Trypanosome morphogenesis involves recruitment of a pleckstrin homology domain protein by an orphan kinesin to the microtubule quartet

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Running title: TbKifX2 and TbPH1 association with the trypanosomal microtubule quartet

ABSTRACT

Kinesins are motor proteins found in all eukaryotic lineages that move along microtubule tracks to mediate numerous cellular processes such as mitosis and intracellular transport of cargo. In trypanosomatids, the kinesin superfamily has undergone a prominent expansion, giving these protists one of the most diverse kinesin repertoires including two trypanosomatid-restricted groups. Here, we characterize in *Trypanosoma brucei* TbKifX2, a hitherto orphaned trypanosomatid-specific kinesin. TbKifX2 tightly interacts with TbPH1, a kinesin-like protein with an inactive motor domain, a rare instance of a kinesin interacting with a protein bearing a catalytically dead kinesin motor domain. TbKifX2 recruits TbPH1 to the microtubule quartet (MtQ), a characteristic but poorly understood cytoskeletal structure that is part of the multipartite flagellum attachment zone (FAZ) and extends from the basal body to the anterior of the cell body. The proximal proteome of TbPH1 is comprised of four proteins that localize to the FAZ, consistent with the notion that the TbKifX2/TbPH1 complex bind the MtQ, a FAZ component. Simultaneous ablation of TbKifX2/TbPH1 leads to formation of prominent protrusions from the cell posterior. Thus, these two trypanosomatid-restricted proteins appear to contribute to morphogenesis in *T. brucei*. Furthermore, TbKifX2 is the first motor protein to be localized to the MtQ.

Keywords: Trypanosoma, kinesin, cytoskeleton, microtubules, microtubule quartet, morphogenesis

37 INTRODUCTION

38 The vermiform morphology of the unicellular parasite *Trypanosoma brucei* that causes African
39 trypanosomiasis in humans and nagana in cattle is defined by a subpellicular array of microtubules
40 (MTs) (Robinson *et al.*, 1995; Gull, 1999; Wheeler *et al.*, 2013). These MTs are cross-linked to each
41 other and the plasma membrane via regularly spaced fibrils (Sherwin *et al.*, 1987). A high order is
42 maintained within the corset with the MT plus ends converging at the parasite's posterior end and
43 the minus ends located at the anterior of the cell (Robinson *et al.*, 1995).

44 The only place where the continuity of the *T. brucei* MT array is interrupted is at the flagellar pocket,
45 the deep invagination where the flagellum exits the cell body and the only place where endo- and
46 exocytoses occur (Field and Carrington, 2009). The opening of the flagellar pocket is encircled by a
47 collar, which influences the overall shape of this cavity. Distal to the collar, the so-called hook
48 complex covers the flagellum as it exits the flagellar pocket, with two lateral arms also flanking the
49 flagellum attachment zone (FAZ) filament and the MT quartet (MtQ) inside the cell (Albisetti *et al.*,
50 2017). The FAZ filament and MtQ are among the interconnected fibres, filaments and junctional
51 complexes of the multipartite FAZ (Lacomble *et al.*, 2009), which together form a lateral attachment
52 for the flagellum as it follows a helical path along the cell body, connecting the flagellar skeleton to
53 the cytoskeleton through both the flagellar and plasma membranes (Sunter and Gull, 2016). The
54 MtQ originates close to the basal bodies, wraps itself around the flagellar pocket and interdigitates
55 within the MT corset next to the FAZ filament. The orientation of MTs in the MtQ is antiparallel to
56 the one found within the MT corset, thus their minus ends are proximal to the basal bodies
57 (Robinson *et al.*, 1995).

58 The trypanosome MT corset is exceptionally stable and only reorganised during cell division and life
59 cycle transitions (Sinclair and de Graffenried, 2019). Cytoskeletal structures (MtQ, MT corset,
60 flagellum, basal bodies, which anchor the flagella within the cell, and FAZ) need to be duplicated and
61 faithfully segregated during the cell division cycle, just like other single-copy organelles. In addition,
62 this has to be coordinated with replication and division of the nucleus and the single mitochondrial
63 DNA network termed the kinetoplast, which is located on the opposite side of the basal bodies
64 relative to the flagellum (Robinson *et al.*, 1995; Wheeler *et al.*, 2019).

65 Basal body duplication and segregation are the first visible steps in the cell division cycle and
66 essential for subsequent cytokinesis (Woodward and Gull, 1990). The new MtQ starts growing just
67 prior to basal body maturation and once the new flagellum has grown enough to invade the flagellar
68 pocket, morphogenetic changes result in two separate pockets enveloping both the new and old
69 flagella (Lacomble *et al.*, 2010). Once the new flagellum has been nucleated and elongated, new MTs
70 are integrated into the array between the old and new flagella, thereby increasing the overall width
71 of the cell (Wheeler *et al.*, 2013).

72 With its antiparallel orientation, the MtQ together with the FAZ filament create an asymmetric seam
73 within the MT corset, which works as a 'cellular ruler' and helps to determine the site of future
74 furrow ingression during cytokinesis (Sunter and Gull, 2016). Any insults to the integrity of these
75 structures result in cytokinesis defects and it is thus tempting to envision the MtQ as a potential
76 specialised 'highway' in charge of transport and delivery of cargo to the site of important cytokinesis
77 events. Furthermore, the endoplasmic reticulum associates with parts of the MtQ, although the
78 importance of this association remains mysterious. The first protein localized to this structure,
79 TbSpf1, is found at the proximal end of the MtQ, which wraps around the flagellar pocket between
80 basal bodies and the flagellar pocket collar (Gheiratmand *et al.*, 2013). Its depletion causes problems
81 with the biogenesis of the MtQ and associated structures as well as motility defects. The minus end

of the MtQ MTs is anchored to the basal bodies by a protein named TbSAF1, found in close proximity to TbSpf1 but localized between the pro and mature basal bodies (Dong *et al.*, 2020). TbSAF1 was found in a proximal proteomics screen of proteins adjoining to and/or directly interacting with TbSpf1, which found three additional proteins that localize to the MtQ between the basal body and flagellar pocket collar (Dong *et al.*, 2020). However, no motor proteins that localise to the MtQ, and which may potentially bring cargo to the cytokinesis site, have thus far been identified.

Kinesins are well-studied motor proteins that regulate MT dynamics, organize MT networks, and transport cargo along MTs. Transporting kinesins can form homo- and heterodimers and consist of a head region containing the kinesin motor domain, which is responsible for ATP hydrolysis and MT binding, and a tail region important for dimerization with a kinesin/kinesin-like partner and cargo binding (Hirokawa and Noda, 2008). In trypanosomatid genomes, kinesins and kinesin-like proteins constitute one of the largest protein superfamilies (Berriman *et al.*, 2005; El-Sayed *et al.*, 2005; Ivens *et al.*, 2005). To date, 47 genes encoding high-likelihood kinesins have been identified in the *T. brucei* genome (Wickstead and Gull, 2006; Wickstead *et al.*, 2010b). Moreover, there is a similar number of kinesin-related proteins, which did not make the threshold due to sequence variance (Berriman *et al.*, 2005). Interestingly, numerous kinesins are members of two major clades named X-1 and X-2 that are restricted to the trypanosomatid lineage (Wickstead and Gull, 2006; Wickstead *et al.*, 2010b). Why the kinesin repertoire has expanded so prominently in trypanosomatids remains mysterious.

Hitherto studied kinesins of *T. brucei* have been shown to be important for various processes occurring in different cellular compartments. KinA and KinB are involved in cell cycle progression and localise to the central spindle during mitosis (Li *et al.*, 2008) while TbKif13-1 localizes either to the nucleus or the mitotic spindle, where it mediates chromosome segregation during mitosis (Wickstead *et al.*, 2010a). KinC and KinD are distributed throughout the cytoskeleton, where they are involved in subpellicular MT organization, basal body segregation and cytokinesis (Hu *et al.*, 2012; Wei *et al.*, 2013).

