The *Trypanosoma brucei* Cytoskeletal Protein KHARON Associates with Partner Proteins to Mediate Both Cytokinesis and Trafficking of Flagellar Membrane Proteins

Marco A. Sanchez, and Scott M. Landfear*

Department of Molecular Microbiology & Immunology, Oregon Health & Science University, Portland, Oregon, USA

*Author for correspondence ([landfear@ohsu.edu](mailto:landfear@ohsu.edu))

**Summary Statement:** This study investigates the essential role in African trypanosomes of the KHARON protein and its molecular partners in trafficking of membrane proteins to the flagellum.
ABSTRACT

In the African trypanosome *Trypanosoma brucei*, the cytoskeletal protein *Tb*KHARON is required for trafficking of a putative Ca\(^{2+}\) channel to the flagellar membrane, and it is essential for parasite viability in both the mammalian stage bloodstream forms and the tsetse fly procyclic forms. This protein is located at the base of the flagellum, in the pellicular cytoskeleton, and in the mitotic spindle in both life cycle forms, and it likely serves multiple functions for these parasites. To begin to deconvolve the functions of KHARON, we have investigated partners associated with this protein and their roles in parasite biology. One KHARON associated protein, *Tb*KHAP1, is a close interaction partner that can be crosslinked to KHARON by formaldehyde and pulled down in a molecular complex, and it colocalizes with *Tb*KHARON in the basal body at the base of the flagellum. Knockdown of *Tb*KHAP1 mRNA has similar phenotypes to knockdown of its partner *Tb*KHARON, impairing trafficking of the Ca\(^{2+}\) channel to the flagellar membrane and blocking cytokinesis, implying that the *Tb*KHARON/*Tb*KHAP1 complex mediates trafficking of flagellar membrane proteins. Two other KHAPs, *Tb*KHAP2 and *Tb*KHAP3, are in close proximity to *Tb*KHARON, but knockdown of their mRNAs does not affect trafficking of the Ca\(^{2+}\) channel. Two different flagellar membrane proteins, which are extruded from the flagellar membrane into extracellular vesicles, are also dependent upon *Tb*KHARON for flagellar trafficking. These studies confirm that *Tb*KHARON acts in complexes with other proteins to carry out various biological functions, and that some partners are involved in the core activity of targeting membrane proteins to the flagellum.

KEYWORDS *Trypanosoma brucei*, cytoskeleton, cytokinesis, flagellar membrane protein trafficking, protein complexes, virulence
INTRODUCTION

African trypanosomes of the species *Trypanosoma brucei* are parasitic protists that cause human African trypanosomiasis and the disease nagana in cattle and are thus of great medical and veterinary importance (Kennedy, 2013). In addition, these parasites have been recognized as valuable models for probing fundamental questions in cell and molecular biology (Cayla et al., 2019). *T. brucei* and related kinetoplastid parasites such as *Trypanosoma cruzi* and *Leishmania* species are flagellated and offer novel insights into the structure and function of flagella (Langousis and Hill, 2014) and the roles of these organelles in infection (Kelly et al., 2020a). Furthermore, the cell division cycle of African trypanosomes has been studied extensively (Farr and Gull, 2012; Vaughan and Gull, 2008; Wheeler et al., 2019), identifying various processes that are important for both proliferative and differentiation-linked cell division.

In previous work on trafficking of integral membrane proteins to flagella, we identified a kinetoplastid-specific protein designated KHARON (KH) that plays a critical role in flagellar targeting of the putative Ca\(^{2+}\) channel *TbCaCh* (*Tb927.10.2880*) in *T. brucei*, originally identified as a flagellar surface protein FS179 (Oberholzer et al., 2011), and the flagellar glucose transporter *LmxGT1* in *L. mexicana* (Tran et al., 2013) (see Table S1 for tabulation of gene IDs and names of *T. brucei* proteins investigated in this study). In both species of parasite (Sanchez et al., 2016), KHARON was localized to three distinct subcellular compartments: the base of the flagellum (Fig. 1A), the subpellicular microtubules that subtend the plasma membrane around the cell body, and the mitotic spindle (Fig. 1B). Application of RNA interference (RNAi) to knock down *TbKH* (*Tb927.10.8940*) revealed that, in addition to preventing flagellar trafficking of *TbCaCh/FS179*, the flagellum attachment zone (FAZ) was disrupted, resulting in detachment of flagella from the cell body (Sanchez et al., 2016), leaving this organelle adherent only through its connection at the flagellar pocket. Interfering with trafficking of *TbCaCh/FS179* to the flagellar membrane is likely to induce disruption of flagellar attachment, as RNAi directed against this channel also results in a similar flagellar detachment phenotype (Oberholzer et al., 2011). In addition, as found for many genetic alterations that disrupt flagellar attachment, these parasites are unable to initiate cell division, generating trypanosomes in which nuclei, kinetoplasts (mitochondrial DNA-containing structures), basal bodies, and flagella have replicated but cytokinesis has not occurred. This phenotype was apparent in both mammalian bloodstream form (BF) and insect stage procyclic form (PF) parasites and was thus lethal to both life cycle stages.

Similar studies in *L. mexicana* have also established a critical role for *LmxKH* (*LmxM.36.5850*) in the life cycle of *Leishmania* parasites. Thus a ∆*lmxkh* null mutant was
generated in insect stage promastigotes, where trafficking of LmxGT1 to the flagellum was strongly impaired (Tran et al., 2013), but cell division and replication of this life cycle stage was not affected. In contrast, Δl mxkh null mutants were unable to undergo cytokinesis after invading host macrophages, resulting in formation of multinucleate multilagellated amastigotes that died over the course of several days (Tran et al., 2013). These null mutants were also avirulent following injection into BALB/c mice (Tran et al., 2015), and studies by others have shown that KHARON null mutants in L. infantum have potential as a live attenuated vaccine (Santi et al., 2018).

KHARON exhibits both similarities and striking differences between T. brucei (TbKH) and L. mexicana (LmxKH). Thus, the two orthologs are relatively divergent in sequence, sharing 27% amino acid identity and differing significantly in length (411 amino acids for TbKH versus 520 amino acids for LmxKH). TbKH is critical for cell division of both mammalian BF and insect stage PF parasites, whereas LmxKH is only essential for division of disease-causing amastigotes. Nonetheless, the three subcellular locations for KHARON are shared between the two parasites, as are functions in cytokinesis and formation of the flagellar membrane.

KHARON proteins do not share significant sequence similarity to proteins outside the Kinetoplastida, nor do they contain conserved sequence motifs that are suggestive of specific biochemical or cellular functions. Furthermore, their residence at multiple subcellular locations suggests that KHARON proteins are likely to be multifunctional, participating in flagellar membrane trafficking, cytokinesis, and spindle function. Additionally, it is possible that distinctions in function could be conferred by association of KHARON with different partner proteins at each of its three subcellular loci. Thus, we hypothesize that there could exist three distinct KHARON Complexes, Complex 1 at the base of the flagellum, Complex 2 at the subpellicular cytoskeleton, and Complex 3 at the mitotic spindle. Furthermore, given the apparent differences in TbKH and LmxKH noted above, there may be similarities and differences between these putative complexes between the two species of parasite.

