STRUCTURE

Structural characterization of KKT4, an unconventional microtubule-binding kinetochore protein

Graphical abstract

Highlights

- Structures of microtubule-binding and BRCT domains in KKT4 are reported
- The microtubule-binding domain consists of a coiled coil and a disordered tail
- KKT4 interacts with microtubules via a basic surface at the coiled-coil N terminus
- KKT4 has a phosphopeptide-binding BRCT domain

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In brief

KKT4 is a unique microtubule-binding kinetochore protein in trypanosomes. Structural analyses by Ludzia and colleagues show that its microtubule-binding domain consists of a coiled coil and a positively charged disordered tail. They also demonstrate that the C terminus of KKT4 is a phosphopeptide-binding BRCT domain.
SUMMARY

The kinetochore is the macromolecular machinery that drives chromosome segregation by interacting with spindle microtubules. Kinetoplastids (such as *Trypanosoma brucei*), a group of evolutionarily divergent eukaryotes, have a unique set of kinetochore proteins that lack any significant homology to canonical kinetochore components. To date, KKT4 is the only kinetoplastid kinetochore protein that is known to bind microtubules. Here we use X-ray crystallography, NMR spectroscopy, and crosslinking mass spectrometry to characterize the structure and dynamics of KKT4. We show that its microtubule-binding domain consists of a coiled-coil structure followed by a positively charged disordered tail. The structure of the C-terminal BRCT domain of KKT4 reveals that it is likely a phosphorylation-dependent protein-protein interaction domain. The BRCT domain interacts with the N-terminal region of the KKT4 microtubule-binding domain and with a phosphopeptide derived from KKT8. Taken together, these results provide structural insights into the unconventional kinetoplastid kinetochore protein KKT4.

INTRODUCTION

Every time a cell divides, it must duplicate and segregate its genetic material accurately into two daughter cells. A key structure involved in chromosome segregation in eukaryotes is the kinetochore, a macromolecular protein complex that assembles onto centromeric DNA and interacts with spindle microtubules during mitosis and meiosis (Mcintosh, 2016). Microtubules are dynamic polymers that change in length by addition or removal of tubulin subunits at the tips (Desai and Mitchison, 1997). Accurate chromosome segregation requires that kinetochores form robust attachments to the dynamic microtubule tips. In addition, kinetochores need to destabilize erroneous attachments to ensure that sister kinetochores bind microtubules emanating from opposite poles (Nicklas, 1997; Biggins, 2013; Cheeseman, 2014; Musacchio and Desai, 2017). Revealing the molecular basis of kinetochore-microtubule attachments and their regulation is key to understanding the mechanism of chromosome segregation.

Commonly studied eukaryotes have a number of microtubule-binding kinetochore proteins, including the Ndc80, Dam1, Ska, and SKAP-Astrin complexes (Cheeseman et al., 2001, 2006; Hanisch et al., 2006; Schmidt et al., 2012; Abad et al., 2014; Friese et al., 2016; Kern et al., 2017). Some of these components are widely conserved among eukaryotes (Meraldi et al., 2006, Van Hooff et al., 2017). However, none of these or other canonical structural kinetochore components has been identified in kinetoplastids, an evolutionarily divergent group of unicellular flagellated eukaryotes, such as parasitic trypanosomatids (e.g., *Trypanosoma brucei*, *Trypanosoma cruzi*, and Leishmania species) and free-living bodonids (e.g., *Bodo saltans*) (Berriman et al., 2005; Cavalier-Smith, 2010). Instead, a number of unique kinetochore proteins have been identified in *T. brucei*, namely 24 kinetoplastid kinetochore proteins (KKT1–20, KKT22–25) and 12 KKT-interacting proteins (KKIP1–12) (Akiyoshi and Gull, 2014, Nerusheva and Akiyoshi, 2016, D’archivio and Wickstead, 2017, Brusini et al., 2019, Nerusheva et al., 2019). These proteins do not appear orthologous to canonical kinetochore proteins, suggesting that kinetoplastids use a distinct set of proteins to build up unique kinetochores. We previously identified KKT4 as a microtubule-binding kinetochore protein in *T. brucei* (throughout this manuscript we refer to KKT4 from *Trypanosoma brucei* unless stated otherwise) (Llauro et al., 2018). KKT4 directly binds to microtubules and maintains load-bearing attachments to both growing and shortening microtubule tips in vitro. Microtubule-binding activities were also found in KKT4 from other kinetoplastids, suggesting that KKT4 plays an important role in the kinetoplastid family (Llauro et al., 2018). Using microtubule co-sedimentation assays, we defined KKT4115–343 as the microtubule-binding domain in *T. brucei*. To date, there is no structural information available for KKT4. It therefore remains unknown how KKT4 forms attachments to microtubules.
and whether its microtubule-binding activity is regulated. Interestingly, KKT4 has a putative BRCA1 C-terminal (BRCT) domain, which is not present in any known kinetochore protein in other eukaryotes (Figure 1) (Akiyoshi and Gull, 2014). The function of this putative BRCT domain is also unknown.

Here, we have used X-ray crystallography and NMR spectroscopy to obtain structural information for KKT4. Its microtubule-binding domain consists of a coiled-coil structure followed by a positively charged disordered tail. A crystal structure of the C-terminal BRCT domain reveals a putative phosphopeptide-binding pocket, which binds a phosphopeptide derived from KKT8. Overall, these analyses show that the KKT4 structure is distinct from any known microtubule-binding kinetochore protein.

**RESULTS**

**KKT4 forms oligomers**

Our previous single-molecule experiments suggested that KKT4 was mostly monomeric but had a tendency to oligomerize even at a low nanomolar concentration (Llauro et al., 2018). To determine the oligomerization state of KKT4, we used size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) (Wen et al., 1996) (Figure S1). Analysis of the full-length protein revealed that KKT4 is mostly tetrameric at higher concentrations and dimeric at lower concentrations (Figures S1B and S1C). To identify the region(s) responsible for oligomerization, smaller fragments of KKT4 were analyzed. The following predicted structural regions of KKT4 are well conserved among kinetoplastids: N-terminal α helix, coiled coil and block of basic residues in the microtubule-binding domain, and the C-terminal BRCT domain (Figures 1 and S2) (Llauro et al., 2018). We found that KKT4463-485, a putative BRCT domain, behaved as a monomer (Figures S1B and S1D), while KKT4485-485, containing the microtubule-binding domain, behaved as a dimer (Figures S1B and S1E). KKT42-224, like the full-length protein, showed characteristics of a tetramer at higher concentrations but of a trimer at lower concentrations (Figures S1B and S1F). Thus, the N-terminal region is responsible for the formation of the KKT4 tetramer. It is likely that KKT42-224 is in a dimer-tetramer equilibrium (rather than trimer-tetramer) because the microtubule-binding domain is a dimer and the full-length protein is likely to be a dimer of dimers. These results suggest that KKT4 has multiple regions that promote oligomerization.

**Crystal structures of T. cruzi KKT4117-218 and L. mexicana KKT4184-284**

To gain structural insights into the microtubule-binding domain of KKT4, we tried to crystalize KKT4115-343. Despite extensive attempts, no suitable crystals were obtained. It is likely that the predicted disordered region in the C-terminal part of KKT4115-343 prevented the formation of diffraction-quality crystals (Figure S2A). We next designed additional constructs that lack the predicted disordered tail. To obtain diffraction-quality crystals from T. brucei, a crystal structure of KKT4117-218 from T. cruzi was solved at 1.9 Å resolution (Table 1). TcKKT4117-218 is homologous to KKT42-224 in T. brucei (Figures 2A and S3). The structure consists of two ~150 Å long parallel α helices organized in a left-handed coiled-coil dimer (Figure 2B); the helical structure starts at L118 and ends at D215. The coiled coil consists of eight regular heptad repeats starting at Y121 and ending at K176. Analysis
with TWISTER (Strelkov and Burkhard, 2002) shows that the six central heptads are characterized by an inter-helical distance of 4.84 ± 0.06 Å and a coiled-coil pitch (the periodicity of the coiled coil) of 136 ± 26 Å; this pitch is close to the theoretical value of 135 Å for a coiled coil. If the first and eighth heptad are included, the pitch increases to 163 ± 60 Å, indicating less supercoiling in these terminal heptads. After K176, the helices move apart (inter-helical distance increases to 5.66 ± 0.15 Å) and the coiled-coil pitch increases significantly, indicating a loss of supercoiling.

To test structural conservation in other kinetoplastid species, we attempted to solve the crystal structure of the KKT4 coiled-coil fragment from *L. mexicana*. We were unable to obtain diffraction-quality crystals of fragments that had the N-terminal residues of the microtubule-binding domain (115–140 in *T. brucei*). Instead, we crystalized and solved a 1.9 Å structure of *Lm*KKT4184–284 (Table 1), which corresponds to residues 141–244 in *T. brucei* (Figure S3). We note that the expressed protein contained an additional 23 residues from the expression vector at its C terminus due to a cloning error (see the STAR Methods for details). Like *Tc*KKT4117–218, *Lm*KKT4184–284 has helices arranged in a parallel coiled-coil fold (Figure 2C).