Some kinesins are associated with the flagellum and adjacent FAZ. KIN2A and KIN2B are kinesins involved in intraflagellar transport, a process that directly affects flagellar assembly and in turn affects cell motility and cytokinesis (Aslett *et al.*, 2009; Bertiaux *et al.*, 2020). The orphan kinesin KIN-E predominantly localises to the flagellar tip and is required for elongation of the new FAZ (An and Li, 2018). Another orphan kinesin named KLIF initially localises to the tip of the new, elongating FAZ and associates with the old FAZ as the cytokinetic furrow ingresses towards the posterior end, thus mediating the final stages of cytokinesis (Hilton *et al.*, 2018). Other kinesins localising to specific cytoskeletal structures include FCP2/TbKinX1 and FCP4/TbKin15, which are found at the flagella connector (FC), a trypanosome-specific structure that connects the outgrowing new flagellum to the old one (Varga *et al.*, 2017). Interestingly, FCP2/TbKinX1 belongs to kinesin clade X-1, one of the two trypanosomatid-restricted kinesin families (Wickstead and Gull, 2006; Wickstead *et al.*, 2010b).

Here we present data on the localisation and function of two members of the kinesin superfamily, TbKifX2 and TbPH1, with the former protein belonging to clade X-2. Functional characterization and the unique localization of these kinesins further expands our knowledge on how these diverse and dexterous proteins affect the biology of these extremely diverse and successful parasites.

123

124 RESULTS

125 TbPH1 is a kinesin-like protein that fractionates with the *T. brucei* cytoskeleton

TbPH1 (Tb927.3.2490) is a 110 kDa protein named after a C-terminal pleckstrin homology (PH) domain, which is followed by a proximate homeodomain-like (HDL) fold and preceded by a coiled coil (CC) domain (Fig. 1). The protein undergoes post-translational modifications, with phosphorylation of a serine at the start of the HDL (Urbaniak *et al.*, 2012) and a methylarginine in the middle of the primary structure (Lott *et al.*, 2013). While TbPH1 has an N-terminal kinesin motor domain, two substitutions of highly conserved amino acids within the Walker A motif (G93P and T/S95R) likely ablate the ATP hydrolase activity. Thus, TbPH1 is unlikely to be a *bona fide* kinesin motor protein.

TbPH1 also belongs to the ~2000 proteins found in the granule-enriched fraction (containing cellular aggregates, nuclei, kinetoplasts and flagella as well as associated cytoskeletal structures) obtained by 'MT sieving', a method originally conceived to purify stress granules from *T. brucei* (Fritz *et al.*, 2015). In brief (see S1 Fig. for 'MT sieving' pipeline), cells are lysed by non-ionic detergent in low ionic strength buffer, which maintains the cytoskeleton, including the MT corset that in turn cages predominantly detergent insoluble cellular structures with at least one dimension >20-30 nm. The MT corset is then disrupted by 300 mM NaCl to release these elements for subsequent analysis.

To verify TbPH1's apparent association with cytoskeletal fractions, we subjected a *T. brucei* cell line in which the endogenous *TbPH1* open reading frame was appended with a C-terminal V5 tag (Dean *et al.*, 2017) to cell fractionation by a procedure identical to 'MT sieving'. The cell equivalents of the ensuing fractions were inspected for the presence of TbPH1-V5 and SCD6, a stress granule marker that served as a control for this method (Fig. 2A). SCD6 was mostly found in the soluble fractions as previously reported for unstressed *T. brucei* (Fritz *et al.*, 2015), with a distribution across the analysed fractions very distinct from that of TbPH1-V5. TbPH1-V5 was well retained in all cytoskeletal fractions P1 and P2, although a fraction was released in the SN1 with other soluble cytosolic proteins upon initial lysis (Fig. 2A). Importantly, an aliquot of P2 fraction that was fixed on microscopic slides for indirect immunofluorescence (IFA) using an anti-V5 antibody shows that TbPH1-V5 is localized as a line extending from the DAPI-stained kinetoplast (Fig. 2B) that decreases in signal intensity distally, suggestive of a cytoskeletal localization. This was confirmed by the observation that the vast majority of TbPH1 was released into the soluble SN3 fraction after high salt disintegration of the cytoskeleton. A small amount of TbPH1-V5 was still found in the final pellet P3, which is consistent with its detection in the cohort of proteins retained by 'MT sieving' (Fritz *et al.*, 2015). Thus, TbPH1 is a kinesin-like protein that fractionates with the trypanosomal cytoskeleton.

TbPH1 strongly interacts with the trypanosomatid-restricted orphan kinesin TbKifX2

Because kinesins tend to form dimers (Marx *et al.*, 2009) and kinesin-like TbPH1 contains a CC domain (Fig. 1), which usually mediates protein-protein interactions (Burkhard *et al.*, 2001), we proceeded to see if TbPH1-V5 has an interaction partner by immunoprecipitation (IP) via its C-terminal epitope tag. To enrich for soluble TbPH1-V5 and augment IP stringency, we followed the previously described fractionation technique and used fraction SN3 as the input (Fig. 3A). A mock IP control using the parental cell line without an expressed V5 epitope was performed in parallel.

After confirming immunocapture of TbPH1-V5 in the first eluate (Fig. 3A), the IP eluate from this and the mock control were resolved on a SDS PAGE gel. Sypro Ruby protein gel staining showed two prominent bands of ~117 kDa (Fig. 3B, B1), the expected size for TbPH1-V5, and of ~80 kDa (Fig. 3B, B2). Importantly, these bands were not observed in the mock control, indicating their presence is not due to unspecific binding to the magnetic beads used to immobilize the anti-V5 antibody. Mass spectrometry analysis of these two excised bands identified TbPH1 itself as expected and a previously uncharacterised kinetoplastid-specific kinesin (Tb927.9.15470) (S1 Dataset), which we call

TbKifX2 based on a previously proposed kinesin nomenclature (Wickstead *et al.*, 2010b). In contrast to TbPH1, TbKifX2's kinesin motor domain contains a conventional Walker A motif (Fig. 1). This motor domain is located on the N-terminus, indicating that TbKifX2 actively moves toward the plus-end of MTs (Hirokawa *et al.*, 2009). It also contains a CC domain and two serine phosphorylation sites (Urbaniak *et al.*, 2012), similarly to TbPH1.

Interaction between these two proteins was further verified by tagging TbKifX2 with a C-terminal HA tag in the TbPH1-V5 cell line. Indeed, TbKifX2 mirrored the fractionation pattern of TbPH1-V5 (Fig. 3C). As before, the SN3 fraction was used as an input for TbKifX2-HA IP, which co-immunoprecipitated TbPH1-V5. Thus, we verified that TbPH1 and TbKifX2 exhibit a strong interaction that persists even in 300 mM NaCl.

Whole cells expressing TbPH1-V5 and TbKifX2-HA were fixed and permeabilized prior to incubation with antibodies recognizing either epitope-tag (Figs. 3D and E). We observed that both proteins possess a considerable cell body signal that appears to be nucleus-excluded, with a prominent signal along the proximal region of the flagellum. This pattern is similar to the TrypTag localization data, which visualized mNeonGreen-tagged proteins in immobilized live cells (Dean *et al.*, 2017). The cell body signal likely represents the cytosolic population of both proteins that was also observed in our fractionation data (Figs. 2A and 3C). It should be also noted that TbPH1-V5 only also displays an increased signal on the anterior tip of the cell body (Fig. 3D, asterisks).

Proximity proteomics reveals TbPH1 and TbKifX2 interactors that are associated with the flagellum attachment zone

Since conventional IP of TbPH1-V5 under high salt conditions identified only TbKifX2 as its major interaction partner, we sought a different method to identify potential cargo and/or interactors of this putative kinesin complex. To do this, we tagged TbPH1 with a C-terminal biotin ligase 'BioID2' (Kim *et al.*, 2016), appended with an HA tag (Pyrih *et al.*, 2020), to facilitate proximity-dependent biotin identification (BioID) of neighbouring proteins. First, we determined by IFA on permeabilized whole cells using anti-HA antibody that TbPH1-BioID2-HA localizes adjacent to the kinetoplast, which was also observed for TbPH1-V5 (*c.f.* Figs. 2B and 4A). TbPH1-BioID2-HA also exhibited biotinylation activity, as visualized by streptavidin-conjugated to Alexa Fluor 488 (Fig. 4A), in contrast to the parental cell line lacking BioID2 (Fig. 4B). Thus, we confirmed that the chimeric protein is properly localized and appears to biotinylate TbPH1 itself or proximal proteins.