To initiate a study of putative KHARON Complexes and their functions, we carried out biotinylation proximity labeling (BioID) (Roux et al., 2012) and tandem affinity purification-mass spectrometry (TAP-MS) (Kaiser et al., 2008) on LmxKH, resulting in the identification of two KHARON Associated Proteins, LmxKHAP1 and LmxKHAP2 (LmxM.32.2440 and LmxM.05.0380, respectively; (Kelly et al., 2020b)). In parallel, we investigated these two KHARON partners in T. brucei and report the results of those studies here. As anticipated, TbKHAP1 and TbKHAP2 exhibit both similarities and notable differences compared to their orthologs in L. mexicana. In addition, T. brucei expresses another KHARON partner related to
TbKHAP2 that we designate TbKHAP3. Furthermore, characterization of additional flagellar membrane proteins suggests that TbKH expression is important for flagellar targeting of multiple such proteins in African trypanosomes, whereas the role of LmxKH in trafficking of flagellar membrane proteins appears to be more restricted. These studies confirm that KHARON proteins in both parasites exist in complexes with various partners and that these partner proteins can play distinct roles in the functions of different KHARON Complexes.
RESULTS

Localization of *Tb*KHAP1, *Tb*KHAP2, *Tb*KHAP3, and *Tb*KH in bloodstream and procyclic African trypanosomes. To facilitate studies on *Tb*KH and its partners, we raised and affinity purified a polyclonal antibody against this protein, anti-*Tb*KH pAb. Western blot analysis indicated that anti-*Tb*KH pAb detects a single protein of ~49 kDa molecular weight, and that an additional band of ~62 kDa appears in parasites also expressing a BirA* fusion on the N-terminus of *Tb*KH (Fig. 1 C), establishing that this antibody is of suitable specificity to employ in localization and biochemical characterization of *Tb*KH. To determine whether *Tb*KHAP1 (Tb927.11.2610), *Tb*KHAP2 (Tb927.10.10360) and *Tb*KHAP3 (Tb927.10.10280) are associated with *Tb*KH in a complex, several complementary approaches were applied. First, each KHAP was tagged at its N-terminus with the triple hemagglutinin peptide tag HA3, and formaldehyde-fixed BF and PF trypanosomes were examined by immunofluorescence deconvolution microscopy (Fig. 2). HA3:*Tb*KHAP1 (Fig. 2A,B, green) overlaps with *Tb*KH (red) at the cell periphery, as demonstrated by the yellow color in this region of both BF and PF parasites. In contrast, there was no apparent overlap of the two signals in the mitotic spindle (central red oval or line marked with a white arrow), indicating that this protein could be associated with *Tb*KH in the subpellicular cytoskeleton but not at the mitotic spindle. Similarly, *Tb*KHAP2::HA3 (Figs. 2C,D) and V53::*Tb*KHAP3 or HA3::*Tb*KHAP3 (Fig. 2E,F) overlap with *Tb*KH at the cell periphery but not at the mitotic spindle. For each of the *Tb*KHAPs, there is also green fluorescence that does not coincide with *Tb*KH so that there is not complete overlap of the signals, and there may thus be populations of each protein that are not associated with each other. However overall, these three *Tb*KHAPs are candidates for *Tb*KH partners that are selective for the subpellicular cytoskeleton versus the mitotic spindle.

To determine whether *Tb*KHAP1, *Tb*KHAP2, or *Tb*KHAP3 might associate with *Tb*KH at the base of the flagellum, flagella were isolated from parasites expressing each HA3-tagged *Tb*KHAP and imaged by deconvolution microscopy. Fig. 3 shows that HA3::*Tb*KHAP1 (Fig. 3A), HA3::*Tb*KHAP2 (Fig. 3B) and HA3::*Tb*KHAP3 (Fig. 3C) overlap significantly with *Tb*KH in the region of the flagellum immediately adjacent to the kinetoplast DNA (kDNA, blue, Fig. 3A), which is close to and physically attached to (Robinson and Gull, 1991) the flagellar basal body. Additional images showing overlap of *Tb*KH with each of the three *Tb*KHAPs at the base of the flagellum are provided in Fig. S1. Overall, these results indicate that *Tb*KHAP1, *Tb*KHAP2, and *Tb*KHAP3 could be partners for *Tb*KH at both the pellicular cytoskeleton and the base of the flagellum. It is noteworthy that *Tb*KHAP3 was previously identified as a basal body protein in a
proteomic study of that subcellular structure (Dang et al., 2017). Hence, it is likely that TbKH, TbKHAP1, TbKHAP2, and TbKHAP3 are all basal body components.

**Molecular association of TbKHAP1, TbKHAP2, and TbKHAP3 with TbKH.** To determine whether the observed subcellular overlap of the fluorescence signals from TbKHAPs and TbKH could indicate physical association in molecular complexes, TbKH was endogenously tagged at its N-terminus with a His10 affinity tag (His10::TbKH) to allow pulldown of this protein, and associated partners, with Ni-NTA magnetic beads, and this affinity tagged protein was expressed in a BF cell line also expressing either HA3::TbKHAP1, TbKHAP2::HA3, or V53::TbKHAP3. Because TbKH is an integral component of the parasite cytoskeleton (Sanchez et al., 2016) and would pulldown many cytoskeletal proteins in an experiment performed under native conditions, we first crosslinked with formaldehyde parasites expressing each pair of tagged proteins. Formaldehyde crosslinks proteins that are in very close proximity (~2-3 Å, reference (Hoffman et al., 2015)), so this treatment will covalently attach close molecular partners of TbKH but not proteins that are more distant partners in a complex or proteins that are in the cytoskeleton but distant from TbKH. Subsequent treatment with strongly denaturing reagents will dissociate peripheral proteins from His10::TbKH while retaining crosslinked partners, and the closely associated partners will thus be purified along with His10::TbKH, following binding and elution from the Ni-NTA beads, and released upon heat-induced reversal of the crosslinks.

Fig. 4A demonstrates that HA3::TbKHAP1 is pulled down with His10::TbKH when parasites are formaldehyde crosslinked (EF*) but not when they are not subjected to crosslinking (EF). As a negative control, another subpellicular cytoskeletal protein, CAP15 (Vedrenne et al., 2002), was HA3 tagged and expressed in His10::TbKH expressing BF parasites, but this protein was not pulled down even from formaldehyde-crosslinked parasites. Parallel experiments were performed using BF parasites expressing TbKHAP2::HA3, or V53::TbKHAP3 and His10::TbKH. However, for reasons that are not clear but possibly having to do with the highly repetitive nature of both proteins (see below), treatment of parasites with formaldehyde followed by immediate dissolution in the strongly denaturing urea buffer employed to disrupt protein interactions led to massive degradation of both TbKHAP2::HA3 and TbKHAP3::HA3. Indeed, the susceptibility of these proteins to degradation has been noted previously (Schneider et al., 1988). Hence, for technical reasons we have not been able to observe pulldowns of either of these two tagged protein with His10::TbKH, thus preventing us from directly demonstrating interactions between these proteins at the 2-3 Å level. Nonetheless, these experiments establish that TbKHAP1 is in very close proximity to TbKH.
Proximity ligation assay (PLA) confirms close proximity of *TbKH* with *TbKHAP1*, *TbKHAP2*, and *TbKHAP3*. To provide another independent examination of whether *TbKH* is in close physical proximity with each *TbKHAP*, we performed the PLA in parasites expressing HA3::*TbKHAP1*, *TbKHAP2::HA3*, and HA3::*TbKHAP3*. In this assay (Fredriksson et al., 2002; Soderberg et al., 2006), cells expressing two partner proteins are first reacted with primary antibodies from different species. Parasites are subsequently incubated with species-specific secondary antibodies directed against each primary antibody, and each of these secondary antibodies contains a unique, covalently attached oligonucleotide. Only if the two target proteins are within ~400 Å of each other, these oligonucleotides can base pair to another linker oligonucleotide and be covalently ligated into a circular substrate that can participate in rolling circle DNA amplification of the cognate sequence. The amplified sequence is then hybridized to a fluorescently labeled DNA probe, resulting in fluorescent puncta within the cell.