### Table 1. Data collection, refinement statistics

| Data collection | *T. cruzi* KKT4117–218 | *L. mexicana* KKT4184–284 | *T. brucei* KKT4163–245 |
|----------------|-----------------------|--------------------------|------------------------|
| Beamline       | Diamond Light Source I03 | Diamond Light Source I24 | Diamond Light Source I24 |
| Wavelength (Å) | 0.9760                | 0.91587                  | 0.96861                |
| Space group (Z) | P 1 2, 1             | P 1 2, 1                | P 2, 1 2, 1           |
| Unit cell |             |                          |                       |
| a, b, c (Å) | 33.62, 25.31, 136.88 | 31.31, 37.71, 122.39 | 46.37, 61.63, 67.78 |
| α, β, γ(°) | 90, 96.81, 90         | 90, 92.17, 90            | 90, 90, 90            |
| Resolution range (Å) | 67.96–1.90 (1.97–1.90) | 61.15–1.90 (1.97–1.90) | 45.60–1.57 (1.63–1.57) |
| Unique reflections | 16,812 (368) | 22,522 (2,211) | 27,032 (2,137) |
| Completeness (%) | 90.4 (20.1) | 98.3 (97.4) | 96.8 (74.7) |
| Multiplicity | 6.1 (4.0) | 6.5 (5.3) | 10.0 (3.8) |
| I/σ | 6.1 (0.1) | 7.1 (0.6) | 14.9 (3.1) |
| Rmerge | 0.095 (7.495) | 0.117 (2.537) | 0.089 (0.549) |
| CC½ | 0.995 (0.274) | 0.997 (0.352) | 0.997 (0.496) |
| Wilson B factor (Å²) | 24.4 | 17.6 | 15.7 |
| Refinement | | | |
| No. of reflections | 16,788 (368) | 22,502 (2,211) | 26,945 (2,109) |
| Rwork | 0.241 (0.347) | 0.199 (0.253) | 0.173 (0.219) |
| Rfree | 0.258 (0.478) | 0.232 (0.264) | 0.192 (0.303) |
| No. of atoms | 1,871 | 1,816 | 1,494 |
| Protein | 1,670 | 1,488 | 1,270 |
| Solvent | 201 | 328 | 224 |
| RMSD | | | |
| Bonds (Å) | 0.006 | 0.014 | 0.011 |
| Angles (°) | 0.97 | 1.45 | 1.46 |
| Ramachandran plot (%) | | | |
| Favored | 100.00 | 100.00 | 98.68 |
| Allowed | 0.00 | 0.00 | 1.32 |
| Outliers | 0.00 | 0.00 | 0.00 |
| Average B factor (Å²) | 36.0 | 30.0 | 22.0 |

*Statistics for the highest-resolution shell are shown in parentheses. RMSD, root-mean-square deviation.*
deviation (RMSD) of 1.03 Å (for 123 Ca) (Figure 2D), confirming the conservation of the coiled-coil structure in these species.

The C-terminal part of the KKT4 microtubule-binding domain is disordered

Due to the failure to crystalize T. brucei KKT4, we employed NMR spectroscopy to probe the structure and dynamics of its microtubule-binding domain. The 2D 1H-15N correlation spectrum of 15N-KKT4115–343 showed a large variation in peak intensities (Figure S5A); this suggests a mixture of structured and disordered regions. The strongest peaks in the spectrum of KKT4115–343 belong to residues 115–118 in the N terminus and 231–343 in the C-terminal half of the fragment (Ludzia et al., 2020). Weaker peaks belong to residues 119–150, while no peaks were observed for residues 151–230 (Ludzia et al., 2020).

Backbone chemical shifts are sensitive indicators of secondary structure (Spera and Bax, 1991; Wishart et al., 1991; Beger and Bolton, 1997; Cornilescu et al., 1999). Analysis of 1Hα, 1HN, 13CA, 13CB, 13CO, and 15N chemical shifts using TALOS-N (Shen and Bax, 2013) predicted no stable secondary structure for residues 231–343 (data not shown). The secondary structure propensity (SSP) score, which is more suitable for identifying structural propensities in disordered proteins (Marsh et al., 2006), also found no SSP greater than 0.25 (Figure 3A), from residues 231–343 in KKT4115–343.

To probe the dynamics of the C-terminus of KKT4115–343, the 1H, 15N heteronuclear NOE, which is sensitive to backbone motions on a timescale (picosecond) faster than the overall tumbling of the molecule (nanosecond), was measured (Kay et al., 1989). A 1H, 15N NOE ratio of less than 0.6, indicating a flexible backbone, was found for all residues from 231 to 343 (Figure 3B). Taken together, the NMR data confirmed disorder in the C-terminal part of KKT4115–343.

The N-terminal part of the KKT4 microtubule-binding domain is structured

The N-terminal region of KKT4115–343 is predicted to adopt a coiled-coil structure (Figure S2B). To gain insights into the structure and dynamics of this region of KKT4, NMR data were collected for a shorter fragment (KKT4115–232) lacking the flexible C-terminal region. However, the spectrum of KKT4115–232 also lacked peaks from residues 151–221; this is likely due to the elongated structure of a coiled coil, which would tumble in a non-uniform way and result in broad 1HN-15N signals (Mackay et al., 1996; Schnell et al., 2005). To overcome this problem, two shorter overlapping constructs were used for further NMR analysis (Figure 1): KKT4115–174, the minimal microtubule-binding domain that retains reduced microtubule-binding activities (Llauro et al., 2018), and KKT4145–232 that was identified as a stable fragment in trypsin digests of KKT4115–343 (Ludzia et al., 2020). The 2D spectra of these constructs contained peaks for all residues (Ludzia et al., 2020), and comparison with the spectrum of KKT4115–232 (Figure S5C) indicates that these shorter constructs retain the structural and dynamical properties observed in the longer fragment. Analysis of the chemical shifts of KKT4115–174 and KKT4145–232 using TALOS-N revealed
significant amounts of secondary structure (Figures 3C and 3D). For KKT4145–232, a continuous helix was observed from 152 to 225 (Figure 3C). For KKT4115–174, two helices, encompassing residues 121–139 and 152–172, separated by an unstructured linker were observed (Figure 3D).

The {1H}-15N NOE ratios for KKT4115–174 and KKT4145–232 indicate dynamics that are consistent with the predicted secondary structure (Figure 3E). {1H}-15N NOE errors here and in (E) were estimated from 500 Monte Carlo simulations using baseline noise as a measure of peak height error.

The helical regions identified in T. brucei KKT4 by NMR match those observed in the crystal structures of TcKKT4117–218 and LmKKT4184–284. However, in both crystal structures, we did not find a flexible linker within the coiled coils that we identified in T. brucei KKT4. Instead, we observed elevated B factors (Figure S4A) for the region where we might expect to find an unstructured linker in the TcKKT4 structure based on the sequence alignment (Figure S3). We speculate that the lack of a flexible linker in T. cruzi and L. mexicana crystal structures may be either due to the stabilizing contacts within the crystal lattice or structural differences of KKT4 between T. brucei and the other two kinetoplastids.

In summary, the microtubule-binding domain of T. brucei KKT4 is composed of two helices, encompassing residues 121–139 and 152–225, separated by a 12-residue flexible linker,
followed by a ~120-residue positively charged disordered region (predicted isoelectric point for residues 226–343 is 10.1).

Modeling of the *T. brucei* KKT4 coiled coil

We next aimed to determine if the helices identified by NMR in *T. brucei* KKT4 are organized into dimeric coiled coils as observed in the crystal structures of the *T. cruzi* and *L. mexicana* homologs. Residual dipolar couplings (RDCs), measured for partially aligned protein samples, are sensitive to N-H bond vector orientation and can be used to distinguish between undistorted and supercoiled helices. In the latter, the helical turns at the packing interface (residues a, d, e, g in the heptad repeat) are slightly compressed, while those facing outside (b, c, f) are stretched; this leads to a periodic variation in the RDCs within the heptad repeat (Schnell et al., 2005). Both helices in KKT4115–174 show large positive RDCs with the periodic variation that is consistent with a coiled-coil structure (Figure 3F). In contrast, the N and C termini and the flexible linker are dynamic, which leads to averaging of their RDCs to values close to 0.

