A centriolar FGR1 oncogene partner-like protein required for paraflagellar rod assembly, but not axoneme assembly in African trypanosomes

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Proteins of the FGR1 oncogene partner (or FOP) family are found at microtubule organizing centres (MTOCs) including, in flagellate eukaryotes, the centriole or flagellar basal body from which the axoneme extends. We report conservation of FOP family proteins, TbFOPL and TbOFD1, in the evolutionarily divergent sleeping sickness parasite Trypanosoma brucei, showing (in contrast with mammalian cells, where FOP is essential for flagellum assembly) depletion of a trypanosome FOP homologue, TbFOPL, affects neither axoneme nor flagellum elongation. Instead, TbFOPL depletion causes catastrophic failure in assembly of a lineage-specific, extra-axonemal structure, the paraflagellar rod (PFR). That depletion of centriolar TbFOPL causes failure in PFR assembly is surprising because PFR nucleation commences approximately 2 μm distal from the basal body. When over-expressed with a C-terminal myc-epitope, TbFOPL was also observed at mitotic spindle poles. Little is known about bi-polar spindle assembly during closed trypanosome mitosis, but indication of a possible additional MTOC function for TbFOPL parallels MTOC localization of FOP-like protein TONNEAU1 in acentriolar plants. More generally, our functional analysis of TbFOPL emphasizes significant differences in evolutionary cell biology trajectories of FOP-family proteins. We discuss how at the molecular level FOP homologues may contribute to flagellum assembly and function in diverse flagellates.

1. Introduction

Coupled, N-terminally located TOF-LisH motifs define a small family of eukaryotic proteins—the FOP family—members of which are required for ciliogenesis in flagellate eukaryotes, and cortical cytoskeleton organization in plant cells. Family members conserved among flagellate eukaryotes are FOP (standing for FGFR1 oncogene partner), OFD1 (mutated in orofaciodigital syndrome 1) and FOR20 (or FOP-related protein of 20 kDa) [1–8]. With regard to localization, in animal cells, OFD1, FOR20 and FOP are all found at the base of cilia associated with basal bodies (or centrioles), either at the level of the triplet microtubule barrel or the transition zone. The microtubule axoneme (the defining structure of all eukaryotic flagella or cilia) extends from the basal body; the transition zone defines the most proximal region of the flagellum and exhibits its own particular architecture, where Y-shaped projections link axoneme outer-doublet microtubules to the flagellar membrane [9]. FOP, OFD1 and FOR20 are all required...
for the formation of a primary cilium, assembled by many types of animal cell in response to appropriate environmental cues [10]. Roles for FOR20 and OFD1 in cilia assembly have been described in the ciliates Paramecium tetraurelia and Tetrahymena thermophila [5,11,12], but we are not aware of any reports regarding functional studies of candidate OFD1 and FOP orthologues in other flagellate protists or fungi.

One member of the FOP protein family is also conserved in at least one group of aflagellate eukaryotes, land-plants. The protein TONNEA1 (or TON1), which is most similar to FOP, interacts with at least one classic protein found at microtubule organizing centers (MTOCs), centrin, and is required for organization of cortical microtubules during cell elongation and division [13]. The absence of TONNEA1 from Arabidopsis, an aflagellate angiosperm, or the evolutionarily more basal bryophyte moss Physcomitrella patens, which deploys flagellate motile sperm for reproduction, results in organelle mis-positioning and defective development [13,14]. Another group of organisms in which a cortical-based microtubule cytoskeleton exerts an overarching and relatively well understood effect on cell morphogenesis and division are the flagellate trypanosomatids [15–17]. Long known as the aetiological agents of a variety of serious tropical diseases (e.g. African sleeping sickness, Chagas disease, leishmaniasis), the parasitic trypanosomatid family belong to the excavate group of protists, which is widely recognized as evolutionarily divergent in comparison with other eukaryotic groups [18,19].

