings of KT–MT attachments and chromosome segregation.

Methods

Expression and purification of recombinant proteins. Ska1135–245 was cloned into a pEC-K-3C-His-GST vector as an N-terminally His-GST-tagged protein with a 3C-cleavage site. Ska1, Ska2 and Ska3 V58I were cloned individually in a pEC-S-CDF-His, pEC-A-HT-His GST and pEC-K-HT-His vectors, respectively, with TEV cleavage sites. Ska1 mutants were generated following the Quickchange site-directed mutagenesis method (Stratagene; primer details are given in Supplementary Table S3). To express the Ska complex containing Ska1Δloop,
Skα1loop (GSSG) and phosphomimetic mutants, Quikchange site-directed mutagenesis was performed on a polycistronic vector (primer details are given in Supplementary Table S3). PETMCN (gift from C. Romier, IGMC, Strasbourg) containing GST-Ska2 (3C-cleavable), untagged Skα1 and Skα3. All protein complexes were expressed in Escherichia coli strain BL21 Gold, either using the polycistronic construct or by cotransforming all the three plasmids containing individual Skα components. Cultures were induced overnight at 18 °C and purified using a similar protocol. Cells were lysed in a buffer containing 20 mM Tris, pH 8, 500 mM NaCl and 5 mM dithiothreitol (DTT). The protein complexes were purified by affinity chromatography in batch mode using glutathione sepharose (GE Healthcare) beads. Protein-bound beads were washed with 20 mM Tris, pH 8, 500 mM NaCl and 5 mM DTT, followed by 20 mM Tris, pH 8, 1 M NaCl, 50 mM KCl, 10 mM MgCl2, 2 mM ATP and 5 mM DTT, then finally with 20 mM Tris, pH 8, 100 mM NaCl and 5 mM DTT. Proteins with 3C-cleavage sites were cleaved, while the proteins were still bound to the beads. TEV cleavable proteins were eluted with 50 mM glutathione, 20 mM Tris, pH 8, 100 mM NaCl and 5 mM DTT, and the tags were removed in solution overnight. Subsequently, proteins/protein complexes were purified by size-exclusion chromatography in 20 mM Tris, pH 8, 100 mM NaCl and 5 mM DTT (Superose 6, GE Healthcare). Ndc80 Bonsai (kindly gifted by Andrea Musacchio) was expressed in E. coli BL21 (DE3) 13. Cells were lysed in lysis buffer containing 20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM DTT and 1 mM EDTA, then finally with 20 mM Tris, pH 8, 100 mM NaCl and 5 mM DTT. Proteins and 3C-cleavage sites were cleaved, while the proteins were still bound to the beads. TEV cleavable proteins were eluted with 50 mM glutathione, 20 mM Tris, pH 8, 100 mM NaCl and 5 mM DTT, and the tags were removed in solution overnight. Subsequently, proteins/protein complexes were purified by size-exclusion chromatography in 20 mM Tris, pH 8, 100 mM NaCl and 5 mM DTT (Superose 6, GE Healthcare). Ndc80 Bonsai (kindly gifted by Andrea Musacchio) was expressed in E. coli BL21 (DE3) 13. Cells were lysed in lysis buffer containing 50 mM Tris, pH 7.6, 300 mM NaCl, 1 mM DTT and 1 mM EDTA. Cleared lysate was incubated with glutathione sepharose beads. After 3 h incubation at 4 °C, beads were washed with 50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM DTT and 1 mM EDTA, then finally with 20 mM Tris, pH 8, 100 mM NaCl, 1 mM DTT and 10% glycerol. Concentrated protein was loaded onto a Superose 6 size-exclusion chromatography column (GE Healthcare) equilibrated with 20 mM Tris, pH 7.6, 100 mM NaCl, 1 mM DTT and 10% glycerol.