Using the *T. cruzi* X-ray structure as a model, we tested different ways of fitting the helices identified by NMR into a coiled-coil structure by optimizing the fit between RDCs predicted from the X-ray structure and the experimental RDCs (Figures S6A and S6B). For both helices in *T. brucei*, good fits were found when they were placed within the first half of the *T. cruzi* sequence, corresponding to the regular coiled-coil structure while poorer agreement was obtained using the less supercoiled C-terminal half of the *T. cruzi* structure. For the first helix, the fit of the experimental RDCs suggests an offset of ~3 residues between the Tb and Tc sequences (Figure S6A), while for the second helix the fit of the RDCs suggests an offset of ~6 residues (Figure S6B). These offsets are in agreement with the alignment of the Tb and Tc sequences (Figure 2A) and place hydrophobic residues in *T. brucei* in positions a/d of the heptad repeat in the *T. cruzi* structure.

A homology model for the two coiled-coil regions of *T. brucei* KKT4115–232 was built using the *T. cruzi* coiled-coil X-ray structure, and the sequence alignments confirmed using the RDC data (Webb and Sali, 2016). Random extended structures, which represent possible conformations that might be sampled, for the flexible N and C termini and inter-helix linker were generated. These coordinates were merged with the coiled-coil homology models to generate an overall model for *T. brucei* KKT4115–232 (Figure 4A).

**Positively charged disordered tail enhances microtubule-binding activity**

Our structural analysis suggested that the KKT4 microtubule-binding domain in *T. brucei* consists of two regions: the N-terminal coiled coil and C-terminal unstructured basic tail (Figures 2 and 3). To evaluate their contribution to KKT4’s affinity for microtubules, the coiled-coil region (KKT4115–232), the basic unstructured tail (KKT4233–343), and the full microtubule-binding domain (KKT4115–343) were purified and tested in microtubule co-sedimentation assays (Figures 5A and 5B). The basic disordered region (KKT4233–343) did not co-sediment with Taxol-stabilized microtubules, consistent with our previous finding using KKT4115–232, we varied the microtubule concentration and calculated their dissociation constants by quantifying the percentage of co-sedimented KKT4 fragments (Figures 5C and 5D). This analysis confirms that KKT4115–343 has higher affinity for microtubules (K0 ~ 0.65 μM) than the coiled-coil region (K0 ~ 1.1 μM).

We previously showed that the KKT4 microtubule-binding domain from different kinetoplastid species (*T. cruzi*, *T. congolesens*, and *Phytomonas*) co-sediments with Taxol-stabilized microtubules (Llauro et al., 2018). Unlike *T. brucei* KKT4, the coiled-coil fragments from *T. cruzi* and *L. mexicana*...
that were used in our structural analysis (TcKKT4\textsuperscript{117–218} and LmKKT4\textsuperscript{194–284}) failed to bind microtubules in our co-sedimentation assay (Figure S7). This suggests that the KKT4 coiled-coil region from different kinetoplastid species may interact with microtubules with different affinities. Indeed, weaker microtubule binding was observed in our previous work of KKT4 microtubule-binding domains from these species (Llauro et al., 2018), which could be explained by minor differences in their structure or surface charges. Further work needs to be done to examine the differences in the microtubule-binding activity of KKT4 in other kinetoplastid species.

**Mapping the microtubule-binding interface of KKT4**

Microtubule interaction is often mediated by the electrostatic effects of surface charges (Ciferri et al., 2008). In fact, we previously showed that a charge-reversal mutant that replaced three basic residues with acidic residues (R123E, K132E, and R154E) severely reduced the microtubule-binding activity of \textit{T. brucei} KKT4\textsuperscript{115–343} (Llauro et al., 2018). To understand the charge distribution of the coiled coil in \textit{T. brucei} KKT4, we used our homology model to calculate the electrostatic surface potential (Jurrus et al., 2018). This revealed positively charged regions in the N terminus of the coiled-coil structure (Figure 4B), with basic residues exposed on the protein surface (e.g., R123, R126, R130, K132, K136, and R140) (Figure 4A). To test the importance of positively charged residues for microtubule binding, we systematically generated single mutants of KKT4\textsuperscript{115–343}, where lysine and arginine within the N-terminal region (115–232) were replaced with glutamic or aspartic acid. Microtubule binding of these mutants was compared with that of wild-type KKT4\textsuperscript{115–343}. We found that mutating any of residues R123, R126, K136, R140, R141, and K144 significantly reduced the microtubule-binding affinity (Figures 6A and 6B). These residues are located within KKT4\textsuperscript{115–174}, which was previously identified as the minimal microtubule-binding domain (Llauro et al., 2018). In contrast, mutating basic residues located in the second, longer helix in the coiled coil of the protein (K154, R164, K166, R167, K179, K198, K199, K204, K206, R217, K218, and R230) had only mild effects on the microtubule-binding activity (Figures 6A and 6B). To test if mutations affected the stability of the structure, 1D 1H NMR spectra were collected for mutants that had reduced affinity to microtubules (R126, K136, R140, R141, and K144); these confirmed that the mutations did not disrupt the coiled-coil structure (data not shown). Together with our previous analysis (Llauro et al., 2018), these results confirmed the importance of positively charged residues for microtubule-binding activities and revealed that the primary microtubule-binding interface of \textit{T. brucei} KKT4 is likely the basic surface located in the N-terminal coiled coil.
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KKT4 has DNA-binding activity

In many species, kinetochore assembly is regulated during the cell cycle. In humans, the constitutive centromere-associated network (CCAN) is composed of 16 chromatin-proximal kinetochore proteins that act as a platform for kinetochore assembly by interacting with CENP-A nucleosomes (Cheeseman and Desai, 2008). Most of the CCAN components constitutively localize at kinetochores and some of them have DNA-binding activities. In contrast, microtubule-binding kinetochore components localize only during mitosis. In *T. brucei*, kinetochore assembly is regulated during the cell cycle. However, the microtubule-binding protein KKT4 localizes at kinetochores in a constitutive manner (Akiyoshi and Gull, 2014). Interestingly, the C terminus of KKT4 is predicted to be a tandem BRCT domain (Akiyoshi and Gull, 2014). BRCT domains are found in a number of prokaryotic and eukaryotic proteins with various functions including DNA or RNA binding (Zhang et al., 1998; Yu et al., 2003; Leung and Glover, 2011). We previously observed significant DNA contamination during the purification of full-length KKT4 from insect cells (Lauro et al., 2018), suggesting that KKT4 might have DNA-binding activity, possibly via its BRCT domain. To test this, we employed fluorescence anisotropy assays (Rossi and Taylor, 2011) using a fluorescently labeled 50-bp double-stranded DNA. We found that full-length KKT4 (KKT42–645) strongly bound DNA (K_D = 11 nM) (Figure 7A), while the KKT4 BRCT domain (KKT4463–645) failed to saturate the DNA signal under the same conditions (Figure 7A). These results suggest that the BRCT domain of KKT4 does not bind DNA tightly and that the high-affinity DNA-binding site is located elsewhere.

Crystal structure of the *T. brucei* KKT4 BRCT domain

To understand the function of the KKT4 BRCT domain, we solved its structure using X-ray crystallography. KKT4463–645 yielded crystals that diffracted to a resolution of 1.6 Å (Table 1). BRCT domains typically comprise ~90–100 residues with the [z]β[β]z[β] secondary structure topology (Leung and Glover, 2011). The structure of KKT4463–645 revealed tandem BRCT domain (Figure 7B). The N-terminal full domain (BRCT1) consists of a central four-stranded β sheet flanked by two α helices on one side of the sheet and one α helix on the opposite side. The smaller domain (BRCT2) consists of two α helices and three β strands, missing a β strand and an α helix in the C terminus (Figure 7B). No electron density was observed for residues 463–473, 519–523, and 617–625, suggesting that these regions are flexible. A search for structural homologs using DALI (Holm, 2019) revealed similarity to several BRCT-containing proteins with breast cancer-associated protein 1 (BRCA1) as one of the top hits (Table S3). The tandem BRCT domains in BRCA1 have a highly conserved phosphopeptide-binding pocket (Clapperton et al., 2004; Shiozaki et al., 2004; Williams et al., 2004). Superposition of KKT4 BRCT1 with the N-terminal BRCT domain of *H. sapiens* BRCA1 showed a good structural match with an RMSD of 1.11 Å (for 65 Ca) (Figure 7C), suggesting that the KKT4 BRCT domain may bind phosphopeptides. In fact, we observed additional electron density in our structure, likely arising from a sulfate ion that may mimic a bound phosphate group. The sulfate ion is coordinated by three residues in the pocket, T494, S495, and K543 (Figure 7D), which correspond to the key residues known to interact with phosphopeptides in other BRCT domains (e.g., S1655, G1656, and K1702 in human BRCA1) (Williams et al., 2004). These results suggest that the BRCT domain of KKT4 likely functions as a phosphorylation-dependent protein-protein interaction domain rather than a DNA-binding domain.