Flagellar assembly and function has been widely studied in trypanosomatids; many facets of this biology are conserved with other flagellate eukaryotes, but there are also notable differences. Visually, the most notable difference is the presence of a complex paraflagellar rod (PFR). Within the flagellar compartment, trypanosomatids and their nearest relatives build an elaborate extra-axonemal PFR structure, but not the axoneme itself. Our data illustrate unexpected functional and evolutionary diversity in the role of conserved centriole-targeted proteins in eukaryotic flagellum assembly and function.

2. Material and methods

2.1. Cell culture and transfection

Procyctic T. brucei (927Smox [27] and S427) were cultured in SDM-79 medium supplemented with 10% v/v fetal bovine serum and haemin [28]. Constitutive expression of YFP- or GFP-tagged proteins and RNAi experiments were performed in 927Smox cells, whereas myc epitope-tagged protein was constitutively expressed in a 427 genetic background. Logarithmic phase cells were transfected and stable transformants selected using 10 μg ml\(^{-1}\) blasticidin (following transfection with pENT6B-derived endogenous tagging plasmids), 50 μg ml\(^{-1}\) hygromycin (following transfection with pPOT endogenous tagging DNA or pDEX377-derived expression plasmids) or 3 μg ml\(^{-1}\) phleomycin (following transfection with p2T7177-derived RNAi plasmids) [29,30]. For the routine culture of 927Smox, 2 μg ml\(^{-1}\) of puromycin was used. Transgenic cultures were kept free of selectable markers for at least 48 h prior to the start of experiments. RNAi was induced by the addition of doxycycline to a final concentration of 1 μg ml\(^{-1}\).

2.2. Plasmid constructs

Fusion proteins were expressed using pEnT or pDEX-based vector systems [30] or the PCR only tagging approach (pPOT [29]). For constitutive expression of N-terminal YFP tagged TbFOPL, TbOFD1 and TbFLAM3 [31] from endogenous chromosomal loci, DNA sequences corresponding to open reading frames (orf) and 3' intergenic regions (igr) were amplified by PCR. Resultant amplicons were digested by XbaI/Xhol (orf) and Xhol/BamHI (igr) prior to three-way ligation into XbaI/BamHI digested pEnT6B-Y. Plasmids were linearized with Xhol prior to transfection. For constitutive expression of C-terminal GFP-tagged TbFOPL from endogenous chromosomal loci, pPOTv2 plasmid DNA was PCR amplified [29] and the resultant amplicon used directly for transfection.

For expression of a myc epitope-tagged TbFOPL, the coding sequence minus stop codon was amplified using a two-step PCR reaction (in order to remove an internal Xhol site in the TbFOPL coding sequence), where the product of the first reaction was used as template for the second. The resultant PCR amplicon was digested with HindIII and Xhol prior to being cloned into a HindIII/Xhol digested pDEX377-myc vector [24]. For TbFOP and TbOFD1, RNAi orf sequences were amplified and the resultant amplicon cloned between opposing head-to-head T7 RNA polymerase promoters in p2T7-177 vector, pre-digested with BamHI and Xhol. Both pDEX- and p2T7-derived plasmids were linearized with NotI prior to transfection. Molecular masses for YFP- and myc-tagged proteins were confirmed by immunoblotting (see Results); correct genmic integration of DNA conferring expression of TbFOPL::GFP was confirmed by southern blotting (not shown).

2.3. Microscopy and immunoblotting

Cells were settled onto coverslips and either fixed directly with 3.7% paraformaldehyde or detergent extracted for 30 s with 1%
Nonidet P40 in 0.1 M PIPES, 2 mM EGTA, 1 mM MgSO₄, 0.1 mM EDTA, pH 6.9 prior to fixation. Fixed cells were placed in methanol for 10 min prior to rehydration in PBS. Indirect immunofluorescence using polyclonal antiserum raised against recombinant TbrRP2 [24] and monoclonal antibodies L8C4 and L3B2 (recognizing PFR and FAZ, respectively [32]), YL1/2 [33] and anti-myc was performed as described previously or as stated in the manufacturer’s instructions (myc; AbCam). Images were captured using an Applied Precision DeltaVision microscope with a Roper Scientific

