

## KHARON is an Essential Cytoskeletal Protein Involved in the Trafficking of Flagellar Membrane Proteins and Cell Division in African Trypanosomes\*

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Running title: *KHARON is essential for African trypanosomes*

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### ABSTRACT

African trypanosomes and related kinetoplastid parasites selectively traffic specific membrane proteins to the flagellar membrane, but the mechanisms for this trafficking are poorly understood. We show here that KHARON, a protein originally identified in *Leishmania* parasites, interacts with a putative trypanosome calcium channel and is required for its targeting to the flagellar membrane. KHARON is located at the base of the flagellar axoneme, where it likely mediates targeting of flagellar membrane proteins, but is also on the subpellicular microtubules and the mitotic spindle. Hence, KHARON is probably a multifunctional protein that associates with several components of the trypanosome cytoskeleton. RNA interference mediated knockdown of *KHARON* mRNA results in failure of the calcium channel to enter the flagellar membrane, detachment of the flagellum from the cell body, and disruption of mitotic spindles. Furthermore, knockdown of *KHARON* mRNA induces a lethal failure of cytokinesis in both

bloodstream (mammalian host) and procyclic (insect vector) life cycle stages, and KHARON is thus critical for parasite viability.

African trypanosomes such as *Trypanosoma brucei* and related subspecies are parasitic protists that cause sleeping sickness in humans and the wasting disease nagana in cattle. Between 2010 and 2013 there were ~7,000 new cases reported per year (1), although considerably larger numbers of people are at risk for infection (2), and disease in domestic livestock represents a major economic burden in impoverished regions of Africa. In addition to the medical and veterinary impact of these parasites, they also exhibit many fascinating and unusual biological features and their study has led to novel discoveries such as glycosylphosphatidylinositol anchors for membrane proteins, trans-splicing of mRNAs, extensive editing of mitochondrial mRNAs, and global polycistronic transcription of gene arrays.

These parasites are flagellated in all life cycle stages, including the bloodstream forms (BFs) that infect the mammalian host and in all of the developmental stages that infect the tsetse fly vector. Given the multiple important roles that flagella play for trypanosomes, these cellular appendages have been studied extensively (3,4). While studies on axonemal and luminal proteins predominated originally, considerable work has been done more recently on proteins of the trypanosome flagellar membrane (5). One of the first such proteins identified was a receptor type adenylate cyclase (6) that is likely involved in sensing the extracellular environment and relaying signals via synthesis of cAMP, and recent work on this family of proteins has uncovered roles for evasion of host innate immunity (7) and in social motility in insect stage procyclic forms (PFs) (8-10). Furthermore, a variety of putative transporters and channels are present in the flagellar membrane (5) where they may play roles in nutrient or ion uptake and potentially in nutrient or solute sensing (11). Other important roles for the flagellar membrane are attachment to the salivary gland epithelium in the tsetse fly (12), flagellar adherence of gametes during mating (13), and attachment of the tip of the new flagellum to the body of the old flagellum during parasite replication, where attachment of flagella is required for cleavage furrow formation and proper cell division (14-16).

While a host of proteins are known that are targeted selectively to the flagellar membrane of trypanosomes or related parasites such as *T. cruzi* or *Leishmania* (17-20), the mechanisms for this selective surface targeting are largely obscure. In studies of the flagellar specific glucose transporter 1 from *L. mexicana*, *LmxGT1*, our laboratory has identified a novel protein KHARON<sup>3</sup> (KH) that interacts with the flagellar targeting signal and mediates trafficking of this permease to the flagellar membrane (21). While this ~57 kD protein did not exhibit detectable sequence similarity to proteins outside the kinetoplastid parasites nor did it possess any recognizable conserved sequence motifs (21), the existence of orthologs of KH in all kinetoplastid parasites whose genomes have been sequenced suggested that this protein might be important for flagellar targeting of multiple membrane proteins across this family of parasites. However to date the role

of KH in other parasites has not been investigated. Furthermore various genetic advantages of *T. brucei*, such as the ability to readily knock down mRNAs by inducible RNA interference (RNAi), as well as the existence of a flagellar membrane proteome (5), suggested that this parasite might represent a particularly facile system with which to study targeting of flagellar membrane proteins. In the present study, we have investigated KH from *T. brucei* (*TbKH*) and established that it is involved in flagellar trafficking of a putative calcium channel, *TbCaCh* (also called FS179) (5). Notably, RNAi-mediated knockdown of *KH* mRNA not only prevented flagellar trafficking of *TbCaCh*, but it was also lethal to both bloodstream form (BF) and insect stage procyclic form (PF) parasites, resulting in detachment of flagella from the cell body via the flagellar attachment zone (FAZ) and failure of the parasites to undergo cytokinesis. Subcellular localization of KH demonstrated that this protein is located at the base of the flagellar axoneme, where it is likely to mediate import of integral membrane proteins into the flagellar membrane, but also on the subpellicular microtubule cytoskeleton and unexpectedly on the mitotic spindle. Hence KH is a central component of flagellar membrane transport machinery and also associates with multiple components of the trypanosome cytoskeleton, where it is likely to serve diverse functions in parasite cell biology. The results reported here establish *T. brucei* as an ideal experimental system in which to study the multiple functions of KH and in particular to dissect the mechanism whereby it mediates trafficking of integral membrane proteins to the flagellar membrane.

## RESULTS

*Identification of TbKH and sub-cellular localization in BF trypanosomes.* Recent studies by our laboratory identified the KHARON protein of *Leishmania mexicana*, *LmxKH*, as a cytoskeletal-associated protein that mediates the translocation of the *LmxGT1* glucose transporter to the flagellar surface membrane (21). Since a BLAST search employing GeneBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and TriTrypDB (<http://tritrypdb.org/tritrypdb>) revealed

the presence of KH orthologs in kinetoplastid parasites but not in other organisms, we hypothesized that KH might play a fundamental biological role in this entire family of protists. Protein sequence alignment of *LmxKH* (gene ID in TriTrypDB: LmxM.36.5850) and *TbKH* (gene ID: Tb927.10.8940) revealed 27% (112/411) identity and an additional 11% similarity, and several segments of sequence that are present in *LmxKH* (520 amino acids) are absent from *TbKH* (411 amino acids). Hence while KH exists in trypanosomes, it is relatively divergent in sequence between these two related parasites, thus underscoring the need to test the biological functions in both organisms. A conserved sequence signature encompassing amino acids 227-235 of *TbKH* (NESVDVLNL) is the largest block of contiguous sequence present in both KH orthologs.

To determine the subcellular localization of *TbKH* in BF parasites, transgenic parasites were generated in which the *TbKH* open reading frame was endogenously tagged with the Ty1 epitope on the N-terminus or with the HA<sub>3</sub> epitope on the C-terminus. To facilitate isolation of separated flagella for some experiments, these transgenic lines were also prepared in a parasite genetic background, the *TbFLAI*<sup>RNAi</sup> line, allowing inducible RNA interference (RNAi) (22,23) against the *TbFLAI* gene (24). Inducing degradation of *TbFLAI* mRNA results in separation of flagellar attachment to the cell body by disrupting the Flagellar Attachment Zone (FAZ), the interface that encompasses parts of both the flagellar and cell body membranes and associated structures where the two membranes adhere (25). This disruption allows the non-adherent flagella to be sheared off the cell body and purified by differential centrifugation (26). Immunofluorescence of the transgenic BFs demonstrated that epitope tagged *TbKH* was located in three discrete compartments. 1) In purified flagella, Ty1::*TbKH* localizes at the base of the flagella (Fig. 1A). 2) In whole cell images, Ty1::*TbKH* localizes in the sub-pellicular microtubule cytoskeleton (Fig 1B) as indicated by the overlap of the *TbKH* signal with  $\alpha$ -tubulin signal. In addition, these two fluorescence signals also overlap in detergent-extracted cytoskeletons from BF parasites (Fig. 1C), consistent with *TbKH* being a cytoskeletal-associated protein. 3)

Unexpectedly, Ty1::*TbKH* also localizes in the mitotic spindle in dividing BF parasites (Fig 1D), where it exhibits intense fluorescence on a tubule that connects two replicated nuclei. To confirm that this localization represents the mitotic spindle, parasites were co-stained with the KMX-1 antibody (Fig. 1E) that preferentially recognizes  $\beta$ -tubulin associated with the spindle (27). These images show strong overlap of the Ty1::*TbKH* and KMX-1 associated signals at the tubule that connects separating nuclei. In addition, spindle morphology has been shown to change during the cell cycle (28), with the rhomboid shaped spindle (Fig. 1E) observed early in mitosis developing into a dense central spindle (Fig. 1D) later in the nuclear replication process, similar to the morphologies observed for Ty1::*TbKH* fluorescence.

To confirm that the localizations observed above did not represent epitope tag mediated mistargeting, similar images were also obtained from parasite lines expressing *TbKH*1 with a complementary epitope tag, the trimeric hemagglutinin epitope HA<sub>3</sub>, fused to the alternate C-terminus of KH. These images confirmed localization at the base of the flagellum (Fig. 2A), where the tagged protein is immediately adjacent to DAPI fluorescence signal that is associated with kinetoplast DNA, the highly catenated mitochondrial DNA that is physically attached to the basal body (29), the subpellicular microtubules (Fig. 2B), and the mitotic spindle (Fig. 2C) for both Ty1::*TbKH* and *TbKH*::HA<sub>3</sub> (compare Fig. 1 and Fig. 2). Whole parasites that are expressing *TbKH*::HA<sub>3</sub> do however also exhibit punctate staining in the interior of the cell in addition to staining on the subpellicular microtubules (Fig. 2B) or the spindle (Fig. 2C). The absence of cross-reacting bands on Western blots of lysates from wild type BF parasites (Fig. 6G, lane 1) developed with anti-HA monoclonal antibody or immunofluorescence signal from wild type BF parasites stained with the same antibody (not shown) suggests that this punctate staining does not represent endogenous cross-reacting material. The additional puncta could represent a proportion of *TbKH*::HA<sub>3</sub> that is mistargeted, indicating that addition of HA<sub>3</sub> to the C-terminus may represent a less optimal tag than addition of Ty1 to the N-terminus.

Although spindle localization had not been detected for *LmxKH* in the original studies on *L. mexicana* (21), subsequent images of hydroxyurea synchronized promastigotes (Fig. 3) demonstrate that *LmxKH* is also associated with the mitotic spindle in that parasite and that this newly discovered localization is thus likely universal. Hydroxyurea synchronization was employed to increase the percentage of parasites captured in mitosis and thus facilitate the detection of mitotic spindles that are associated with *LmxKH*, but similar images were also observed with lower frequency using non-synchronized parasites (data not shown). *L. mexicana* promastigotes assumed an oval morphology following exposure to hydroxyurea, rather than the elongated shape of untreated promastigotes, but *LmxKH* was nonetheless apparent on the mitotic spindle that separates dividing nuclei.

*TbKHARON* is essential for viability of the BF parasites. To determine whether *TbKH* is essential for BF parasites, we inhibited the expression of *TbKH* by RNAi. The transgenic BF/pHD1313 line (30), which expresses the tetracycline repressor, was transfected with the *TbKH*<sup>RNAi</sup> construct employing the p3666 vector (22) that transcribes the hairpin loop RNA driven by the EP procyclin promoter fused to four tetracycline operators. Induction of expression of this hairpin loop RNA with doxycycline for 24 h reduced the level of *TbKH* mRNA by at least 6-fold (Fig. 4A, upper panel), as determined by densitometry, while *T. brucei* hexose transporter 1 (*TbHT1*) mRNA remained unchanged (Fig. 4A, bottom panel). A time course for expression of *TbKH::HA<sub>3</sub>* following induction of RNAi against *TbKH* mRNA (Fig. 4B) showed that the protein was almost completely eliminated by 48 h post induction. Upon induction of RNAi parasite growth was severely compromised (Fig. 4C, empty circles), resulting in death of BFs by 72 h. Whereas uninduced parasites exhibited normal morphology upon staining with anti- $\alpha$ -tubulin antibody and DAPI (Fig. 4E), parasites where RNAi had been induced for 12 h (Fig. 4F), 24 h (Fig. 4G) or 48 h (Fig. 4H) continued to replicate nuclear and kinetoplast DNA, as determined by the multiple DAPI staining foci, but failed to undergo cell division, resulting in cells with multiple nuclei, kinetoplasts and flagella. These parasites are similar to the ‘monster’ cell

phenotype originally described for RNAi directed against mRNAs encoding various flagellar axoneme proteins (31). Microscopic quantification of parasites with a normal complement of kinetoplasts and nuclei (1K1N) or with altered numbers of both genomes (e.g., 2K1N, 2K2N, etc.) revealed that 1N1K parasites are reduced rapidly following induction of RNAi and that monster cells like those in Fig. 4G began to dominate the population increasingly from 0-48 h (Fig. 4C). Additionally, the flagella became detached from the cell body along their length as early as 12 h. Microscopic observation of live cells revealed that detached flagella remained motile even after 48 h of RNAi induction, similar to the phenotype reported upon RNAi of *TbFLA1* (24,28).

BF trypanosomes induced for RNAi against *TbKH* for 24 or 48 h were also examined by electron microscopy. Images show a number of abnormalities, including multiple flagellar pockets, multiple axonemes within single flagellar membranes (Fig. 5A,B), axonemes without flagellar membranes (Fig. 5C), enlarged flagellar pockets (Fig. 5D), multiple nuclei in close apposition (Fig. 5E), and nuclei with apparent membranous inclusions, which represent invaginations of the nuclear envelope (Fig. 5F-H).