The KKT4 BRCT domain is a phosphopeptide-binding domain

To identify potential binding partners for KKT4BRCT domain (KKT4463, sequences of kinetochore proteins were searched for the BRCT consensus motif (pS/pT)-x-x-(F/Y/L) (Manke et al., 2003; Yu et al., 2003). Among those proteins that co-purified with KKT4 (Akiyoshi and Gull, 2014), we identified possible motifs in KKT7 (SVTF, residues 65–68), KKT8 (SVRY, residues 381–384), and KKT12 (SILL, residues 192–195), which are highly conserved among kinetoplastids (Figure 7E and data not shown). Fluorescently labeled phosphopeptides derived from these proteins were tested for KKT4BRCT binding using a fluorescence anisotropy assay. The peptide derived from KKT8 bound KKT4BRCT with a K_D of ~30 μM (Figure 7F), while the other two peptides failed to bind KKT4BRCT with a similar affinity (data not shown). Importantly, we found that the non-phosphorylated KKT8 peptide bound KKT4BRCT with significantly weaker affinity (Figure 7F). Furthermore, replacement of K543, located in the putative phosphopeptide-binding site in KKT4BRCT with alanine, decreased the binding affinity by roughly an order of magnitude (Figure 7F). These results show that KKT4BRCT is a phosphopeptide-binding domain and identify KKT8 as a potential interaction partner.

The KKT4 BRCT domain interacts with the microtubule-binding region

To obtain further structural information on KKT4, we used cross-linking mass spectrometry (XL-MS), which can identify interaction surfaces between partner proteins or within the same molecule (Mattson et al., 1993; Leitner et al., 2016). Crosslinking on full-length KKT4 was carried out using two different crosslinkers: (1) BS3, a homo-bifunctional crosslinker that reacts with primary amines and covalently links pairs of lysines that are within 26–30 Å on the protein surface and (2) zero-length EDC and Sulfo-NHS that activates carboxyl groups for reaction with primary amines. XL-MS of KKT4 resulted in numerous crosslinks across the molecule, and similar patterns of crosslinks were observed for both crosslinkers (Figure 7G). Interestingly, a number of crosslinks were identified between the BRCT domain and the...
Figure 7. KKT4 BRCT domain is a phosphopeptide-binding domain

(A) Measured anisotropy is plotted against KKT42–645 and KKT4463–645 protein concentrations in the fluorescence anisotropy assay using a 50-bp DNA probe, showing that full-length KKT4, but not the BRCT domain, binds DNA. The DNA sequence (~36% GC content) used in this assay is part of the centromeric (legend continued on next page)
microtubule-binding domain using both BS² (K115/K499, K115/K521, K115/K543, K132/K618, K199/K521, K206/K521, K218/K499, K218/K510, K218/K521, K218/K618) and EDC/Sulfo-NHS (K115/D645, K132/E575, K132/D645, E178/K521, K204/D645, K206/E573, K206/D645 K218/E573, K218/D645). These results suggested that the KKT4 BRCT domain interacts with the microtubule-binding domain.

To confirm this, we monitored the effect of adding unlabeled KKT4 BRCT domain (Figures S8A and S8B) to 15N-KKT4115–232 using 2D NMR. Several residue-specific chemical shifts changes were observed, allowing identification of the BRCT interaction site on KKT4115–232 (Figure S8B). The largest perturbations are observed for residues 115–123 (Figures S8A and S8B). It is interesting that one of the perturbed residues, K115, crosslinked with K543 from the BRCT domain. We repeated the experiment with a shorter KKT4 construct, KKT4115–174, and obtained similar results (Figure S8B). Although crosslinks were also observed between the BRCT domain and residues E178, K199, K204, K206, and K218, these residues did not give observable peaks in the spectra of KKT4115–232, so we could not confirm the interaction by NMR using KKT4115–232. When similar experiments were performed with 15N-KKT4145–232 and KKT4BRCT, no significant changes in chemical shift were observed (Figure S8B). These results suggest that an interaction between the BRCT domain and the microtubule-binding domain of KKT4 involves residues at the N terminus of the microtubule-binding domain.

The observed interaction between the BRCT domain and the microtubule-binding domain could be a potential regulation mechanism for the microtubule-binding activity of KKT4. To test this possibility, we purified KKT4115–645, which contains both domains. We found that KKT4115–645 and KKT4115–343 interacted with microtubules with a similar affinity (Figures S8C–S8E). This suggests that the BRCT domain does not influence the interaction between KKT4 and Taxol-stabilized microtubules, at least in this assay. In the future, it will be interesting to analyze whether the observed interaction can regulate other activities of KKT4.

**DISCUSSION**

Many kinetochore-localized microtubule-binding proteins, such as the Ndc80, Ska, and Dam1 complexes, SKAP/Astrin, CENP-E, CENP-F, MCAK, INCENP, XMAP215, and dyneins, have been characterized in other model organisms (Maiato et al., 2004; Foley and Kapoor, 2013; Musacchio and Desai, 2017). Besides folded domains, many microtubule-binding proteins have predicted disordered regions that enhance their binding affinity (Guimaraes et al., 2008; Friese et al., 2016; Volkov, 2020). It is noteworthy that the predicted disorder has not been experimentally confirmed in most cases. Similarly, KKT4 has a predicted disordered segment at the microtubule-binding domain C terminus, which is not sufficient to bind microtubules but enhances the binding affinity (Lauro et al., 2018). In this study, we used NMR to confirm that this region is indeed disordered and also found that the N-terminal half of the KKT4 microtubule-binding domain has an elongated coiled-coil fold (Figure 8). Although a number of kinetochore proteins have coiled coils, microtubule-binding domains are typically located elsewhere, such as the calponin-homology domain for Ndc80/Nuf2 (Wei et al., 2007; Ciferri et al., 2008). In the case of SKAP/Astrin, which also has predicted coiled coils, it has been shown that the coiled-coil segment is unable to interact with microtubules on its own and that microtubule binding requires the N-terminal disordered fragment (Friese et al., 2016). Our mutagenesis analysis suggested that, in *T. brucei*, KKT4 binds microtubules through the N-terminal basic surface of the coiled coil. In the future, it will be important to directly visualize the microtubule-binding interface using methods such as electron microscopy.

It remains unknown whether (and how) microtubule-binding activities of KKT4 are regulated. Interestingly, we found that the KKT4 BRCT domain interacts with the N-terminal part of the microtubule-binding domain but does not modulate the microtubule-binding activities of KKT4, at least in the absence of phosphorylation (Figure S8D,E). Alternatively, the observed interaction might regulate other activities of KKT4. KKT4 co-purifies with the APC/C subunits (Akiyoshi and Gull, 2014), so we speculate that the interaction between the BRCT domain and the microtubule-binding domain might be governed by the attachment status, which in turn controls APC/C activities and cell-cycle progression.

In other eukaryotes, the Aurora B kinase plays an important role in regulating kinetochore-microtubule attachment by phosphorylating microtubule-binding kinetochore proteins, including Ndc80 and the Ska complexes (Cheeseman et al., 2002; Tien et al., 2010; Chan et al., 2012; Reddi et al., 2016). Although Aurora B is conserved in kinetoplastids, it remains unclear whether it
regulates kinetochore-microtubule attachments (Tu et al., 2006). Our preliminary in vitro kinase assay failed to find evidence that T. brucei Aurora B phosphorylates KKT4 (data not shown). In contrast, we previously showed that KKT4 is phosphorylated by the KKT10 kinase, which localizes at kinetochores until the onset of anaphase and promotes the metaphase-to-anaphase transition (Ishii and Akiyoshi, 2020). A phospho-deficient KKT4 S477A mutant failed to rescue the growth defect caused by KKT4 RNAi (Ishii and Akiyoshi, 2020). Although the underlying molecular mechanism remains unknown, it is noteworthy that S477 is located just before the BRCT domain. In this study, we identified KKT8 as a putative binding partner for the KKT4 BRCT domain. It will be important to identify which kinase(s) phosphorylates the KKT8 S381 site to promote the interaction. Because kinetochore localization of the KKT10 kinase depends on the KKT8 complex (composed of KKT8, KKT9, KKT11, and KKT12) (Ishii and Akiyoshi, 2020), it is possible that KKT10’s role in regulating the metaphase-to-anaphase transition is controlled by the KKT4-KKT8 interaction. These hypotheses will need to be tested in the future to better understand the mechanism of chromosome segregation in trypanosomes.

**STAR METHODS**

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

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**QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.str.2021.04.004.

