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TFK1, a basal body transition fibre protein that is essential for cytokinesis in *Trypanosoma brucei*

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MS TITLE: TFK1, a basal body transition fibre protein that is essential for cytokinesis in Trypanosoma brucei

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from reviewers.

In terms of additional experiments, there could be some consideration to examining other markers e.g. those used by the other labs named but I would not consider this an essential revision. If you have further work you could include or could easily conduct some further comparative analysis then this would improve the manuscript. Otherwise some additional discussion of the choice of marker and relationship to those used by others should suffice. I agree with the other substantive points raised but consider that these can be addressed by changes to the text and figures.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This work describes the characterisation of a new component of the transitional fibres, TFK1. Apparently kinetoplastid specific, the authors use widefield fluorescence microscopy, ultra-expansion-microscopy (U-ExM) and immunogold labelling to localise TFK1 to the distal transitional fibres between CEP164C, a location consistent with the transitional fibre matrix. The authors express TFK1 domains in a Y2H analysis and show that the coiled-coils are necessary and sufficient for interaction with the full length protein, and ectopic expression in procyclic trypanosomes to show that the coiled-coils are necessary and sufficient to localise to the transitional fibres (in the genetic context of parasites expressing the wildtype protein), together suggesting that the coiled-coils mediate a TFK1-TFK1 interaction that is required for localisation.
Phenotypic analysis of RNAi mutants gives no clues to function in procyclics, but suggest an important function in bloodstream form cells. The authors use fluorescence microscopy of DNA stained RNAi mutants and SEM of RNAi mutants to show that TFK1 is required for correct cytokinesis, a function that is distinct from that of the other trypanosome transitional fibre proteins, CEP164C and RP2.
This is a well performed study with high quality data (especially the U-ExM) that ascribes a new function to a highly conserved structure (the transitional fibres) and will be of interest to cell biologists and parasitologist studying the cytoskeleton.

Comments for the author

I feel that some more detailed characterisation of the furrow phenotype could be done using markers that have been published by the de Graffenreid / Sunter / Li labs. However, I appreciate that lab access can be challenging in COVID times and leave it in the hands of the authors to decide whether this is necessary for this paper.
The authors must clearly and unambiguously outline how they discovered TKP1 in the results section.
Some additional detail on how they determined that TFK1 is kinetoplastid specific (e.g. BLAST cutoffs etc).
Did they also try HMMs as a more sensitive ortholog detection method? PSI-BLAST? Trypanosomes are divergent and detecting non-kinetoplastid orthologs can be challenging and require more sensitive methods.
General point: the discussion is rather long, at times unfocussed, and contains a few unclear sentences.

Specific points:
Line 55: the basal bodies are NOT connected to the kinetoplast via the transitional fibres Line 150 and Figure 1B and Figure 1 legend: 4 bands are detected in the anti TFK1 western on bsf, but the text only describes two. This looks as if the anti TFK1 antibody is picking up a tagged and untagged copy of TFK1 but the F1 legend states these are WT cells. Please could the authors clarify this issue.
Line 155: here and other places in the text “mNeon Green” should be “mNeonGreen”.
Line 247 and Figure 5: the authors state that deletion mutants T1::myc and T3::myc are extracted using NP40 but this data is not in Figure 5.
Line 421: “bioinformatics analysis in the TriTrypDB resource” is overly ambiguous; rephrase this sentence for clarity and precision.
Line 440: rephrase for clarity.
Line 783: can the authors confirm they used a p value cutoff of 0.005 (if so, great).

Line 695: typo “anti-BILBO1 1-110”.
Line 696: please detail the source of the anti mNeonGreen antibody (and also any other primary antibodies not detailed elsewhere).
Acknowledgements: Several unpublished pPOT plasmids have been used in this study (e.g. 10xTY1, line 168 line 574). Could the authors acknowledge the source of these?
Acknowledgements: It would be courteous to also acknowledge Prof Keith Gull as the original source of the BB2 antibody and hybridoma.

Reviewer 2

Advance summary and potential significance to field

Ramanantsalama and colleagues characterise the localisation and function of transition fibre protein TFPK1 in PCF and BSF in Trypanosoma brucei. This is a nicely presented paper, which reveals a unique and interesting function of the transition fibre component in cytokinesis and furrow ingression in BSF trypanosomes. The experiments are well carried out in general except for a few issues that are outlined below.

Comments for the author

1. In the introduction (p5), the authors mention the MTs of the BB are followed by the transition zone and then transition fibres. This is a little misleading as the transition fibres are distal to the TZ. The order of wording would ideally match the structure position proximal to distal or vice versa e.g BB, followed by the TF and then TZ (or TZ, followed by TF and BB) I recommend changing the wording to Transition fibres followed by the transition zone.
2. Lin90-97 are not clear in terms of linking TF with distal appendages. Do the authors consider them analogous structurally and functionally? It appears so in the discussion. They should make this clearer in the intro. There are some nice papers on distal appendage proteins that should be introduced here and included to compare with functions in this organism.
3. In the introduction the term TF does not appear after the original long name of transition fibres.
4. On p7 for the co-labelling experiments, authors mention their marker proteins BLD-10, FTZC and BILBO1 without much explanation of why these proteins were chosen out of many possible marker proteins. A short explanation on their localisation cell cycle pattern etc would be good, particularly with BLD-10. The use of CEP164C and RP2 is obvious given they are the only TF proteins that have been published. It would also be helpful to label the mature basal body and pro-basal bodies with an arrow/arrowhead to guide the reader.
5. ExM (p8) the diameter of TFK1 was shown to be 477nm. This seems quite wide given the ultrastructural analysis by Trepout et al., 2018 who suggested that cross sectional diameter of TFs by EM is around 330nm. Sample of n=6 is quite small. Does this align with immunogold measurements? Suppl. Table 1 is not conclusive as only measures from gold particle to edge of BB (TFs are angled at 50° (trepout et al. 2018) so diameter from this would not be the sum of 120 (Sup. 1) x2 + 200 (bb diameter)). Variation is quite high in these measurements (some as high as 170nm and others 83nm so concerns on the orientation of slices used for EM). Measuring the distance between gold particles in figure 3C (lateral view) would give you an indication of whether 477nm is a reasonable measurement.
6. ExM (Figure 2A) showed that CEP164C and TFK1 puncta do not overlap. There is a mention that they are in different compartments of the TF. TF is a single structure that is unlikely thick enough for two proteins to be completely offset (even by U-ExM). Likely one protein is on the blade and one protein is within the matrix. RP2 does not exhibit 9 distinct puncta like CEP164C and TFK1 yet it is a transition fibre protein. Is there an explanation for this?
7. Green signal in Figure 2A is maybe slightly overexposed. In Figure 2B e the cell shown with DAPI and phase looks morphologically abnormal.
8. The reviewer realises that immuno-EM is not an easy set of experiments however there are not really very convincing images on the TFs, rather in the position where one might expect them to be?
9. Figure 7: Is KNKN orientation is not unusual in PCF form trypanosomes.