*Role of TbKHARON in trafficking of flagellar membrane proteins.* To determine whether *TbKH* could be involved in trafficking of flagellar membrane proteins, we tested the potential role of this protein in localization of several flagellar membrane proteins that had been identified in the flagellar membrane proteome and localized by immunofluorescence in BF parasites (5): a putative  $\text{Ca}^{2+}$  channel (*TbCaCh*, FS179, Tb927.10.2880), a *bona fide* (32)  $\text{Ca}^{2+}$  ATPase, (*TbCaATPase*, FS60, Tb927.8.1200), a membrane bound adenylate cyclase (FS33, Tb927.8.7940), and an integral membrane protein of unknown function (FS133, Tb927.2.1700). Upon knockdown of *TbKH* mRNA, normal flagellar trafficking of the *TbCaCh* to the region of the flagellum that adheres to the cell body ((5) and Fig. 6A) was blocked, resulting in BF parasites with *TbCaCh* in only a few (Fig. 6B,C) of the multiple flagella, as, indicated by white arrowheads that mark flagella that stain with anti- $\alpha$ -tubulin but not with antibody directed against the HA<sub>3</sub> epitope on *TbCaCh::HA<sub>3</sub>*. Isolation of



sheared flagella followed by immunoblotting using antibody against the HA<sub>3</sub> tag also confirmed that *TbCaCh* was largely absent from the flagella under *TbKH* RNAi conditions (Fig. 6E, lane 1) but not when RNAi was directed against *TbFLA1* mRNA (Fig. 4D; Fig. 4E, lane 2), a different knockdown that also results in detachment of flagella from the cell body (24). Hence, mistargeting of *TbCaCh* is a specific consequence of down-regulating *TbKH* and not simply an outcome of flagellar detachment.

To examine whether *TbKH* may interact with flagellar membrane proteins that it targets to the flagellar membrane, we monitored possible co-immunoprecipitation of Ty1::*TbKH* and HA-tagged flagellar membrane proteins. In BFs expressing Ty1::*TbKH*, cells were crosslinked with formaldehyde and sonicated to disrupt cytoskeletons, followed by immunoprecipitation with BB2 monoclonal antibody directed against the Ty1 epitope. This procedure was carried out employing BF clones that also expressed *TbCaCh*::HA<sub>3</sub>. As negative controls, similar experiments were done in BF parasites expressing *TbCaATPase*::HA<sub>3</sub>, FS33::HA<sub>3</sub> and FS133::HA<sub>3</sub>, proteins that do not depend on *TbKH* for trafficking to the flagellar membrane (data not shown). *TbCaCh*::HA<sub>3</sub> co-immunoprecipitated along with Ty1::*TbKH* (Fig. 6F, upper panel lane 2) while none of the other tested flagellar membrane proteins did so (Fig. 6F, upper panel lanes 1,3-5). Control immunoblots of input lysates before immunoprecipitation (Fig. 6G) demonstrated that these control lines did express the relevant tagged proteins, supporting the notion that the absence of signals in the blot in Fig. 6F was due to failure or any of these proteins to interact with *TbKH* and not simply to failure of the cell line to express the tagged flagellar membrane protein. Immunoblots of the immunoprecipitates were also probed with BB2 antibody (Fig. 6F, middle panel) to demonstrate that similar amounts of Ty1::KH had been captured in the immunoprecipitation step. Tubulin, the major component of the subpellicular microtubule cytoskeleton, co-precipitated in all samples (Fig. 6F, lower panel in all lanes). Although tubulin could be a contaminant resulting from the abundance of tubulin in cell lysates, it would also be expected to be physically associated with *TbKH*, since sonication disrupts cytoskeletons but

likely leaves short microtubule fragments associated with *TbKH*.

Loss of *TbCaCh* from flagella following induction of *TbKH*<sup>RNAi</sup> was due to failure to target this channel to flagellar membranes and not simply to degradation of the channel. Thus immunoblots (Fig. 6H, *TbKH*<sup>RNAi</sup>) of lysates from parasites that had been induced for RNAi against *TbKH* mRNA for 24 or 48 h showed that *TbCaCh* was still present at levels similar to that seen before induction of RNAi (0 h). Similar results were obtained for parasites where RNAi had been induced against *TbFLA1* mRNA (Fig. 6H, *TbFLA1*<sup>RNAi</sup>).

Localization of *TbCaATPase*, a protein that traffics to puncta in both the flagellar membrane and the cell body (Fig. 7A), was also determined following induction of RNAi against *TbKH* mRNA. This flagellar membrane protein continued to traffic to the flagellar membrane when RNAi was induced against either *TbKH* (Fig. 7B) or *TbFLA1* (Fig. 7C) mRNA. Immunoblots of isolated flagella probed with antibody against the HA<sub>3</sub> tag also demonstrated that *TbCaATPase* was still present in flagella following induction of RNAi against either *TbKH* or *TbFLA1* mRNA (Fig. 7D, upper panel lanes 1 and 2, respectively). This result demonstrates that induction of RNAi against *TbKH* mRNA does not cause global disintegration of the flagellar membrane and/or non-specific removal of all flagellar membrane proteins but rather specifically impairs flagellar trafficking of *TbCaCh*.

*TbKHARON* is a functionally important component of the mitotic spindle in BF parasites. To investigate the potential role of *TbKH* in the mitotic spindle of BF parasites, the mitotic spindle fate was followed by immunofluorescence microscopy, employing KMX-1 antibodies, following the induction of RNAi against *TbKH* mRNA for 12, 24, or 48 h (Fig. 8A-D). Prior to induction of RNAi, 3 ± 1% (n = 2, 100 cells counted per sample) of BF parasites had observable spindles (Fig. 8E). Following induction of RNAi against *TbKH*, the percentage of BF parasites with spindles first increased to 17 ± 2% at 12 h post induction, but at 24 h and 48 h, no spindles were observed (Fig. 8C,D and Fig. 8E, asterisks). This observation suggests that *TbKH* not only localizes to the mitotic spindles but is also necessary for their integrity and/or stability.

Moreover, 24 h RNAi induced parasites have multiple nuclei that appeared to be well separated (Fig. 8C) while parasites induced for 48 h had multiple clumped nuclei (Fig. 8D), suggesting that nuclear segregation may be impaired following sufficient reduction in the level of *TbKH* mRNA. In addition, synchronization of Ty1::TbKH parasites using hydroxyurea (33) resulted in an increase in the number of parasites showing specific Ty1::TbKH fluorescence signal on the mitotic spindle of dividing cells, from ~5% of total cells in asynchronous to ~20% in synchronous parasites, as determined by examining 100 cells by immunofluorescence microscopy. This observation further confirms the association of *TbKH* with the mitotic spindle.

*TbKHARON* is a cytoskeletal protein essential for viability of the PF parasites. To establish the subcellular localization of *TbKH* in PF parasites, a PF line was generated that carried a copy of *TbKH* ORF endogenously tagged on the N-terminus with Ty1. *TbKH* localization in PFs was essentially the same as that observed in BF. In isolated flagellar cytoskeletons (31), Ty1::TbKH was detected at the base of the flagella adjacent to the DAPI stained kinetoplast DNA (Fig. 9A). In whole PF parasites, Ty1::TbKH was observed in the subpellicular microtubule cytoskeleton by overlapping fluorescence with the  $\alpha$ -tubulin signal (Fig. 9B), and in the mitotic spindles of dividing PF (Fig. 9C, right) similar to the signal observed in parasites stained with KMX-1 antibody (Fig. 9C, left). In Fig. 9C, separate cells were stained with each primary antiserum, because the same secondary antiserum is employed to detect both primary monoclonal antibodies. Induction of RNAi resulted in loss of detectable *TbKH* mRNA within 48 h (Fig. 10A), and while parasite growth arrested by this time, PF cell death was not complete until 12.5 days (Fig. 10B), much longer than for BF parasites. Induction of RNAi also resulted in formation of poly-nucleated and poly-flagellated PF parasites with disrupted attachment of the flagella to the cell body (Fig. 10C,E, F), unlike uninduced PFs (Fig. 10D). Hence, the phenotypes upon induction of RNAi against *TbKH* mRNA are similar in BF and PF parasites. This observation is notably distinct from the monster cell phenotype originally observed using RNAi directed against components of the flagellar axoneme (31), where the

cytokinesis defect was only observed for BF but not PF trypanosomes. Of note, both BF and PF parasites undergoing RNAi against *TbKH* were still motile, as determined by phase contrast microscopy, even after these dramatic morphological changes.

*Rescue of L. mexicana  $\Delta$ lmxkh[pGT1::GFP] phenotype by TbKH.* To determine whether *TbKH* could substitute in *L. mexicana* for *LmxKH* regarding trafficking of *LmxGT1* to the flagellar membrane and support growth of intracellular amastigotes, the *\Delta*lmxkh[pGT1::GFP] knockout line was complemented with the *TbKH* ORF expressed from an episome. Partial restoration of *LmxGT1* flagellar membrane targeting (Fig. 11A,B) and growth of intracellular amastigotes (Fig. 11C) was observed. These results indicate that *TbKH* can function, albeit sub-optimally, in trafficking of flagellar membrane proteins in *L. mexicana*, despite the fact that sequence identity for the KH proteins is relatively low between the two species of parasite.

## DISCUSSION

Cilia and flagella are multifunctional organelles that are represented broadly in biology, from unicellular eukaryotes to differentiated mammalian cells. Over the past decade or so, the role of cilia and flagella as sensors of the extracellular environment has emerged, with a corresponding emphasis on the membrane proteins that mediate these sensory processes (34). In parallel, a burgeoning interest has developed in the mechanisms whereby membrane proteins are selectively targeted to cilia and flagella (35).

Recent studies on African trypanosomes have uncovered novel unexpected functions for flagella in these parasites. Szempruch and colleagues (36) have reported that BF trypanosomes generate extracellular vesicles that originate from the flagellar membrane, contain flagellar proteins and virulence determinants such as serum resistance-associated protein, and deliver these vesicles to host cells including erythrocytes. This observation further underscores the importance of flagellar membrane proteins in the biology and virulence of African trypanosomes. Furthermore, flagellar membranes of PF trypanosomes can fuse with each other and

transfer both membrane bound and cytosolic proteins between the two flagellar-attached parasites (37), suggesting that flagella may be organelles involved in cell-cell communication. In addition, flagellar adenylate cyclases are involved in the process of social motility (8-10), a coordinated motility process that is important for migration of parasites within the tsetse fly and for successful colonization of this insect vector (38). Hence, understanding the function of parasite flagella and the processes for targeting integral membrane proteins to these organelles is central to the biology of these parasites and the diseases they cause.

The current characterization of *TbKH* has resulted in a number of novel discoveries that extend beyond the initial discovery of KH in *L. mexicana*. First, KH is essential for viability of both BF and PF trypanosomes, establishing the critical role of this protein in a second kinetoplastid parasite, in this case in both mammalian and insect life cycle stages. Second a novel putative  $\text{Ca}^{2+}$  channel is dependent upon *TbKH* for flagellar targeting, demonstrating that this conserved trafficking machinery mediates flagellar targeting of at least two distinct membrane proteins in diverse parasites. Third, a new location for KH has emerged, namely its association with the mitotic spindle.

*TbKH* is involved in flagellar targeting of the putative  $\text{Ca}^{2+}$  channel *TbCaCh*, and this function is likely mediated by *TbKH* located at the base of the flagellum. During biosynthesis integral membrane proteins are first incorporated into the surface membrane at the flagellar pocket (39), from where they migrate to the plasma membrane surrounding the cell body or the flagellar membrane. The presence of KH in close apposition to the flagellar pocket membrane may thus mediate, by a still unknown mechanism, the entry of *TbCaCh* and *LmxGT1* into the adjacent flagellar membrane. This function of *TbKH* in targeting flagellar membrane proteins is likely to be essential for viability of BF and PF parasites. Thus an early phenotype accompanying induction of RNAi against *TbKH* RNA is detachment of the flagellum from the cell body. Previous studies have also demonstrated that induction of RNAi against *TbCaCh* mRNA results in detachment of the flagella from the cell body and parasite death (5,40). This observation suggests that failure of

*TbKH*<sup>RNAi</sup> knockdown parasites to target *TbCaCh* to its normal location in the component of the flagellar membrane that associates with the cell body would be sufficient to disrupt this junction between the flagellar and cell body membranes. Failure of BF parasites to divide, resulting in multinucleate, multiflagellated monster cells, was previously observed by Gull and colleagues following induction of RNAi against mRNAs encoding proteins of the flagellar axoneme (31). These authors suggested that proper flagellar function is required for establishment of the cleavage furrow during mitosis, thus establishing an intimate link between flagella and cytokinesis. A number of other studies (41-44) have also underscored a role for the flagellum in cytokinesis and parasite viability. Hence, it is not surprising that disruption of targeting for flagellar membrane proteins, such as *TbCaCh* and possibly others, following RNAi against *TbKH* mRNA would induce failure of cytokinesis and a lethal phenotype in BF parasites. It is however notable that the essentiality of flagellar function noted in the previous studies on other flagellar proteins was typically reserved to the BF stage, but induction of RNAi in the PF forms was not lethal. The role of *TbKH* is distinct in this regard, as RNAi induced against *TbKH* mRNA is lethal in both life cycle stages.