Indirect immunofluorescence using polyclonal antiserum placed in methanol for 10 min prior to rehydration in PBS. 0.1 mM EDTA, pH 6.9 prior to fixation. Fixed cells were

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[38]. Tb927.11.3090 encodes an FOR20 orthologue and localizes

orthologues of all three genes are present in all trypanosomatid

proteins with coupled N-terminal TOF-LisH motifs:

Tb

3.2. TbFOPL protein is required for paraflagellar rod assembly but not axoneme formation

Following TbFOPL RNAi induction, levels of YFP::TbFOPL declined (figure 2b) and abnormal cells appeared within 24 h. By 48 h, cell growth had slowed and very few cells presented with a normal morphology (figures 2bc, 3 and 4; electronic supplementary material, figure S3); cells at this stage varied in size and distinct intra-flagellar swelling was frequently observed (figure 3). Swelling was typically observed either at the very distal end of the flagellum (figure 3d; arrow) or within the flagellum (figure 3e–g; arrows). Cells decorated for immunofluorescence microscopy with the monoclonal antibody L8C4 (which detects PFR2; one of the two major proteins that form the PFR) indicated the variable width of the flagellum noted in the SEM images was the result of

3. Results

3.1. Divergent FGR1 oncogene partner family proteins in trypanosomatids

Additional to TbrRP2, three further genes in T. brucei encode proteins with coupled N-terminal TOF-LisH motifs: Tb927.11.3090, Tb927.5.4090 and Tb927.10.3000. Syntenic orthologues of all three genes are present in all trypanosomatid species for which genome sequences are available at EukPathDB [38]. Tb927.11.3090 encodes an FOR20 orthologue and localizes to both pro- and basal bodies [4]. By contrast, the predicted proteins encoded by Tb927.5.4090 and Tb927.10.3000 are not immediately recognizable as orthologous to any particular FOP family protein. In that context, we also note failure to correctly predict a trypanosome FOP orthologue in both a published bioinformatics survey of centriole/basal body evolution, and within the phylogenomic co-occurrence survey that is a part of the ‘STRING’ programme [37,39]. Given the importance of a microtubule corset in defining trypanosome cell morphology, and involvement of an FOP-related protein to organizing the cortical cytoskeleton in acientriolar plant cells, we made no assumption regarding the localization of proteins encoded by Tb927.5.4090 and Tb927.10.3000. Thus, we expressed both as N-terminal fusions with YFP from their endogenous chromosomal loci (and thus under the regulatory control of the endogenous 3’ intergenic sequence; in trypanosomatids, 3’ intergenic sequences are accepted as exerting the dominant influence on gene expression). In these experiments, YFP fluorescence was compared to the indirect immunofluorescence signal obtained using polyclonal affinity-purified anti-TbRP2 antibody [24]. As shown in figure 1 and electronic supplementary material, figure S1, proteins encoded by Tb927.5.4090 (figure 1) and Tb927.10.3000 (electronic supplementary material, figure S1), and tagged at the N-terminus with YFP, each co-localized with the mature basal body marker TbRP2 but were not detectable at other MTOCs or other cellular locales at any point during the cell cycle.

Returning to the interrogation of T. brucei FOP family candi- dature, BLAST analyses revealed human OFD1 identified Tb927.10.3000 as the top hit, albeit with an e-value below an e-10 threshold and requiring the insertion of numerous gaps to produce an alignment with moderate identity and similarity. These trypanosome and human proteins also differ in length by over 200 amino acids (electronic supplementary material, figure S2a,b). Nevertheless, gene-specific RNAi provided further evidence for the TbOFD1 candidature of Tb927.10.3000 (electronic supplementary material, figure S2–m). By contrast, HsFOPL and divergent FOP-like proteins from Tetrahymena (THERM_00357420, or TtFop1 [40]; THERM_00305510; THERM_00699980) failed to identify candidate orthologues from trypanosomatids with expectancy values above even an e-05 threshold; here, both differences in size and an overall shorter protein length influence analysis outcomes. However, with the acceptance of three insertions, HsFOPL and the Tb927.5.4090 gene product align with reasonable identity and similarity along their length (figure 2a). Based on our analyses, we believe Tb927.5.4090 encodes an FOP-related protein, but following published reports of HsFOPL and TtFop1 [1,40], we conservatively refer to the trypanosome gene as FOP-like (or TbFOPL). We also prefer the designation FOP-like because of the surprising RNAi phenotype described below.