Do flagella assemble to the correct length? Transition fibres are known to play a critical role in flagellar assembly in other organisms. Is CEP164c and RP2 labelling maintained in TFK1 RNAi cells? This would indicate whether transition fibre assembly is affected.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

This work describes the characterisation of a new component of the transitional fibres, TFK1. Apparently kinetoplastid specific, the authors use widefield fluorescence microscopy, ultra-expansion-microscopy (U- ExM) and immunogold labelling to localise TFK1 to the distal transitional fibres between CEP164C, a location consistent with the transitional fibre matrix. The authors express TFK1 domains in a Y2H analysis and show that the coiled-coils are necessary and sufficient for interaction with the full length protein, and ectopic expression in procyclic trypanosomes to show that the coiled-coils are necessary and sufficient to localise to the transitional fibres (in the genetic context of parasites expressing the wildtype protein), together suggesting that the coiled-coils mediate a TFK1-TFK1 interaction that is required for localisation.
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I feel that some more detailed characterisation of the furrow phenotype could be done using markers that have been published by the de Graffenreid / Sunter / Li labs. However, I appreciate that lab access can be challenging in COVID times and leave it in the hands of the authors to decide whether this is necessary for this paper.

Author response (blue font)

We thank the reviewer for this comment and agree that the furrow phenotype is of interest. We have planned to look at this in future work, but we also acknowledge that currently COVID related issues have reduced our ability to do the work in a reasonable amount of time or recruit individuals to do it.

The authors must clearly and unambiguously outline how they discovered TKP1 in the results section.

Author response

We thank the reviewer for raising this point; TFK1 was identified with a moderate confidence in the interaction in a yeast two-hybrid genomic library screen by Hybrigenics using BILBO1 as bait (Albisetti et al., 2017). This suggests a low probability potential interaction between BILBO1 and TFK1. The characterization of the BILBO1-TFK1 interaction is ongoing and the focus of another piece of work.

We have clarified as amended in the text (line 147): “Tb927.6.1180 protein (ProteinID: XP_845240.1) was first identified as a potential BILBO1 binding partner, with a low interaction reliability predicted biological score, in *T. brucei* 927 genomic yeast two-hybrid screen (Hybrigenics), using BILBO1 as bait (Albisetti et al., 2017). Here, we identified Tb927.6.1180 as a novel basal body protein and designated it as TFK1 for Transition Fibres Kinetoplastid- specific protein 1.”

Some additional detail on how they determined that TFK1 is kinetoplastid specific (e.g. BLAST cutoffs etc). Did they also try HMMs as a more sensitive ortholog detection method? PSI-BLAST? Trypanosomes are divergent and detecting non-kinetoplastid orthologs can be challenging and require more sensitive methods.

Author response

We thank the reviewer for this comment and we agree that the detection of non-kinetoplastid orthologs can be difficult and requires more sensitive methods. This is why we had performed ortholog searches with other algorithms and software:

(i) PSI-BLAST (Position-Specific Iterated BLAST) with no significant similarity found, using default parameters (Expect Evalue 0.05, Hitlist size 500, Gapcosts 11,1, Matrix BLOSUM62,with low Complexity Filter, Filter string L; Genetic Code 1, Threshold 11, Composition-based stats 2)

(ii) HMMER v3.3.2 (biosequence analysis using profile hidden Markov models) comparing a profile-HMM
-with the reference proteomes database, the last outcomes retained are "Q4QGT1_LEIMA Leishmania major E-value 1.6e-49" and then"A0A7L1E995_OENON Oenanthe oenanthe E-value 0.0081",
-with the UniProtKB database the last outcomes retained are "Q4QGT1_LEIMA Leishmania major E- value 5.4e-49" and then "A0A7S4CGM1_9EUGL Eutreptiella gymnastica E-value 1.9e-09" and "A0A2W6RL65_9CAUL Brevundimonas sp. E-value 0.004",
-and with the SwissProt and the Pfam database no hits were found.

We used the default parameters (Hit E-value threshold (reporting) (domE) of 1, sequence E-value threshold (incE) of 0.01, Hit E-value threshold of 0.03 (incdomE), BLOSUM62 matrix, Gap extend penalty (pextend) of 0.4, Gap open penalty (popen) of 0.02)

These analyses show that the orthologs are found only in kinetoplastida and were not found in other organisms including mammals. We have detailed here in lines 159: "Orthologs searches using BLAST-P, PSI-BLAST and HMMER (Altschul, 1997; Finn et al., 2011), identified orthologs but only in the Kinetoplastea class with a conserved gene synteny."

The required details have been added in Materials and Methods (In silico analysis section) (line 552): "TFK1 orthologs were identified by walking BLAST-P, PSI-BLAST and HMMER ((Altschul, 1997; Altschul et al., 1990; Altschul et al., 2005; Finn et al., 2011) using default parameters (standard databases, non-redundant protein sequences database, maximum target sequences 100, with short queries, expect threshold E-value of 0.05 (and 0.01), no maximum matches in a query range)".

General point: the discussion is rather long, at times unfocussed, and contains a few unclear sentences.

Author response

Upon we agree with the reviewer and have shortened some of the discussion points.

Specific points:
Line 55: the basal bodies are NOT connected to the kinetoplast via the transitional fibres

Author response

We apologies for this inaccuracy. We corrected it as follows (line 56): "The basal bodies (BBs) are connected to the mitochondrial genome (kinetoplast) through the tripartite attachment complex (TAC) but also with the FP membrane through the transition fibres (TFs)."

Line 150 and Figure 1B and Figure 1 legend: 4 bands are detected in the anti TFK1 western on bsf, but the text only describes two. This looks as if the anti TFK1 antibody is picking up a tagged and untagged copy of TFK1, but the F1 legend states these are WT cells. Please could the authors clarify this issue.

Author response

We thank the reviewer for pointing out this issue. Indeed, on figure 1B, we observe 2 major bands, one at the expected size of TFK1 (141.1 kDa) and a lower band which is particularly visible in BSF. We confirm that the BSF and PCF samples used for the WB are wild-type whole cell extracts. Both of these major bands have weaker associated bands that are very close in size - ultimately producing two doublet signals.
Analysis for protease-recognized sites revealed two potential sites (Enterokinase and Thrombin cleavage sites) in the C-terminal region of TFK1 that could be recognized by endogenous proteases during cell lysis. Cleavage at these sites could results in the lower bands when probed with the monoclonal antibody. Further, phospho-proteomic data from BSF indicate the presence of four phosphorylated serines - S898, S906, S1104 (Urbaniak et al., 2013) and S919 (Nett et al., 2009). These phosphorylations could be responsible for the two upper bands at approximately 141.1kDa (full-length) and the two lower bands.

As requested by the reviewer, and in order to clarify the description, we modified the sentence (line 172) as follows: "The anti-TFK1 labelling revealed a major band at the expected size but also several other bands in both PCF and BSF (Fig. 1B). These bands diminished after TFK1 RNA interference (RNAi) knockdown experiments (described below) (Fig. S3C, D) and could reflect the phosphorylation status of the serines S898, S906, S1104 and S919 identified in phosphoproteomic analyses ((Nett et al., 2009; Urbaniak et al., 2013), or due to proteolysis during cell lysis due to the presence of potential cleavage sites in the C-terminal domain of TFK1."