If *TbCaCh* is a *bona fide* calcium channel, it may also play an important role in flagellar physiology that is dependent upon flagellar localization. One example of such an organelle-dependent function for  $\text{Ca}^{2+}$  channels is provided by the cilia of mammalian kidney tubule cells, where several of these channels are thought to be involved in sensing fluid flow within this organ (45). We anticipate that some other flagellar membrane proteins will likely be dependent upon *TbKH* for targeting to this organelle. It should be possible to identify other potential cargo for *TbKH* by performing quantitative proteomics to detect proteins whose relative abundance in isolated flagella decreases after induction of RNAi directed against *TbKH* mRNA.

While the specific functions of *TbKH* at the mitotic spindle and the subpellicular microtubules are not yet clear, this protein could also be required for critical processes at those loci. RNAi targeting of *TbKH* mRNA impairs mitotic spindle formation, a deficiency that would be

expected to be lethal. However, the effect of *TbKH* RNAi on spindle integrity is only detected at 24 h, after the detachment of flagella and failure of cytokinesis, which are already detected at 12 h. Hence, loss of spindles is probably not the primary lethal defect of knocking down *TbKH* mRNA. Similarly, association of *TbKH* with the subpellicular microtubules might mediate critical functions for this extensive cytoskeletal network. In principle, lethality could be due to disrupted KHARON function at the base of the flagellum, on the mitotic spindle, or in the subpellicular cytoskeleton, or loss of KHARON at all three loci may contribute to the lethal phenotype, potentially with different kinetics. Hence one challenge will be to dissect the potentially distinct functions of *TbKH* at each of its subcellular loci. Experiments employing biotin proximity labeling (46) (unpublished data of K.D. Tran and M.A. Sanchez) suggest that both *TbKH* and *LmxKH* may associate with multiple additional subunits to form KHARON Complexes, and this possibility is being further investigated. These potential separate complexes may share KH and other subunits, but they may also incorporate other distinct subunits that are unique to the subcellular localization and specific function of each complex. If this model of KH structure and function is correct, then targeting complex-specific subunits using RNAi may allow dissection of the distinct functions of each complex and insight into the role of KH and its associated subunits at each of its three subcellular locations.

## EXPERIMENTAL PROCEDURES

**Growth and transfection of *T. brucei* cell lines.** BF Lister 427 and PF TREU927 *T. brucei* cell lines were grown as described previously (30). For T7 RNA polymerase-independent driven expression (22), BF or PF parasites transfected with the pHD1313 plasmid expressing the tetracycline repressor, kindly provided by Dr. Christine Clayton (47), were generated and grown in 2.5  $\mu\text{g ml}^{-1}$  phleomycin. Subsequently, 5-10  $\mu\text{g}$  of linearized plasmid DNA was used to transfect mid-log phase parasites as described (48). Transfectants were isolated by limiting dilution according to published protocols (49,50).

### *Hydroxyurea synchronization.*

Synchronization of BF Ty1::TbKH cell line with hydroxyurea was performed as described by Forsythe et al. (33). Briefly, parasites were incubated in HMI-9 (51) culture medium containing 10  $\mu\text{g ml}^{-1}$  of hydroxyurea for 6 h at 37 °C in 5% CO<sub>2</sub>. Later, parasites were pelleted by centrifugation at 1600 X g for 10 min at room temperature to remove hydroxyurea, resuspended in fresh HMI-9 culture medium, and centrifugation was repeated. Finally, parasites were resuspended in 10 ml of fresh HMI-9 and incubated for 3 h at 37 °C in 5% CO<sub>2</sub>. Then parasites were processed for fluorescence microscopy as indicated.

**RNA isolation and Northern Blots.** Total RNA from *T. brucei* parasites was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Northern blot analysis was performed as described elsewhere (52). Non-radioactive detection employing DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) was used according to manufacturer's instructions, and the Image Quant LAS 400 (GE Healthcare) was employed for image acquisition. Image J software (NIH) was used for densitometry analysis and Adobe Photoshop CS3 and Adobe Illustrator CS3 to create figures.

**Primary amino acid sequence analysis.** For DNA sequence analysis of *TbKH* and amino acid sequence alignment with the *LmxKH* open reading frame (ORF), MacVector software (Intelligenetics) was employed. Sequence similarity was analyzed using the BLAST search and GeneBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) or TritypDB (<http://tritypdb.org/tritypdb>).

**Inhibition of gene expression by RNAi.** To inhibit the expression of *TbKH* in both BF and PF parasites, a construct was generated based on the p3666 vector harboring the blasticidin (BSD) resistance marker (22) that integrates into the rRNA gene repeat locus and generates a hairpin RNA derived from the *TbKH* ORF. A 521 bp gene fragment corresponding to nts 340-860 of the *TbKH* ORF (Tb927.10.8940) was employed, identified as an optimal fragment for RNAi using RNAi software (<http://trypanofan.path.cam.ac.uk/software/RNAi.html>). The *TbKH*<sup>RNAi</sup> construct was linearized and transfected into BF or PF/pHD1313 cell lines as described (30) to generate BF or PF/*TbKH*<sup>RNAi</sup>.



clonal lines. RNAi clones were selected by resistance to  $2.5 \mu\text{g ml}^{-1}$  phleomycin and  $5 \mu\text{g ml}^{-1}$  ( $10 \mu\text{g ml}^{-1}$  for PF) blasticidin, and expression of hairpin loop RNAi was induced by addition of  $1 \mu\text{g ml}^{-1}$  doxycycline. Similarly, a *TbFLA1*<sup>RNAi</sup> hairpin loop construct was generated using a 539 bp gene fragment corresponding to nts 692-1230 bp of the *TbFLA1* ORF (Tb927.8.4010), identified by RNAi software, and the p3666 plasmid as described above. A BF/*TbFLA1*<sup>RNAi</sup> clonal line was produced as described for the *TbKH*<sup>RNAi</sup> clones. RNAi was induced by adding  $1 \mu\text{g ml}^{-1}$  doxycycline to the medium.

**Endogenous epitope tagging.** For endogenous tagging of *TbKH* at the N-terminus with the Ty1 epitope tag (53), a DNA segment representing sequence 500 bp upstream of the start codon of the *TbKH* ORF was ligated to the *BSD/BB2* module (54) followed by another segment representing the first 170 bp corresponding to the N-terminal open reading frame. For endogenous tagging of *TbKH* at the C-terminus with a trimeric hemagglutinin (*HA*<sub>3</sub>) tag, 500 bp corresponding to C-terminal coding region of the *TbKH* ORF was ligated to the pMOTag2H plasmid (55), followed by a 500 bp segment downstream of the stop codon. Constructs for expression of flagellar membrane proteins FS33, FS60, FS133 and FS179 tagged with a *HA*<sub>3</sub> epitope at the C-terminus were kindly provided by Dr. Kent Hill (5). Parasites were transfected with  $5 \mu\text{g}$  of linear construct as described (48), and selected using  $5 \mu\text{g ml}^{-1}$  blasticidin or  $0.1 \mu\text{g ml}^{-1}$  puromycin and cloned by limiting dilution.

**Immunofluorescence microscopy.** For immunofluorescence microscopy, parasites ( $5 \times 10^6$ ) were centrifuged at 700 X g for 4 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 10 mM glycine in PBS and once with PBS, resuspended in 100  $\mu\text{l}$  PBS, spotted onto poly-L-lysine coated coverslips, and 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: 1:500 dilution anti-*HA*.11 clone 16B12 monoclonal antibody (mAb) (BioLegend, Cat. # MMS-101R, Lot # D13FF01558), 1:1000 dilution anti-*HA* polyclonal antibody (pAb) (Sigma-Aldrich, Cat. # H6908, Batch # 083M4837V), 1:500 dilution anti-Ty1 (BB2) mAb (53), 1:1000 dilution anti- $\alpha$ -tubulin mAb (Sigma-Aldrich, Cat. # T5168, Batch # 104M4790V), 1:1000 dilution anti- $\alpha$ -tubulin pAb (Sigma-Aldrich, Cat. # SAB3501071, Lot # 75971302), 1:1000 anti- $\beta$ -tubulin clone KMX-1 mAb (Millipore, Cat. # MAB3408, Lot # 2646950) and 1:1000 anti-GFP pAb (Molecular Probes, Cat. # A11122, Lot # 847818). 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, Lot # 1572559), Alexa Fluor® 595 goat anti mouse IgG (H+L) (Cat. # A11005, Lot # 1524907), Alexa Fluor® 594 goat anti rabbit IgG (H+L) (Cat. # A11012, Lot # 84B2-1) and Alexa Fluor® 488 goat anti rabbit IgG (H+L) (Cat. # A11008, Lot # 84B1-1), 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 acquired 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 in a 1024 X 1024 format and deconvolved with 9 iterations using SoftWoRx software. Adobe Photoshop CS3 and Adobe Illustrator CS3 (Adobe Systems Inc.) were used to create image compositions.

Images that included differential interference contrast (DIC) were acquired on a high resolution wide field Core DV system (Applied Precision™). This system is an Olympus IX71 inverted microscope with a proprietary XYZ stage enclosed in a controlled environment chamber; differential interference contrast (DIC) transmitted light and a solid state module for fluorescence. The camera is a Nikon Coolsnap ES2 HQ. Each image was acquired as Z- stacks in a 1024x1024 format with a 60x 1.42 NA PlanApo lens in 3 colors: Alexa Fluor® 488, Alexa Fluor®

594, and DAPI. The pixel size in XYZ was 0.107 x 0.107 x 0.2 microns. The images were deconvolved with the appropriate optical transfer function using an iterative algorithm of 10 iterations. The histogram was optimized for the most positive image and applied to all the other images for consistency before saving the images as 24-bit merged TIFF. A reference DIC image was acquired from the middle of the Z-stack.

*Flagellar protein purification, formaldehyde crosslinking, co-immunoprecipitation, and Western blot assay.* Flagellar purification was performed as described (26) with slight changes. Briefly, BF/TbKH<sup>RNAi</sup> clones or BF/TbFLA1<sup>RNAi</sup> clones were grown in 1 µg ml<sup>-1</sup> doxycycline for 48 h or 72 h respectively, and pelleted at 420 X g for 20 min. Cell pellets were washed with buffer A (25 mM Na<sup>+</sup>-tricine, pH 7, 1% BSA, 0.1 mM CaCl<sub>2</sub>, 0.2 mM EDTA, 5 mM MgCl<sub>2</sub> 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 10<sup>8</sup> parasites ml<sup>-1</sup> 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 1600 X g for 10 min at 4 °C. Pellets containing the isolated flagella were resuspended in 25 µl of PBS and used for Western blot assays. Also, flagellar cytoskeleton purification from wild type BF and PF clones was performed by isolation of cytoskeletons using Nonidet P-40 extraction, followed by 1M NaCl treatment according to Broadhead *et al.* (31) to dissociate the subpellicular cytoskeleton. The remaining flagellar cytoskeletons were processed for immunofluorescence microscopy as indicated.

For formaldehyde crosslinking and co-immunoprecipitation, cell lines were prepared that expressed TbKH endogenously tagged with the Ty1 epitope at the N-terminus (Ty1::TbKH) and also co-expressed a flagellar membrane protein of interest endogenously tagged at its C-terminus with an HA<sub>3</sub> epitope (FS33::HA<sub>3</sub>; FS60::HA<sub>3</sub>; FA133::HA<sub>3</sub>; FS179::HA<sub>3</sub>) (5). Parasites were washed once with PBS and pelleted at 1000 X g for 10 min, and cell pellets were resuspended in 9.37 ml PBS plus 0.63 ml 16% formaldehyde-EM grade (Polysciences, Inc) and incubated at 37 °C for 10 min. Subsequently, 1 ml of 2.5 M glycine in

PBS was added to the crosslinked samples and incubated at 37 °C for 5 min to terminate crosslinking, and crosslinked parasites were pelleted at 1000 X g for 10 min and washed twice with PBS. Finally, cell pellets were resuspended in 500 µl of Tween-20/PBS buffer (0.2% tween-20 in 1x PBS, pH 7.2) containing 1x of Halt™ Protease Inhibitor Single-Use Cocktail (Thermo Fisher). Crosslinked samples were sonicated on ice using 2 X 15 sec pulses at maximum output (Branson Sonifer 150). After disruption, samples were centrifuged at 16000 X g for 20 min at 4 °C. Supernatants were transferred to new Eppendorf tubes and incubated overnight with 1:50 dilution of BB2 mAb at 4 °C on a rocker. Later, 30 µl of PureProteome™ Protein A/G Mix Magnetic beads (Millipore) were incubated for 30 min at room temperature on a rocker to capture Ty1::TbKH proteins that had been crosslinked to other partner proteins. Two final washes of the magnetic beads for 30 s at room temperature with RIPA buffer were performed to increase stringency and reduce background. Co-immunoprecipitated proteins were eluted and crosslinking reversed by adding 30 µl 1x NUPAGE®-LDS sample buffer (Life Technologies) containing 10 mM DTT followed by heating at 70 °C for 30 min. Eluted protein samples were used for Western blot analysis.