Fig. 4B,C shows a positive PLA signal (left panels) for BF and PF trypanosomes expressing HA3::*TbKHAP1* and probed with anti-*TbKH* rabbit and anti-HA murine mAb. In contrast, when the anti-HA murine antibody is not employed (right panels), the PLA signal is absent, demonstrating the dependency of the signal on detection of both closely associated proteins. Similar results confirm that *TbKH* is in close physical proximity to *TbKHAP2* (Fig. 4D,E) and *TbKHAP3* (Fig. 4F,G).

Predicted properties of *TbKHAP1*, *TbKHAP2*, and *TbKHAP3*. Bioinformatic analysis of the 50.9 kDa *TbKHAP1* sequence indicates that it is a protein apparently unique to kinetoplastid protists for which there are orthologs widely distributed among Kinetoplastida. A BLASTP search (tritrypdb.org) revealed several coiled-coil proteins such as neurofilament proteins and tropomyosin as being significantly, although not closely, related (E values of 2.6e-08 – 5.4e-12). Prediction of protein disorder using the PrDOS web server (Ishida and Kinoshita, 2007) (http://prdos.hgc.jp/cgi-bin/top.cgi) generated a strong prediction of disorder (probability >0.9) over the C-terminal region from amino acids 314 – 461. Indeed, this sequence is rich in E residues, which predispose such regions to intrinsic disorder (Uversky, 2013), and this property suggests that this region could be involved in protein-protein interactions through induced folding (Zhang et al., 2013). InterPro (Mitchell et al., 2015) (https://www.ebi.ac.uk/interpro/search/sequence/) predicted coils between amino acids 7 – 31 and 196 – 241 and a disordered region from residue 332 - 461, and PSIPRED V4.0 (McGuffin et al., 2000) (http://bioinf.cs.ucl.ac.uk/psipred/) predicted the sequence to be largely helix or coil. Overall, computational analyses suggest that *TbKHAP1* is a coiled-coil protein with an
intrinsically disordered C-terminus, both properties that are consistent with formation of multi-
protein complexes.

*TbKHAP2* is the 374 kDa microtubule-associated repetitive protein 1, MARP-1, and
*TbKHAP3* is the 267 kDa MARP-2 that have been studied previously by Seebeck and
colleagues (Affolter et al., 1994; Hemphill et al., 1992; Schneider et al., 1988) and will hereafter
be referred to as *TbKHAP2/MARP-1* and *TbKHAP3/MARP-2* to indicate both their association
with *TbKH* and their previously demonstrated roles in microtubule binding. Each sequence
contains short unique N- and C-terminal domains, and the remainder of the sequence consists
of 38-amino acid repeats that are largely conserved within each sequence but ~50% identical
between the two proteins. The unique C-terminal domains (95% identical between the two
proteins) bind to microtubules (Affolter et al., 1994), and the proteins decorate the subpellicular
cytoskeleton (Schneider et al., 1988), but their specific biological functions have not been
elucidated. In addition, these proteins have also been localized to the basal body and
*TbKHAP3/MARP-2* was designated *TbBBP268* (Dang et al., 2017).

**Phenotypes of BF trypanosomes following knockdown of *TbKHAP1* RNA.** We have
previously demonstrated that knockdown of *TbKH* RNA by inducible RNAi results in a lethal
phenotype on both BF and PF trypanosomes (Sanchez et al., 2016). In these parasites, the
flagellum detaches from the cell body along the flagellum attachment zone (FAZ), and the
parasites are blocked in cytokinesis, resulting in accumulation of multi-nucleated, multi-
flagellated ‘monster cells’ that are not viable in the long term. To assess the roles of *TbKHAP1*,
*TbKHAP2/MARP-1*, and *TbKHAP3/MARP-2* in the biology of BF parasites, we targeted by RNAi
*TbKHAP1* mRNA, using a unique RNAi probe, and *TbKHAP2/TbKHAP3* mRNAs jointly, using a
500 nt probe covering the conserved C-termini, and assessed the consequent phenotypes.

Induction of RNAi against *TbKHAP1* in BFs using doxycycline resulted in rapid reduction in
the level of this protein (Fig. 5A). Furthermore, RNAi-induced parasites stopped growing almost
immediately and were largely dead by 72 h (Fig. 5B). Quantification via microscopy of the
percentage of cells with different numbers of nuclei and kinetoplasts (Fig. 5C) showed that
following induction of RNAi over 48 h, the percentage of 1N1K parasites dropped dramatically,
while those with multiple nuclei and kinetoplasts (XNYK) increased and began to predominate
the population. In comparison to the normal morphology of pre-induced parasites (Fig. 5D),
induction of RNAi for 20 h (Fig. 5E) or 48 h (Fig. 5F) resulted in parasites with multiple nuclei
and/or kinetoplasts and tadpole-like morphology (white arrowhead) or duplicated flagella located
at opposite poles of the cell body (yellow arrowhead).
Notable among cells in RNAi induced populations are those with multiple flagella located at various relative positions around the cell (e.g., the two parasites in Fig. 5F). Such parasites have initiated cytokinesis and cleavage furrow formation, as the two duplicated flagella have moved apart from the initial position they would have following flagellar duplication. However, the cleavage furrow did not progress to separate the duplicated nuclei and kinetoplasts as it would in normal cell division. In summary, these observations suggest that loss of \textit{TbKHAP1} protein from BF parasites results in a block in progression of cleavage furrow rather than an inability to initiate cleavage furrow ingression.

These results are further enhanced by more refined time course studies shown in Fig. S2 following the progression of nuclear content and cell morphologies between 0 – 48 h after induction of RNAi against \textit{TbKHAP1} RNA. At 4 h (Fig. S2B) and 8 h (Fig. S2C) most parasites had morphologies similar to that preceding induction of RNAi (0 h, Fig. S2A). However, by 12 h (Fig. S2D), multi-flagellated parasites with ingression furrows appeared, and by 24 h and 48 h (Fig. S2E,F), many parasites had incompletely resolved ingression furrows and multiple nuclei.

**Phenotypes of parasites following knockdown of \textit{TbKHAP2/MARP-1} and \textit{TbKHAP3/MARP-2} RNAs.** Induction of RNAi jointly against \textit{TbKHAP2/MARP-1} and \textit{TbKHAP3/MARP-2} resulted in complete loss of HA\textsubscript{3}::\textit{TbKHAP2/MARP-1} protein by 24 h (Fig. 6A). Depletion of \textit{TbKHAP2/MARP-1} and \textit{TbKHAP3/MARP-2} impaired growth of BF parasites, resulting in an ~50-fold reduction in parasite number by 120 h (Fig. 6B) post-induction, but growth inhibition was not nearly as pronounced as it is for \textit{TbKHAP1} RNAi (Fig. 5B). Compared to uninduced parasites (Fig. 6C), images of parasites following 4 d RNAi (Fig. 6D) still showed many parasites with normal morphology similar to that of uninduced parasites, but some parasites rounded up and showed multiple flagella (Fig. 6D, white arrowhead, flagella on opposite sides of the cell body in DIC image). By 4 d post-RNAi, parasites with 1N2K and XNYK began to accumulate (Fig. 6E), but the proportion was not nearly as great as for RNAi directed against \textit{TbKHAP1} (Fig. 5C).