2.4. Electron microscopy

Fixation was by addition of glutaraldehyde (2.5% final concentration, 5 min) to cultures. Cell pellets were re-suspended in 0.1 M PBS (pH 7.4) for 10 min, followed by 2.5% glutaraldehyde, 2% paraformaldehyde and 0.1% tannic acid in 0.1 M phosphate buffer (pH 7.0) for 2 h at room temperature. Pellets were washed with 0.1 M phosphate buffer (pH 7.0) and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.0) for 1 h at room temperature. Samples were rinsed and stained en bloc for 40 min in 2% uranyl acetate, dehydrated in an ascending acetone series and embedded in Agar 100 resin (Agar Scientific). Thin sections were examined by electron microscopy using a Hitachi H-7650, operated at 120 kV.

2.5. Bioinformatics

Protein sequences were aligned by CLUSTAL OMEGA [36], and the STRING database [37] was used to identify known and predicted interactions between human FOP and other proteins.
defective PFR assembly (figure 4). Instead of the uniform PFR2 signal seen along the length of the flagellum, from the point of cell body exit in normal cells (figure 4a), we typically observed cells where PFR2 signal was absent, except for the accumulation of PFR2 protein at a point coincident with the end of the cell body and/or the distal tip of the flagellum (figure 4b–h). We also observed cells where sometimes a faint PFR2 signal was present in the proximal region of the flagellum but the signal was then lost, indicating an initiation of PFR assembly but subsequent failure of PFR assembly within the same flagellum (figure 4b,f,h). In cells where the PFR of a pre-existing flagellum was fully formed, we saw no evidence for subsequent PFR loss, although assembly of PFR in new elongating flagella was perturbed (figure 4c,e). In such cells, an accumulation of PFR2 at the end of the cell body and/or flagellar tip was often evident; in some of these cells, detachment of the flagellum from the cell body was also evident in the regions lacking PFR2 (figure 4d–g). The representative images shown in electronic supplementary material, figure S3 illustrate how PFR formation was compromised in virtually all (more than 95%) cells 48 h post-RNAi induction. We reported previously that for calmodulin (CaM) RNAi mutants, where PFR formation also fails completely, the default status is for flagellum–cell body attachment and that flagellum detachment occurs some time later [22]. Examples of cells with attached flagella that lacked PFR were observed in RNAi-induced TbFOPL mutants (figure 4c,h). However, by 48 h post-induction of TbFOPL RNAi, considerable heterogeneity in cell morphology was evident (figure 4; electronic supplementary material, figure S3). Included in this heterogeneity were ‘cells’ or, perhaps more accurately, cell ‘slivers’ apparently lacking flagella and of varying size. These were the likely consequence of asymmetric cell division; the higher resolution afforded by SEM emphasized the irregularities in cell morphogenesis that could occur following TbFOPL depletion (figure 3d–g).

Depletion of centriolar FOP in cultured mammalian cells results in loss of ciliogenesis [6,7]. By contrast, flagellum assembly was maintained post-TbFOPL RNAi induction, albeit with flagella that were often detached from the cell body. Indication that an intact axoneme was assembled within these flagella