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Structure Article

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AUTHOR CONTRIBUTIONS

P.L. purified recombinant proteins, solved crystal structures, and performed all experiments and data analysis. E.D.L. and G.M. assisted in solving crystal structures of KKT4-184–284 and LmKKT4-184–284. S.M. analyzed crosslinking mass spectrometry data. P.L. and C.R. performed and analyzed NMR experiments. P.L., C.R., and B.A. designed experiments and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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**STAR METHODS**

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| 1-Hexanol | Sigma-Aldrich | H1,330-3 |
| Ammonium Chloride (15N, 99%) | Goss Scientific | NLM-467 |
| Anti-FLAG M2 affinity gel | Sigma-Aldrich | A2220 |
| Benzonase Nuclease | Sigma-Aldrich | E1014 |
| BS3-d0 | Thermo Fisher | SK256507 |
| Cellfectin II | Invitrogen | 10362-100 |
| D-Glucose (13C6, 99%) | Goss Scientific | CLM-1396 |
| Deuterium oxide | Sigma-Aldrich | 151882 |
| E-64 | Peptide Institute Inc. | 4096 |
| EDC | Thermo Fisher | PG82079 |
| EDTA | Sigma-Aldrich | 324503 |
| EGTA | Sigma-Aldrich | E3889 |
| 3xFLAG peptide | Sigma-Aldrich | F4799 |
| Glycerol | Sigma-Aldrich | G5516 |
| Heparin 1 ml chromatography column | GE-Healthcare | 17-0406-01 |
| HEPES | Sigma-Aldrich | H3375 |
| Hexaethylene glycol monododecyl ether (C12E6) | Sigma-Aldrich | 52044 |
| HiLoad 16/600 Superdex 200 pg | GE-Healthcare | 28-9893-35 |
| HiLoad 16/600 Superdex 75 pg | GE-Healthcare | 28-9893-33 |
| Imidazole | Sigma-Aldrich | 56750 |
| Index crystallisation screen | Hampton Research | HR2-134 |
| KCl | Sigma-Aldrich | P9541 |
| Leupeptin | EMD Millipore Corp. | 3158107 |
| MgCl₂ | Sigma-Aldrich | M8266 |
| Morpheus II HT-96 crystallisation screen | Molecular Dimensions | MD1-92 |
| Na₂HPO₄ | Sigma-Aldrich | S0876 |
| NaCl | Sigma-Aldrich | S9888 |
| NaH₂PO₄ | Sigma-Aldrich | S0751 |
| Pepstatin A | EMD Millipore Corp. | 516481 |
| PIPES | Sigma-Aldrich | P6757 |
| PMSF | Sigma-Aldrich | P7626 |
| Porcine brain tubulin | Cytoskeleton, Inc | T240 |
| ProPlex crystallisation screen | Molecular Dimensions | MD1-42 |
| Resource S 6 ml cation exchange chromatography column | GE-Healthcare | 17-1180-01 |
| SF-900 II SFM media | Invitrogen | 10902104 |
| SimplyBlue Safe Stain | Invitrogen | 46-5034 |
| Sulfo-NHS | Thermo Fisher | 24510 |
| Superdex 200 10/300 | GE-Healthcare | 17-5175-01 |
| Superose 6 10/300 | GE-Healthcare | 17-5172-01 |
| Synthetic KKT8 phosphopeptide (BA_peptide_2 (DBS1831-1)): 5-FAM/DDICGISGQ(pSer)VRYSND-NH₂ | Designer Bioscience, Cambridge | N/A |
| Synthetic KKT8 peptide (BA_peptide_5 (DBS1838-1)): 5-FAM/DDICGISGQSVRYSND-NH₂ | Designer Bioscience, Cambridge | N/A |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| TALON Metal Affinity Resin | Takara | 635503 |
| TCEP | Sigma-Aldrich | C4706 |
| Zeba Spin Desalting Columns, 7K MWCO, 5 ml | Thermo Fisher | 89891 |

**Deposited data**

- *Trypanosoma cruzi* KKT4177–218 crystal structure: This study, PDB: 6ZPM
- *Leishmania mexicana* KKT4184–286 crystal structure: This study, PDB: 6ZPK
- *Trypanosoma brucei* KKT4693–645 crystal structure: This study, PDB: 6ZPK

- KKT4115–343 chemical shifts: (Ludzia et al., 2020), BMRB: 50229
- KKT4115–174 chemical shifts: (Ludzia et al., 2020), BMRB: 50215
- KKT465–232 chemical shifts: (Ludzia et al., 2020), BMRB: 50228
- Crosslinking mass spectrometry raw data: This study, PRIDE: PDXD020229

**Experimental models: organisms/strains**

- *Escherichia coli*: BL21(DE3) Novagen 69450
- *Spodoptera frugiperda*: Sf9 Thermo Fisher 12659017

**Recombinant DNA**

- Synthetic dsDNA (BA3098): 6-FAM/CAATATGTAAGGTTTGGTGTAACACCGATCTTCGCATAACATGCAMultiBac
- Custom synthesis by Integrated DNA Technologies, Inc N/A

| Plasmid | Source | Identifier |
|---------|--------|------------|
| prSFduet-1 | Novagen | 71341 |
| pNIC28-Bsa4 | (Gileadi et al., 2008) | N/A |
| pACEBac1 | Geneva Biotech | MultiBac |
| pACEBac2 | Geneva Biotech | MultiBac |
| pIDK | Geneva Biotech | MultiBac |
| pIDS | Geneva Biotech | MultiBac |
| KKT15 in pIDK (pBA336) | This study | N/A |
| KKT14 in pIDK (pBA485) | This study | N/A |
| KKT14, KKT15 in pIDK (pBA515) | This study | N/A |
| 3FLAG-KKT4 in pACEBac2 (pBA818) | (Ishii and Akiyoshi, 2020) | N/A |
| 3FLAG-KKT4 (pBA826) | (Ishii and Akiyoshi, 2020) | N/A |
| SNAP-6HIS-3FLAG-KKT4 (codon optimised for expression in *Spodoptera frugiperda*) in pACEBac2 (pBA925) | (Llauro et al., 2018) | N/A |
| 6HIS-KKT4115–646 (pBA987) | This study | N/A |
| 6HIS-KKT4115–343 (pBA1065) | (Llauro et al., 2018) | N/A |
| Synthesised gene: KKIP1 (codon optimised for expression in *Spodoptera frugiperda*) (pBA1166) | This study | N/A |
| 6HIS-KKT4115–174 (pBA1171) | (Llauro et al., 2018) | N/A |
| KKT4 (codon optimised for expression in *Spodoptera frugiperda*) in pIDP (pBA1207) | This study | N/A |
| 6HIS-KKT4115–343 R123E (pBA1328) | This study | N/A |
| 6HIS-KKT4115–343 K132E (pBA1329) | This study | N/A |
| 6HIS-KKT4115–343 K154E (pBA1330) | This study | N/A |
| 6HIS-KKT4101–352 (pBA1393) | (Llauro et al., 2018) | N/A |
| 3FLAG-KKT4, KKT14, KKT15 in pACEBac2 (pBA1371) | This study | N/A |
| 3FLAG-KKT4, KKT14, KKT15 (pBA1388) | This study | N/A |
| 6HIS-KKT4142–114 (pBA1413) | (Llauro et al., 2018) | N/A |
| 6HIS-KKT4146–232 (pBA1441) | This study | N/A |
## REAGENT or RESOURCE SOURCE

| REAGENT or RESOURCE ID | SOURCE |
|------------------------|--------|
| Plasmid: KKIPI1 (codon optimised for expression in *Spodoptera frugiperda*) in pACEBac1 (pBA1469) | This study |
| Plasmid: 6HIS-KKT41463-645 (pBA1513) | Ishii and Akiyoshi, 2020 |
| Bacmid: KKIPI1 (pBA1540) | This study |
| Plasmid: 6HIS-KKT4115-232 (pBA1601) | This study |
| Plasmid: 6HIS-LmKKT4184-284 (pBA1618) | This study |
| Plasmid: 6HIS-TcKKT4117-128 (codon optimised for expression in *E. coli*) (pBA1753) | This study |
| Plasmid: 6HIS-KKT4115-343 R167D (pBA2036) | This study |
| Plasmid: 6HIS-KKT4115-343 K218E (pBA2037) | This study |
| Plasmid: 6HIS-KKT4115-343 R230D (pBA2038) | This study |
| Plasmid: 6HIS-KKT4115-343 K204E (pBA2042) | This study |
| Plasmid: 6HIS-KKT4115-343 R230D (pBA2043) | This study |
| Plasmid: 6HIS-KKT4115-343 K204E (pBA2044) | This study |
| Plasmid: 6HIS-KKT4115-343 R230D (pBA2045) | This study |
| Plasmid: 6HIS-TcKKT4117-218 (codon optimised for expression in *E. coli*) (pBA2151) | This study |
| Plasmid: 6HIS-KKT4115-343 R126D (pBA2244) | This study |
| Plasmid: 6HIS-KKT4115-343 R130D (pBA2245) | This study |
| Plasmid: 6HIS-KKT4115-343 R126D (pBA2246) | This study |
| Plasmid: 6HIS-KKT4115-343 K130D (pBA2247) | This study |
| Plasmid: 6HIS-KKT4115-343 K130D (pBA2248) | This study |
| Plasmid: 6HIS-KKT4115-343 K130D (pBA2249) | This study |
| Plasmid: 6HIS-KKT4115-343 R140D (pBA2252) | This study |
| Plasmid: 6HIS-KKT4115-343 R164D (pBA2253) | This study |
| Plasmid: 6HIS-KKT4115-343 K198E (pBA2254) | This study |
| Plasmid: 6HIS-KKT4115-343 R127D (pBA2255) | This study |
| Plasmid: 6HIS-KKT4115-343 K179E (pBA2256) | This study |
| Plasmid: 6HIS-KKT4115-343 K190E (pBA2257) | This study |
| Plasmid: 6HIS-KKT4463-645 K543A (pBA2264) | This study |
| Plasmid: 6HIS-KKT4233-343 (pBA2380) | This study |