Flagellar protein extracts or co-immunoprecipitated protein extracts from 2.5 X 10<sup>7</sup> parasites per lane were resolved on NUPAGE® 4-12% Bis-Tris Mini Gels using the XCell SureLock® Mini-Cell system following the manufacture's instructions (Life Technologies) and proteins were transferred onto PVDF membranes (Millipore). Anti-HA.11 clone 16B12 monoclonal antibody (mAb) (BioLegend, Cat. # MMS-101R, Lot # D13FF01558) 1:2500 dilution, BB2 mAb (53) 1:500 dilution and anti α-tubulin mAb (Sigma-Aldrich, Cat. # T5168, Batch # 104M4790V) 1:10000 dilution were used as primary antibodies, and goat anti mouse-HRP-conjugated (Jackson ImmunoResearch Laboratories, Cat. # 115-03-174, Lot # 117119) 1:15000 dilution was employed as secondary antibody. SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher) was used for detection and an Image Quant LAS 400 (GE Healthcare) scanner was employed to acquire luminescent images. Adobe Photoshop CS3 and Adobe Illustrator CS3 (Adobe Systems Inc.) were

used to create image compositions. Immunoblots of total BF cell lysates, such as those in Figs. 4B and 6H, were performed by the same method, and ImageJ was employed for quantification of band densities.

*Functional complementation of Leishmania mexicana kh null mutant ( $\Delta lmxkh$ ) with TbKH, and macrophage infections.* Transgenic  $\Delta lmxkh$  overexpressing  $LmxGT1::GFP$ , previously generated (21), was transfected with the *TbKH* ORF cloned into the pX63HYG expression vector (56) for episomal expression. Stable transfectants were selected on bleomycin ( $50 \mu\text{g ml}^{-1}$ )/puromycin ( $50 \mu\text{g ml}^{-1}$ )/neomycin ( $100 \mu\text{g ml}^{-1}$ )/hygromycin ( $80 \mu\text{g ml}^{-1}$ ) and tested for their ability to translocate  $LmxGT1::GFP$  to the flagellar membrane as monitored by immunofluorescence microscopy as indicated above.

Macrophage infections were performed by infecting differentiated macrophages from the human acute leukemia monocyte cell line (THP-1) in triplicate samples, as described (57). Briefly,  $3 \times 10^5$  differentiated THP-1 cells were infected with stationary phase *L. mexicana* (10:1 promastigotes:macrophages ratio) for 4 h. Infected macrophages were then washed exhaustively to remove extracellular promastigotes. Cultures were incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  environment and infected macrophages stained using the HEMA 3 STAT PACK staining kit as described by the manufacture (Fisher Scientific) at 1 and 7 days post-infection. Stained infected macrophages were examined using a Nikon Eclipse 50i microscope equipped with a 100x 1.25 NA oil objective (Nikon Instruments). The number of parasites per 100 macrophages was determined by counting 100 cells in each of the triplicate experiments.

*Electron microscopy.* BF/*TbKH*<sup>RNAi</sup> clone was grown in the presence of  $1 \mu\text{g ml}^{-1}$  doxycycline to induce RNAi against *TbKH* mRNA and parasite samples were harvested at 0 h, 18 h, 24 h and 48 h postinduction. Parasites were fixed in 2.5% glutaraldehyde by addition of 1/10 volume of 25% glutaraldehyde directly to the culture and incubated at room temperature for 5 min. Fixed cells were subsequently collected and centrifuged for 10 min at 800 X g. The cells were resuspended in 1ml of 0.1M sodium phosphate buffer, pH7.4, containing 50mM sucrose, and pelleted for 10 min at 800 X g. The cells were then resuspended in

1ml of buffered fixative (2.5% glutaraldehyde, 2% formaldehyde, and 50mM sucrose in 0.1 M phosphate buffer, pH 7.4), pelleted for 1 min at 21,000 X g in a micro centrifuge, and stored at  $4^\circ\text{C}$ .

Fixed samples were prepared for transmission electron microscopy (TEM) and serial block-face scanning electron microscopy (SBF-SEM). TEM samples were prepared and imaged as previously described (57). SBF-SEM samples were washed in 0.1M PIPES buffer and then embedded in a mix of 4% low melting point agarose and 4% gelatin from porcine skin. Excess agarose/gelatin was trimmed away and samples were prepared according to Wilke et al. (58) with minor modifications: samples were incubated at  $4^\circ\text{C}$  for 1h in a 1:1 mix of 3% potassium ferricyanide in 0.2M PIPES buffer and 4% aqueous osmium tetroxide. Samples were washed with double distilled (dd)  $\text{H}_2\text{O}$  between each of the subsequent heavy metal staining steps, which consisted of 1% thiocarbohydrazide in dd $\text{H}_2\text{O}$  for 20 min at room temperature, 2% aqueous osmium tetroxide for 30 min at  $4^\circ\text{C}$ , 1% uranyl acetate overnight at  $4^\circ\text{C}$ , and lead aspartate (0.02M lead nitrate and 0.03M aspartic acid) for 30 min at room temperature. Final washes in dd $\text{H}_2\text{O}$  were performed prior to dehydration through a graded series of ethanol and acetone incubations. The samples were then infiltrated with and embedded in Durcupan ACM epoxy resin. Serial block face images were captured with a Zeiss Merlin Compact Field Emission Gun Scanning Electron Microscope (FEG-SEM) with Variable Pressure (VP) mode and a Gatan 3View System using Digital Micrograph (www.gatan.com) with an accelerating voltage of 3 kV, variable pressure of 45 Pa, aperture size of 30  $\mu\text{m}$ , 4.7 nm lateral resolution and 50 nm axial resolution and processed using Image J (59).

*Synchronization of L. mexicana with hydroxyurea.* Promastigotes of *L. mexicana* were synchronized with hydroxyurea as described by Santos da Silva et al. (60). Briefly, mid-log phase promastigotes were incubated in RPMI culture medium containing 5 mM hydroxyurea for 14 h at  $26^\circ\text{C}$ . Later, parasites were pelleted by centrifugation at 1600 X g for 10 min at room temperature to remove hydroxyurea, resuspended in fresh RPMI and incubated for 7 h at  $26^\circ\text{C}$ .

Subsequently, parasite samples were processed for fluorescence microscopy as indicated.

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*Conflict of Interests*—The authors declare that they have no conflict of interest with the contents of this article.

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## FOOTNOTES

<sup>3</sup>The *L. mexicana* protein was originally designated KHARON1, or KH1, to allow subsequent numbering of additional subunits in a KHARON Complex. However, for simplicity we now refer to KHARON1 as KHARON or KH for both *L. mexicana* and *T. brucei*, and associated proteins, currently under investigation, will be designated KHARON Associated Proteins or KHAPs.

## FIGURE LEGENDS

**Figure 1. Sub-cellular localization of *TbKH* in BF parasites using an N-terminal Ty1 epitope tag.** (A) Flagellar axonemes prepared from Ty1::*TbKH* expressing BF trypanosomes by selective depolymerization of subpellicular microtubules using high salt. The sample was immunostained with anti- $\alpha$ -tubulin pAb (Tub, red) and anti-Ty1 (BB2) mAb (Ty1::*TbKH*, green). White arrowheads denote localization of Ty1::*TbKH* at the base of the flagellum. Scale bars (white) in all figures represent 4  $\mu$ m. (B) The Ty1::*TbKH* cell line was immunostained with anti-Ty1 (BB2) mAb (Ty1::*TbKH*, green) and anti- $\alpha$ -tubulin pAb (Tub, red) and demonstrates staining of Ty1::*TbKH* on the subpellicular microtubules, indicated by white arrowheads. (C) Detergent-extracted cytoskeletons from BF parasites expressing Ty1::*TbKH* were stained with BB2 mAb (*TbKH*, green) and with anti- $\alpha$ -tubulin antibody (Tub, red). (D) The Ty1::*TbKH* cell line was stained with BB2 mAb (*TbKH*, green). This image displays a parasite in which Ty1::*TbKH* staining is associated with both the subpellicular microtubules and the mitotic spindle that connects the two nuclei late during karyokinesis. (E) Another Ty1::*TbKH*-expressing parasite captured earlier during karyokinesis than the cell shown in (D). In this image, the KMX-1 anti- $\beta$ -tubulin antibody (red) was employed, which stains spindle microtubules as well as other microtubules (61), and displays the rhomboid spindle that converges at two poles at opposite ends of dividing nuclei (62). All preparations were stained with DAPI that detects both nuclear and kinetoplast DNA (blue). White arrowheads in (D) and (E) point to the mitotic spindle.

**Figure 2. Sub-cellular localization of *TbKH* in BF parasites using a C-terminal HA<sub>3</sub> epitope tag.** Localization of *TbKH*::HA<sub>3</sub> in (A) isolated flagella, (B) subpellicular microtubules, and (C) mitotic spindles and subpellicular microtubules, prepared as in Fig. 1 but employing anti-HA mAb for immunostaining.

**Figure 3. Overlap of *LmxKH* with the mitotic spindle.** Promastigotes of *L. mexicana* expressing *LmxKH*::HA<sub>3</sub> were synchronized with hydroxyurea, released from the cell cycle block by washing out hydroxyurea, and cultured in hydroxyurea-free RPMI medium for 7 h. (A), (B), and (C) show images of parasites that were stained with anti- $\alpha$ -tubulin pAb (red), anti-HA antibody pAb (green), and with the red, green, and blue (DAPI stained) images merged (merged). White arrowheads mark mitotic spindles that stain with both anti- $\alpha$ -tubulin and anti-HA antibodies.

**Figure 4. Depletion of *TbKH* is lethal for BF parasites.** (A) Northern blot of total RNA from *TbKH*<sup>RNAi</sup> parasites grown in the presence (+) and absence (-) of doxycycline (dox) for 24 h were hybridized against a probe recognizing *TbKH* (upper panel) or the hexose transporter *TbHT1* (lower panel). Arrows indicate the positions of the relevant mRNAs. Molecular weight markers are indicated in nucleotides (nt). (B) Time course for loss of *TbKH*::HA<sub>3</sub> protein following induction of RNAi against *TbKH* mRNA. BF parasites expressing *TbKH*::HA<sub>3</sub> and carrying the *TbKH*<sup>RNAi</sup> construct were induced (+dox) or not induced (-dox) with doxycycline for the times indicated (h). Cell lysates (20  $\mu$ g) were resolved by SDS-PAGE, blotted, and probed with anti-HA (top panels) or anti- $\alpha$ -tubulin (bottom panels, Tub) antibodies. Molecular weight markers are indicated at the left of each panel in kD units. The numbers below the upper right panel indicate the relative signal intensity for *TbKH*::HA<sub>3</sub> (Expression level), first normalized to the  $\alpha$ -tubulin signal at each time point and then normalized to the signal at 0 h (set to 1). (C) Growth curve of induced (empty circles) and non-induced (filled circles) *TbKH*<sup>RNAi</sup> cell line. Parasite density was quantified by phase contrast microscopy using a hemacytometer. Points represent the means of two biological replicates, but error bars are too small to be visible on the logarithmic scale employed for the y-axis. (D) Frequency (%) of cells with different numbers of nuclei (N) and kinetoplasts (K) at different times following induction of RNAi against *TbKH* mRNA. Results represent the average and range of two independent experiments. (E-H) Representative *TbKH*<sup>RNAi</sup> cells undergoing RNAi were stained with DAPI (blue) and immunostained with anti- $\alpha$ -tubulin mAb (Tub,



red) at (E) 0 h, (F) 12 h (G) 24 h and (H) 48 h post-RNAi induction. DIC indicates images collected by differential interference contrast microscopy.

**Figure 5. Abnormal subcellular structures in BF *T. brucei* following induction of RNAi against *TbKH* mRNA.** Transmission electron micrographs (A-G) and serial block face scanning electron micrographs (H i-iv) show: (A), multiple flagella (white arrowheads) in multiple flagellar pockets; note presence of paraflagellar rods in several profiles. (B), multiple flagellar axonemes within a single flagellar membrane. (C), flagellar axoneme (white arrowhead) with associated PFR but without a flagellar membrane within the cell body. (D), enlarged flagellar pocket (FP) with a single flagellum (black arrowhead). (E), two nuclei (N) abnormally close together. (F), nucleus with several lobes. (G), nucleus with apparent membranous inclusion (white arrowhead). H (i-iv), sequential sections through a nucleus showing that apparent membranous inclusions represent invaginations of the nuclear envelope. All scale bars represent 500 nm. Induction of RNAi against *TbKH* mRNA was as follows: A=24 h, B=18 h, C=24 h, D=48 h, E=24 h, F=24 h, G=25 h, H=48 h).