Figure 1. Localization of Tb927.5.4090 gene product (TbFOPL) in procyclic T. brucei. (a,b) Localization of YFP::TbFOPL at the mature basal body in 1K1N (a) and 2K mitotic (b) cells. Images show detection of YFP::TbFOPL relative to TbRP2 in whole cells (a–c); insets show the same localizations at higher magnification. 6-Diamidino-2-phenylindole (DAPI) was used to detect nuclear DNA (N) and the mitochondrial genome (or kinetoplast, K). (c) TbFOPL::GFP localization at the mature basal body in a 1K1N procyclic T. brucei cell; inset shows TbFOPL::GFP and TbRP2 localization at higher magnification. (d,e) Retention of YFP::TbFOPL (d) and TbFOPL::GFP (e) in detergent extracted cytoskeletons. Scale bars in all main panels indicate 5 μm and in the inset panels 1 μm.
Figure 2. RNAi knockdown of TbFOPL results in severe morphological defects. (a) Cartoon representation of human FOP and T. brucei FOPL, showing insertions necessary to achieve maximal alignment of amino acid sequences, and amino acid alignment of Homo sapiens FOP (accession number CAJ7020.1) and T. brucei FOPL. (b) Effect of TbFOPL RNAi induction on trypanosome growth (diamonds) compared to RNAi non-induced controls (triangles); immunoblotting with monoclonal antibody BB2 (detecting an N-terminal Ty-epitope) with time post-induction (h). 

3.3. Localization and flagellum exclusion of TbFOPL::myc

With the TONNEAU1 connection to acinetoriblant MTOCs and the microtubule-dominant organization of cell form in trypanosomes in mind, we questioned further the localization of TbFOPL by expression of protein tagged at the C-terminus with GFP (TbFOPL::GFP) and myc-tagged (TbFOPL::myc) protein. TbFOPL::GFP expressed from an endogenous locus, albeit under the regulation of a PFR2 3' intergenic region, gave the same localization pattern as TbFOPL::myc expressed from a strong RNA Polymerase I promoter. Open Biol. 2018. 8: 170218

organelles and overall cell morphogenesis in T. brucei [16]. The diameter of flagella seen from SEM analysis of TbFOPL RNAi mutants was indicative of IFT-dependent axoneme elongation, rather than sleeve formation. Nonetheless, mindful of the importance of FOP for axoneme formation in ciliated mammalian cells [6,7], we looked at axoneme ultrastructure in our TbFOPL RNAi mutants. In 50 transverse sections (out of a total of 51 analysed), axoneme structure looked normal, irrespective of whether PFR was absent, or the axoneme partially surrounded by an accumulation of PFR protein(s) (figure 5b,c). This contrasts with the loss of outer-doublet and/or central pair microtubule integrity associated with T. brucei flagellum RNAi mutants depleted for radial spoke, central pair or nexin-dynein regulatory complex components, and selected, cultured, fixed and prepared for electron microscopy using the same protocols as the current study [43–45]. This further emphasizes TbFOPL is not required for axoneme formation per se. Transmission electron microscopy (TEM) analysis revealed that PFR protein accumulated and assembled as an amorphous structure rather than the elaborate ordered lattice observed in normal cells (compare figure 5a with b–d). In the one section where a defect in axoneme structure was evident, displacement of an outer doublet microtubule (figure 5d) was conceivably the consequence of excessive accumulation of PFR proteins.