Line 155: here and other places in the text "mNeon Green" should be "mNeonGreen".

Author response

This was corrected throughout the manuscript.

Line 247 and Figure 5: the authors state that deletion mutants T1::myc and T3::myc are extracted using NP40 but this data is not in Figure 5.

Author response

We thank the reviewer to point this issue and we have added to the figure S5B the immunofluorescence of the detergent-extracted cytoskeleton cells for truncations T1::myc, T3::myc and T5::myc. The figure legend has been modified as well as the figures indicated in the manuscript (line 279 to line 294).

Line 421: "bioinformatics analysis in the TriTrypDB resource" is overly ambiguous; rephrase this sentence for clarity and precision.

Author response

We have modified the sentence as follows (line 463): "Using BLAST-P, we searched for the FBF1 (isoform 1) human protein sequence (Q8TES7-1) ortholog in kinetoplastids and identified Tb926.6.4100 (Protein ID: XM_840435.1) with 11.6% similarity and 7.2% identity. Interestingly, Tb926.6.4100 was localized at the basal bodies by the TrypTag resource (Dean et al., 2016, Dean et al., 2017)."

Line 440: rephrase for clarity.

Author response

We have modified the sentence as follows (line 483):" Our data demonstrate once again that the essentiality of a protein such as TFK1 can vary from one life-cycle stage to another in *T. brucei*. "

Line 783: can the authors confirm they used a p value cutoff of 0.005 (if so, great).

Author response

We confirm that we used a p value cutoff of 0.005 in all statistical tests performed in this study as mentioned in line 820.

Line 695: typo “anti-BILBO1 1-110”.

Author response

The typology has been modified as follows” anti-BILBO1” (line 737).

Line 696: please detail the source of the anti mNeonGreen antibody (and also any other primary antibodies not detailed elsewhere).

Author response

We apologize for this inaccuracy and the source of the primary antibodies (and other primary antibodies) is now specified in the material and methods.

Acknowledgements: Several unpublished pPOT plasmids have been used in this study (e.g. 10xTY1, line 168, line 574). Could the authors acknowledge the source of these?
Acknowledgements: It would be courteous to also acknowledge Prof Keith Gull as the original source of the BB2 antibody and hybridoma.

Author response

We deeply apologize for these omissions. Dr. Samuel Dean is now acknowledged for the invaluable pPOT plasmid series. Pr. K. Gull (University of Oxford) and Dr. P. Bastin are acknowledged for the anti-TY1 antibody and the hybridoma.

Reviewer 2 Advance Summary and Potential Significance to Field:Ramanantsalama and colleagues characterise the localisation and function of transition fibre protein TFPK1 in PCF and BSF in Trypanosoma brucei. This is a nicely presented paper, which reveals a unique and interesting function of the transition fibre component in cytokinesis and furrow ingression in BSF trypanosomes. The experiments are well carried out in general except for a few issues that are outlined below.

Reviewer 2 Comments for the Author:

1. In the introduction (p5), the authors mention the MTs of the BB are followed by the transition zone and then transition fibres. This is a little misleading as the transition fibres are distal to the TZ. The order of wording would ideally match the structure position proximal to distal or vice versa e.g BB, followed by the TF and then TZ (or TZ, followed by TF and BB) I recommend changing the wording to Transition fibres followed by the transition zone.

Author response

We apologize for this inaccuracy and it is now modified line 117.“The trypanosome mature BB is composed of nine microtubule triplets followed by the transition fibres (TF) (Lacomble et al., 2010; Trépout et al., 2018; Vaughan and Gull, 2015) and the transition zone (TZ) (Vaughan and Gull, 2015).

2. Lin90-97 are not clear in terms of linking TF with distal appendages. Do the authors consider them analogous structurally and functionally? It appears so in the discussion. They should make this clearer in the intro. There are some nice papers on distal appendage proteins that should be introduced here and included to compare with functions in this organism.

Author response

We thank the reviewer to point out this issue and we have modified this part in the introduction (line 91 to 115):
"In eukaryotic cells, centrioles are structures with a diameter of approximately 250 nm and with a length ranging between 150-500 nm (Winey and O'Toole, 2014). In a pair of centrioles, only the mother centriole (MC, i.e the mature centriole) bears distal appendages distal (DAs) and sub-distal appendages (SDAs) (Hatch and Stearns, 2010). The DAs are shaped as radial nine-fold twisted arrangement structures with fibrous located centrally and symmetrically at the distal end of the mother centriole. Each DA is associated with one of the centriole triplets with an angle of about 50 degrees (Uzbekov and Alieva, 2018). SDAs are conical-shaped structures linked to the centrosomal barrel by two axonemal microtubules triplets ((Paintrand et al., 1992; Uzbekov and Alieva, 2018; Winey and O'Toole, 2014). These appendages (DA and SDA) are required for ciliogenesis and MC transformation into a basal body (BB) ((Hall and Hehnly, 2021; Jana et al., 2014; Paintrand et al., 1992). MC and BB are similar in structure and function and the transition fibers that correspond to the DAs of the MC extend from the BB and connect to the plasma membrane to initiate ciliogenesis ((Hall and Hehnly, 2021; Kobayashi and Dynlacht, 2011; Kumar et al., 2021; Paintrand et al., 1992; Pearson, 2014). The TF provide a physical demarcation of the flagellum/cilium compartment from the rest of the cell, allowing the recruitment and selective transport of proteins by the IFT machinery, essential for axoneme elongation and maintenance (Reiter et al., 2012b; Wei et al., 2015). TF/DA components are well preserved among ciliated organisms such as CEP83, CEP89, CEP164 (centrosomal proteins), SCLT1 (sodium channel and clathrin linker 1), ANKRD26 (ankyrin repeat domain containing 26) that forms the backbone of blades, and FBF1 (Fas-Binding Factor 1) that localizes within the distal appendage matrix (DAM) between the appendage blades in proximity to the ciliary membrane ((Bowler et al., 2019; Chong et al., 2020; Graser et al., 2007; Greenan et al., 2020; Hodges et al., 2010; Joo et al., 2013; Tanos et al., 2013; Tischer et al., 2021; Wei et al., 2013; Yang et al., 2018). Recently, a bioinformatics analysis identified orthologs of CEP164 and ANKRD26 in T. brucei (Tischer et al., 2021)."

3. In the introduction the term TF does not appear after the original long name of transition fibres.

Author response

The sentence line 56 now reads as : "The basal bodies (BBs) are connected to the mitochondrial genome (kinetoplast) through the exclusion zone filament of the tripartite attachment complex (Baudouin et al., 2020; Robinson and Gull, 1991) but also with the FP membrane through the transition fibres (TFs) (Ogbadoyi et al., 2003; Vaughan and Gull, 2015)."

4. On p7 for the co-labelling experiments, authors mention their marker proteins BLD-10, FTZC and BILBO1 without much explanation of why these proteins were chosen out of many possible marker proteins. A short explanation on their localisation cell cycle pattern etc would be good, particularly with BLD-10. The use of CEP164C and RP2 is obvious given they are the only TF proteins that have been published. It would also be helpful to label the mature basal body and pro-basal bodies with an arrow/arrowhead to guide the reader. It would also be helpful to label the mature basal body and pro-basal bodies with an arrow/arrowhead to guide the reader.