**Figure 6. *TbCaCh* depends on *TbKH* for translocation to the flagellar membrane in BF parasites.** *TbCaCh::HA<sub>3</sub>/TbKH<sup>RNAi</sup>* or *TbCaCh::HA<sub>3</sub>/TbFLA1<sup>RNAi</sup>* cell lines were induced with 1 µg ml<sup>-1</sup> doxycycline (dox). Parasites were stained with DAPI and immunostained with anti-HA mAb (*TbCaCh*, green) and anti- $\alpha$ -tubulin pAb (Tub, red). (A) Non-induced *TbCaCh::HA<sub>3</sub>/TbKH<sup>RNAi</sup>* parasites (-dox). (B,C) *TbCaCh::HA<sub>3</sub>/TbKH<sup>RNAi</sup>* parasites induced for 48 h (48 h dox). (D) *TbCaCh::HA<sub>3</sub>/TbFLA1<sup>RNAi</sup>* parasites induced for 72 h (72 h dox). White arrowheads in (B,C) indicate flagella where *TbCaCh* is absent, and the yellow arrowhead in (D) indicates a flagellum where *TbCaCh* is present. (E) Immunoblot of isolated flagella from *TbCaCh::HA<sub>3</sub>/TbKH<sup>RNAi</sup>* (lane 1) and *TbCaCh::HA<sub>3</sub>/TbFLA1<sup>RNAi</sup>* (lane 2) cells, induced for 48 h and 72 h respectively, and probed with anti-HA mAb (*TbCaCh*, upper panel), and anti- $\alpha$ -tubulin mAb (Tub, lower panel) as loading control. (F) Immunoblot of coimmunoprecipitated proteins from Ty1::TbKH (lane 1), *TbCaCh::HA<sub>3</sub>/Ty1::TbKH* (lane 2), *TbCaATPase::HA<sub>3</sub>/Ty1::TbKH* (lane 3), FS33::HA<sub>3</sub>/Ty1::TbK1 (lane 4) and FS133::HA<sub>3</sub>/Ty1::TbKH (lane 5) expressing parasites. Immunoprecipitation was performed with BB2 mAb directed against the Ty1 epitope to precipitate Ty1::TbKH, and the blot of the immunoprecipitated material was probed (upper panel) with antibody directed against the HA epitope to detect coimmunoprecipitated proteins. The lower panel shows the blot probed with BB2 mAb to detect the immunoprecipitated Ty1::TbKH. (G) Immunoblot of input total protein lysates from Ty1::TbKH expressing lines used for coimmunoprecipitation experiments in (F). Lanes were spliced from different sections of the blots in panels (F) and (G), according to the order in (F), and delineated by black bars. (H) Immunoblot of total protein lysates (20 µg) from *TbCaCh::HA<sub>3</sub>/TbKH<sup>RNAi</sup>* or *TbCaCh::HA<sub>3</sub>/TbFLA1<sup>RNAi</sup>* cell lines induced with 1 µg ml<sup>-1</sup> doxycycline (dox) at various time points, to determine the level of *TbCaCh::HA<sub>3</sub>* following induction of RNAi. Protein blots were probed with anti-HA mAb (*TbCaCh*, upper panel), and anti- $\alpha$ -tubulin mAb (Tub, lower panel) as loading control.

**Figure 7. *TbCaATPase* translocation to the flagellar membrane is *TbKH* independent.** *TbCaATPase::HA<sub>3</sub>/TbKH<sup>RNAi</sup>* or *TbCaATPase::HA<sub>3</sub>/TbFLA1<sup>RNAi</sup>* clones were induced with doxycycline for 48 h and 72 h, respectively, stained with DAPI (blue) and immunostained with anti-HA mAb (*TbCaATPase*, green) and anti- $\alpha$ -tubulin pAb (Tub, red). (A) Non-induced *TbCaATPase::HA<sub>3</sub>/TbKH<sup>RNAi</sup>* clone (-dox). (B) *TbCaATPase::HA<sub>3</sub>/TbKH<sup>RNAi</sup>* clone induced for 48 h (48 h dox). (C) *TbCaATPase::HA<sub>3</sub>/TbFLA1<sup>RNAi</sup>* clone induced for 72 h (72 h dox). (D) Immunoblot of isolated flagella from *TbCaATPase::HA<sub>3</sub>/TbKH<sup>RNAi</sup>* (lane 1) and *TbCaATPase::HA<sub>3</sub>/TbFLA1<sup>RNAi</sup>* (lane 2) cells, induced for 48 h and 72 h respectively, was immunodetected with anti-HA mAb (*TbCaATPase*, upper panel), and anti- $\alpha$ -tubulin mAb (Tub, lower panel) as loading control.

**Figure 8. Loss of mitotic spindle in BF parasites upon induction of RNAi against *TbKH*.** (A-D) *TbKH*<sup>RNAi</sup> cells were grown in the presence of doxycycline for times between 0 – 48 h, stained with DAPI (blue) and immunostained with KMX-1 mAb (red). The white arrowheads point to mitotic spindles that were visible in uninduced cells and in parasites for which RNAi against *TbKH* had been induced for 12 h, but spindles were not visible in cells that had been induced for 24 or 48 h. (E) Percent of BF parasites showing mitotic spindles prior to induction (-dox) and 4, 8, 12, 24 or 48 h after induction with doxycycline. Numbers represent the mean and range of two independent experiments for which 100 cells were counted. No mitotic spindles were detected at 24 and 48 h as indicated by asterisks.

**Figure 9. Sub-cellular localization of *TbKH* in PF parasites.** (A) Flagellar axonemes were isolated from Ty1::TbKH-expressing PF parasites by detergent lysis and incubation with NaCl (Materials & Methods) to disrupt the subpellicular microtubules, and preparations were stained with antibodies against  $\alpha$ -tubulin (red) and Ty1 (BB2 mAb, green) and with DAPI to visualize kDNA (blue). The white arrowhead indicates the green fluorescence representing Ty1::TbKH at the base of the flagella. (B) Ty1::TbKH PF parasites were immunostained with BB2 mAb (*TbKH*, green) and anti- $\alpha$ -tubulin (Tub, red) to show localization of *TbKH* that partially overlaps with the subpellicular microtubules (white arrowhead). (C) Ty1::TbKH PF parasites were stained with DAPI (blue) and immunostained with BB2 mAb (*TbKH*, green) or KMX-1 mAb (Tub, red). The white arrowhead indicates KMX-1 antibody staining on the spindle between two nuclei (left image) and *TbKH* on the mitotic spindle (right image) in parasites undergoing karyokinesis.

**Figure 10. Effect of *TbKH* depletion on PF parasites.** (A) Northern blot of total RNA from parasites grown in the presence (+) and absence (-) of 1  $\mu$ g ml<sup>-1</sup> doxycycline (dox) for 48 h was hybridized against probes for *TbKH* (upper band) or *TbHT1* (lower band). Molecular weight markers are indicated in nucleotides (nt) and the positions of the relevant mRNAs are indicated by arrows. (B) Growth curve of induced (empty circles) and non-induced (filled circles) *TbKH*<sup>RNAi</sup> PF parasites. Data represent the average of two independent experiments; error bars are too small to see on the logarithmic scale employed for the y-axis. (C) Frequency (%) of cells with different numbers of nuclei (N) and kinetoplasts (K) following induction of RNAi for 1-7 d. Data represent the mean and range of two independent experiments for which 100 cells were counted. The absence of the black filled bar for 1N1K and 2N2K samples indicates that no parasites with these characteristics were observed on day 7. (D-F) *TbKH*<sup>RNAi</sup> PF parasites grown in the presence and absence of doxycycline were stained with DAPI (blue) and immunostained with anti- $\alpha$ -tubulin mAb (red). Parasite samples were processed at different time points, (D) 48 h minus doxycycline (48h -dox), (E) 48 h plus doxycycline (48h dox) and (F) 72 h plus doxycycline (72h dox).

**Figure 11. Functional complementation of *Leishmania kharon* null mutant by *TbKH*.** *L. mexicana*  $\Delta$ *lmxkh* null mutants ( $\Delta$ *kh*) expressing the *LmxGT1* flagellar glucose transporter tagged with GFP at its C-terminus [pGT1::GFP] were transfected with an episomal expression vector encoding the *TbKH* gene [p*TbKH*].  $\Delta$ *lmxkh*[pGT1::GFP] ( $\Delta$ *kh*) and  $\Delta$ *lmxkh*[pGT1::GFP]/p*TbKH* ( $\Delta$ *kh*+ [p*TbKH*]) were stained with DAPI (blue), and immunostained with anti-GFP pAb (GFP, green) and anti- $\alpha$ -tubulin mAb (Tub, red). (A) Quantification of *LmxGT1*::GFP localization (%) in *L. mexicana* lines. Data for wild type *L. mexicana* (WT),  $\Delta$ *kh* and  $\Delta$ *kh*[p*KH*] ( $\Delta$ *kh* null mutants complemented with an episomal expression vector encompassing the *LmxKH* gene) were previously published by Tran *et al.* (21) and are reproduced here for comparison. The key at the bottom shows the labeling scheme for quantification of parasites that contain GT1::GFP in the flagellar membrane (FM), flagellar membrane plus pellicular plasma membrane (FM+PPM), flagellar pocket (FP), and pellicular plasma membrane only (PPM only). The number of parasites examined in each quantification was: WT=210,  $\Delta$ *kh*=241,  $\Delta$ *kh*+ [p*KH*]=139 and  $\Delta$ *kh*+ [p*TbKH*]=249. (B) Examples of *LmxGT1*::GFP localization in non-complemented ( $\Delta$ *kh*, upper panel) and complemented ( $\Delta$ *kh*+ [p*TbKH*]) mutants. Yellow arrowheads in the upper panel indicate GT1::GFP localized in the flagellar pocket. White arrowheads in the lower panel indicate GT1::GFP in

the flagellum (C) Quantification of THP-1 infection with wild type (WT),  $\Delta kh$  mutant, and  $\Delta kh$  complemented with an episomal copy of *TbKH* ( $\Delta kh+[pTbKH]$ ) promastigotes. Day1 and Day7 indicate the time elapsed following infection. Data represent the averages and standard deviations of triplicate infections (n = 3), each counted in triplicate and averaged. The statistical significance for comparison of the null mutant and complemented null mutant on Day 7 was determined by two tailed Student's t-test ( $P<0.05$ ).