## Software and algorithms

- **ARCIMBOLDO LITE** *(Rodriguez et al., 2009)*
  - [Website](http://www.ccp4.ac.uk/)
- **ASTRA**
  - Wyatt Technology
  - [Website](https://store.wyatt.com/shop/viscostar/viscostar-iii/astra-software/)
- **Buccaneer** *(Cowtan, 2006)*
  - [Website](http://www.ccp4.ac.uk/)
- **CCPNmr** *(Vranken et al., 2005)*
  - [Website](https://www.ccpn.ac.uk)
- **COILS server** *(Lupas et al., 1991)*
  - [Website](https://embnet.vital-it.ch/software/COILS_form.html)
- **COOT** *(Emsley et al., 2010)*
  - [Website](http://www2.mrc-lmb.cam.ac.uk/Personal/pemsley/coot/)
- **DALI server** *(Holm, 2019)*
  - [Website](http://ekhidna2.biocenter.helsinki.fi/dali/)
- **Diffraction Anisotropy Server** *(Strong et al., 2006)*
  - [Website](http://services.mbi.ucla.edu/anisoascale/)
- **DisEMBL** *(Linding et al., 2003)*
  - [Website](http://dis.embl.de)
- **HMMER web server** *(Potter et al., 2018)*
  - [Website](https://www.ebi.ac.uk/Tools/hmmer/)
- **ImageJ** *(Schneider et al., 2012)*
  - [Website](https://imagej.nih.gov/ij/)
- **Jalview** *(Waterhouse et al., 2009)*
  - [Website](http://www.jalview.org/)
- **MAFFT** *(Katoh et al., 2019)*
  - [Website](https://mafft.cbrc.jp/alignment/server/)
- **MODELLER** *(Webb and Sali, 2016)*
  - [Website](https://salilab.org/modeller/)

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead contact, Bungo Akiyoshi (bungo.akiyoshi@bioch.ox.ac.uk).

Material availability
Plasmids generated in the course of this study can be requested from the Lead contact, Bungo Akiyoshi (bungo.akiyoshi@bioch.ox.ac.uk).

Data and code availability
Data generated during this study are included in the manuscript and supplemental information. Protein coordinates have been deposited in the RCSB Protein Data Bank (http://www.rcsb.org/) with accession codes PDB: 6ZPM (Trypanosoma cruzi Sylvio X10 KKT4117–218), PDB: 6ZPJ (Leishmania mexicana KKT4184–284) and PDB: 6ZPK (Trypanosoma brucei KKT4463–645). The chemical shift assignments for KKT4 have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under the accession numbers 50215 (Trypanosoma brucei KKT4115–174), 50228 (Trypanosoma brucei KKT4145–232) and 50229 (Trypanosoma brucei KKT4115–343). All raw files relating to crosslinking mass-spectrometry have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2019) with the dataset identifier PXD020229.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial culture
Bacterial strains and insect cell lines used in this study are listed in the key resources table. Bacterial growth conditions can be found in method details.

Cell culture
Spodoptera frugiperda Sf9 cells were cultured in SF-900 SFM media (Gibco) at 27°C with shaking (160 rpm).

METHOD DETAILS

Plasmids
KKT4 fragments used in this study were amplified from Trypanosoma brucei genomic DNA and cloned into the pNIC28-Bsa4 expression vector using ligation-independent cloning (Gileadi et al., 2008) or cloned into the RSFDuet-1 vector (Novagen) using NEBuilder HiFi DNA Assembly Kit (New England Biolabs). All constructs were sequence verified. LmKKT4184–284 was cloned from Leishmania mexicana genomic DNA (kindly provided by Richard Wheeler), which contained an R218Q mutation. Due to a cloning error, which
failed to place a stop codon after the LmKKT4\textsuperscript{184–284} coding sequence, an additional 23 residues (EFELGAPGRQACGRMLKSN) from the vector were inserted at the C-terminus. To KK4\textsuperscript{177–218} was cloned from a synthetic Trypanosoma cruzi KKT4 gene fragment, codon optimisation for expression in \textit{E. coli} (Llauro et al., 2018). Point mutants of the microtubule-binding domain were created using site-directed mutagenesis using PrimeSTAR Max DNA polymerase (Takara Bio).

\textbf{Protein expression and purification}

Expression and purification of KKT4 fragments used for SEC-MALS, crystallographic studies and fluorescence anisotropy assays was done as follows. Transformed \textit{E. coli} BL21(DE3) cells were inoculated into 5 ml of 2xTY medium containing 50 \( \mu \)g/ml kanamycin and grown overnight at 37°C. The next morning, 1 l of 2xTY medium with 50 \( \mu \)g/ml of kanamycin was inoculated with 5 ml of the overnight culture and grown at 37°C with shaking (200 rpm) until the OD\textsubscript{600} reached \( \sim 0.6 \). Protein expression was induced with 0.2 mM IPTG for \( \sim 16 \) hr at 16°C. Cells were spun down at 3,400 g at 4°C and resuspended in lysis buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, and 10% glycerol) supplemented with protease inhibitors (20 \( \mu \)g/ml leupeptin, 20 \( \mu \)g/ml pepstatin, 20 \( \mu \)g/ml E-64 and 0.4 mM PMSF), benzonase nuclease (500 U/1 l culture), and 0.5 mM TCEP. All subsequent steps were performed at 4°C. Bacterial cultures were mechanically disrupted using a French press (1 passage at 20,000 psi) and the soluble fraction was separated by centrifugation at 48,000 g for 30 min. Supernatants were loaded on TALON beads (Takara Bio) pre-equilibrated with lysis buffer (1 ml of beads per 1 l of bacterial culture). Next, the beads were washed with lysis buffer without protease inhibitors and proteins were eluted with 50 mM sodium phosphate pH 7.5, 500 mM NaCl, 10% glycerol, 250 mM imidazole and 0.5 mM TCEP. To cleave off the His-tagged, samples were incubated with TEV protease in 1:50 w/w ratio overnight while being buffer-exchanged into 50 mM sodium phosphate, 500 mM NaCl, 10% glycerol, 5 mM imidazole, and 0.5 mM TCEP by dialysis. To increase the sample purity and remove the His-tag, samples were re-loaded on TALON beads pre-equilibrated with dialysis buffer and the flow-through was collected. Next, the samples were further purified using either two-step (ion exchange and size exclusion chromatography) or one-step (size exclusion chromatography) purification. To promote binding of proteins to the ion exchange column, samples were diluted with buffer A (25 mM HEPES pH 7.5 and 0.5 mM TCEP) to achieve the final NaCl concentration of 50 mM. Ion exchange chromatography was performed using either a 6 ml RESOURCE S or RESOURCE Q column (GE Healthcare) pre-equilibrated with 5% of buffer B (25 mM HEPES pH 7.5, 1 M NaCl and 0.5 mM TCEP). Proteins were eluted with a linear gradient from 5% to 100% of buffer B, concentrated using 3- or 10-kD MW Amicon concentrators (Millipore), and loaded on Superdex 75 or Superdex 200 16/60 (GE Healthcare) columns to further purify and buffer exchange into 25 mM HEPES pH 7.5, 150 mM NaCl with 0.5 mM TCEP. Fractions containing KKT4 were pooled, concentrated using a 3- or 10-kD MW Amicon concentrator (Millipore), and flash-frozen in liquid nitrogen for –80°C storage.