\textit{TbKHAP1}, but not \textit{TbKHAP2/MARP-1} or \textit{TbKHAP3/MARP-2}, is required for targeting \textit{TbCaCh/FS179} to the flagellar membrane. The observation that \textit{TbKHAP1} is located at the base of the flagellum (Fig. 3A), likely in the basal body, raises the question of whether it could play a role in the function of \textit{TbKH} in mediating trafficking of the putative Ca\textsuperscript{2+} channel, \textit{TbCaCh/FS179}, to the flagellar membrane. To test this possibility, we induced RNAi against \textit{TbKHAP1} RNA in parasites expressing \textit{TbCaCh/FS179::HA\textsubscript{3}} tagged at the C-terminus, which localized to the flagellar membrane prior to RNAi (Fig. 7A). BF parasites induced for RNAi for 24 h (Fig. 7B) or 48 h (Fig. 7C) exhibited flagella that were devoid of \textit{TbCaCh::HA\textsubscript{3}} (white...
arrowheads). These results suggest that a complex of \( TbKH/TbKHAP1 \), and potentially other currently unknown partners located at the base of the flagellum, is involved in trafficking this channel to the flagellar membrane. Since both proteins are also located in the subpellicular cytoskeleton, it is not possible to definitively ascribe this flagellar trafficking phenotype to the complex at the base of the flagellum; complexes at both locations will be downregulated by \( TbKHAP1 \) RNAi. However, integral membrane proteins are first delivered to the flagellar pocket membrane during biosynthesis (Manna et al., 2014). Hence, the presence of a protein complex located close to the interface between the flagellar pocket and flagellar membrane, and for which downregulation of both known partners inhibits trafficking of a protein into the flagellar membrane, suggests that this complex may mediate trafficking of \( TbCaCh/FS179 \) from the flagellar pocket membrane into the flagellar membrane. Such trafficking would presumably be mediated by a direct interaction between \( TbKH \) and the cargo, \( TbCaCh/FS179 \), and such a molecular interaction has been demonstrated to occur (Sanchez et al., 2016).

In contrast, RNAi directed against \( TbKHAP2/MARP-1 \) and \( TbKHAP3/MARP-2 \) RNAs did not prevent trafficking of \( TbCaCh/FS179::HA3 \) into the flagellar membrane, where it is located prior to RNAi (Fig. 7D). At both 4 d (Fig. 7E) and 7 d (Fig. 7F) post RNAi, BF parasites with multiple flagella still trafficked this channel into the flagellar membrane (green arrowheads).

**TbKH-dependent trafficking of other flagellar membrane proteins.** \( TbKH \) is required for trafficking of \( TbCaCh/FS179 \) to the flagellar membrane of BF trypanosomes, and the two protein interact with each other, as demonstrated by crosslinking pulldown assays (Sanchez et al., 2016). Is \( TbKH \) important for flagellar trafficking of other membrane proteins? To address this question, we monitored the dependency of other flagellar membrane proteins on \( TbKH \) for targeting to that organelle. The TrypTag project (http://tryptag.org/) has defined the subcellular location of a large number of trypanosome proteins in PF parasites (Dean et al., 2017), employing live cell microscopy of parasites expressing mNeonGreen fluorescent protein fusions, and this endeavor has identified a cohort of flagellar membrane proteins.

One such flagellar membrane protein is \( Tb927.7.4270 \), a 25 kDa protein predicted to have a N-terminal signal sequence and a single transmembrane domain (TMD) near its C-terminus. This protein is one of four paralogous proteins (\( Tb927.7.4230, 4260, 4270, \) and 4280) studied previously by Shimogawa et al. (Shimogawa et al., 2015) and designated the Fam79 proteins (Fam79.1, 79.2, 79.3, and 79.4, respectively). To address potential dependency upon \( TbKH \) for flagellar targeting, we monitored the localization of the C-terminal mNG fusion of \( Tb927.7.4270/Fam97.3 \) in both formaldehyde fixed and live PF parasites immobilized in CyGEL (MacLean et al., 2013). As shown in Fig. 8A (0 h RNAi), this fusion protein is present in flagella
and also in filaments and vesicles that emerge from the flagella, and we designate Tb927.7.4270 as extracellular vesicle membrane protein 1 or TbEVMP1/Fam79.3. Multiple investigators have observed filaments and vesicles emerging from various parts of trypanosomes (Baudieri and Tomassini, 1962; Ellis et al., 1976; Molloy and Ormerod, 1964; Schepilewsky, 1912; Vickerman and Luckins, 1969; Wright and Lumsden, 1970), including the flagella, and a recent study by Szempruch et al. (Szempruch et al., 2016a; Szempruch et al., 2016b) has investigated such structures from BF parasites in detail and concluded that the flagellum-derived nanotubes and resulting extracellular vesicles (EVs) incorporate a cohort of parasite proteins. Furthermore, delivery of parasite-derived EVs to host red blood cells or to other trypanosomes can mediate pathogenic processes, such as erythrocyte clearance and anemia in the mammalian host or delivery of innate immune factors from a resistant to a sensitive strain of trypanosome. Hence, understanding the process for delivery of parasite proteins to these EVs is of importance for deciphering mechanisms of parasite virulence. Notably, when PF parasites expressing TbEVMP1::mNG were subjected to RNAi directed against TbKH for 24 h, they were strongly impaired in trafficking of this fusion protein to flagella or nanotubes (Fig. 8A, 24 h RNAi), and fluorescence often accumulated within the parasite cell body. White arrows indicate flagella that are devoid of fluorescence and which thus exhibit a trafficking defect. This result indicates that TbEVMP1/Fam79.3 is dependent upon TbKH, either directly or indirectly, for trafficking to the flagellum and subsequently for release into EVs. Furthermore, this trafficking defect occurs after 24 h of RNAi directed against TbKH, but these PF parasites do not exhibit significant loss of viability until ~10 days of continuous RNAi (Sanchez et al., 2016), indicating that the effects of TbKH RNAi upon flagellar trafficking are not due to global loss of cellular functions. Notably, Fam79.1 (Tb927.7.4230) was also detected by proteomic analysis in EVs of BF trypanosomes by Szempruch et al. (Szempruch et al., 2016b), indicating that multiple members of this family are delivered to the membranes of EVs during the parasite life cycle.

A second flagellar membrane protein localized in TrypTag is Tb927.11.1830, designated here as TbEVMP2. This 62 kDa protein has 6 predicted TMDs and is widely distributed among the kinetoplastid protists but does not have obvious orthologs outside that order, nor does it possess conserved Pfam domains (Sonnhammer et al., 1998), except for the TMDs and one predicted coiled coil. The TbEVMP2::mNG fusion protein is also localized to the flagellar membrane, nanotubes, and extracellular vesicles in PF parasites (Fig. 8B, 0 h RNAi), but induction of RNAi directed against TbKH also inhibits trafficking to these flagellar structures (Fig. 8B, 24 h RNAi), albeit less strongly than for TbEVMP1. Although fluorescence is visible in some
flagella after induction of RNAi (green arrows, righthand image for 24 h RNAi in Fig. 8B), there are some flagella that exhibit little if any fluorescence (white arrows). We designate this protein TbEVMP2 and suggest that it is a second such protein that is dependent upon TbKH for efficient trafficking to the surface of the flagellum.