# Ty1::TbKH

Figure 1

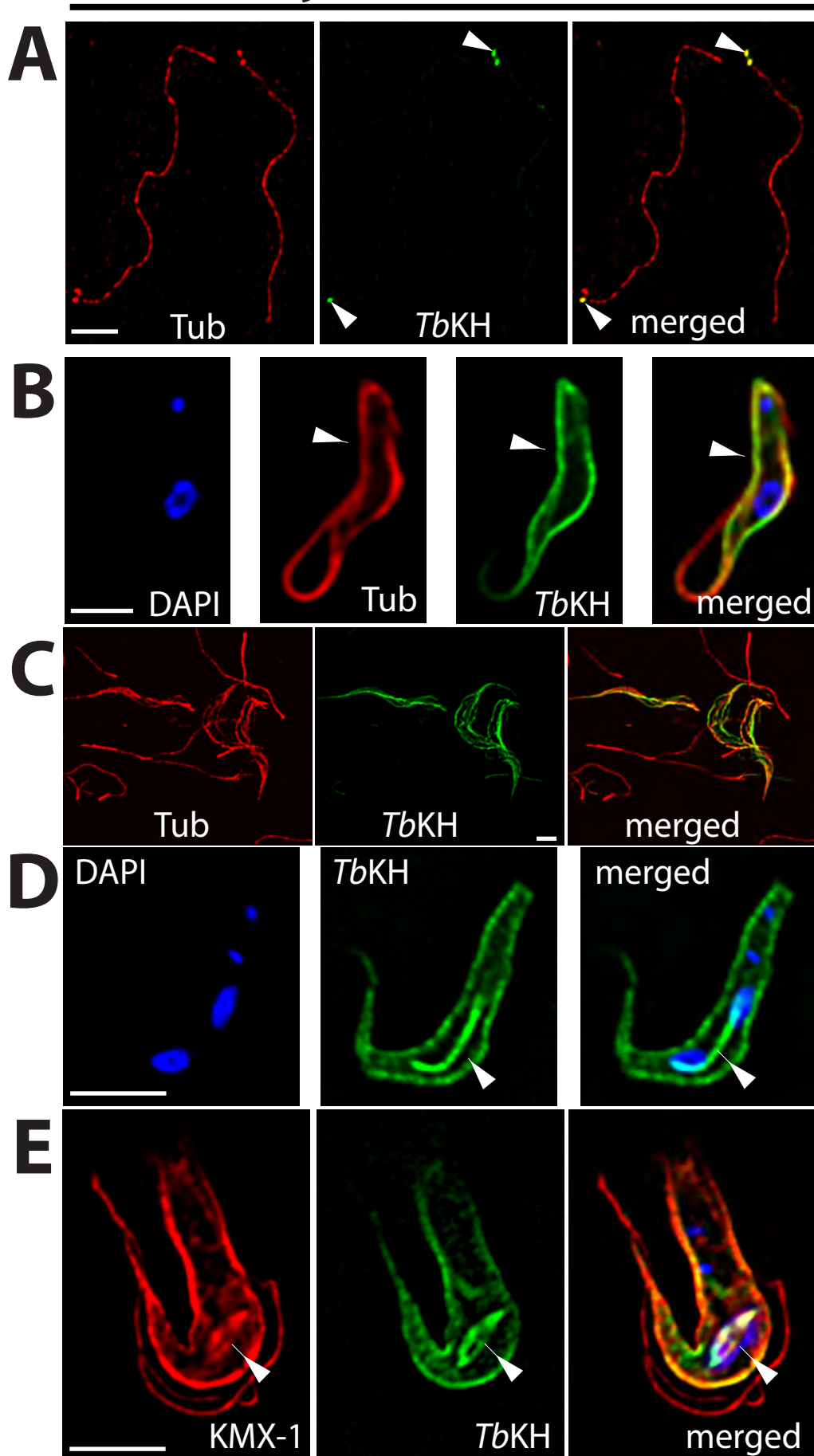




Figure 2

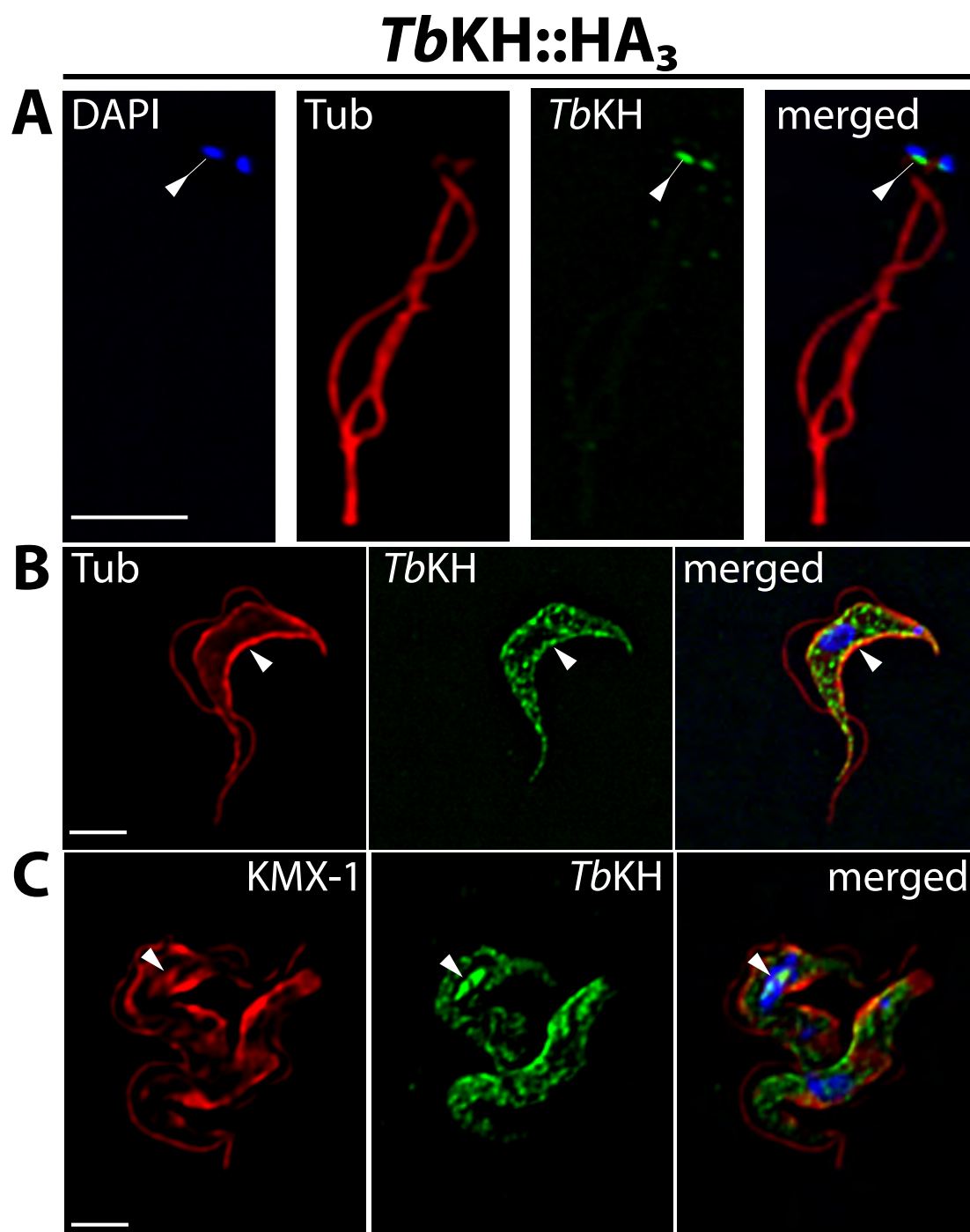
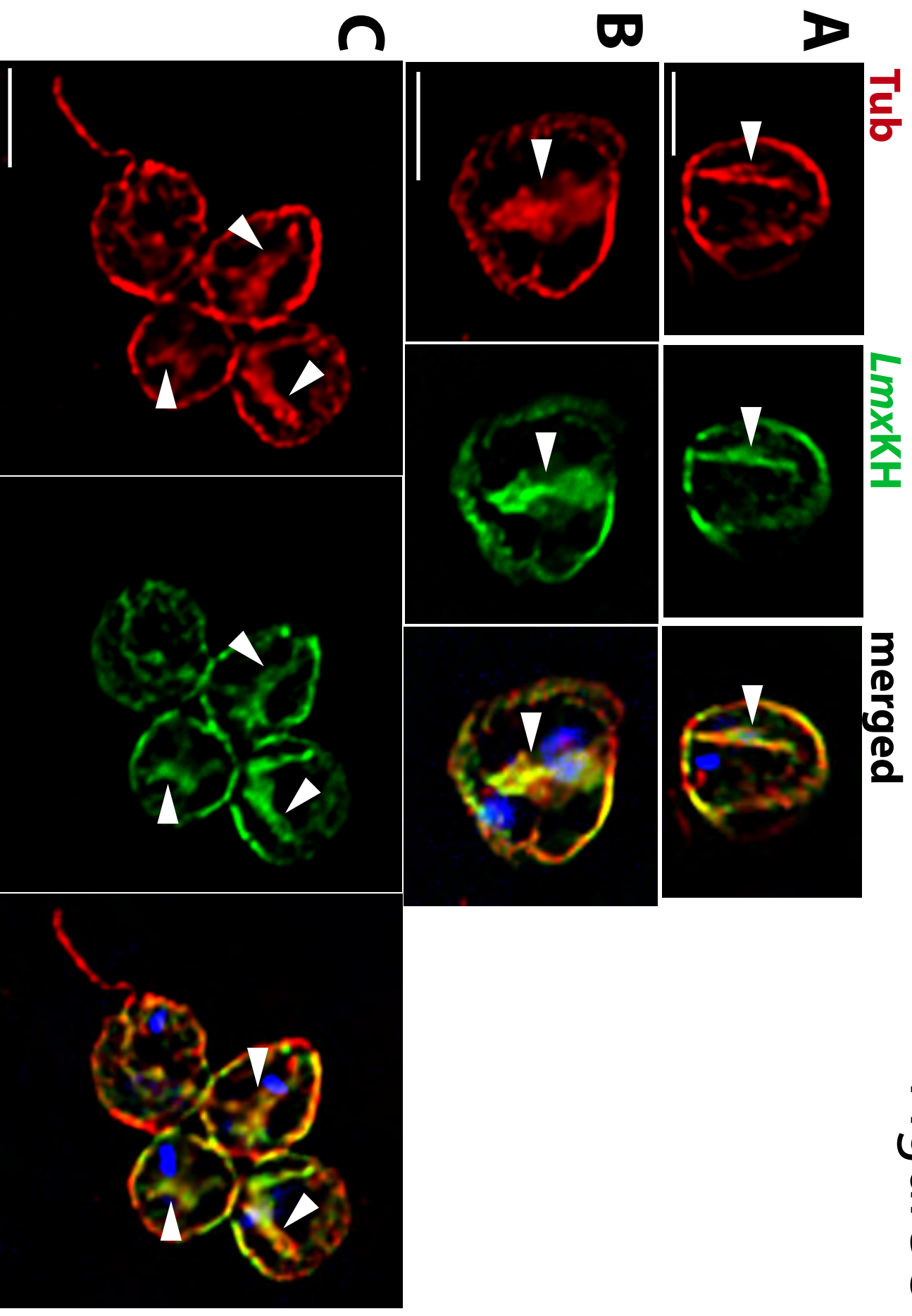
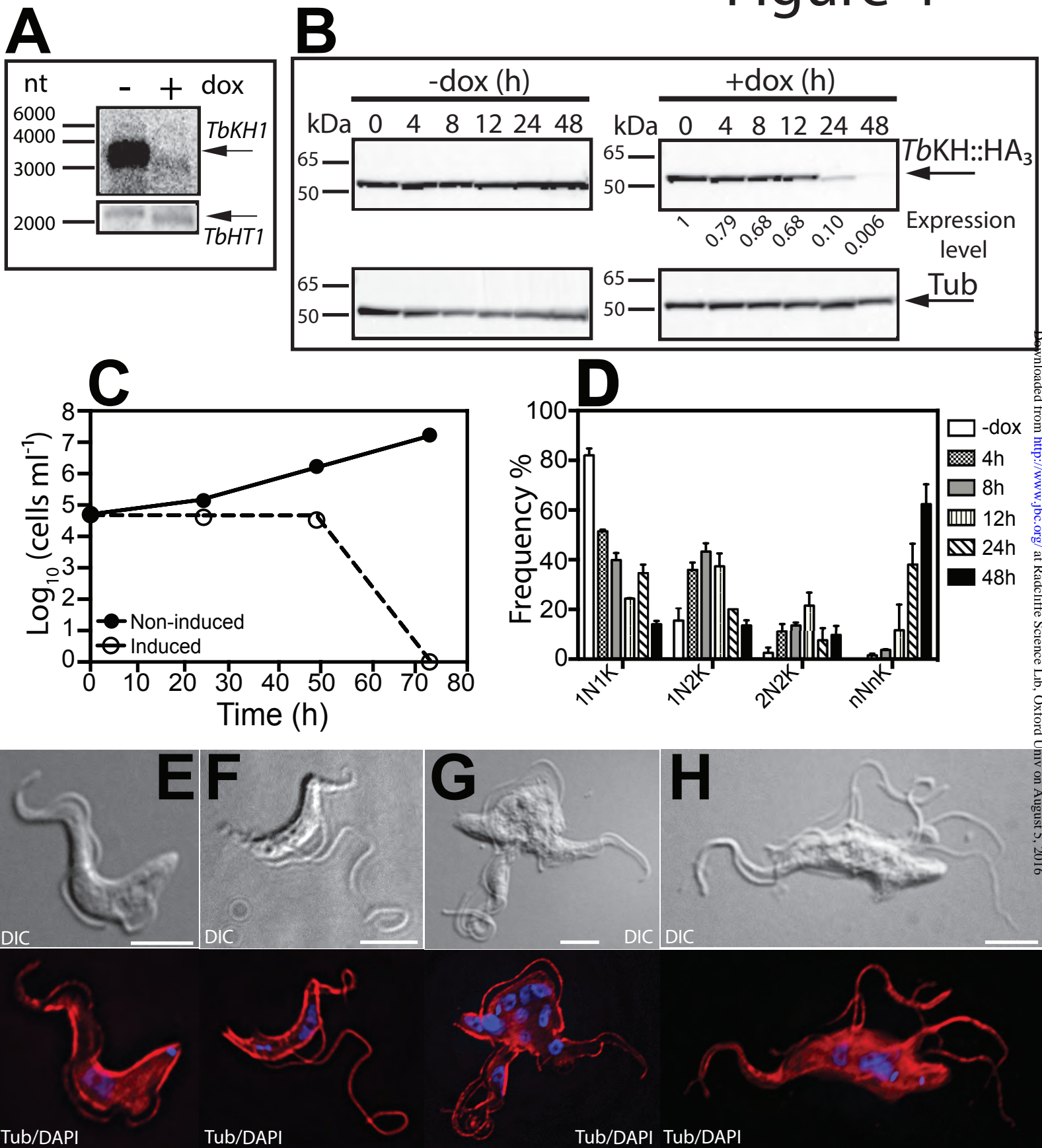