Author response

We thank the reviewer for noticing this. We now described in more details BLD-10, FTZC, TbRP2, CEP164C and BILBO1 (lines 188 to 207): "Further analysis of the immuno-labelling using the anti-TFK1 showed that TFK1 is present throughout the cell cycle at the base of both the old and the new flagellum in PCF and BSF (Fig. 1C). We thus wanted to co-label TFK1 with the BBs and flagellum-associated cytoskeleton protein markers BLD10, FTZC, CEP164C, TbRP2 and BILBO1. BLD10 is a conserved cartwheel protein ((Carvalho-Santos et al., 2010) that stabilizes the 9-fold symmetry of the centriole in *Chlamydomonas* ((Hiraki et al., 2007; Matsuura et al., 2004) and controls the formation of the flagellum central pair in *Drosophila* spermatozoid (Carvalho-Santos et al., 2012). BLD10 was also identified by bioinformatics and proximity-dependent biotin identification as a *T. brucei* basal body component (Dang et al., 2017). BLD10 localizes at both the mature and the immature BB throughout the cell cycle ((Dang et al., 2017; Geimer and Melkonian, 2004). FTZC (for flagellar transition zone component) is a *T. brucei* specific protein localized at the perimeter of the transition zone (TZ) and thus can be used to localize the mature BB ((Bringaud et al., 2000; Gorilak et al., 2021). CEP164C is a TFs proteins that plays a role in the regulation of the

T. brucei old flagellum length (Atkins et al., 2021) and TbRP2 is an alpha-tubulin binding and TFs protein involved in flagellum structure (Stephan et al., 2007)) and in targeting of the ciliary gates TbMKS1 and TbMKS6 proteins ((Andre et al., 2014). TbBILBO1 is one of the components of the flagellar pocket collar (FPC), a cytoskeleton structure distal to the transition zone that maintains the flagellar pocket enclosed around the flagellum (Bonhivers et al., 2008). BILBO1 is also present at the BB and most probably along the MtQ between the BBs and the FPC ((Broster Reix et al., 2021; Perdomo et al., 2022)."

As indicated by the reviewer and to guide the reader, we have indicated in Figure 2 the mature basal body and pro- basal bodies with an arrow and arrowhead respectively and modified the legend.

5. ExM (p8) the diameter of TFK1 was shown to be 477nm. This seems quite wide given the ultrastructural analysis by Trepout et al., 2018 who suggested that cross sectional diameter of TFs by EM is around 330nm. Sample of n=6 is quite small. Does this align with immunogold measurements? Suppl. Table 1 is not conclusive as only measures from gold particle to edge of BB (TFs are angled at 50° (trepout et al. 2018) so diameter from this would not be the sum of 120 (Sup. 1) x2 + 200 (bb diameter)). Variation is quite high in these measurements (some as high as 170nm and others 83nm so concerns on the orientation of slices used for EM). Measuring the distance between gold particles in figure 3C (lateral view) would give you an indication of whether 477nm is a reasonable measurement.

Author response

We would like to thank the reviewer for their comments, which we will answer to point-by-point.

- To compare our TFK1 protein labelling diameter measurements with the data of Trépout et al (2018) and Bowler et al (2019), we repeated our TFK1 immunogold labelling distance measurements, this time from the BB's center on iEM cross-section. Our results indicate an average distance of 172.3±26 nm (n=28) away from BB's center. This measurement is in agreement with the data of Bowler et al (2019) (PMID: 30824690) of ~ 177 nm away from centriole's center on mammalian centrioles. These measurements are also supported by the data from Trépout et al (2018), who suggested a diameter of TFs by EM around 330 nm (as mentioned by the reviewers) corresponding to our measurements of 344.6±51 nm (2 x 172.3 nm).

These new measurements have now replaced the previous ones in supplementary Table 1 and the legend has been modified. The location of the white arrow indicating the measurement from BB's center has been changed in Figure 3C. In order to determine the exact location, we measured the distance between the basal body's center and the immuno-gold labelling on transversal sections revealing a length of 172.3±26 nm (n=28, white arrow in Fig. 3C and Table S1

We have modified the sentences as follows:
(line 264): "In order to determine the exact location, we measured the distance between the basal body's center and the immuno-gold labelling on transversal sections revealing a length of 172±26 nm (n=28, white arrow in Fig. 3C and Table S1);
and (line 445): "In addition, we show that the TFK1 immuno-gold labelling is located at 172±26 nm away from the BB's center, in agreement with the same distance of ~ 177 nm measured for mammalian centrioles ((Bowler et al., 2019). This distance (or radius) is equivalent to a diameter of 344±51 nm. The diameter of the dense TF structures (the blades) observed in *T. brucei* by EM is in the range of 338 nm to 388 nm (Trépout et al., 2018) suggesting that TFK1 localizes at the distal end of the blades structure in the DAM as described for FBF1 ((Wei et al., 2013; Yang et al., 2018)."

- We agree that measuring the distance between gold particles in figure 3A (longitudinal section) would give us accurate diameter. However, there are statistically very few cross-sectional images including both (i) the center of the BB and the center of the axoneme (with a central pair of MTs visible) and (ii) showing two TFK1 immunogold labeling on either side of the BB. Only one micrograph has been obtained (shown in figure 3A), and indeed the TFK1 diameter measurements (3 values, 379± 29 nm) are in agreement our data of 344±51 nm.
- We agree that the TFs are angled at 50° (Trépout et al. 2018), our 28 new measurements, presented now in the modified Table S1 (mean value of 172±26 nm from BB's center), take account

for all the immunogold visible in the cross-sectional iEM micrographs. The statistical analysis graph below, for reviewers only, indicates a median of 169 nm with a range observations between 160-185 nm. This analysis strengthens the mean value of 172±26 nm presented in the manuscript (Table S1).

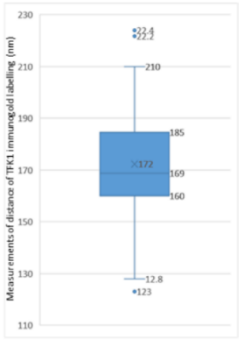

Figure for reviewers. The statistics box-plot of measurements of distance of TFK1 immunogold: The lower (Q1): 160 nm and upper (Q3) quartile: 185 nm, representing observations outside the 128 –210 nm percentile range. The diagram shows the median: 169 nm and mean: 172 nm

In addition, our side view of IF images by U-ExM (as well as the cross-sectional iEM immunogold images Fig 3A) indicated that TFK1 and CEP164C labelling is localized in a much thinner plane (Fig 2Ae-g) in contrast to TbRP2 presenting widespread labelling along the BB (Fig 2Ag).