In contrast, the mNG tagged FLA1 binding protein TbFLA1BP::mNG, which is in the flagellar membrane component of the FAZ (Sun et al., 2013), traffics efficiently to the FM both before and after induction of RNAi (Fig. 8C, 0 h, 24 h, and 48 h RNAi), even in PF parasites that have multiple flagella. Hence, TbFLA1BP does not require TbKH for targeting to the FM, implying that there are both TbKH-dependent and TbKH-independent FM proteins in this parasite.
DISCUSSION

KHARON is a cytoskeletal protein that plays multiple roles in the biology of both *T. brucei* and *L. mexicana*. In trypanosomes, *TbKH* is critical for flagellar trafficking of *TbCaCh/FS179* to the FM, and failure to traffic this channel to the flagellar component of the FAZ causes flagellar detachment and arrest of cytokinesis. Nonetheless, *TbKH* may affect cell viability by other mechanisms as well. In addition to its localization at the base of the flagellum, the protein is also present in the subpellicular cytoskeleton and mitotic spindles where it could also play roles vital to parasite viability.

In the current study, we have investigated three potential partners of *TbKH*: *TbKHAP1* and the two related proteins *TbKHAP2/MARP1* and *TbKHAP3/MARP2*. *TbKHAP1* can be crosslinked to *TbKH* by formaldehyde indicating that the two proteins associate within 2-3 Å of each other. *TbKHAP2* and *TbKHAP3* are close enough to *TbKH* to give a consistently positive signal using the PLA, that is within 400 Å, but the degradation of these two *TbKHAPs* under conditions of crosslinking and solubilization has prevented us from definitively demonstrating their interaction at the near atomic level. The knockdown of *TbKHAP1* RNA has the most pronounced phenotype, strongly arresting division of BF parasites, inhibiting progression of the cleavage furrow during cytokinesis, and impairing trafficking of *TbCaCh/FS179* to the FM. Hence, the phenotypes of RNAi for both *TbKH* and *TbKHAP1* are similar. In contrast, efficient knockdown of *TbKHAP2/3* RNAs slows growth of BF parasites but has a much less severe effect on cell division than knockdown of *TbKHAP1* RNA. In addition, trafficking of *TbCaCh/FS179* to the FM is not impaired, even after 7 days of knockdown in BFs. These results imply that although both types of protein likely associate with *TbKH* in the cytoskeleton, they play different roles. These distinctions in functions could either result from separate complexes between *TbKH* and each partner or from different roles that each partner plays in the same complex. The association of all partners with the cytoskeleton complicates this issue, as one cannot readily separate different complexes from each other, as would be possible for cytosolic multi-protein complexes. Nonetheless, these studies confirm that *TbKH* associates with other partner proteins that mediate its activities in different ways.

One central activity for *TbKH* is to traffic *TbCaCh/FS179* to the FM, a process that is critical for integrity of the FAZ and for parasite viability. The localization of many PF proteins to their subcellular sites achieved in the TrypTag.org project (Dean et al., 2017) has identified some additional FM proteins, along with some others that were identified previously as FM components from various targeted studies (Kelly et al., 2020a), and one question of relevance is how many of these flagellar surface components rely upon *TbKH* for organellar trafficking. For
TbCaCh/FS179, TbKH appears to be directly involved in trafficking, since the two proteins can be crosslinked by formaldehyde and isolated as molecular partners (Sanchez et al., 2016), but it is possible that others depend upon TbKH either directly or indirectly via the ability of this protein to affect various cellular processes. In this study, we have shown that two additional FM proteins, TbEVMP1/Fam79.3 (Tb927.7.4270) and TbEVMP2 (Tb927.11.1830) are present in both the FM and in EVs secreted from the FM in PF trypanosomes. Both proteins exhibit dependency upon TbKH for trafficking to the FM, as RNAi directed against TbKH reduces the efficiency of their localization to this organelle. In contrast, FLA1BP, which participates in adhesion of the flagellum to the cell body by binding to the FLA1 protein in the cell body component of the FAZ (Sun et al., 2013), is not dependent upon TbKH to reach the FM, confirming that both TbKH-dependent and TbKH-independent FM proteins exist.

EVs released from the cell body and FM of BF trypanosomes play important roles in virulence of African trypanosomes, including lysis of host erythrocytes leading to anemia, a major mechanism of trypanosome-mediated pathogenesis (Szempruch et al., 2016a; Szempruch et al., 2016b). One might anticipate that surface components of EVs could play important roles in either formation of the EV membrane or interaction of EVs with mammalian or tsetse fly tissues. TbEVMP1 and TbEVMP2 mRNAs are both preferentially expressed in PF trypanosomes (tritrypdb.org), but paralogs of TbEVMP1, such as Tb927.7.4230 and Tb927.7.4260, are expressed at higher levels in BFs compared to PFs, suggesting potential roles for such EVMPs in both life cycle stages.

The TbKH partners discovered in this study are associated primarily with the subpellicular microtubules, but there are likely to be other partners that may reside principally at the base of the flagellum or in the mitotic spindles and could be associated with distinct activities at those sites. A BioID study by Zhou et al. (Zhou et al., 2018) identified five spindle-associated proteins, NuSAP1, NuSAP2, Kif13-1, TbMIP2, and TbAUK1, that are in proximity to TbKH and are thus candidates for molecular partners at the mitotic spindle. In addition, Akiyoshi and Gull (Akiyoshi and Gull, 2014) identified kinetoplast kinetochore proteins (KKTs) that associate with KHARON by CoIP/MS experiments, suggesting a possible role of KHARON in faithful chromosome segregation in kinetoplastid parasites. Molecular interaction studies of the type carried out here will be required to determine which of these proteins may be bona fide molecular partners with TbKH and what roles KHARON complexes may be playing at the spindle. Similarly, the ability to isolate flagella with associated kinetoplast DNA and basal bodies (Oberholzer et al., 2011; Robinson and Gull, 1991; Subota et al., 2014) should facilitate identification by either BioID or TAP-MS of additional TbKH partners at the base of the flagellum. Thus, it should be possible to
achieve a comprehensive understanding of the role of KHARON in the cytoskeleton and the
distinct functions it carries out in association with different partner proteins.
MATERIALS AND METHODS

Growth and transfection of *T. brucei* cell lines. BF and PF *T. brucei* cell lines were grown as described previously (Sanchez, 2013). For T7 RNA polymerase-independent driven expression the BF/pHD1313 or PF/pHD1313 clones were employed (Sanchez et al., 2016). For T7 RNA polymerase driven expression in BF, *T. brucei* 427 parasites transfected with the pSmOx (Poon et al., 2012) plasmid expressing the tetracycline repressor and T7 RNA polymerase were generated and grown in 0.1 µg/ml puromycin. For T7 RNA polymerase driven expression in PF, *T. brucei* 427 13-6 clone expressing TETR and T7 RNA polymerase was used (Wirtz et al., 1999). Linear plasmid or PCR DNA amplicons were used to transfect mid-log phase parasites as described (Sanchez et al., 2016). Transfected clones were obtained by limiting dilution according to published protocols (Burkard et al., 2007; McCulloch et al., 2004).