Purification of biotinylated proteins was performed as described elsewhere (Morriswood *et al.*, 2013) (S2 Fig.). Western blotting revealed that the vast majority of TbPH1-BioID2-HA was present in the soluble S1 fraction (Fig. 4C), which was used for subsequent analysis. At first glance, this result appears to disagree with previous findings from the 'MT sieving' fractionation, which employs comparable amounts of non-ionic detergent and buffers of low ionic strength; however, we should emphasise that 'MT sieving' fractionation uses a different detergent for a shorter incubation time and at a lower temperature in comparison to the BioID purification (see Methods), which likely explains this difference.

Next, the S1 fraction was incubated with streptavidin-coated magnetic beads to capture biotinylated proteins, which were subsequently trypsinized on beads prior to liquid chromatography-tandem mass spectroscopy (LC-MS/MS). A mock control using the parental cell line lacking BioID2 expression was processed in parallel. A total of 1,131 proteins were detected with high confidence in at least one of three biological replicates (Andromeda Protein Score ≥ 20 ; >1 unique peptide per protein) (S2 dataset). Following imputation of missing values, seven proteins were shown to be preferentially

biotinylated by TbPH1-BioID2-HA, as visualised by plotting the -10 log t-test p value versus the t test difference in a volcano plot (Fig. 4D). Statistically significant hits are found above the cut-off curve in this plot.

TbKifX2 was the most enriched protein based on both parameters, consistent with its strong interaction with TbPH1. As expected, TbPH1 is also among the enriched proteins, indicating that TbPH1-BioID2-HA biotinylated itself and/or a nearby untagged, endogenous form. The sub-cellular location of the five novel proteins was determined using the TrypTag protein localization resource (Dean *et al.*, 2017). This allowed us to already exclude one of them (Tb927.10.12110) as a contaminant, given its nuclear localization. A putative kinesin with a C-terminal PH domain (Tb927.6.2880) is enriched in the basal body. Two proteins are enriched in the flagellar pocket: a dynamin-like protein 1/2 (DLP2, Tb927.3.4760; DLP1, Tb927.3.4720) and a flagellar pocket protein FP45 (Tb927.9.9730). DLP1/2 is involved in clathrin-mediated endocytosis (Morgan *et al.*, 2004; Benz *et al.*, 2017) as well as in mitochondrial scission (Chanez *et al.*, 2006; Benz *et al.*, 2017). Interestingly, FP45 has also been demonstrated to be a soluble protein that associates with the membrane surrounding the flagellar pocket by immunogold labelling in an independent study (Gheiratmand *et al.*, 2013). Furthermore, TrypTag localization of FP45 demonstrates a line emanating from the flagellar pocket toward the anterior in many cells, similar to TbPH1. Only a putative flavin-trafficking protein (Tb927.8.1950) does not have a TrypTag localization entry at this time. However, this protein was found among those that interact with TOEFAZ1 (alias CIF1, (Zhou *et al.*, 2018)), a protein that associates with the tip of the extending FAZ (Fig. 4E) (Hilton *et al.*, 2018). Furthermore, Hilton and colleagues N-terminally tagged Tb927.8.1950 to determine its localization by IFA, which exhibited an enriched signal in the vicinity of the kinetoplast. Also, both TbPH1 and TbKifX2 were found within the TOEFAZ1-BioID proximity proteome with high statistical significance (P=0.001138 and 0.0032861, respectively), along with two other proteins biotinylated by TbPH1-BioID2-HA (Fig. 4E-F) (Hilton *et al.*, 2018).

We also compared the TbPH1 proximity proteome to those of TbMORN1, a protein that is part of the hook complex (Morriswood *et al.*, 2013), and TbSpf1, a protein localized to the MtQ (Dong *et al.*, 2020) (Fig. 4F). Tellingly, there was no overlap among any of these proteomes. We conclude that none of members of the TbPH1 proximity proteome are part of the hook complex and that TbSpf1 and TbPH1 are not near each other given that the radius of biotinylation of adjacent proteins by the BioID biotin ligase is approximately 10 nm (Kim *et al.*, 2014).

These observations confirm the tight interaction between TbPH1 and TbKifX2. They also suggest that these proteins associate with the basal body, a cytoskeletal element near the flagellar pocket, as well as the FAZ given the localization of the biotinylated proteins. Thus, we proceeded to test the basal body and FAZ localization suggested by the TbPH1-BioID (Fig. 4) and IFA (Figs. 2B, 3D and E) results.

TbPH1 and TbKifX2 localize to the microtubule quartet within the cytoskeleton

We next decided to address whether TbPH1 and TbKifX2 localize to or near the FAZ, as was suggested by the TbPH1 proximity proteome. Cytoskeletons were detergent-extracted from the TbPH1-V5 and TbKifX2-HA expressing cells to visualize any potential localization to discrete cytoskeletal structures. Indeed both proteins co-localized to a long structure running along the long-axis of the cytoskeleton adjacent to the flagellum (Figs. 5A and S3A). Furthermore, detergent extraction solubilized the cell body signal (Figs. 3D and E), confirming it represented the cytosolic population of TbPH1 and TbKifX2 (Figs 2A and 3C). The specificity of the antibodies recognizing V5

and HA on extracted cytoskeletons was verified by a lack of signal in the parental cell line lacking either of these epitopes (Fig. S3B).

We proceeded to identify specifically which cytoskeletal element TbPH1 and TbKifX2 associate with. We used antibody recognizing TbKifX2-HA as a proxy for the TbKifX2-TbPH1 complex because of their demonstrated tight interaction (Fig 3), and their co-localization on detergent-extracted cytoskeletons. We note that in contrast to the TbPH1 co-localization assay depicted in Figs. 5A and S3A, we used rabbit antibody recognizing the HA tag to allow us to counterstain with antibodies raised in mouse and rat.

We found TbKifX2-HA exhibited a strong signal further toward the proximal region of the flagellum that tapered in signal intensity in a line juxtaposed with the FAZ filament (Figs. 5B and S3D). This region of strong TbKifX2-HA signal co-localized with the basal bodies (Figs. 5C and S3C). This pattern led us to investigate whether the kinesin was bound to the MtQ, as it originates close to the basal body and is considered a part of the FAZ (Lacomble *et al.*, 2009; Sunter and Gull, 2016). To this end, we used the monoclonal antibody 1B41 recognizing the MtQ (Gallo *et al.*, 1988), a somewhat contentious claim that is belied by its use as a MtQ marker in subsequent studies (Rotureau *et al.*, 2011) (see Experimental Procedures for more details about the 1B41 batch used here). Indeed, TbKifX2-HA shows co-localization with 1B41 (Figs. 5D and S3E), suggesting that TbKifX2 may associate with the MtQ.

To verify that TbKifX2 indeed binds the MtQ, we employed expansion microscopy (ExM), which enlarges cells by embedding them within a swellable polyacrylamide matrix prior to immunolabelling to allow examination of cellular structures in greater detail (Gambiarotto *et al.*, 2019). ExM has recently been implemented in *T. brucei* (Gorilak *et al.*, 2021; Amodeo *et al.*, 2021), and accentuates the part of MtQ that wraps around the flagellar pocket upon immunolabeling with antibody recognizing MT-incorporated, acetylated α -tubulin (Fig 6A) (Gorilak *et al.*, 2021). Additionally, this procedure allows the proximal MtQ to be distinguished from the nearby hook complex, which lacks MTs (Esson *et al.*, 2012).