- As requested by the reviewer, the number of diameter measurements of TFK1 labelling, as nine dots on U-ExM IFs, was increased from 6 to 10. In addition, and in order to compare these new data with previous studies of the ultrastructure of the centriole and the basal body done by Yang et al 2018 (PMID: 29789620), Bowler et al 2019 (PMID: 30824690), Chong et al 2020 (PMID: 32242819) and Katoch et al 2020 (PMID: 32726175), we measured the inner and outer diameters of the circles around the nine-dots radial labelling of TFK1 and have modifed Fig S4. The mean diameters obtained (after correction of expansion factors) are 356±28 nm and 558±56 nm for the inner and outer respectively. These diameters align with our immunogold measurements and data from Trépout et al of 366±25 nm (mean value of 388, 338 and 374 nm, Table 1, Trépout et al, 2018).

We have modified the sentence as follows :
-line 222:"This circular arrangement had average diameters of 1617.6 nm (inner) and 2533 nm (outer) which, after taking into account the expansion factor, indicates average diameters of 356.8 nm (inner) and 558.6 nm (outer) (n=10) (Fig. S4B)."

-and line 439:" Our measurements reveal that the nine TFK1 dots define an inner and an outer diameter of 356±28 nm and 558±58 respectively, similar to the diameters found for the human orthologs CEP164 (568±18 nm to 219±14 nm) and FBF1 (269±33 nm to 496±26 nm) on DAs' ((Bowler et al., 2019; Katoh et al., 2020; Yang et al., 2018)"

Furthermore, these new measurements align with the FBF1 and CEP164 protein diameter measurements obtained in the previously mentioned studies, which are summarized in this table for the reviewers:

		Diameter Katoh et al (nm)	Diameter Yang et al (nm)	Diameter Chong et al (nm)	Diameter Bowler et al (nm)
FBF1	**outer**	461 ± 17	429 ± 23	429 ± 23	496 ± 26
	inner	326 ± 15			269 ± 33
CEP164	**outer**	484 ± 15	475 ± 39	475 ± 39	568 ± 18
	inner	219 ± 14	381 ± 70	381 ± 70	241 ± 19

- Finally, our correlative iEM and U-ExM data shows that TFK1 is localized at the end of the dense structures of FTs and extended beyond the electron dense signal, filling the distal appendage matrix (DAM) between the electron dense distal appendages composed by CEP164C.

6. ExM (Figure 2A) showed that CEP164C and TFK1 puncta do not overlap.There is a mention that they are in different compartments of the TF. TF is a single structure that is unlikely thick enough for two proteins to be completely offset (even by U-ExM). Likely one protein is on the blade and

one protein is within the matrix. RP2 does not exhibit 9 distinct puncta like CEP164C and TFK1 yet it is a transition fibre protein. Is there an explanation for this?

Author response

The several recent ultrastructure studies of the centriole and basal body by super resolution shown that one structure (or compartment) is also thick enough to contain several proteins that are very close but do not co-localize together and moreover do not belong to the same structure, as is the case here for TFK1 presents in the DAM between the CEP164C .(by dSTORM (Chong et al., 2020; Chong et al., 2020), by STORM with 2D and 3D EM analysis (Bowler et al., 2019) and by U-ExM with super resolution microscopy (Katoh et al., 2020).

Concerning TbRP2 the reviewer underlines a very interesting point in the light of recent studies of TFs/ DA.
It is assumed that TbRP2 is a protein present on the TFs as defined by Stephan et al (PMID: 17645436). However, these authors' iEM immunolabelling show (in the figure below [NOTE: We have removed a figure which was provided for the referees. It showed Fig. 3 F-H from **Stephan et al.** (2007) An Essential Quality Control Mechanism at the Eukaryotic Basal Body Prior to Intraflagellar Transport. *Traffic* **8 (10)**, 1323-1330. (DOI: 10.1111/j.1600-0854.2007.00611.x)]), in longitudinal view in Figure 3 (micrographs G and H), labelling all along and at the base of the mature BB. The cross-section (micrograph F) shows labelling around the BB but it is not possible to establish that the section passes through the TFs (DA) (it could also be through the SDA.) On micrographs G and H, we can see some labelling that could correspond to the location of the TFs (DA) but this remains a minority.

Interestingly, human RP2 has been shown to localize to the primary cilium (Hurd et al., 2010) and is implicated in trafficking proteins from the Golgi to the ciliary membrane ((Evans et al.). Localization is also described by iEM of Human RP2 at the BB (but not at the level of the TFs) ((Evans et al.) (PMID: 20106869)). This location along the BB is also reported by (Harmer et al., 2017) (PMID: 28822909) and in C. elegans, by (Reiter et al., 2012) (PMID: 22653444). Moreover, none of the super-resolution studies indicated the presence of the human RP2 ortholog at the distal and sub-distal appendages of the centriole and BB ((Yang et al., 2018) (PMID: 29789620), (Bowler et al., 2019) (PMID: 30824690), (Chong et al., 2020) (PMID: 32242819) and (Katoh et al., 2020) (PMID: 32726175)).
All these data give a more consistent overview of the localization of RP2, which could vary according to the organisms that express the TbRP2 orthologs. However, our data and others may question whether TbRP2 belongs to the TFs/DAs as they are defined today and in particular to the localization of the TFK1 protein. TbRP2 may be part of the sub-distal appendages or directly related to the MTs that make up the BB.

7. Green signal in Figure 2A is maybe slightly overexposed. In Figure 2B e the cell shown with DAPI and phase looks morphologically abnormal.

Author response

The TFK1 immunolabelling in U-ExM experiments was rather weak which sometimes required increasing the contrast to picture the whole labelled area. However, as requested by the reviewer, overexposition has been reduced in Figure 2A.

We also changed the image in figure 2Be to a new cell presenting normal morphology.

8. The reviewer realises that immuno-EM is not an easy set of experiments, however there are not really very convincing images on the TFs, rather in the position where one might expect them to be?

Author response

We agree with the reviewer that we had to test several iEM conditions, with low fixation conditions, in order for the anti-TFK1 monoclonal to recognise its TFK1 epitope, and to obtain the micrographs presented in the manuscript (Figure 3). The immunogolds localized in agreement with the previous localization of the TFs (Vaughan and Gull, 2015) (Figure 1a, PMID: 26862392)). Indeed,

the TFs are located at the junction between the transition zone and the BB at the level of the MT doublets/triplets. The immunogold visible in Figure 3 A and B are in agreement with the location of the TFs according to (Vaughan and Gull, 2015) (Figure 1a, below).

As a complement for the reviewers, we present correlative images to visualize together the TF structures and an immunogold labelling at the tip of the FTs. [NOTE: We have removed a figure which was provided for the referees. It showed Fig. 1 A-B from **Vaughan and Gull** (2015) Basal body structure and cell cycle-dependent biogenesis in *Trypansoma brucei*. *Cilia* **5**, 5. (DOI: 10.1186/s13630-016-0023-7). The authors compared Transition Fibres marked by a black arrow in their figure, with those marked with white arrow 4 in the image cited above.]