Crystallization and data collection. Crystallization trials were performed using a nanolitre crystallization robot at the Edinburgh Protein Production Facility. Crystals of form I (C2221) were grown by vapour diffusion method using Mor- nanolitre crystallization robot at the Edinburgh Protein Production Facility. Crystals of form II (P32) were grown in mother liquor containing 24% glycerol. Crystals of form I (C2221) were grown by vapour diffusion method using Mor-

Figure 7 | Schematic model. Schematic model summarizing the mode of MT binding of Skα1 and its implications for maintaining stable KT-MT attachments.
Chemical crosslinking and MS analysis. Crosslinking experiments were carried out using a zero-length crosslinking agent, EDC (Thermo Fisher Scientific) in the presence of N-hydroxysuccinimide (Thermo Fisher Scientific). Ska complex (6 μM) and 10 μM MTs were incubated with 10 μg EDC and 22 μg N-hydroxysuccinimide in a final volume of 20 μl. The reaction mixture was incubated for 90 min at 25 °C and was quenched by adding Tris-Cl to a final concentration of 100 mM. The reactions were resolved by SDS-PAGE (4–12% Bis-Tris NuPAGE, Invitrogen) gel separation and stained using Instant Blue (Expedeon). The bands corresponding to crosslinked complexes were excised and the proteins therein were reduced using 10 mM DTT for 30 min at room temperature and digested using 13 ng μl−1 trypsin (sequencing grade; Promega) overnight at 37 °C. The digested peptides were desalted using C18-StageTips59 and analysed on a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific)60. An analytical column with a spray emitter (75 μm inner diameter, 8 μm opening, 250 μm length; New Objectives) that was packed with C18 material (ReproSil-Pur C18-AQ 3 μm; Dr Maisch GmbH, Ammerbuch-Entringen, Germany) using an air pressure pump (ProXeon Biosystems). Mobile phase A consisted of water with 0.1% formic acid. Mobile phase B consisted of 80% acetonitrile with 0.1% formic acid. Peptides were loaded onto the column with 1% B at 600 μl h−1 flow rate and eluted at 300 nl min−1 flow rate, with a linear gradient increased from 5 to 35% acetonitrile in 0.1% formic acid in 150 min to elute peptides. Peptides were analysed using a high/high strategy; both MS spectra and MS2 spectra were acquired in the Orbitrap61. Mass spectra were recorded at 100,000 resolution. The eight highest intensity peaks with a charge state of three or higher were selected in each cycle for ion-trap fragmentation. The fragments were produced using collision-induced dissociation with 35% normalized collision energy and detected by the Orbitrap at 7,500 resolution. Dynamic exclusion was set to 50 s and repeat count was 1. The data were processed, generating peak lists by MaxQuant58 and matching crosslinked peptides to spectra using in-house developed X!-dC (2014).
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Author contributions
A.A.J conceived and designed the project. M.A.A., B.M., A.A.J., A.S., J.Z., C.P.-H., A.M., U.I. and P.M.R. performed the experiments and analysis. J.R. and E.A.N. provided the computational resources. We also thank the authors for their generous support during the initial stages of the project. A.A.J. provided the computational resources. We also thank Bill Earnshaw, Kevin Hardwick, Sutapa Chakrabarti, Laksmi Subramanian and Esben Lorentzen for critical reading of the manuscript. The Wellcome Trust generously supported this work through a Wellcome Trust Career Development Grant to A.A.J. (095822), a Senior Research Fellowship to J.R. (084229), a Wellcome Trust Centre Core Grant (092076) and an instrument grant (091020). A.A.J. also acknowledges the support by the EC for the Marie-Curie career integration grant (FP7). A.S. and E.A.N. acknowledge support from the University of Basel, the Swiss Cancer League (02657-08-2010) and the Swiss National Science Foundation (310030B_149641). A.M. thanks the Department of Biotechnology (DBT), India, for funding.

Additional information
Accession codes: Coordinates and structural factors have been deposited in the protein data bank under accession codes 4CFY (C222) form and 4CA0 (P31 form).
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