Performing ExM allowed us to localize TbPH1-V5 or TbKifX2-HA to the part of the MtQ that spirals around the flagellar pocket, as demonstrated by the co-localization of each epitope tag with acetylated α -tubulin in both 3D reconstruction of expanded cells and individual confocal focal planes (Figs. 6A-F). The associated V5 or HA signal was not discernible beyond the point of the MtQ joining the corset MTs, where the signal of FAZ constituents, such as ClpGM6 (Hayes *et al.*, 2014), starts (*c.f.* Figs. 6A and C with Fig. S4). The fact, that the signal of TbPH1-V5 or TbKifX2-HA was apparent only in the part of the MtQ around the flagellar pocket is consistent with this being the region of the highest concentration of both proteins in the cell based on IFA (Figs. 3D-E), as well as with a lower sensitivity of ExM due to dilution of epitopes stemming from specimen expansion. Taken altogether, ExM reveals that TbPH1 and TbKifX2 localize to the MtQ element of the *T. brucei* cytoskeleton. Additionally, we observed a punctate signal of an anti-V5 or anti-HA antibody in the expanded cell body (Figs. 6C-F), consistent with the significant cytosolic fraction of both proteins (Figs. 2A, 3C-E).

Depletion of TbPH1 and TbKifX2 causes prominent morphological defects to the posterior end of *T. brucei*

To assay the function of the novel kinesin TbKifX2 and its associated kinesin-like protein TbPH1, we depleted their expression by doxycycline-induced RNAi in the cell line expressing TbPH1-V5 and TbKifX2-HA. The downregulation of either protein alone only marginally affected the growth of *T. brucei* in comparison to the non-induced cells, despite efficient depletion of each of the respective

tagged proteins (Fig. 7A, Fig. S5A). After 6 days of RNAi-silencing of TbKifX2, we fractionated the cytoskeleton from the cytosol under low salt conditions (Fig. 7B). Consistent with 'MT sieving' fractionation (Figs. 2A and 3C) and IFA (Figs 3D-E and 5A), western blot detection revealed TbKifX2-HA and TbPH1-V5 in both cytoskeletal and cytosolic fractions in cells grown in the absence of tetracycline, in which neither protein was downregulated (Fig. 7B). However, TbPH1 was preferentially depleted from the cytoskeleton fraction upon TbKifX2 silencing. This result indicates that TbKifX2 recruits TbPH1-V5 to the MtQ (Fig. 7B).

To get a more robust RNAi phenotype for phenotypic analysis, we generated cell lines in which both proteins were downregulated, which showed more pronounced growth inhibition than the single knock-downs (Fig. 7A). Growth slowed after 3 days of RNAi induction and did not reach levels of the uninduced counterparts at any point during the 7 day time course (Fig. S5B). Depletion of both proteins was confirmed by western blotting (Fig. S5B, inset), although neither protein was decreased below the detection limits of this assay, perhaps explaining the lack of a more severe growth phenotype.

Because both TbPH1 and TbKifX2 are modified in a way suggestive of post-translational regulation (Fig. 1), plus both associate with proteins that may regulate the cell cycle (*e.g.* FP45) (Gheiratmand *et al.*, 2013), we wondered whether the growth inhibition of the double RNAi knockdown is due to a cell cycle defect. Thus, we assayed the proportion of cell cycle stages present over a course of 7 days of TbPH1/TbKifX2 depletion, sorting individual DAPI-stained cells into different cell cycle stages by scoring their number of nuclei (N) and kinetoplasts (K) (Robinson *et al.*, 1995). The proportion of 1N1K cells decreased while 2N2K cells doubled at the same time from around 10% of the population to about 20% (Fig. S5C). The number of zoids (cells with 1 kinetoplast and lacking a nucleus) reached a peak at day 5, making up about 7% of the population, which may suggest problems with cytokinesis. However, we did not observe the consequent emergence of 2N1K or multinucleated cells, arguing against a specific cytokinesis defect (Robinson *et al.*, 1995). Thus, we conclude that either TbKifX2 and TbPH1 do not play a direct role in cell cycle progression and cytokinesis, or the partial depletion of these proteins did not reach the threshold needed to affect cytokinesis. Thus we looked for other phenotypes that would explain the moderately decreased fitness of these cells.

We assayed the MtQ and FAZ by IFA after 5 days of RNAi depletion and observed no defects in either of these structures, indicating that TbPH1 and TbKifX2 do not play a role in their biogenesis (Fig. S5D). However, by phase contrast microscopy we noticed the emergence of two morphological phenotypes, namely multiple cells joined together on their posterior end, a phenotype resembling abscission defects (Ralston *et al.*, 2006; Benz *et al.*, 2012) and single cells with protrusions from the posterior end (Fig. 7C).

To better visualize these phenotypes, we observed these cells by scanning electron microscopy (Fig. 7D). While control cells showed normal trypomastigote morphology (Fig. 7D), we regularly observed cells with multiple protrusions (arrows) originating from the posterior end in the induced population (Fig. 7D). The occurrence of these cell types was also corroboratively found in transmission electron microscopy, which in addition provided evidence that these protrusions appear to contain normal cellular contents and were surrounded by subpellicular MTs (Fig. S6A-B).

This effect of TbPH1/TbKifX2-depletion resembled the RNAi-phenotype of two tubulin-tyrosine-ligase-like (TTLL) proteins, which post-translationally modify the C-terminal tails of α - and β -tubulin by addition of polyglutamate side chains (Jentzsch *et al.*, 2020). Upon depletion of either TTLL, protrusions were observed emanating from the posterior end, which prompted the authors to name this morphological change the 'glove' phenotype. To determine whether TbPH1/TbKifX2

downregulation truly phenocopies that of each TTLL, we immunolabelled cells 5 days post RNAi-induction with antibodies recognizing glutamyl side chains (GT335 antibody) and tyrosinated α -tubulin (YL 1/2 antibody) (Fig. 5C and S3C); the latter antibody was used previously to visualize the basal bodies, where it additionally cross-reacts with TbRP2 (Andre *et al.*, 2014). Upon depletion of either TTLL, polyglutamylation was decreased and α -tubulin tyrosination increased in the posterior end, from which the protrusions emerged (Jentzsch *et al.*, 2020). We did not observe either of these changes in post-translational modifications upon TbPH1/TbKifX2 silencing by observing them on individual cells by IFA (Fig. S6C) or the whole cell population by western blot analysis (Fig. S6D), suggesting the mechanism underlying the emergence of posterior-end protrusions differs from that of the TTLLs.

DISCUSSION

Kinesins and related kinesin-like proteins constitute one of the largest protein families encoded by the *T. brucei* genome (Berriman *et al.*, 2005) and are represented by diverse gene repertoires in other trypanosomatids as well (El-Sayed *et al.*, 2005; Ivens *et al.*, 2005; Wickstead and Gull, 2006; Wickstead *et al.*, 2010b). Among the eukaryotes, trypanosomatids have experienced one of the largest expansions of the kinesin motor superfamily, suggesting these proteins endow trypanosomes with unique biological properties, many of which still await elucidation (Berriman *et al.*, 2005; Wickstead and Gull, 2006; Wickstead *et al.*, 2010b). Indeed, this expansion has resulted in the emergence of two trypanosomatid-specific clades of kinesins, named X-1 and X-2. A kinesin belonging to the former, named FCP2/TbKinX1, has been shown to maintain a connection of a new flagellum's distal tip along the side of the old flagellum, thus facilitating the unique mechanism of templated flagellar replication in *T. brucei* (Varga *et al.*, 2017).

In this work, we have shed light on the function of a member of the trypanosomatid-specific X-2 clade, which we named TbKifX2. We show that TbKifX2 is localized to the MtQ component of the cytoskeleton while also maintaining a cytosolic fraction. Thus, TbKifX2 is the first motor protein that has been demonstrated to localize to the MtQ, a cytoskeletal component that is considered to be part of the FAZ and restricted to trypanosomatids (Sunter and Gull, 2016).

TbKifX2 recruits TbPH1, a likely catalytically-dead, kinesin-like protein with a C-terminal PH domain, to the MtQ. Given that TbPH1 remains in the cytosolic fraction upon TbKifX2 depletion argues that both proteins do not always interact. This notion is also consistent with the enrichment of TbPH1 on the anterior tip of the cell, which was not observed in KifX2. However, when TbKifX2 and TbPH1 do interact on the MtQ, the part of the cytoskeleton where both proteins co-localize, they interact with a strong affinity that persists in high salt concentrations, a condition that usually disrupts non-covalent interactions between the CC domains (Grigoryan and Keating, 2008).