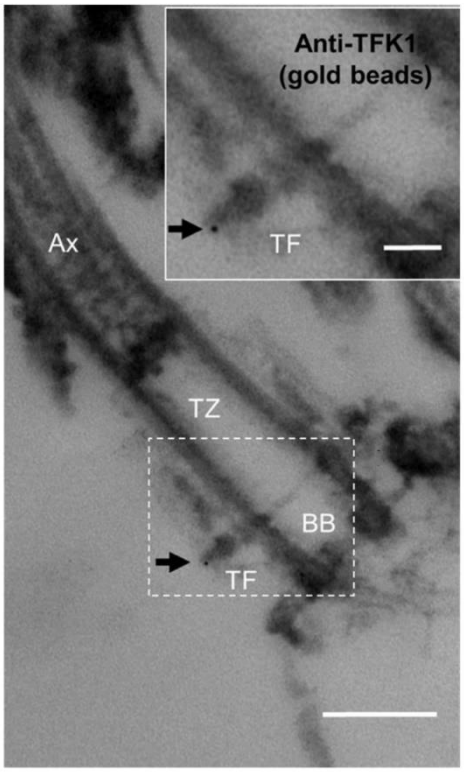

Figure for the reviewer:
TFK1 is localized at the tip of the transition fibres
Immuno-gold labeling on thin section of PCF whole cells showing the localization of TFK1 using anti-TFK1 (gold beads: black arrow). Longitudinal section of the mature basal body. BB: basal body, TF: transition fibres, TZ: transition zone, Ax: axoneme. Scale bars: 200 nm, insert 100 nm.

9. Figure 7: Is KNKN orientation is not unusual in PCF form trypanosomes. Do flagella assemble to the correct length? Transition fibres are known to play a critical role in flagellar assembly in other organisms. Is CEP164c and RP2 labelling maintained in TFK1 RNAi cells? This would indicate whether transition fibre assembly is affected.

Author response

We agree with the reviewer that KNKN organization is not unusual in PCF. However, this organization is unusual and undescribed in the bloodstream form, these results have been presented in Figure 7 for phenotypes induced by TFK1 RNAi. There is no phenotype observed in the PCF form when TFK1 is depleted. Unfortunately, we have tried several times, but failed with no particular reasons, to generate BSF cell lines inducible for TFK1 RNAi in tagged CEFP164C or RP2 background (and vice versa).

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Second decision letter

MS ID#: JOCES/2022/259893

MS TITLE: TFK1, a basal body transition fibre protein that is essential for cytokinesis in Trypanosoma brucei

AUTHORS: Miharisoa Rijatiana Ramanantsalama, Nicolas Landrein, Elina Casas, Bénédicte Salin, Corinne Blancard, Mélanie Bonhivers, Derrick R Robinson, and Denis Dacheux
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. There are no substantive comments that would require any further changes at this stage.

Reviewer 1

Advance summary and potential significance to field

I am satisfied with the changes the authors have made.

Comments for the author

None.

Reviewer 2

Advance summary and potential significance to field

Original reviewer response and new response below:

Ramanantsalama and colleagues characterise the localisation and function of transition fibre protein TFPK1 in PCF and BSF in Trypanosoma brucei. This is a nicely presented paper, which reveals a unique and interesting function of the transition fibre component in cytokinesis and furrow ingression in BSF trypanosomes. The experiments are well carried out in general except for a few issues that are outlined below.

Reviewer 2 Comments for the author
1. In the introduction (p5), the authors mention the MTs of the BB are followed by the transition zone and then transition fibres. This is a little misleading as the transition fibres are distal to the TZ. The order of wording would ideally match the structure position proximal to distal or vice versa e.g BB followed by the TF and then TZ (or TZ, followed by TF and BB) I recommend changing the wording to Transition fibres followed by the transition zone.

Author response

We apologize for this inaccuracy and it is now modified line 117. “The trypanosome mature BB is composed of nine microtubule triplets followed by the transition fibres (TF) (Lacomble et al., 2009; Treépout et al., 2018; Vaughan and Gull, 2016) and the transition zone (TZ) (Vaughan and Gull, 2016).

Reviewer 2 response Happy with this change.

2.Lin90-97 are not clear in terms of linking TF with distal appendages. Do the authors consider them analogous structurally and functionally? It appears so in the discussion. They should make this clearer in the intro. There are some nice papers on distal appendage proteins that should be introduced here and included to compare with functions in this organism.

Author response

We thank the reviewer to point out this issue and we have modified this part in the introduction (line 91 to 115):
“In eukaryotic cells, centrioles are structures with a diameter of approximately 250 nm and with a length ranging between 150-500 nm (Winey and O’Toole, 2014). In a pair of centrioles, only the mother centriole (MC, i.e the mature centriole) bears distal appendages distal (DAs) and sub-distal appendages (SDAs) (Hatch and Stearns, 2010). The DAs are shaped as radial nine-fold twisted arrangement structures with fibrous located centrally and symmetrically at the distal end of the mother centriole. Each DA is associated with one of the centriole triplets with an angle of about 50 degrees (Uzbekov and Alieva, 2018).
SDAs are conical-shaped structures linked to the centrosomal barrel by two axonemal microtubules triplets (Paintrand et al., 1992; Uzbekov and Alieva 2018; Winey and O’Toole, 2014). These appendages (DA and SDA) are required for ciliogenesis and MC transformation into a basal body (BB) (Hall and Hehnly 2021; Jana et al., 2014; Paintrand et al., 1992). MC and BB are similar in structure and function and the transition fibers that correspond to the DAs of the MC extend from the BB and connect to the plasma membrane to initiate ciliogenesis (Hall and Hehnly, 2021; Kobayashi and Dynlacht, 2011; Kumar and Reiter, 2021; Paintrand et al., 1992; Pearson, 2014). The TF provide a physical demarcation of the flagellum/cilium compartment from the rest of the cell allowing the recruitment and selective transport of proteins by the IFT machinery, essential for axoneme elongation and maintenance (Reiter et al., 2012b; Wei et al., 2015). TF/DA components are well preserved among ciliated organisms such as CEP83, CEP89, CEP164 (centrosomal proteins), SCLT1 (sodium channel and clathrin linker 1), ANKRD26 (ankyrin repeat domain containing 26) that forms the backbone of blades, and FBF1 (Fas-Binding Factor 1) that localizes within the distal appendage matrix (DAM) between the appendage blades in proximity to the ciliary membrane (Bowler et al., 2019; Chong et al., 2020; Graser et al., 2007; Greenan et al., 2020; Hodges et al., 2010; Joo et al., 2013; Tanos et al., 2013; Tischer et al., 2021; Wei et al., 2013; Yang et al., 2018). Recently, a bioinformatics analysis identified orthologs of CEP164 and ANKRD26 in T. brucei (Tischer et al., 2021).”

Reviewer response: This modification provides a good explanation to the nomenclature regarding TFs and DAs

3.In the introduction the term TF does not appear after the original long name of transition fibres.

Author response

The sentence line 56 now reads as : “The basal bodies (BBs) are connected to the mitochondrial genome (kinetoplast) through the exclusion zone filament of the tripartite attachment complex (Robinson 1991, Baudouin 2020) but also with the FP membrane through the transition fibres (TFs) (Ogbadoyi et al., 2003; Vaughan and Gull, 2016).“

Reviewer response: This modification is more accurate in describing the proximal-distal organisation of the ciliary base.