Figure 3

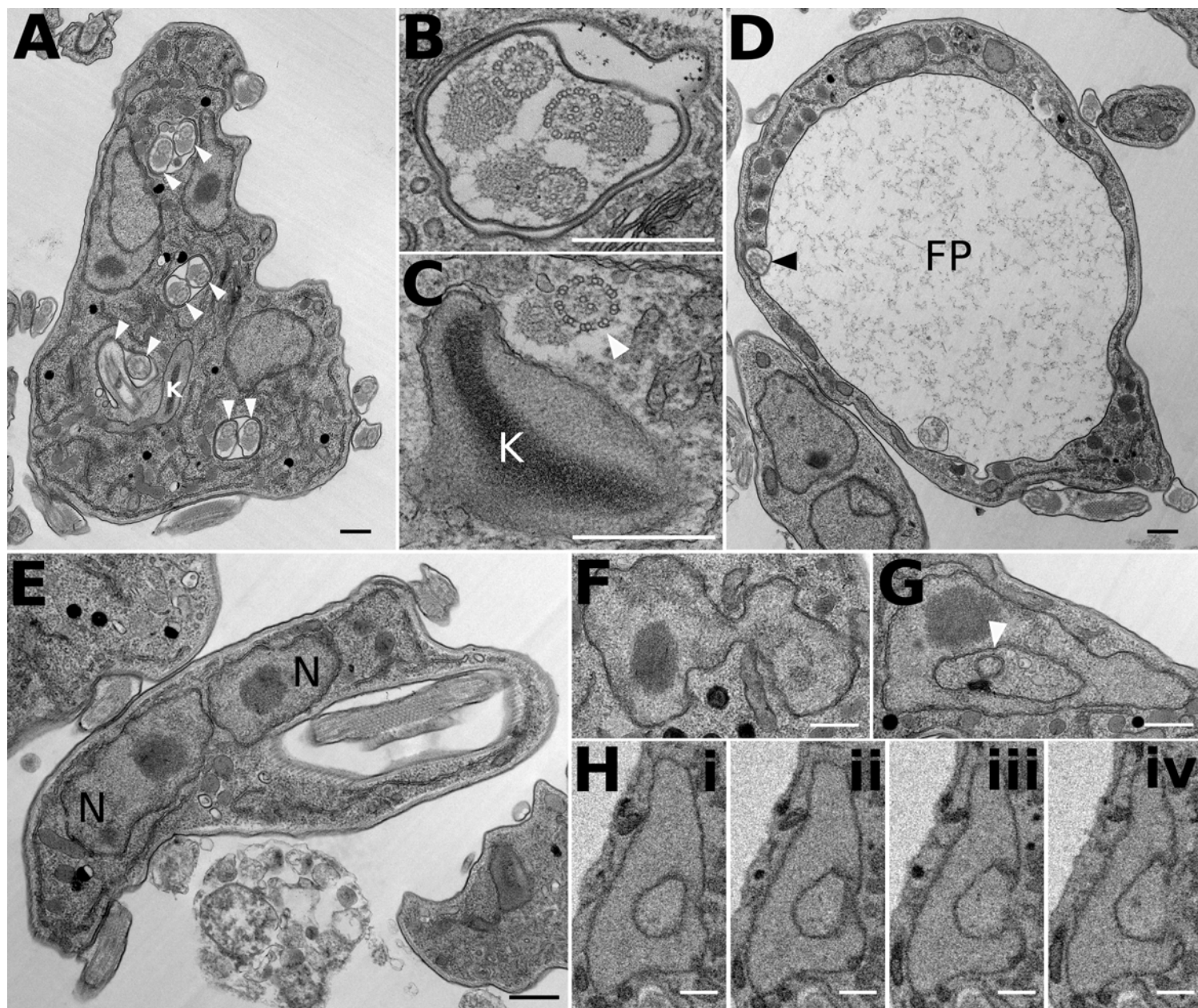


# Figure 4





**Fig. 5**





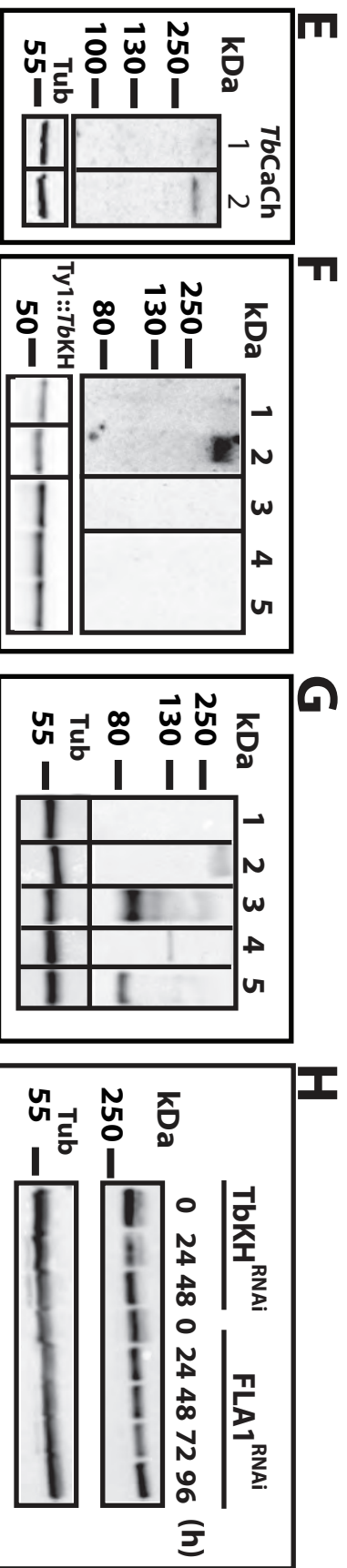
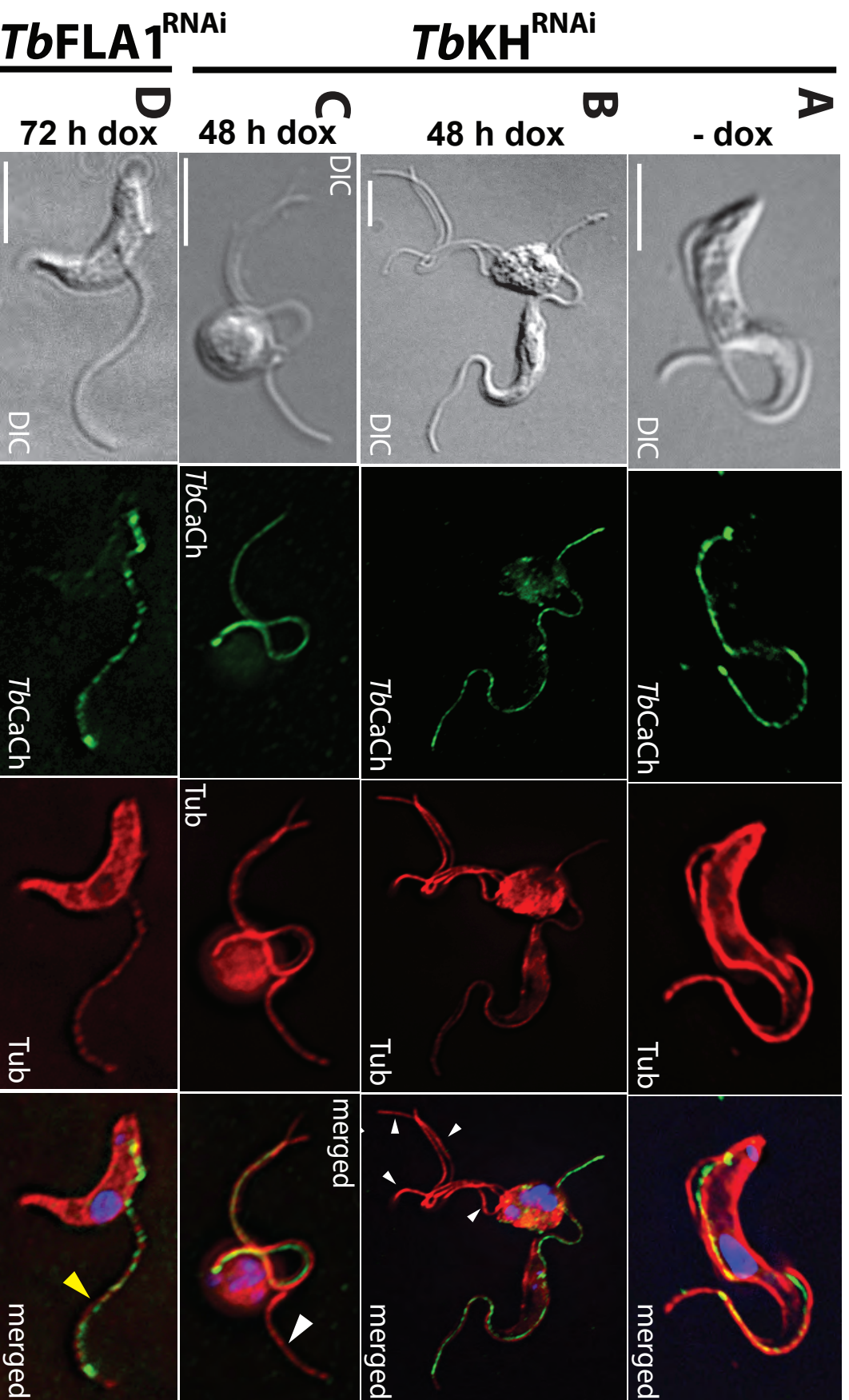
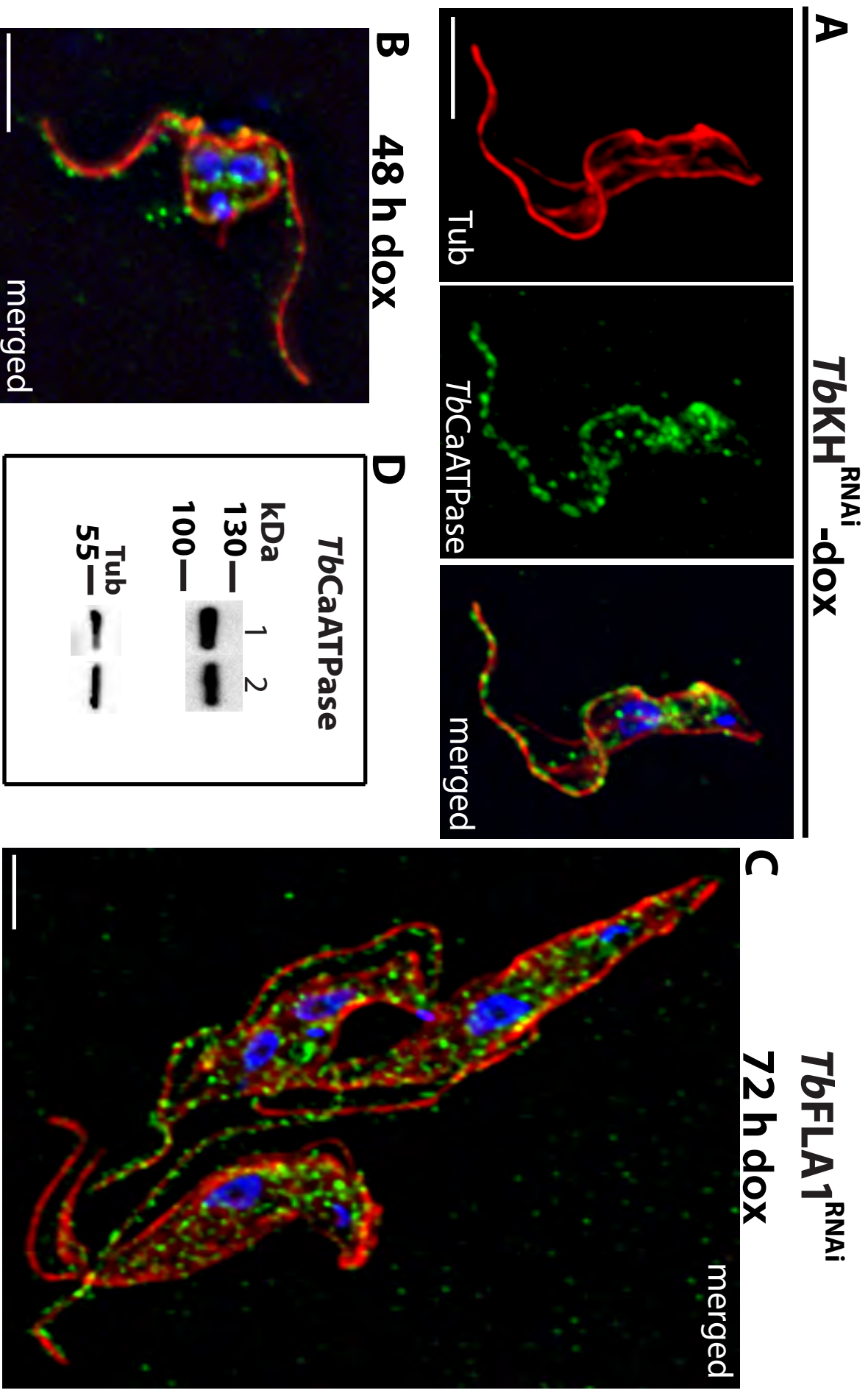