**Primary amino acid sequence analysis.** For DNA and amino acid sequence analysis of *TbKHAPs*, ExPASy, via the SIB Bioinformatics Resource Portal (http://expasy.org), and GeneBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or TritrypDB (http://tritrypdb.org/tritrypdb) were used.

**Inhibition of gene expression by RNAi.** To inhibit the expression of *TbKH*, *TbKHAPs* or *TbFLA1* a number of cell lines were generated employing different genetic backgrounds. The BF and PF *TbKH* RNAi and *TbFLA1* RNAi clones, for which expression is T7 polymerase-independent, were previously described (Sanchez et al., 2016), and RNAi was induced by adding doxycycline (1 µg/ml) to the culture medium. The BF and PF *TbKHAP1* RNAi clones were generated by subcloning the first 500 bp of the *TbKHAP1* (Tb927.10.1026) ORF into the pZJM RNAi vector, where expression is driven by two opposing T7 promoters (Wang et al., 2000). Similarly, BF and PF *TbKHAP2/3* RNAi clones were generated by subcloning the last 500 bp of *TbKHAP2* (Tb927.10.10360) that is almost identical to the *TbKHAP3* (Tb927.10.10280) ORF into the pZJM RNAi vector. RNAi clones were selected by resistance to 2.5 µg/ml phleomycin and 0.1 µg/ml puromycin, and expression of dsRNA was induced by addition of 1 µg/ml doxycycline. To verify inhibition of *TbKHAP1-3* expression, total cell lysates were obtained from parasite cultures induced for RNAi and subjected to Western blot experiments as indicated below.

**Endogenous epitope tagging.** For endogenous tagging of *TbKH*, *TbKHAP1*, *TbKHAP2*, *TbKHAP3*, CAP-15, *TbCaCh* (FS179), *TbEVMP1*, *TbEVMP2* and *TbFLA1BP* at the N-terminus or C-terminus, different epitopes were employed as indicated in the text, following the protocol described (Dean et al., 2015). Briefly, epitope-tagging cassettes were generated by using two specific 100 nt oligonucleotides containing ~80 nt each that are homologous to the ORF region
to be tagged and ~20 nt homologs to the plasmid pPOTV4 template flanking the drug resistance marker cassette, and using the universal PCR settings. Epitope tagging PCR cassettes were ethanol precipitated and resuspended in 10 µl of nucleofection buffer (Wang et al., 2000), then parasites were transfected with the purified tagging cassettes as described (Dean et al., 2015) and selected using 1.5 µg/ml G418 (15 µg ml\(^{-1}\) for PF) or 0.1 µg/ml puromycin (1 µg/ml for PF), and cloned by limiting dilution.

**Generation of Rabbit anti-\(Tb\)KH antibody.** A custom rabbit anti-\(Tb\)KH polyclonal antibody (pAb) was generated by GenScript, using their 49-day antibody generation protocol. Briefly, two rabbits were injected with 200 µg of His\(_6::\)\(Tb\)KH, representing amino acids 43 - 411, emulsified in Freund’s complete adjuvant. The rabbit was boosted 3 times at 14-day intervals with 200 µg of His\(_6::\)\(Tb\)KH emulsified in Freund’s incomplete adjuvant. Antibody specificity for \(Tb\)KH was evaluated by western blot comparing the reactivities of the rabbit serum from immunized rabbits to \(T. brucei\) protein lysates from wild-type cells and N-BirA*::\(Tb\)KH cell line (Fig 3A). Proteins were immunodetected using 1:2500 dilution of the rabbit anti-\(Tb\)KH polyclonal antibody and 1:15,000 dilution of goat anti-rabbit-HRP antibody (Sigma-Aldrich). The chemiluminescent protocol was used for developing as indicated below.

**Immunofluorescence microscopy.** For immunofluorescence microscopy, 5 X 10\(^6\) parasites were centrifuged at 1000 X g for 5 min and washed twice at room temperature with phosphate buffered saline pH 7.2 (PBS) containing 10 mM glucose. The cell pellet was resuspended in 4% paraformaldehyde in PBS, pH 7.2 and incubated for 15 min at room temperature, cells were centrifuged as described above and washed once with PBS, resuspended in 100 µl PBS, spotted onto poly-L-lysine coated coverslips, permeabilized with 0.1% Triton X-100 in PBS for 1 min, and washed 3X with PBS. Then parasites were blocked with 2% goat serum, 0.01 sodium azide, 0.01 saponin in PBS (blocking solution) for 1 h at room temperature, rinsed 3 X with PBS, and incubated with primary antibodies for 1 h at room temperature. The following primary antibodies were employed: 1:250 dilution rabbit anti-\(Tb\)KH pAb (reported in this work), 1:500 dilution mouse anti-HA monoclonal antibody (mAb) (BioLegend, Cat # MMS-101R), and 1:1000 dilution mouse anti-\(\alpha\)-tubulin mAb (Sigma-Aldrich, Cat. # T5168). Subsequently, cells were rinsed as before and incubated with a 1:1000 dilution of secondary antibodies coupled to Alexa Fluor dyes (Molecular Probes) as follows: Alexa Fluor® 488 goat anti-mouse IgG (H+L) (Cat. # A11001), Alexa Fluor® 594 goat anti-mouse IgG (H+L) (Cat. # A11005), Alexa Fluor® 594 goat anti-rabbit IgG (H+L) (Cat. # A11012) and Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Cat. # A11008), as indicated, in blocking solution for 1h at room temperature in the dark. Cover slips were rinsed 3 X with PBS and mounted onto slides using...
DAPI Fluoromount-G (SouthernBiotech). Fluorescence images were obtained using a wide field deconvolution system (Applied Precision Instruments, Inc.) consisting of an inverted Nikon TE 200 Eclipse microscope, a Kodak CH350 CCD camera, and the Deltavision operating system. Images were acquired using a 60 X objective and 1.25 X magnification in a 1024 X 1024 format, and deconvolved using SoftWoRx software. Adobe Photoshop CC and Adobe Illustrator CC (Adobe Systems Inc.) were used to create image compositions.

**Flagellar protein purification, formaldehyde crosslinking, pull down, and Western blot assay.** Flagellar purification was performed from BF parasites (Fig. 3A,B and Fig. S1A,B) as described (Subota et al., 2014) with slight changes. Briefly, BF *TbKH1RNAi*, *TbFLA1RNAi*, *TbKHAP1RNAi* and *TbKHAP2/3RNAi* clones were grown in 1 µg/ml doxycycline for the indicated times and pelleted at 420 X g for 10 min. Cell pellets were washed with buffer A (25 mM Na+-tricine, pH 7, 1% BSA, 0.1 mM CaCl₂, 0.2 mM EDTA, 5 mM MgCl₂ and 12 mM β-mercaptoethanol) containing 0.32 M sucrose and centrifuged at 420 X g for 10 min. Cell pellets were gently resuspended at 3 X 10⁸ parasites/ml in buffer A plus 0.3 M sucrose, transferred into Eppendorf tubes and vortexed for 5 min or until microscopic verification of flagellum detachment, followed by centrifugation at 420 X g for 10 min. Supernatants were recovered and centrifuged at 16,000 X g for 20 min at 4 °C. Pellets containing the isolated flagella were resuspended in 200 µl of PBS and used for immunofluorescence microscopy. Also, flagellar purification from PF clones without induction of RNAi (Fig. 3C and Fig. S1C) was performed by isolation of cytoskeletons followed by treatment with 1 mM CaCl₂, according to Imhof et al. (Imhof et al., 2019).