Expression of KKT4 mutants used in microtubule co-sedimentation assays was done as described above with the following modifications. After overnight expression at 16°C, cells were spun down at 3,400 g at 4°C and resuspended in lysis buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, and 10% glycerol) supplemented with protease inhibitors (20 \( \mu \)g/ml leupeptin, 20 \( \mu \)g/ml pepstatin, 20 \( \mu \)g/ml E-64 and 0.4 mM PMSF), benzonase nuclease (500 U/1 l culture), and 0.5 mM TCEP. All subsequent steps were performed at 4°C. Bacterial cultures were sonicated on ice (three rounds of 15 sec pulse and 1 min pause) and the soluble fraction was separated by centrifugation at 48,000 g for 30 min. Supernatants were incubated with TALON beads (Takara Bio) pre-equilibrated with lysis buffer and the flow-through was collected. Next, the samples were further purified using either two-step (ion exchange and size exclusion chromatography) or one-step (size exclusion chromatography) purification. To promote binding of proteins to the ion exchange column, samples were diluted with buffer A (25 mM HEPES pH 7.5 and 0.5 mM TCEP) to achieve the final NaCl concentration of 50 mM. Ion exchange chromatography was performed using either a 6 ml RESOURCE S or RESOURCE Q column (GE Healthcare) pre-equilibrated with 5% of buffer B (25 mM HEPES pH 7.5, 1 M NaCl and 0.5 mM TCEP). Proteins were eluted with a linear gradient from 5% to 100% of buffer B, concentrated using 3- or 10-kD MW Amicon concentrators (Millipore), and loaded on Superdex 75 or Superdex 200 16/60 (GE Healthcare) columns to further purify and buffer exchange into 25 mM HEPES pH 7.5, 150 mM NaCl with 0.5 mM TCEP. Fractions containing KKT4 were pooled, concentrated using a 3- or 10-kD MW Amicon concentrator (Millipore), and flash-frozen in liquid nitrogen for –80°C storage.

Expression and purification of isotopically labelled KKT4 fragments

Transformed \textit{E. coli} BL21(DE3) cells were plated on agar plates containing 50 \( \mu \)g/ml kanamycin and incubated at 37°C overnight. After overnight incubation, a few colonies were inoculated into 5 ml of 2xTY medium containing 50 \( \mu \)g/ml kanamycin and grown at 37°C for 6 hr. Next, 5 ml of M9 minimal medium containing 50 \( \mu \)g/ml kanamycin supplemented with 1g/l \( ^{15} \)NH\textsubscript{4}Cl and 4g/L \( ^{13} \)C–D-glucose (CIL) as the sole nitrogen source was inoculated with 500 \( \mu \)l of bacterial culture. Cell growth was continued overnight at 37°C. Next, 5 ml of overnight culture was inoculated into 1l of M9 minimal medium supplemented with 1g/l \( ^{15} \)NH\textsubscript{4}Cl, 4g/L \( ^{13} \)C–D-glucose and 50 \( \mu \)g/ml kanamycin. Cells were grown at 37°C to an OD\textsubscript{600} of \( \sim 0.8 \). Protein expression was induced by 0.4 mM IPTG.
and incubated overnight at 16°C with shaking (200 rpm). To purify isotopically labelled proteins, we followed the same protocol as for samples used in crystallography, which is described above.

Expression and purification of full length KKT4 from insect cells
To express full-length SNAP-6HIS-3FLAG-KKT4 (Llauro et al., 2018), 500 ml of Sf9 cell culture at 1–1.2 million cells/ml was infected with P3 baculovirus for ~72 hr before harvesting. Subsequent steps were performed at 4°C. Cells were pelleted at 700 g for 10 min, washed once with PBS, and resuspended in 10 ml BH0.25 (25 mM HEPES, pH 7.5, 0.2% NP-40, 2 mM MgCl$_2$, 0.1 mM EDTA, 0.5 mM EGTA, 10% glycerol, and 250 mM NaCl) supplemented with 2× protease inhibitors (20 μg/ml leupeptin, 20 μg/ml pepstatin, 20 μg/ml E-64, and 0.4 mM PMSF) and benzonase nuclease (1000 U/1 ml culture). Cells were lysed on ice using sonicator (three rounds of 15 sec pulse and 1 min pause) followed by centrifugation for 30 min at 45,000 g. The supernatant was incubated with 2 ml of anti-FLAG M2 affinity gel (Sigma) for 3 hr with constant rotation, followed by five washes with BH0.25 supplemented with 0.5 mM TCEP (10 ml each). Proteins were eluted from the beads with gentle agitation of beads in 2 ml BH0.25 containing 0.5 mg/ml 3FLAG peptide (Sigma) and 1× protease inhibitors. The sample was further purified using 1 ml HiTrap Heparin HP column preequilibrated with 5% of buffer B (buffer A: 25 mM HEPES, pH 7.5, with 0.5 mM TCEP; buffer B: 25 mM HEPES, pH 7.5, and 1 mM NaCl with 0.5 mM TCEP) and eluted with a linear gradient from 5% to 100% of buffer B. Fractions containing SNAP-tagged KKT4 were pooled and concentrated by a 10-kD MW Amicon concentrator (Millipore). For crosslinking experiments with BS$^3$, FLAG-KKT4 was immunoprecipitated from insect cells transfected with baculovirus prepared from pBA1388 (3FLAG-KKT4, KKT14, KKT15), whereas crosslinking with EDC/Sulfo-NHS was performed on FLAG-KKT4 that was purified from insect cells transfected with baculoviruses prepared from pBA826 (3FLAG-KKT4) and pBA1540 (KKIP1), both purified according to the protocol described above.

Size Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS)
MALS experiments were performed during size exclusion chromatography on analytical Superose 6 and Superdex 200 HR10/300 columns (GE Healthcare) equilibrated with 25 mM HEPES pH 7.5, 150 mM NaCl and 0.5 mM TCEP (for SNAP-6HIS-3FLAG-KKT4, 25 mM HEPES pH 7.5, 340 mM NaCl and 0.5 mM TCEP was used). Elution was monitored via online static light-scattering (DAWN HELOS B+, Wyatt Technology), differential refractive index (Optilab T-rEX, Wyatt Technology) and UV (SPD-20A, Shimadzu) detectors. Data were analysed using the ASTRA software package (Wyatt Technology).

Crystallization
All crystals were obtained in sitting drop vapour diffusion experiments in 96-well plates, using drops of 200 nl overall volume, mixing protein and mother liquor in a 1:1 ratio. Crystals of *Trypanosoma cruzi* (Sylvio X10) KKT4$^{117-218}$ (10.0 mg/ml) were grown at 18°C in Morpheus II HT-96 G3 solution (Molecular Dimensions) containing 0.1 M buffer system 4 (MOPSO, Bis-Tris) pH 6.5, 50% v/v precipitant mix 7 (20% w/v PEG 8000, 40% v/v 1,5-Pentanediol) and 100 mM amino acids II (0.2 M DL-Arginine hydrochloride, 0.2 M DL-Threonine, 0.2M DL-Histidine monohydrate monohydrate, 0.2 M DL-Hydroxylysine hydrochloride, 0.2 M trans-4-hydroxy-L-proline). Mother liquor served as a cryoprotectant. Crystals of *Leishmania mexicana* KKT4$^{184-284}$ (13.5 mg/ml) were grown at 4°C in ProPlex crystallisation screen (Molecular Dimensions) solution containing 0.1 M imidazole pH 7.0 and 50% v/v MPD. Mother liquor served as a cryoprotectant. Crystals of *Trypanosoma brucei* KKT4$^{463-645}$ (26.5 mg/ml) were grown at 4°C in Index crystallisation screen (Hampton Research) solution containing 0.1 M bis-Tris pH 5.5 and 2.0 M ammonium sulphate. Crystals were briefly transferred into mother liquor prepared with addition of 23% glycerol prior to flash-cooling by plunging into liquid nitrogen.

Diffraction data collection and structure determination
X-ray diffraction data from *Trypanosoma cruzi* (Sylvio X10) KKT4$^{117-218}$ and *Leishmania mexicana* KKT4$^{184-284}$ were collected at the I03 and I24 beamlines respectively, at the Diamond Light Source (Harwell, UK). The structures were solved using ab initio macromolecular phasing software, ARICIMBOLDO LITE optimised for coiled coils (Rodríguez et al., 2009, 2012) followed by initial model building with BUCCANEER (Cowtan, 2006). Further manual model building and refinement were completed iteratively using COOT (Emsley et al., 2010) and PHENIX (Liebschner et al., 2019). The data sets used for the final refinement were scaled to the high-resolution limit of 1.9 Å and processed using anisotropic scaling (Strong et al., 2006).

X-ray diffraction data from *Trypanosoma brucei* KKT4$^{463-645}$ were collected at the I24 beamline at the Diamond Light Source (Harwell, UK). The structure was solved using ARICIMBOLDO LITE (Rodríguez et al., 2009, 2012) followed by initial model building with BUCCANEER (Cowtan, 2006). The further model building and refinement were completed using COOT (Emsley et al., 2010) and PHENIX (Liebschner et al., 2019).

The final refinement statistics for three structures are summarised in Table 1. All structure figures were prepared using PyMOL (Delano, 2002). Protein coordinates have been deposited in the RCSB Protein Data Bank (http://www.rcsb.org/) with accession codes: PDB: 6ZPM (*Trypanosoma cruzi* (Sylvio X10) KKT4$^{117-218}$), PDB: 6ZPJ (*Leishmania mexicana* KKT4$^{184-284}$) and PDB: 6ZPK (*Trypanosoma brucei* KKT4$^{463-645}$).