Figure 7



# Figure 8

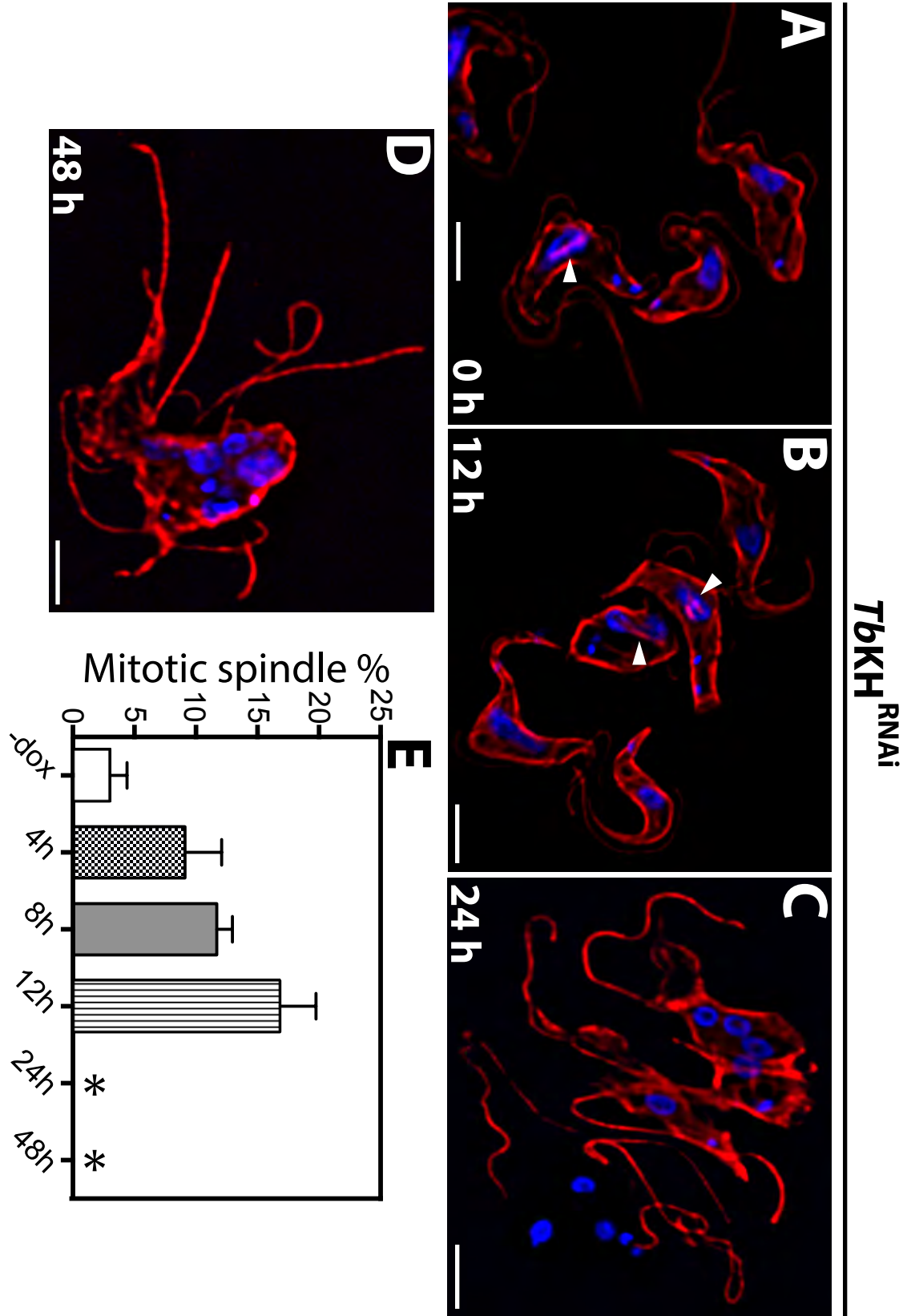


Figure 9

# Ty1::TbKH

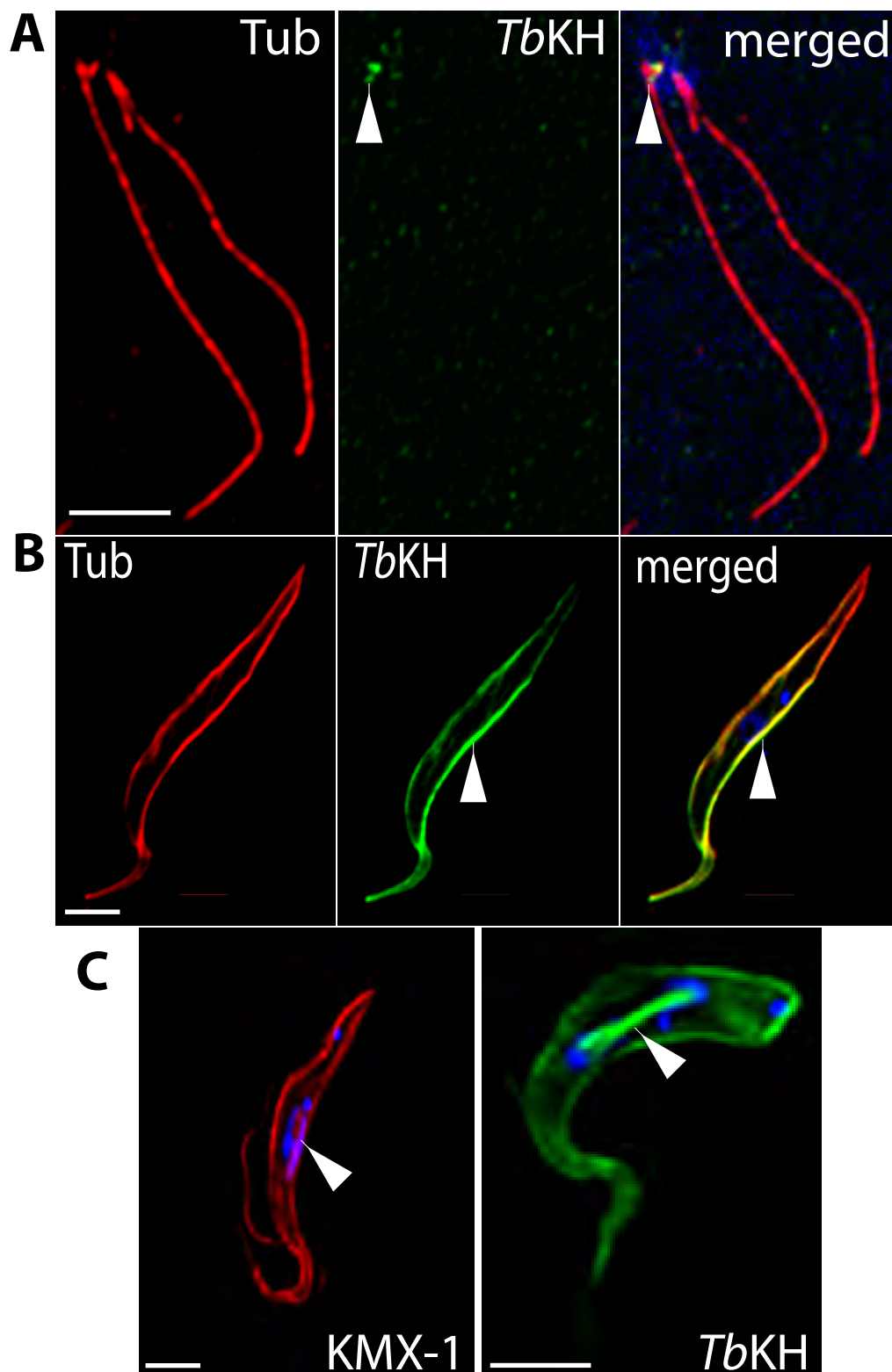




Figure 10

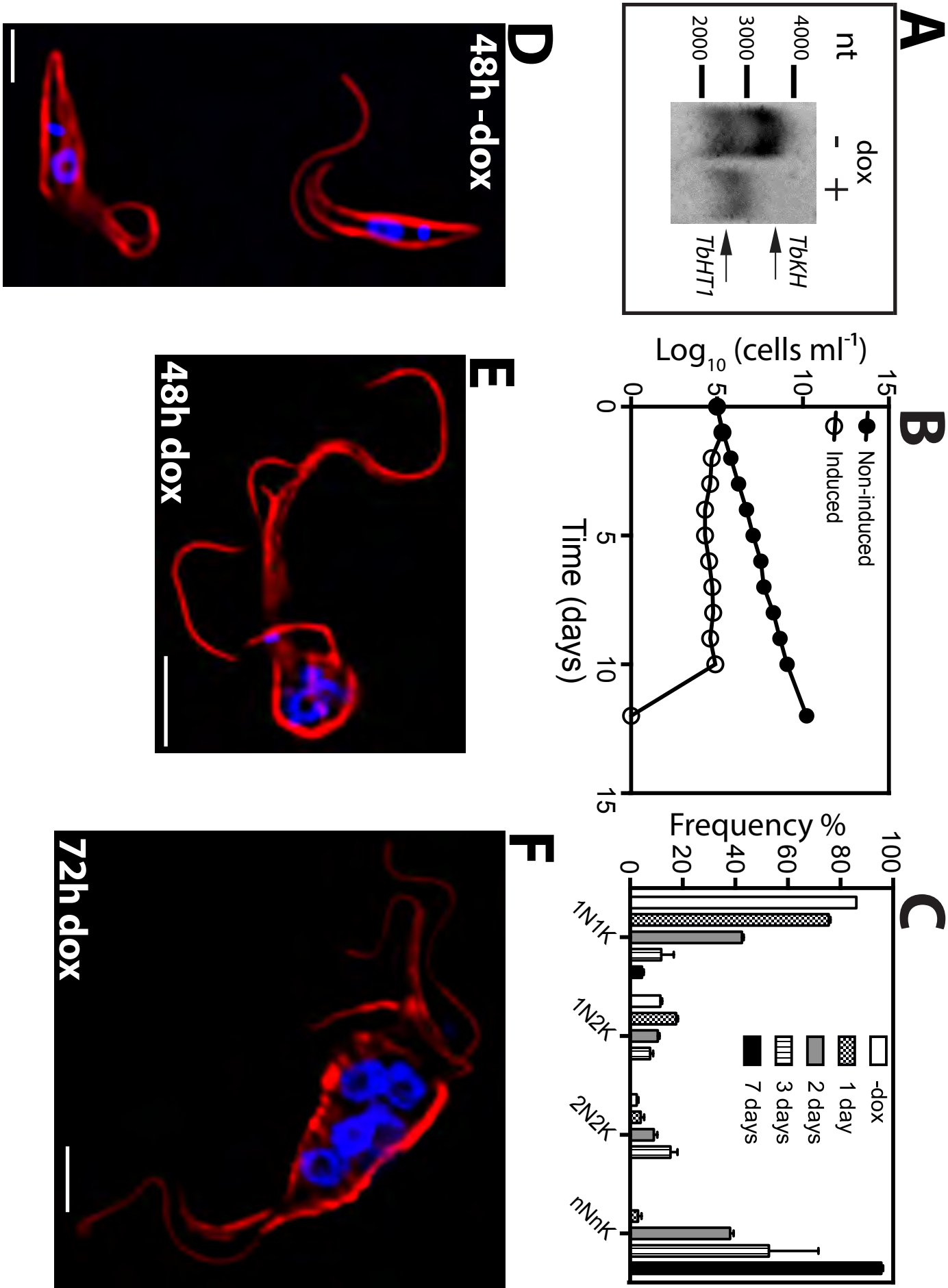
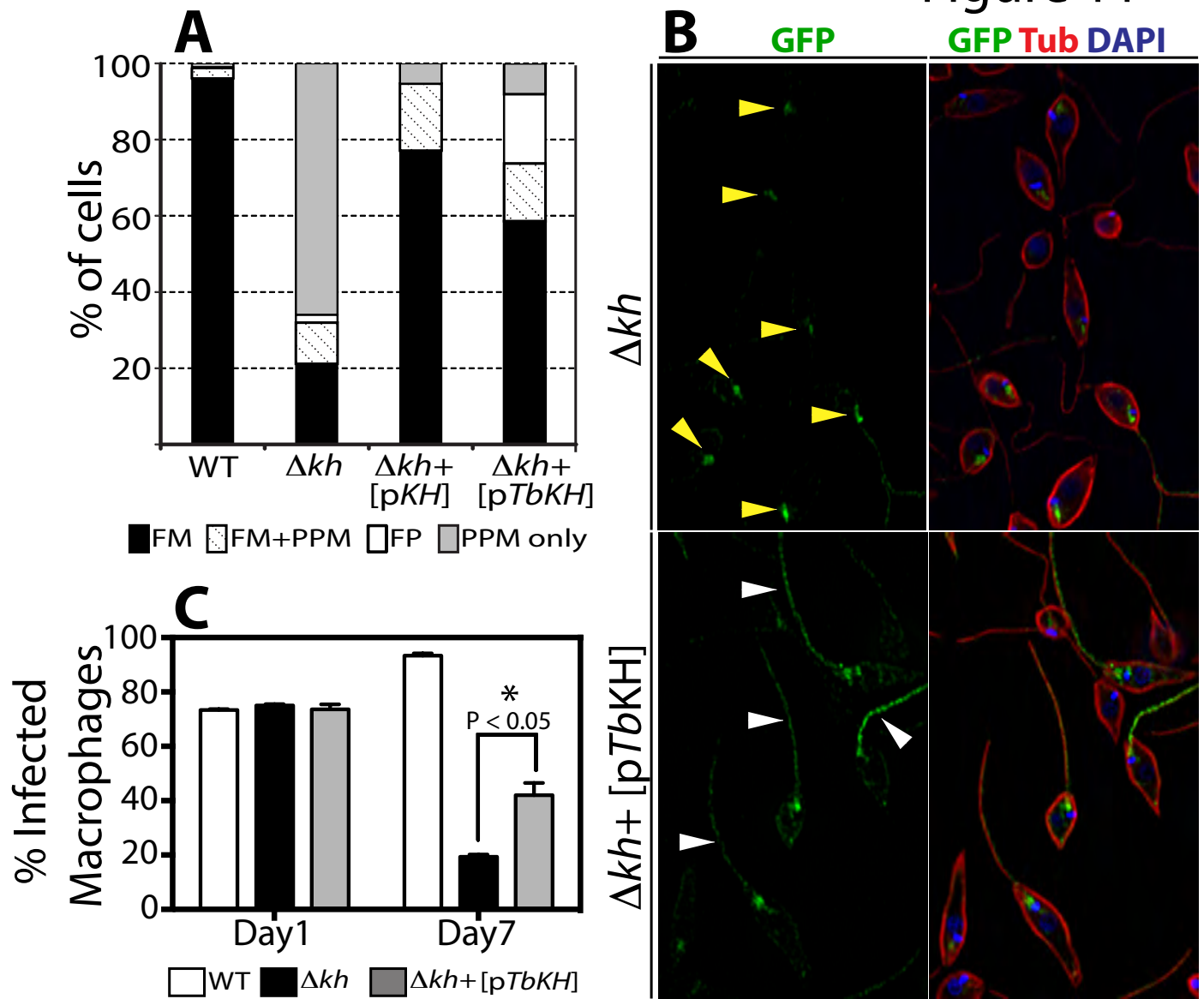


Figure 11



**KHARON is an Essential Cytoskeletal Protein Involved in the Trafficking of Flagellar Membrane Proteins and Cell Division in African Trypanosomes**

Marco A. Sanchez, Khoa D. Tran, Jessica Valli, Sam Hobbs, Errin Johnson, Eva Gluenz and Scott M. Landfear

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