4.On p7 for the co-labelling experiments, authors mention their marker proteins BLD-10, FTZC and BILBO1 without much explanation of why these proteins were chosen out of many possible marker proteins. A short explanation on their localisation cell cycle pattern etc would be good, particularly with BLD-10. The use of CEP164C and RP2 is obvious given they are the only TF proteins that have been published. It would also be helpful to label the mature basal body and pro-basal bodies with an arrow/arrowhead to guide the reader. It would also be helpful to label the mature basal body and pro-basal bodies with an arrow/arrowhead to guide the reader.

Author response

We thank the reviewer for noticing this. We now described in more details BLD-10, FTZC, TbRP2, CEP164C and BILBO1 (lines 188 to 207): “Further analysis of the immuno-labelling using the anti-TFK1 showed that TFK1 is present throughout the cell cycle at the base of both the old and the new flagellum in PCF and BSF (Fig. 1C). We thus wanted to co-label TFK1 with the BBs and flagellum-associated cytoskeleton protein markers BLD10, FTZC, CEP164C, TbRP2 and BILBO1. BLD10 is a conserved cartwheel protein (Carvalho-Santos 2010) that stabilizes the 9-fold symmetry of the centriole in Chlamydomonas (Hiraki 2007, Matsuur 2004) and controls the formation of the flagellum central pair in Drosophila spermatozoid (Carvalho-Santos 2012). BLD10 was also identified by bioinformatics and proximity-dependent biotin identification as a T. brucei basal body component (Dang 2017). BLD10 localizes at both the mature and the immature BB throughout the cell cycle (Dang 2017, Geimer 2004). FTZC (for flagellar transition zone component) is a T. brucei specific protein localized at the perimeter of the transition zone (TZ) and thus can be used to localize the mature BB (Bringaud 2000, Gorilak 2021). CEP164C is a TFs proteins that plays a role in the regulation of the T. brucei old flagellum length (Atkins et al., 2021) and TbRP2 is an alpha-tubulin binding and TFs protein involved in flagellum structure (Stephan et al., 2007) and in targeting of the ciliary gates TbMKS1 and TbMKS6 proteins (Andre et al 2014). TbBILBO1 is one of the components of the flagellar pocket collar (FPC), a cytoskeleton structure distal to the transition zone that maintains the flagellar pocket enclosed around the flagellum (Bonhivers et al., 2008). BILBO1 is also present at the BB and most probably along the MtQ between the BBs and the FPC (Perdomo 2022, Broster Reix 2021).”

Reviewer response: The explanation of these different markers is well written and helps the author to justify the use of said markers.

As indicated by the reviewer and to guide the reader, we have indicated in Figure 2 the mature basal body and pro-basal bodies with an arrow and arrowhead respectively and modified the legend.

Reviewer response: The arrows help guide the reader and we are happy with this change.

5. ExM (p8) the diameter of TFK1 was shown to be 477nm. This seems quite wide given the ultrastructural analysis by Trepout et al., 2018 who suggested that cross sectional diameter of TFs by EM is around 330nm. Sample of n=6 is quite small. Does this align with immunogold measurements? Suppl. Table 1 is not conclusive as only measures from gold particle to edge of BB (TFs are angled at 50° (trepout et al. 2018) so diameter from this would not be the sum of 120 (Sup. 1) x2 + 200 (bb diameter)). Variation is quite high in these measurements (some as high as 170nm and others 83nm so concerns on the orientation of slices used for EM). Measuring the distance between gold particles in figure 3C (lateral view) would give you an indication of whether 477nm is a reasonable measurement.

Author response

We would like to thank the reviewer for their comments, which we will answer to point-by-point.

- To compare our TFK1 protein labelling diameter measurements with the data of Trépout et al (2018) and Bowler et al (2019), we repeated our TFK1 immunogold labelling distance measurements, this time from the BB’s center on iEM cross-section. Our results indicate an average distance of 172.3±26 nm (n=28) away from BB’s center. This measurement is in agreement with the data of Bowler et al (2019) (PMID: 30824690) of ~ 177 nm away from centriole's center on mammalian centrioles. These measurements are also supported by the data from Trépout et al

(2018), who suggested a diameter of TFs by EM around 330 nm (as mentioned by the reviewers) corresponding to our measurements of 344.6±51 nm (2 x 172.3 nm).
These new measurements have now replaced the previous ones in supplementary Table 1 and the legend has been modified. The location of the white arrow indicating the measurement from BB's center has been changed in Figure 3C. In order to determine the exact location, we measured the distance between the basal body's center and the immuno-gold labelling on transversal sections revealing a length of 172.3±26 nm (n=28, white arrow in Fig. 3C and Table S1

We have modified the sentences as follows:
(line 264):"In order to determine the exact location, we measured the distance between the basal body's center and the immuno-gold labelling on transversal sections revealing a length of 172±26 nm (n=28, white arrow in Fig. 3C and Table S1); and (line 445): "In addition, we show that the TFK1 immuno-gold labelling is located at 172±26 nm away from the BB's center, in agreement with the same distance of ~ 177 nm measured for mammalian centrioles (Bowler et al., 2019). This distance (or radius) is equivalent to a diameter of 344±51 nm. The diameter of the dense TF structures (the blades) observed in T. brucei by EM is in the range of 338 nm to 388 nm (Trépout et al., 2018) suggesting that TFK1 localizes at the distal end of the blades structure in the DAM as described for FBF1 (Wei et al., 2013; Yang et al., 2018)."

- We agree that measuring the distance between gold particles in figure 3A (longitudinal section) would give us accurate diameter. However, there are statistically very few cross-sectional images including both (i) the center of the BB and the center of the axoneme (with a central pair of MTs visible) and (ii) showing two TFK1 immunogold labeling on either side of the BB. Only one micrograph has been obtained (shown in figure 3A), and indeed the TFK1 diameter measurements (3 values, 379± 29 nm) are in agreement our data of 344±51 nm.
- We agree that the TFs are angled at 50° (Trépout et al. 2018), our 28 new measurements, presented now in the modified Table S1 (mean value of 172±26 nm from BB's center), take account for all the immunogold visible in the cross- sectional iEM micrographs. The statistical analysis graph (see PDF "Response to Reviews", Reviewer 2, point 5), for reviewers only, indicates a median of 169 nm with a range observations between 160-185 nm. This analysis strengthens the mean value of 172±26 nm presented in the manuscript (Table S1).

In addition, our side view of IF images by U-ExM (as well as the cross-sectional iEM immunogold images Fig 3A) indicated that TFK1 and CEP164C labelling is localized in a much thinner plane (Fig 2Ae-g) in contrast to TbRP2 presenting widespread labelling along the BB (Fig 2Ag).