As far as we know, there are not many reports of kinesin interactions with a protein with a kinesin-like structure, but still being catalytically inactive. The most extensively characterized example being the budding yeast Kar3 kinesin and Vik1, which confers to the Kar3/Vik1 complex a strong interaction with a microtubule (Allingham *et al.*, 2007). We hypothesize that the TbKifX2/TbPH1 complex may represent an adaptation for binding the MtQ. How the TbKifX2/TbPH1 complex preferentially interacts with the MtQ over the corset microtubules is an intriguing question. It has been suggested that the MtQ contains β -tubulin with a unique but still unknown modification that is recognized by the 1B41 monoclonal antibody (Gallo *et al.*, 1988; Gallo and Precigout, 1988), and could have an increased affinity to the kinesin complex investigated here. Alternatively, the kinesins

could be activated at a specific site, for example at the proximal end of the MtQ, similar to the yeast kinesin Kip2 being recruited to the minus ends of microtubules by the activity of the spindle pole bodies (Chen *et al.*, 2019). However, at this juncture, we do not know whether TbKifX2 and TbPH1 interaction occurs prior to or after the proteins bind the MtQ.

The TbPH1 proximity proteome obtained *via* BioID labelling reassuringly revealed a protein cohort that is enriched around the proximal end of the MtQ. Among these were another kinesin with a PH domain that was localized to the basal bodies. Also, two proteins were found to be associated with the flagellar pocket, including FP45 (Gheiratmand *et al.*, 2013). The other protein is interestingly a putative flavin-trafficking protein, containing an ApbE-like domain, which is common in prokaryotes but a rare occurrence in eukaryotic proteins (Schenk *et al.*, 2021). What an enzyme potentially influencing redox is doing in association with the MtQ is another open question that has been uncovered in this study.

The TbPH1 proximity proteome was also informative in terms of proteins that were missing. One group of absent proteins are those identified to be part of the hook complex (Morriswood *et al.*, 2013), allowing us to conclude that TbPH1 does not associate with this cytoskeletal structure which lacks MTs (Esson *et al.*, 2012). Also, the eleven high confidence proteins found to be in the TbSpf1 proximity proteome were not found in our study (Dong *et al.*, 2020). This is quite surprising given that TbSpf1 also localized to the proximal MtQ (Gheiratmand *et al.*, 2013). This may indicate that these two proteins maintain a constant spatial separation, even if they both seemingly localize to the same, constrained part of the cell. Indeed the biotin ligase fused to TbPH1 to mediate BioID biotinylates proteins within a 10 nm radius (Kim *et al.*, 2014), suggesting that TbSpf1 and TbPH1, and perhaps other MtQ-binding proteins, may be partitioned at nanoscales. Consistent with this notion, only one of the eleven proteins in the TbSpf1 proximity proteome was found in that of TOEFAZ, namely the known FAZ protein named FAZ 10 (Moreira *et al.*, 2017). The higher degree of overlap between the proximity proteomes of TOEFAZ, which is enriched at the distal tip of the FAZ (Hilton *et al.*, 2018; Zhou *et al.*, 2018), and TbPH1 in comparison to the TbSpf1 proximity proteome indicates that perhaps TbPH1 is more distal to TbSpf1 in the MtQ.

Simultaneous depletion of TbPH1 and TbKifX2 does not affect the overall morphology of MtQ or FAZ, indicating they are likely not involved in the biogenesis of these cellular structures. As these single copy organelles must be faithfully replicated during cell proliferation, it is consistent with the double knockdown having a minimal impact on the cell cycle. Nevertheless, TbKifX2/ TbPH1 downregulation has a striking morphogenesis phenotype, namely the accumulation of protrusions from the posterior end of *T. brucei*. The intracellular contents of these protrusions appear to be comparable to the rest of the cell and they are also surrounded by subpellicular MTs. Interestingly, these protrusions are reminiscent of the ‘glove phenotype’ observed upon depletion of two TTLL enzymes, which are responsible for post-translational modifications appending the C-terminal tails of α - and β -tubulin (Jentzsch *et al.*, 2020).

However, we can now only speculate about the nature of these posterior extrusions. For one, simultaneous TbPH1 and TbKifX2 depletion did not affect polyglutamylation or tyrosination of tubulins, indicating that these proteins do not play a role in regulating either of these post-translational modifications as is the case for either TTLL (Jentzsch *et al.*, 2020). Malfunction of regulated polyglutamylation and tyrosination led to mislocalization of EB1, a MT-plus-end-binding protein that binds to the MT corset at the posterior tip (Sheriff *et al.*, 2014; Jentzsch *et al.*, 2020). It was proposed that EB1 proteins may dock onto tubulins with the aforementioned post-translational modifications (Jentzsch *et al.*, 2020). So, the malformations seen in TbPH1/TbKifX2 may affect EB1 or other such MT-plus-end-binding proteins, leading to uncontrolled MT growth or aberrant MT

organization. However, it should be noted that EB1 was not identified in the TbPH1 proximity proteome, so any effect on this protein may be an indirect consequence of RNAi-silencing. The protrusions may also be a consequence of delayed or uncoordinated cytokinesis or represent mechanical damage to the posterior ends due to faulty separation of daughter cells at the final stage of cytokinesis. However, arguing against protrusions arising from a putative cytokinesis defect is that they were not observed in *T. brucei* unable to pull apart due to motility defects (Ralston *et al.*, 2006).

The finding that the depletion of proteins that associate with the MtQ can affect the gross morphology of *T. brucei* was unexpected. Unfortunately, the proteins that were identified in the TbPH1 proximity proteome do not provide an obvious explanation that would link the phenotype to the localization of TbKifX2 and TbPH1. Nevertheless, we have uncovered an intriguing relationship between the MtQ and *T. brucei* morphogenesis that is worthy of further exploration.

EXPERIMENTAL PROCEDURES

Cells

Procyclic SmOxP9 cells (Poon *et al.*, 2012) were grown in SDM79 with 1 µg/ml of puromycin. Cell lines bearing epitope tags were grown with 50 µg /ml hygromycin (TbPH1-V5), 15 µg /ml G418 (TbKifX2-HA) and 10 µg /ml blasticidin (TbPH1-BioID2-HA). Selection for the RNAi constructs was with 2.5 µg /ml phleomycin (TbKifX2 RNAi, TbPH1 RNAi and TbKifX2+TbPH1 RNAi).

Plasmids and generation of cell lines

For epitope tagging at the endogenous locus with HA and V5, long PCR products were generated with primers TbPH1V5 FW (TTTCTTCCGGTCTTGACTTATTCCGCTCAGGGAAGCTTATGATTTCTTATGCGA AAAGAGAGTCATACCGCTCCCGTACgggttctgtagtggttc) and TbPH1V5 RV (ATAATAAAGAGGAAGGGAAG GTAAAGTTCAAGAAACAAATTCTGTTGGCTCCTGATAACACTCTCATATTTCTTCACCGCccaatttgagagacctgtg c) and primer TbKifX2HA FW (AACTACAGTTGCAAATGTGCATGATAGGAAGAAGTTGCCTCACGATGGAGC GTTGGAAGCGGAACATGTCGGAACCGCAGgttctgtagtggttc) and TbKifX2HA RV (CATTTTAAACAATTC TCCT TCCCTCTTTTTCACTCATTCAATTCATTCAATTCACCACCTTCTTTTTTTTTTGCTcctgtgc) using pPOT-V5-HygR and pPOT-HA-NeoR vectors as templates (based on (Kaurov *et al.*, 2018) and modified from pPOTv4 (Dean *et al.*, 2017)). For tagging with the BioID2 ligase (38), a modified version of pPOTv7-Blast-mNG (Pyrih *et al.*, 2020) was used with the aforementioned primers for TbPH1 endogenous locus tagging. 50 µl PCR reactions were directly transfected into procyclic SmOxP9 cells using the Amaxa Nucleofactor electroporator and selection occurred approximately 16 hours after electroporation.