For formaldehyde crosslinking and pull down, cell lines were prepared that expressed *TbKH1* endogenously tagged with the His₁₀ epitope at the N-terminus (His₁₀::*TbKH1*) and also co-expressed with a protein of interest endogenously tagged at its N- or C-terminus with a HA₃ or V₅₃ epitope (HA₃::*TbKHAP1*, HA₃::*TbKHAP2*, *TbKHAP2::HA₃*, V₅₃::*TbKHAP3*, HA₃::*TbKHAP3* and *TbCAP15::HA₃*). Parasites were washed once with PBS and pelleted at 1000 X g for 10 min, and cell pellets resuspended in 9.37 ml PBS plus 0.63 ml 16% formaldehyde-EM grade (Polysciences, Inc) and incubated at room temperature for 10 min. For non-crosslinked control samples, PBS was added instead of formaldehyde. Subsequently, 1 ml of 2.5 M glycine in PBS was added to the crosslinked samples and incubated at room temperature for 5 min, crosslinked parasites were pelleted at 1000 X g for 10 min and washed twice with PBS. Then, cell pellets with or without crosslinking were resuspended in 1 ml of Buffer 1 (8 M urea, 300 mM NaCl, 0.5% Nonidet P-40, 50 mM NaH₂PO₄, 50 mM Tris, pH 7.0, 10 mM imidazole) on ice. Samples were sonicated 3 times on ice at 50% max amplitude (Sonic
Dismembrator, 500W, Fisher Scientific) for 10 s with 30 s between pulses. A 2.5% aliquot of this protein lysate was saved as the protein lysate fraction (LF).

The remainder of the protein was incubated with 50 µl of Ni-NTA Magnetic Beads (New England Biolabs, Inc.) on a rocker for 45 min at room temperature. The beads were washed three times according to the manufacturer’s instructions using Buffer 1 plus 20 mM imidazole. Finally, bound protein complexes were eluted with 100 µl Buffer 1 containing 500 mM imidazole. Crosslinking was reversed by boiling protein samples for 30 min in 1X Bolt™-LDS sample buffer (Life Technologies) containing 10 mM DTT.

Protein extracts were prepared and analyzed by Western blot employing Bolt™ 4-12% Bis-Tris Mini Protein Gels or NuPage™ 3-8% Tris-acetate gels, Mini Gel Tank and Mini Blot Module following the manufacturer’s instructions (Life Technologies). Proteins were transferred onto PVDF membranes (Millipore). Protein immunodetection was done using rabbit anti-\(TbKH\) pAb at 1:2500 dilution, mouse anti-HA mAb (BioLegend, Cat # MMS-101R) 1:2500 dilution, mouse anti-V5 mAb (Invitrogen, Cat # MA5-15253) 1:2500 dilution, and mouse anti-\(\alpha\) tubulin mAb (Sigma-Aldrich, Cat. # T5168) 1:10,000 dilution. Goat anti-rabbit-HRP (Sigma-Aldrich) 1:15,000 dilution and goat anti-mouse-HRP (Jackson ImmunoResearch Laboratories, Cat. # 115-03-174, Lot # 117119) were used as secondary antibodies and Western blots were developed using the SuperSignal™ West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific) and an Image Quant LAS 400 (GE Healthcare) scanner was employed to acquire luminescent images. Adobe Photoshop CC and Adobe Illustrator CC (Adobe Systems Inc.) were used to create image compositions.

Proximity ligation assay (PLA). Mid-log BF or PF parasites were harvested by centrifugation at 1000 x g for 10 min, washed once in PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Fixed cells were attached to cover slips and permeabilized as indicated above for immunofluorescence analysis. Subsequently the PLA protocol was followed according to the Duolink In Situ Red Starter Kit Mouse/Rabbit (Millipore Sigma) instructions. Briefly, after blocking cells were incubated with 1:250 dilution of rabbit anti-\(TbKH\) pAb and 1:500 dilution of mouse anti-HA mAb (BioLegend, Cat# MMS-101R). As a negative control, cells were incubated only with 1:250 dilution of the rabbit anti-\(TbKH\) pAb. Then PLA species-specific secondary antibodies with minus and plus oligonucleotide probes were added, followed by ligation, amplification and hybridization with specific red-fluorescent oligonucleotides to allow detection by fluorescence microscopy. Samples were mounted and imaged as described for immunofluorescence analysis.
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Competing interests

The authors declare no competing or financial interests.

Author contributions


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References


**FIGURE LEGENDS**

**Fig. 1.** *TbKH* localizes in three distinct subcellular compartments. (A) Flagellar preparation from *TbFLA1*<sup>RNAi</sup> cell line, isolated after 72 h of induction with 1 µg/ml doxycycline (dox), stained with DAPI (*blue*) and immunostained with anti-*TbKH* pAb (*TbKH*, *red*). *TbKH* localization at the base of the flagellum is indicated by the *red arrowhead*. (B) Wild type BF parasites were immunostained with anti-*TbKH* pAb (*TbKH*, *red*) and stained with DAPI. These images display a parasite in which *TbKH* staining is associated with both the subpellicular microtubules (*right arrowheads*) and the mitotic spindle that connects the two nuclei late during mitosis (*left arrowheads*). DIC indicates images collected by differential interference contrast microscopy. (C) Western blot of total protein lysates from BF trypanosomes that are either wild type (*WT*) or expressing BirA*::*TbKH fusion protein (*A*<sup>+</sup>). Blot was probed with anti-*TbKH* pAb and developed by chemiluminescence. Specific immunodetected proteins corresponding to the native *TbKH* and the BirA*::*TbKH fusion protein are shown. Relative protein molecular weights are shown in kDa, as determined by mobility relative to molecular weight markers.

**Fig. 2.** Subcellular localization of *TbKHAP*s in BF and PF *T. brucei*. (A, B) The HA<sub>3</sub>::*TbKHAP1* cell line was immunostained with anti-HA mAb (*HA<sub>3</sub>::*TbKHAP1*, *green*) and anti-*TbKH* pAb (*TbKH*, *red*). (C, D) The *TbKHAP2*::HA<sub>3</sub> cell line was stained with anti-HA mAb (*TbKHAP2*::HA<sub>3</sub>, *green*) and anti-*TbKH* pAb (*TbKH*, *red*). (E) The BF 3V5::*TbKHAP3* and (F) PF HA<sub>3</sub>::*TbKHAP3* cell lines were immunostained with (E) anti-V5 mAb (3V5::*TbKHAP3*, *green*) or (F) anti-HA mAb (HA<sub>3</sub>::*TbKHAP3*, *green*) and anti-*TbKH* pAb (*TbKH*, *red*). All preparations were stained with DAPI, which detects both nuclear and kinetoplast DNA (*blue*). DIC images were also acquired from all samples.

**Fig. 3.** KHARON and KHAPs colocalize at the base of the flagellum in *T. brucei*. (A) Whole isolated flagella from the BF HA<sub>3</sub>::*TbKHAP1*/*TbFLA1*<sup>RNAi</sup> clone induced for RNAi for 72 h, were immunostained with anti-HA mAb (HA<sub>3</sub>::*TbKHAP1*, *green*) and anti-*TbKH* pAb (*TbKH*, *red*) and stained with DAPI (*blue*). (A, left panel) shows an immunofluorescence/DIC image of an isolated flagellum, *white punctuated* box indicates the magnified area depicted in (A, center-right, center-left and left) panels. *Green arrowheads* indicate HA<sub>3</sub>::*TbKHAP1* localization, *red arrowheads* indicate *TbKH* localization and *yellow arrowheads* indicate overlapping signals, when channels are merged, near the kinetoplast (*blue*). (B) Flagella were isolated from BF trypanosomes expressing HA<sub>3</sub>::*TbKHAP2* and imaged as described in (A). (C) Flagellar cytoskeletons were isolated from PF trypanosomes expressing HA<sub>3</sub>::*TbKHAP3* and imaged as described in (A).