- As requested by the reviewer, the number of diameter measurements of TFK1 labelling, as nine dots on U-ExM IFs, was increased from 6 to 10. In addition, and in order to compare these new data with previous studies of the ultrastructure of the centriole and the basal body done by Yang et al 2018 (PMID: 29789620), Bowler et al 2019 (PMID: 30824690), Chong et al 2020 (PMID: 32242819) and Katoch et al 2020 (PMID: 32726175), we measured the inner and outer diameters of the circles around the nine-dots radial labelling of TFK1 and have modifed Fig S4. The mean diameters obtained (after correction of expansion factors) are 356±28 nm and 558±56 nm for the inner and outer respectively. These diameters align with our immunogold measurements and data from Trépout et al of 366±25 nm (mean value of 388, 338 and 374 nm, Table 1, Trépout et al, 2018).

We have modified the sentence as follows :
-line 222:"This circular arrangement had average diameters of 1617.6 nm (inner) and 2533 nm (outer) which, after taking into account the expansion factor indicates average diameters of 356.8 nm (inner) and 558.6 nm (outer) (n=10) (Fig. S4B)."
-and line 439:" Our measurements reveal that the nine TFK1 dots define an inner and an outer diameter of 356±28 nm and 558±58 respectively, similar to the diameters found for the human orthologs CEP164 (568±18 nm to 219±14 nm) and FBF1 (269±33 nm to 496±26 nm) on DAs' (Yang et al., 2018; Bowler et al., 2019; Katoh et al., 2020)."
Furthermore, these new measurements align with the FBF1 and CEP164 protein diameter measurements obtained in the previously mentioned studies, which are summarized in the table for the reviewers (see PDF "Response to Reviews", Reviewer 2, point 5)

• Finally, our correlative iEM and U-ExM data shows that TFK1 is localized at the end of the dense structures of FTs and extended beyond the electron dense signal, filling the distal appendage matrix (DAM) between the electron dense distal appendages composed by CEP164C.

Reviewer comment: Measuring from the centre of the BB is a good solution and n=28 is a more suitable number of measurements. The concordance between the 3 papers improves the confidence of these measurements and we feel our queries have been addressed suitably.

6.ExM (Figure 2A) showed that CEP164C and TFK1 puncta do not overlap. There is a mention that they are in different compartments of the TF. TF is a single structure that is unlikely thick enough for two proteins to be completely offset (even by U-ExM). Likely one protein is on the blade and one protein is within the matrix. RP2 does not exhibit 9 distinct puncta like CEP164C and TFK1 yet it is a transition fibre protein. Is there an explanation for this?

Author response

The several recent ultrastructure studies of the centriole and basal body by super resolution shown that one structure (or compartment) is also thick enough to contain several proteins that are very close but do not co-localize together and moreover do not belong to the same structure, as is the case here for TFK1 presents in the DAM between the CEP164C .(by dSTORM (Yang et al 2018, Chong et al 2020), by STORM with 2D and 3D EM analysis (Bowler et al 2019) and by U-ExM with super resolution microscopy (Katoch et al 2020).

Concerning TbRP2 the reviewer underlines a very interesting point in the light of recent studies of TFs/ DA.
It is assumed that TbRP2 is a protein present on the TFs as defined by Stephan et al (PMID: 17645436). However, these authors' iEM immunolabelling show in the figure (see PDF “Response to Reviews”, Reviewer 1, point 6), in longitudinal view in Figure 3 (micrographs G and H), labelling all along and at the base of the mature BB. The cross-section (micrograph F) shows labelling around the BB but it is not possible to establish that the section passes through the TFs (DA)
(it could also be through the SDA.) On micrographs G and H, we can see some labelling that could correspond to the location of the TFs (DA) but this remains a minority.

Interestingly, human RP2 has been shown to localize to the primary cilium (Hurd et al. 2011) and is implicated in trafficking proteins from the Golgi to the ciliary membrane (Evans et al. 2010). Localization is also described by iEM of Human RP2 at the BB (but not at the level of the TFs) (Evans et al 2010 (PMID: 20106869)). This location along the BB is also reported by Harmer et al 2017 (PMID: 28822909) and in C. elegans, by Reiter et (2012)(PMID: 22653444).
Moreover, none of the super-resolution studies indicated the presence of the human RP2 ortholog at the distal and sub-distal appendages of the centriole and BB (Yang et al 2018 (PMID: 29789620), Bowler et al 2019 (PMID: 30824690), Chong et al 2020 (PMID: 32242819) and Katoch et al 2020 (PMID: 32726175)).
All these data give a more consistent overview of the localization of RP2, which could vary according to the organisms that express the TbRP2 orthologs. However our data and others may question whether TbRP2 belongs to the TFs/DAs as they are defined today and in particular to the localization of the TFK1 protein.
TbRP2 may be part of the sub-distal appendages or directly related to the MTs that make up the BB.

Reviewer response: The reviewer is happy with this response and the comments around the localisation of RP2 is intriguing, which I’m sure will be researched further.
7.Green signal in Figure 2A is maybe slightly overexposed. In Figure 2B e the cell shown with DAPI and phase looks morphologically abnormal.

Author response
The TFK1 immunolabelling in U-ExM experiments was rather weak which sometimes required increasing the contrast to picture the whole labelled area. However, as requested by the reviewer, overexposition has been reduced in Figure 2A.
We also changed the image in figure 2Be to a new cell presenting normal morphology.

Reviewer response: Good update.

8.The reviewer realises that immuno-EM is not an easy set of experiments however there are not really very convincing images on the TFs, rather in the position where one might expect them to be?

Author response

We agree with the reviewer that we had to test several iEM conditions, with low fixation conditions, in order for the anti-TFK1 monoclonal to recognise its TFK1 epitope, and to obtain the micrographs presented in the manuscript (Figure 3).
The immunogolds localized in agreement with the previous localization of the TFs (Vaughan and Gull 2015 (Figure 1a, PMID: 26862392)). Indeed, the TFs are located at the junction between the transition zone and the BB at the level of the MT doublets/triplets. The immunogold visible in Figure 3 A and B are in agreement with the location of the TFs according to Vaughan and Gull 2015 (see PDF
"Response to Reviews", Reviewer 2, point 8, Figure 1a).
As a complement for the reviewers, we present correlative images to visualize together the TF structures and an immunogold labelling at the tip of the FTs
(see PDF "Response to Reviews", Reviewer 2, point 8, Figure for the reviewer)

9.Figure 7: Is KNKN orientation is not unusual in PCF form trypanosomes. Do flagella assemble to the correct length? Transition fibres are known to play a critical role in flagellar assembly in other organisms. Is CEP164c and RP2 labelling maintained in TFK1 RNAi cells? This would indicate whether transition fibre assembly is affected.
Author response We agree with the reviewer that KNKN organization is not unusual in PCF. However, this organization is unusual and undescribed in the bloodstream form these results have been presented in Figure 7 for phenotypes induced by TFK1 RNAi. There is no phenotype observed in the PCF form when TFK1 is depleted.
Unfortunately, we have tried several times, but failed with no particular reasons, to generate BSF cell lines inducible for TFK1 RNAi in tagged CEFP164C or RP2 background (and vice versa).

Reviewer response: We are satisfied that the authors have attempted this experiment and understand that this experiment is not required to support the conclusions made in this manuscript (although interesting nonetheless). Overall we are satisfied that the authors have addressed our comments and think the manuscript is a nice piece of work with some very interesting results.

Comments for the author

No further revisions are suggested and happy for this to be published.