To create a vector to simultaneously downregulate both TbPH1 and TbKifX2, a fragment of each (as suggested by RNAit2 software (<https://dag.compbio.dundee.ac.uk/RNAit/>) (Redmond *et al.*, 2003)) was PCR amplified using primers TbPH1-RNAi-Fw (TAATtctagaGTTGCGTGATGAGCTTCAAA), TbPH1-RNAi-Rv (TATAaagcttCTCCTCTAGCACCGTTTTGC), TbKifX2-RNAi-Fw (TAATggatccCACCGCCATTCTGCA AACAA) and TbKifX2-RNAi-Rv (TATAtctagaGTCATTGCGTGGGCGATAC) and cloned into BamHI-HindIII sites of p2T7-177 (Wickstead *et al.*, 2002) in a three way ligation using XbaI as the restriction site in between the two RNAi fragments. The plasmid was linearised with NotI before electroporation.

RNAi and growth curves

Procyclic form cell lines for depletion of either TbPH1 or TbKifX2 were induced at a density of 2×10^6 cells/ml with 1 µg/ml doxycycline and growth monitored daily with a Z2 Coulter Counter Analyzer (Beckman Coulter). Cells were diluted back to the initial cell density every day. Cell lines carrying the

vector for simultaneous depletion of TbPH1 and TbKifX2 were induced at a density of 5×10^5 cells/ml with 1 μ g/ml doxycycline and growth monitored daily with a Neubauer counting chamber. Cells were diluted back to the initial cell density every two days.

DAPI counts

For cell cycle analysis, cells either induced or not for depletion of TbKifX2 and TbPH1 were spread on glass slides, the cell suspension allowed to air-dry and the slides then fixed in methanol at -20 °C. Slides were rehydrated for 5 minutes in PBS before a drop of ProLong™ Gold antifade reagent with DAPI (ThermoScientific) was added, a coverslip applied and the slide sealed. At least 200 cells were scored according to the number of nuclei and kinetoplasts per slide.

Cell fractionation via 'microtubule sieving'

To entrap non-soluble and cytoskeletal proteins within the MT corset, a MT sieving technique as described in (Fritz *et al.*, 2015) was employed. The experiment was conducted exactly as described before in (Fritz *et al.*, 2015) (see also S1 Fig.).

V5 immunoprecipitation

For immunoprecipitation (IP) of TbPH1-V5 from procyclic trypanosome lysates, the V5 antibody (Thermo Fisher) was coupled and cross-linked to protein G coated dynabeads (Thermo Scientific) using 6 mg/ml dimethyl pimelidate dihydrochloride in 0.2M triethanolamine. Beads were then washed in IP wash buffer (20 mM Tris-HCl, pH 7.7, 100 mM KCl, 3 mM MgCl₂, 0.5% TritonX-100, cOmplete™ EDTA-free protease inhibitor (Roche)) and stored at 4 °C before further usage. For proteomic analysis of TbPH1 interactions, 1×10^9 PCF cells were pre-fractionated using the MT sieving technique described above. The SN3 lysate was incubated with V5-coupled dynabeads for 24 hours at 4 °C. Following several washes in IP wash buffer, proteins were eluted using low pH glycine buffer. Aliquots were taken of the different steps for western blot analysis.

Western blots

Cell lysates were prepared by boiling in 1x SDS sample buffer and the equivalent of 2×10^6 cells (RNAi lines) or 1×10^7 cells (IP samples) was loaded per well. Following western blotting, PVDF membranes were blocked for 1 hour in 5% milk-PBS. Incubation with primary antibodies (anti-V5 at 1:1,000; ThermoScientific, anti-HA at 1:1,000; ThermoScientific, anti-tubulin at 1:5,000; Sigma) was performed at 4 °C overnight. Secondary antibodies (HRP-coupled anti-mouse or -rabbit IgG; Sigma) were used at a concentration of 1:2,000 and signals were visualised using Clarity Western ECL substrate (Bio-Rad) on a ChemiDoc MP (Bio-Rad).

SYPRO Ruby staining

Following electrophoresis, polyacrylamide gels were placed into fixing solution (7% glacial acetic acid, 50% methanol) for 30 minutes at room temperature. Following another, identical fixation step, the gel was incubated with SYPRO Ruby solution overnight at room temperature. A washing step in washing solution (7% glacial acetic acid, 10% methanol) for 30 minutes at room temperature was followed by three 5 minute washes in milliQ water before imaging on a ChemiDoc MP (Bio-Rad).

Immunofluorescence and cytoskeleton extraction

Approximately 2×10^6 cells were used per slide. Cells were washed in PBS and fixed in 2.3% PFA in PBS for approximately 30 minutes before being transferred to microscopy slides (ThermoScientific). Following neutralisation in 0.1M glycine in PBS, slides were incubated in methanol at -20° C

overnight for permeabilisation. Slides were then rehydrated in PBS and blocked in 1% BSA in PBS for 1 hour followed by incubation with primary antibody for 1 hour in a humid chamber. The slides were then washed three times with PBS before incubation in AlexaFluor-conjugated secondary antibody (goat anti-rabbit/mouse, used at 1:1,000; Invitrogen). Following three further washes in PBS, a drop of ProLong Gold Antifade reagent with DAPI (ThermoScientific) was added, a coverslip applied and the slide sealed with nail polish. Slides were imaged with a Zeiss AxioScope or an Olympus FluoView Fv1000 confocal microscope.

For immunofluorescence analysis of cells extracted using the 'MT sieving' fractionation method described above the pellet fraction P2 was fixed in 2.3% PFA as described. All subsequent steps were the same as for whole cell immunofluorescence.

To extract cytoskeletons conventionally, approximately 1×10^7 PCF cells were collected and centrifuged at room temperature for 5 min at 800 x g and the supernatant discarded. Thereafter, the cell pellet was washed once in PBS and after resuspension in PBS applied in a drop-wise fashion to Superfrost plus® slides (ThermoScientific). The cells were left to settle before the addition of PEME buffer (100 mM Pipes, pH 6.9, 1 mM MgSO₄, 2 mM EGTA, 0.1 mM EDTA) containing 0.5% (v/v) NP-40 (Igepal) for 10 seconds. This cytoskeleton extraction was followed by a 10 minute fixation step in 4% PFA. All subsequent steps were as described above for whole cell immunofluorescence.

Antibodies used in immunofluorescence

Antibody	Dilution	Origin
anti-HA (produced in rabbit)	1:1,000	Sigma, Catalog #: H6908
anti-V5 (produced in mouse)	1:1,000	ThermoScientific, Catalog #: 377500
1B41 (MtQ) ¹	1:1,000	(Gallo <i>et al.</i> , 1988)
L3B2	1:10	(Kohl <i>et al.</i> , 1999)
YL1/2	1:50	(Sherwin and Gull, 1989)
GT335	1:2000	Adipogen Life Sciences, Catalog #: AG-20B-0020
¹ IgM purified from hybridoma supernatant and not mouse ascitic fluid as in original report.		

Scanning and transmission electron microscopy

For TEM, cell pellets were high-pressure frozen, freeze substituted in the presence of 2% OsO₄ in acetone, and embedded into Spi-Pon812 resin (SPI) as described previously (Kaurov *et al.*, 2018). For SEM of the TbPH1/TbKifX2 RNAi cell line, cells were fixed in 2.5% glutaraldehyde in 100 mM PBS overnight at 4°C and then spotted onto poly-L-lysine-coated glass cover slips. The cells were post-fixed in 2% osmium tetroxide in 100 mM PBS for 1 h at room temperature and finally washed in the same buffer. After dehydration cells were critical-point dried, coated with gold palladium and imaged.