**Fig. 4.** *TbKH* is closely associated with *TbKHAP1*, *TbKHAP2*, and *TbKHAP3*. (A) Western blot of protein samples from pull down of HA<sub>3</sub>::*TbKHAP1* using His<sub>10</sub>::*TbKH* as a bait and
CAP15::HA3 as negative control. LS and EF, lysate and elution fractions without formaldehyde crosslinking; LS* and EF*, lysate and elution fraction with formaldehyde crosslinking. Protein blots were developed by chemiluminescence, bands are indicated by protein names, and migration of molecular weight markers are designated in kDa. (B, C) PLA employing BF or PF HA3::TbKHAP1/His10::TbKH cell lines, as indicated. For these and all subsequent sections of this figure, the left panel shows results using both anti-HA mAb and anti-TbKH pAb, and the right panel shows results using only the anti-TbKH pAb, as the negative control. Red puncta indicate positive HA3::TbKHAP1-TbKH interaction. DIC images were acquired from all samples. (D, E) PLA employing BF or PF TbKHAP2::HA3 cell lines, as indicated. Red puncta indicate positive TbKHAP2-TbKH interaction. (F, G) PLA employing BF or PF HA3::TbKHAP3 cell lines, as indicated using. Red puncta indicate positive TbKHAP3-TbKH interaction.

**Figure 5. Depletion of TbKHAP1 is lethal for T. brucei parasites.** (A) Western blot of total protein lysates from TbKHAP1RNAi BF parasites grown in the presence of doxycycline (+dox) and immunodetected with anti-HA mAb, anti-TbKH pAb. The approximate molecular weights in kDa of HA3::TbKHAP1 and TbKH are indicated as determined by mobility compared to weight markers. Numbers under the blot represent the relative intensity (RI) of HA3::TbKHAP1 protein normalized to the TbKH protein level. (B) Cell density for induced (empty circles) and non-induced (filled circles) TbKHAP1RNAi BF cell lines. Parasite density was quantified by phase contrast microscopy using a hemocytometer. Data represent the averages and standard deviations of two experiments, each employing an independently isolated TbKHAP1RNAi clonal cell line, and technical replicates were also performed for each biological replicate. Standard deviations are too small to be visible. (C) Microscopic analysis of nuclei and kinetoplast numbers of BF TbKHAP1RNAi parasites following induction of RNAi. Data represent frequency (%) of cells with different numbers of DAPI stained nuclei (N) and kinetoplasts (K). Results represent the average and range of two independent experiments. (D-F) TbKHAP1RNAi BF parasites were stained with DAPI (blue) and immunostained with anti-α-tubulin mAb (Tub, red) at (D) 0 h, (E) 20 h and (F) 48 h post-RNAi induction. In (E) the white arrowhead indicates a cell with a tadpole-like morphology and the yellow arrowhead indicates a cell with two flagella at opposite poles of the cell body.

**Fig. 6. Phenotypes resulting from RNAi directed against TbKHAP2 and TbKHAP3.** (A) Western blot of total protein lysates of BF parasites expressing HA3::TbKHAP2 following induction of RNAi (1 µg ml⁻¹ doxycycline, dox) directed against TbKHAP2/3 RNAs. Protein blots were probed with anti-HA mAb and anti-TbKH pAb as loading control. Molecular weight markers are indicated in kDa. (B) Growth curve of induced (empty circles) and non-induced (filled circles)
TbKHAP2/3RNAi cell line. Parasite density was quantified by phase contrast microscopy using a hemocytometer. The data represent two biological replicate experiments, but the standard deviations are too small to see in the figure. Representative TbKHAP2/3RNAi cells induced for RNAi were stained with DAPI (blue) and immunostained with anti-TbKH pAb (TbKH, red) at (C) 0 h and (D) 4 days post-RNAi induction. White arrowhead in (D) indicates a cell showing aberrant morphology. DIC images were obtained for all samples. (E) Microscopic analysis of NK in BF parasites undergoing RNAi. Frequency (%) of cells with different numbers of nuclei (N) and kinetoplasts (K) at different times following induction of RNAi against TbKHAP2/3 mRNA. Results represent the average and range of two independent experiments.

**Fig. 7. Knockdown of TbKHAP1, but not TbKHAP2 or TbKHAP3, impairs trafficking of TbCaCh::HA3 to the flagellum.** (A-C) TbCaCh::HA3/TbKHAP1RNAi BF cell line was induced with 1 µg/ml doxycycline (dox). Parasites were stained with DAPI (blue) and immunostained with anti-HA mAb (TbCaCh::HA3, green) and anti-TbKH pAb (TbKH, red). (A) Non-induced TbCaCh::HA3/TbKHAP1RNAi parasites (0 h dox), (B) parasites induced for 24 h (24 h dox) and (C) parasites induced for 48 h (48 h dox). Green arrowheads in (A-C) indicate flagella where TbCaCh::HA3 is present, and white arrowheads in (B-C) indicate flagella where TbCaCh::HA3 is absent. (D-F) TbCaCh::mNG/TbKHAP2/3RNAi BF cell line was induced with 1 µg/ml doxycycline (dox). Parasites were stained with DAPI (blue) and anti-TbKH pAb (TbKH, red), and mNG endogenous fluorescence was also acquired (TbCaCh::mNG, green). Parasites were induced with doxycycline for (D) 0 days (0 d dox), (E) 4 days (4d dox), or (F) 7 days (7d dox). Green arrowheads indicate flagella where TbCaCh::mNG is present. DIC images are indicated. The scale bar shown in (A) applies to all images.

**Fig. 8. Trafficking of TbEVMP1, TbEVMP2, and TbFLA1BP in TbKHRNAi BF parasites.** (A) Parasites expressing TbEVMP1::mNG before (0 h RNAi) and after (24 h RNAi) induction of RNAi against TbKH mRNA. Green arrowheads indicate mNG fluorescence in the FM (all panels) or in extracellular vesicles (0 h RNAi, right panel). White arrowheads indicate flagella without mNG fluorescence. (B) Parasites expressing TbEVMP2::mNG before (0 h RNAi) and after (24 h RNAi, 48 h RNAi) induction of RNAi against TbKH mRNA. (C) Parasites expressing TbFLA1BP::mNG before (0 h RNAi) and after (24 h RNAi, 48 h RNAi) induction of RNAi against TbKH mRNA. The left-most images in A, B, and C represent formaldehyde fixed parasites, whereas for the other images, live parasites were suspended in CyGel, which facilitates visualization of secreted extracellular vesicles.
<table>
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Figure 2
Figure 3

A  
DIC  

7 µm  

TbKHAP1  
TbKH  
merged  

B  
DIC  

7 µm  

TbKHAP2  
TbKH  
merged  

C  
DIC  

4 µm  

TbKHAP3  
TbKH  
merged
Figure 8

A  *TbEVMP1* DAPI  

B  *TbEVMP2*  

C  *TbFLA1BP*  

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