NMR spectroscopy and analysis of NMR data
All NMR samples were prepared in 25 mM HEPES pH 7.2, 150 mM NaCl, 0.5 mM TCEP and 95% H$_2$O/5% D$_2$O. All NMR spectra were acquired using a 750 MHz spectrometer equipped with a Bruker Avance III HD console and a 5 mm TCI CryoProbe. All NMR data were processed using NMRPipe (Delaglio et al., 1995) and analysed using CCPN Analysis (Vranken et al., 2005).
**Modelling of *T. brucei* KKT4**

Homology models for the two coiled-coil regions of *T. brucei* KKT4 were generated using Modeller 9 v24 (Webb and Sali, 2016), the X-ray structure of the *T. cruzi* KKT4, and the sequence alignments of *T. brucei* and *T. cruzi* derived from the RDC data collected for KKT4. Modeller was run using the fully automated comparative modelling mode. Random extended structures for the N and C termini of KKT4 and for the inter-helix linker were generated using X-PLOR (Brünger, 1992); these represent possible conformations that these residues might sample in solution. These coordinates were merged with the two coiled-coil dimer models to generate an overall model for *T. brucei* KKT4. The model for KKT4 was created by merging the model for KKT4, the X-ray structure of the BRCT domain (KKT4), and two random structures for disordered residues 233–473 generated using X-PLOR (Brünger, 1992). The two random structures selected to represent 233–473 were chosen from a group of ten structures to illustrate possible conformations in which the BRCT domain is in close proximity to the N-terminal coiled coil and is more distant from this region.

**Microtubule co-sedimentation assay**

Taxol-stabilized microtubules were prepared by mixing 2.5 μl of 100 μM porcine tubulin (Cytoskeleton) resuspended in BRB80 (80 mM Pipes-KOH pH 6.9, 1 mM EGTA, and 1 mM MgCl₂) with 1 mM GTP, 1.25 μl of BRB80, 0.5 μl of 40 mM MgCl₂, 0.5 μl of 10 mM GTP, and 0.25 μl DMSO, and incubated for 20 min at 37°C. Then, 120 μl of pre-warmed BRB80 containing 12.5 μM Taxol (paclitaxel; Sigma) was added to the sample to achieve 2 μM microtubule solution. To achieve higher concentrations of microtubules, the protocol described above was scaled up. Prior to the assay, KKT4 fragments were buffer-exchanged into BRB80 with 100 mM KCl using Zeba desalting spin columns (Thermo Fisher). For the microtubule co-sedimentation assay, 20 μl of KKT4 fragments (4 μM) were mixed with 20 μl of microtubules (2 μM) and incubated for 45 min at room temperature. For a no-microtubule control, KKT4 fragments were mixed with BRB80 with 12.5 μM Taxol. The samples were spun at 20,000 g at room temperature for 10 min, and the supernatant was collected. To the tube with a pellet, we added 40 μl of chilled BRB80 with 5 mM CaCl₂ and incubated on ice for 5 min to depolymerise microtubules. Following the incubation, samples were boiled for 5 min before analysis by SDS-PAGE gels stained with SimplyBlue Safe Stain (Invitrogen).

**Fluorescence anisotropy assay**

The DNA-binding analysis of KKT4 was performed in binding buffer (25 mM HEPES pH 7.5, 50 mM NaCl and 0.5 mM TCEP) using a 50-bp DNA probe BA3098 (~36% GC content), which is part of the *Trypanosoma brucei* centromere CIR147 sequence (Obado et al., 2007), labelled at the 5’ end with 6-carboxyfluorescein (6-FAM). Prior to the assay, KKT4 proteins were buffer-exchanged into the binding buffer using Zeba spin desalting columns (Thermo Fisher). KKT4 (0.67 μM) and KKT4 (1 μM) samples were mixed with DNA probe in the binding buffer to a final DNA concentration of 1 nM. Next, the proteins were serially diluted in the binding buffer containing 1 nM DNA in the 2:3 v/v ratio. The binding reactions were incubated for 30 min at room temperature and fluorescence anisotropy was measured using a PHERAstar FS next-generation microplate reader (BMG LABTECH). Equilibrium dissociation constants (K_D) were calculated by fitting the data in SigmaPlot (Monks, 2002). The phosphopeptide-binding experiments were carried out using fluorescently-labelled phosphorylated (DICGIGSQ(pS)VRYSLND) and unphosphorylated peptide probes.
(DICGSGQSVRYSLND), KKT4463–640 (170 μM) was mixed with the probe (100 nM) and serially diluted in a 2:3 v/v ratio. Incubation of the samples and measurements were done as described above.

**Chemical crosslinking mass spectrometry (XL-MS)**

Prior to the experiment, bis(sulfosuccinimidyl)suberate, BS3 (Thermo Fisher), crosslinker was equilibrated at room temperature for 2 hr and then resuspended to 0.87 mM in distilled water. Immediately after, 2 μl of the crosslinker was mixed with 18 μl of ~5 μM KKT4 in 25 mM HEPES pH 8.0, 2 mM MgCl₂, 0.1 mM EDTA, 0.5 mM EGTA-KOH, 10% glycerol, 250 mM NaCl, and 0.1% NP40. The crosslinking reaction was incubated on ice for 60 min. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS) were resuspended in distilled water to 4 mM and 10 mM respectively. Immediately after, 1 μl of EDC and 1 μl of Sulfo-NHS were mixed with 18 μl of ~5 μM KKT4. The crosslinking reaction was incubated at room temperature for 60 min. Following the incubation, all the crosslinking samples were boiled for 10 min and resolved on a NuPAGE 4–12% gradient polyacrylamide gel (Invitrogen). The gel pieces were then washed with 5% formic acid and then acetonitrile. Both washes were combined with the initial supernatant and the mixture was then dried down.

Peptides were resuspended in 5% formic acid and 5% DMSO and analysed by LC-MS with an Ultimate 3000 UHPLC system (Thermo Fischer Scientific) coupled to a QExactive mass spectrometer (Thermo Fischer Scientific) through an EASY-Spray nano-electrospray ion source (Thermo Fischer Scientific). The peptides were trapped on a C18 PepMap 100 pre-column (300 m, 120 Å, Dr. Maisch GmbH) using a linear gradient (length: 45 min, 15% to 50% solvent B (acetonitrile with 0.1% formic acid)). Acquisition was performed in data-dependent mode (DDA). Full scan MS spectra were acquired in the Orbitrap (scan range 350–1,500 m/z, resolution 70,000, AGC target 3 x 10^6, maximum injection time 50 ms) followed by 10 MS/MS events at 30% NCE (resolution 17,500, AGC target 5 x 10^4, maximum injection time 120 ms, isolation window 1.5 m/z) with first fixed mass at 180 m/z. Charge exclusion was selected for unassigned 1+ and 2+ ions.

MS data were converted into mgf format using pParse and searched by the pLink software (Chen et al., 2019) (version 1 for BS3 dataset and version 2 for EDC dataset) using FASTA databases containing KKT1–4, 6, 7–11, 14, 15, 20, and α/β tubulins without KKI1 (for BS3 dataset) or with KKI1 (for EDC dataset). Search parameters were as follows: maximum number of missed cleavages = 2, fixed modification = carbamidomethyl-Cys, variable modification 1 = Oxidation-Met, variable modification 2 = Glu to pyro-Glu. Crosslinks that have score < 1 x 10⁻⁷ were visualised using xiNET (Combe et al., 2015) (Tables S4 and S5).

**Interaction of the microtubule-binding and BRCT domains**

¹H–¹⁵N BEST TROSY spectra were collected at 20 °C for 0.2 mM KKT4115–174, KKT4115–232 and 30 °C for 0.2 mM KKT4145–232 alone and in the presence of 0.22 mM KKT4BRCT. Following addition of KKT4BRCT the samples were incubated at room temperature for ~30 min. Interaction was monitored by comparing peak positions; the combined chemical shift changes of ¹H and ¹⁵N are reported in Hz in Figures S8A and S8B.

**Multiple sequence alignment**

Protein sequences and accession numbers for KKT4 and KKT8 homologues used this study were retrieved from the TriTryp database (Aslett et al., 2010), UniProt (UniProt, 2019), or a published study (Butenko et al., 2020). Searches for homologous proteins were done using BLAST in the TriTryp database (Aslett et al., 2010) or Jackhmmer on the UniProtKB proteome database using a default setting (HMMER web version 2.24) (Potter et al., 2018). Multiple sequence alignment was performed with MAFFT (L-INS-i method, version 7) (Katoh et al., 2019) and visualised with the Clustalx colouring scheme in Jalview (version 2.10) (Waterhouse et al., 2009).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Intensities of gel bands in the microtubule co-sedimentation assays were calculated using ImageJ (Schneider et al., 2012). All measured P intensities were subtracted by the intensity of the P fraction at 0 μM microtubules. Fraction bound was calculated using the following equation: \[\frac{P}{S+P}\]. S and P stand for supernatant and pellet respectively. Standard deviation was used to generate error bars for all graphs in this study by evaluating data from at least N=3 experiments.