Expansion microscopy

Expansion microscopy samples were prepared as described previously (Gorilak *et al.*, 2021). In brief, 1×10^6 cells were fixed overnight in a solution containing 4% formaldehyde and 4% acrylamide in PBS and adhered to a poly-L-lysine (Sigma, P4707) coated coverslip. The cells were gelated for 30 min at 37°C in PBS supplemented with 19% sodium acrylate, 10% acrylamide, and 0.1% N, N'-Methylenebisacrylamide, 0.5% N, N', N'-tetramethylethylenediamine, and 0.5% ammonium persulfate. The specimens were further denatured by a 2 hour incubation at 95°C in a buffer

consisting of 50 mM tris(hydroxymethyl)aminomethane-hydrochloride, 200 mM sodium chloride, 200 mM sodium dodecyl sulphate, pH 9.0, and expanded by three 20 min incubations with 15 ml of ultrapure water.

Thereafter, the gels were incubated overnight first with a mixture of anti-HA (Cell Signaling Technology, 3724S; used at 1:250) and C3B9 ((Woods *et al.*, 1989), used at 1:10), anti-V5 (Sigma, V8137-2MG; used at 1:250) and C3B9 or anti-ClpGM6 ((Hayes *et al.*, 2014), used at 1:200) and C3B9 antibodies, followed by washes and an overnight incubation with a mixture of secondary antibodies (Invitrogen, A11001 and A21428; used at 1:500). Both primary and secondary antibodies were diluted in PBS supplemented with 2% bovine serum albumin. Finally, the gels were washed with ultrapure water.

Prior to imaging pieces of gels containing stained expanded cells was transferred to a glass-bottom dish coated with poly-L-lysine, and imaged using a Leica TCS SP8 confocal microscope with an HC PL apochromatic 63x/NA 1.40 oil immersion objective. Z-stacks were acquired with the step size of 100 nm. Confocal z-stacks were processed in Fiji (Schindelin *et al.*, 2012). 3D reconstructions were performed in the 3D viewer plugin.

BioID

PCF cells (TbPH1-BioID2-HA and SmOxP9 as controls) were grown in the presence of 50 μ M biotin for 24 hours. For proximity-dependent biotin identification (BioID), 10^9 PCF cells were extracted in PEME buffer (100 mM Pipes, pH 6.9, 1 mM MgSO₄, 2 mM EGTA, 0.1 mM EDTA) containing 0.5% (v/v) NP-40 (Igepal) for 15 minutes at room temperature resulting in extract E1. Following centrifugation at 3,400 x g for 2 minutes, supernatant S1 was created and pellet P1 was further processed by extraction in lysis buffer (0.4% SDS, 500 mM NaCl, 5 mM EDTA, 1 mM DTT, 50 mM Tris-HCl, pH7.4). Another centrifugation step at 16,000 x g for 10 minutes created supernatant S2. Both supernatants, S1 and S2, were then incubated with streptavidin-conjugated Dynabeads (company) for 4 hours at 4 °C. An aliquot of flow through samples F1 and F2 were retained for western blotting and the dynabeads were washed five times with PBS. A small sample of the beads was then resuspended in 2x SDS PAGE buffer and boiled, while the remainder of the beads was stored at -80 °C until further processing for mass spectrometry analysis.

Mass spectroscopy analysis of captured biotinylated proteins

Trypsin-digestion of captured biotinylated proteins was performed on bead prior to liquid chromatography-tandem mass spectroscopy (LC-MS/MS) as previously described (Pyrih *et al.*, 2020). Data was processed using MaxQuant (Cox and Mann, 2008) version 1.6.14 which incorporates the Andromeda search engine (Cox *et al.*, 2011). Proteins were identified by searching a protein sequence database containing *T. brucei brucei* 927 annotated proteins (Version 51, TriTrypDB (Aslett *et al.*, 2009), <http://www.tritrypdb.org/>) supplemented with frequently observed contaminants. Search parameters specified an MS tolerance of 6 ppm, an MS/MS tolerance at 0.5 Da and full trypsin specificity, allowing for up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine and N-terminal protein acetylation were allowed as variable modifications. The experimental design included matching between runs for biological replicates. Peptides were required to be at least 7 amino acids in length, with false discovery rates (FDRs) of 0.01 calculated at the levels of peptides, proteins and modification sites based on the number of hits against the reversed sequence database. The obtained data was subsequently processed in Perseus version 1.6.14 as described in (Zoltner *et al.*, 2020).

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD025802.

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Author contributions

Conceptualization (CB, NM, SK, VV, HH), formal analysis (CB, NM, SK, HV, MV, VV, HH), investigation (CB, NM, SK, HV, MV), visualization (CB, VV, HH), writing – original draft (CB, HV, JL, VV, HH), funding acquisition (JL, VV, HH), project administration (HH).

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FIGURE LEGENDS

Fig. 1: Domain architecture of TbPH1 and TbKifX2. Schematic depiction includes phosphorylation and arginine methylation sites. The consensus Walker A motif is shown in between the two schemata for comparison of the respective motifs within the kinesin motor domains of TbPH1 and TbKifX2. Key residues in ATP hydrolysis shown in red and mutations in purple. The likely inactive state of the TbPH1 ‘kinesin motor domain’ is highlighted by striped pattern. CC: coiled coil domain, PH: pleckstrin homology domain, HDL: homeodomain-like fold.

Fig. 2: TbPH1-V5 fractionates with the cytoskeleton. **(A)** Western blot of-trypanosome cell fractions. Fractionation procedure is summarized in S1 Fig. Antibodies used were anti-V5 and anti-SCD6 (Kramer *et al.*, 2008). T: total lysate, SN: supernatant, P: pellet. **(B)** IFA of on an aliquot of fraction P2 placed on slides for fluorescence microscopy. Cells were labelled with anti-V5 antibody (white) and DAPI (magenta). The scale bar corresponds to 5 µm.

Fig. 3: TbPH1 interacts with TbKifX2, a kinetoplastid-specific kinesin. **(A)** Western blot verifying immunoprecipitation of TbPH1-V5. The blot was probed with anti-V5 and anti-α-tubulin antibodies. The parental cell line SmOxP9 was used as a control. T: total lysate, SN3: supernatant 3, FT: flow through, E: elution. **(B)** SyproRuby-stained gel of elution fractions of TbPH1-V5 IP and mock control (SmOxP9). B1 and B2: bands excised and by mass spectrometry identified to be TbPH1 and TbKifX2, respectively. Asterisks: band corresponding to denatured immunoglobulin light chain that was incidentally eluted. **(C)** Western blot showing interaction of TbPH1-V5 and TbKifX2-HA. TbKifX2-HA was immunoprecipitated in the same way as PH1-V5. T: total lysate, SN: supernatant, P: pellet, FT: flow through, E: elution. **(D)** IFA on whole cells detecting TbPH1-V5 (white) and counterstained with DAPI to visualize DNA (magenta). Arrowheads denote increased signal in the area along the proximal part of the flagellum. Asterisks demark an increased signal at the anterior cell end. Merge of DAPI

and Phase contrast images shown on bottom. Scale bar, 10 μ m. (E) IFA on whole cells detecting TbKifX2-HA (white). Images depicted as in D.

Fig. 4: The proximal proteome of TbPH1. (A) IFA on whole cells showing localisation of TbPH1-BioID2-HA (white) and biotinylated proteins (white) and counterstained with DAPI (magenta). Arrowheads denote signal in vicinity of kinetoplast, near the proximal part of the flagellum. DIC on right to show observed cell. Scale bar, 5 μ m. (B) IFA as in A of parental cell line lacking TbPH1-BioID2-HA. (C) Western blot (top panel) and fluorescently-detected proteins resolved on SDS-PAGE as a loading control (bottom panel, stain-free gel) of a representative BioID experiment. The blot was probed with anti-HA to confirm the presence of TbPH1-BioID2-HA in the respective fractions. E: extract, SN: supernatant, F: flow through, B: boiled beads. (D) Volcano plot with $-\log_{10}$ t-test p value plotted against the t-test difference between TbPH1-BioID2-HA sample and mock (SmOxP9 cells) from three biological replicates. Statistically significant hits are found above the curve in the top right quadrant and their identity is indicated. Accession numbers are given with additional annotations in parentheses; these are color coded based on their localization determined by TrypTag (Dean *et al.*, 2017). (E) Heat map of SAINTq score (*i.e.* P value) of proteins interacting with TOEFAZ1 (Hilton *et al.*, 2018), the relative enrichment of proteins also found in the TbPH1 proximal proteome are indicated. a: Tb927.3.2490 (TbPH1), b: Tb927.8.1950 (ApbE-like domain), c: Tb927.9.15470 (TbKifX2), d: Tb927.3.4720/60 (DLP1/2). PG, protein groups. (F) Venn diagram comparing the proximity proteome of TbPH1 with those of the TOEFAZ (as depicted in E), the hook complex protein TbMorn1 (all 79 biotinylated proteins (Morriswood *et al.*, 2013)), and the 11 high confidence proteins in the TbSpef1, a MtQ binding protein (Dong *et al.*, 2020). Proteins exclusive to the TbPH1 proximity proteome are highlighted in green while magenta highlights those that are found to also be in proximity with TOEFAZ.

Fig. 5: Localization of cytoskeleton-associated TbKifX2 and TbPH1. (A) IFA on extracted cytoskeletons probing localisation of TbPH1-V5 (green) and TbKifX2-HA (white). (B) IFA on extracted cytoskeletons probing co-localisation of TbKifX2-HA (white) with the FAZ (green), the latter labelled with L3B2 antibody. (C) IFA on extracted cytoskeletons probing co-localisation of TbKifX2-HA (white) with basal bodies (green), the latter labelled with YL1/2 antibody (D) IFA on extracted cytoskeletons probing co-localisation of TbKifX2-HA (white) with the MtQ (green), the latter labelled with 1B41 antibody. In all panels, merge images depict co-localization in magenta and DIC on right showing observed extracted cytoskeleton. Scale bar, 5 μ m.

Fig 6: TbKifX2 and TbPH1 localize to the MtQ. Ultrastructure expansion microscopy images depicted as 3D projections (A-D) and representative confocal image sections used to make projections (E-F) of *T. brucei* expressing TbPH1-V5 and TbKifX2-HA. (A) 3D reconstruction of the area of the cell in the vicinity of the basal body and flagellum exit from the flagellar pocket looking from outside the MT corset. Top shows signal from anti-V5 (green) and anti-acetylated α -tubulin (white) antibodies. Bottom is the same image of anti-acetylated α -tubulin signal only with relevant structures marked. Tick marks at 5 μ m increments (A-C). (B) Same as A but showing anti-HA antibody signal (green) and a view shown from inside the MT corset. (C) 3D reconstruction of the posterior part of the cell. Signal colors correspond to A. (D) 3D reconstruction of whole dividing *T. brucei* cell. Signal colors correspond to B. Box highlights region of interest shown in F. Tick marks at 10 μ m increments. (E) Confocal images from the vicinity of the basal body showing from left to right signal of anti-V5 and anti-acetylated α -tubulin antibodies (both white) plus an overlay of both signals with V5 signal in green. Left image shows 5 μ m scale bar. Middle image marks relevant structures. (F) Same as in E except two sections being shown, focusing on new (Top) and old MtQ (Bottom), and the left images

showing anti-HA antibody signal. Solid arrowheads point to overlay of epitope antibody signal and MtQ. Hollow arrowheads point to representative epitope antibody signals found in the cytosol.

Fig. 7: Depletion of TbPH1 and TbKifX2 affects posterior end remodelling. **(A)** Bar chart showing population doubling times for TbPH1, TbKifX2 and TbPH1/TbKifX2 depletion cell lines. -/+ : +/- doxycycline to induce RNAi, the average difference in hours from three experiments is shown above the respective bars. **(B)** Western blot of cellular fractionation experiment following TbKifX2 RNAi. The blot was probed with anti-HA (to detect TbKifX2), anti-V5 (to detect TbPH1) and anti- α -tubulin as a loading control. WCL: whole cell lysate. **(C)** Phase contrast images of TbPH1/TbKifX2 RNAi cells. + tet: induced for 5 days. The scale bar corresponds to 5 μ m. Arrows indicate morphological defects. **(D)** Scanning electron microscopy of TbPH1/TbKifX2 RNAi cells. + tet: induced for 5 days. Arrows pointing to posterior end protrusions. The scale bar corresponds to 10 μ m.

S1 Fig.: Schematic depiction of microtubule sieving technique. According to (Fritz *et al.*, 2015).

S2 Fig.: Schematic depiction of proximity-dependent biotinylation (BioID). According to (Morriswood *et al.*, 2013). Only steps in black were performed in our study.

S3 Fig.: Wider field of view of multiple extracted cytoskeletons co-localizing TbKifX2 and TbPH1 to basal body **(C)**, FAZ **(D)** and MtQ **(E)**. Images depicted as in Fig 5 with scale bar corresponding to 5 μ m. **(A)** Co-localization (merge, magenta) of TbPH1-V5 (green) and TbKifX2-HA (white). **(B)** Parental cell line lacking TbPH1-V5 and TbKifX2-HA, incubated with antibodies recognizing V5 and HA epitopes used to acquire other IFA images.

S4 Fig.: Ultrastructure expansion microscopy of a cell labelled with anti-ClpGM6 (to detect the FAZ, green) and anti-acetylated α tubulin antibody C3B9 (grey). Only the region around the pocket and flagellum exit shown.

S5 Fig.: TbPH1/TbKifX2 RNAi. **(A)** Growth curves of TbPH1 and TbKifX2 RNAi cell lines. Filled circles: TbKifX2 – tet, open circles: TbKifX2 + tet, filled triangles: TbPH1 – tet, open triangles: TbPH1 + tet. Standard deviation of measurements at each time point (n=3) is not visible at this scale. Insets show western blots probed with anti-HA (to detect TbKifX2), anti-V5 (to detect TbPH1) and anti- α -tubulin as a loading control. **(B)** Growth curve of TbPH1/TbKifX2 RNAi cell line. Filled circles: TbPH1/TbKifX2 – tet, open circles: TbPH1/TbKifX2 + tet. Standard deviation of measurements at each time point (n=3) is not visible at this scale. Inset depicts western blot showing depletion of TbPH1/TbKifX2. Samples were taken at the same time as the growth curve in B was performed. The blot was probed with anti-HA (to detect TbKifX2), anti-V5 (to detect TbPH1) and anti- α -tubulin as a loading control. **(C)** Bar chart depicting different cell cycle phases during TbPH1/TbKifX2 RNAi time course. DAPI counts were performed in triplicate with at least 200 cells counted per time point. N: nucleus, K: kinetoplast. **(D)** IFA of TbPH1/TbKifX2 RNAi cells. + tet: induced for 5 days. Extracted cytoskeletons were labelled with L3B2 (recognising the FAZ filament, white), mAB 1B41 (recognising the MtQ, white) and DAPI (magenta). Scale bar, 5 μ m.

S6 Fig.: Transmission electron microscopy and IFA of TbPH1/TbKifX2 RNAi cell line. Cells were induced for 5 days. **(A)** Image depicting posterior end protrusions (arrow). Scale bar, 1 μ m. **(B)** Close-up of protrusion encapsulated by microtubule corset (arrowheads). The scale bar corresponds to 500 nm. **(C)** IFA on cytoskeletons probing effects of TbPH1/TbKifX2 depletion post-translational modifications of tubulin. Cells were counterstained with GT335 (red, staining glutamyl side chains) and YL1/2 (green, staining tyrosinated α -tubulin). The scale bar corresponds to 5 μ m. **(D)** Western blots taken after 3 to 5 days (d) of growth in presence (+) or absence (-) of doxycycline probed with antibodies recognizing GT335, YL1/2 and α -tubulin, the last as a loading control.

944 **S1 Dataset:** Mass spectrometry analysis of excised bands B1 and B2 from Fig. 3B.

945 **S2 Dataset:** Mass spectrometry data of TbPH1 BioID experiment. Data with and without imputation
 946 and filtered for Andromeda scores >20 and presence of >1 peptide, which was used to
 947 generate the volcano plot in Fig. 4D.

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Fig. 1

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Suppl. Fig. 2

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