To interrogate further the observation of flagellum detachment from the cell body, we queried the localization of flagellum attachment zone (FAZ) components following TbFOPL RNAi induction. On the cell body side of the FAZ, we observed a normal localization of FAZ1, a component of the fibres radiating from membrane junctional complexes [46], even in the absence of flagellum attachment (figure 6a). This was consistent with initial flagellum–cell body attachment in cells where no PFR is built [22]. On the intraflagellar side of the FAZ, we queried the localization of the high molecular weight protein TbFLAM3 [47,48]. Here, the normal localization was lost following RNAi induction, with YFP::FLAM3 co-localizing with the aberrant intraflagellar accumulation of PFR2 at the distal end of the cell body (figure 6b). In these experiments, detergent-extracted cytoskeletons, rather than intact cells, were examined; the retention of L8C4 signal and YFP fluorescence indicated a stable association of bulky, amorphous ‘PFR’ components with the cytoskeleton rather than the more labile accumulation of PFR components seen in some flagellar RNAi mutants [49].
**TbFOPL** to question why a basal body-localized protein was so critical for specifying correct assembly of an extra-axonemal structure approximately 2 μm distal to the basal body. Specifically, we considered whether there was a pool of intraflagellar **TbFOPL** not seen when analysing the localization of fluorescent-tagged FOPL protein. There is indication that the transition zone limits protein access into the flagellum compartment on the basis of size (where only small proteins, less than 4.5 nm Stokes radii or 40 kDa, enter the flagellum by diffusion [50]). **TbFOPL::myc** (predicted molecular mass approx. 30 kDa) fell comfortably beneath this threshold limit (cf. YFP-tagged **TbFOPL**, molecular mass greater than 50 kDa). Notably, **TbFOPL::myc** was excluded from the flagellum and the nucleus in whole cells (figure 7a). This leaves open the question of how a basal body-localized protein, **TbFOPL**, critically influences the asymmetric assembly of the extra-axonemal PFR, which is built only after the flagellum exits its flagellar pocket, a distance of approximately 2 μm from the point where the axoneme initially extends from its basal body. Curiously, careful examination of detergent-extracted cytoskeletons revealed **TbFOPL::myc** localization at the poles of the mitotic spindle in early mitotic cells (figure 7c(iii,iv)).

This was in addition to basal body localization throughout the cell cycle. However, the nuclear signal was absent from cells fixed later in mitosis (figure 7c(v)). Re-examination of YFP::**TbFOPL** localization did not reveal any indication of nuclear localization. The severe morphological abnormalities of **TbFOPL** RNAi-induced cells meant it was not realistic to sensibly ascertain whether spindle formation was also compromised by an absence of **TbFOPL** protein.

**4. Discussion**

The combination of N-terminal localized TOF-LisH motifs is a seldom used but highly effective means of localizing proteins to MTOCs: FOP family proteins including FOR20 and OFD1 are centriolar proteins conserved in diverse eukaryotes, and in humans, chromosomal translocation results in TOF-LisH-dependent retargeting of the tyrosine kinase domain of the FGFR1 receptor to the centrosome, and an atypical myeloproliferative disorder [1,2].

In trypanosomatids, and their free-living relative *Bodo saltans*, the presence of N-terminal TOF-LisH motifs in the...
GTPase activating protein RP2 provides a lineage-specific elaboration within the FOP family, and ensures basal body localization of the protein (in animals, basal body localization of RP2 is dependent on N-terminal acylation [26]). Curiously, although the evolutionary context is for N-terminal TOF-LisH motifs in eukaryotes, the single exception that we found in our bioinformatics analysis was of a gene model encoding a 688 amino acid protein with a candidate C-terminal TOF-LisH motif combination in the centric diatom *Thalassiosira pseudonana* (accession no. XP_002286300.1). In all four trypanosome proteins that use a TOF-LisH motif combination, N-terminal fusion to YFP does not compromise localization to mature basal bodies, or in the case of *Tb*FOR20 localization to mature and associated pro-basal bodies [4]. Here, the more significant and unanticipated characteristic of the trypanosome FOP protein family is that *Tb*FOPL is not required for axoneme elongation but is essential for the assembly of extra-axonemal PFR. This contrasts strikingly with the essentiality of the mammalian FOP homologue at the earliest stages of ciliogenesis [6,7].

Similar to the *T. brucei* calmodulin (CaM) RNAi mutant, where PFR assembly is also totally compromised [22], a failure of PFR assembly in the *Tb*FOPL-depleted cells leads to flagellum—cell body detachment and abnormalities in cell morphogenesis. However, *Tb*CaM is present in both the PFR lattice and struts linking the PFR and outer doublet microtubules of the axoneme. Thus, the *Tb*CaM PFR assembly defect can be readily explained. By contrast, *Tb*FOPL appears not to be present within the flagellum compartment, even when expressed from a strong transcription promoter, thus raising the question as to how a basal body located protein is essential for PFR assembly.

We found no indication of problems in axonemal assembly within *Tb*FOPL-depleted trypanosomes—flagellum length was normal and only seldom was there a discernible defect in axoneme ultrastructure. This indicated that IFT was not lost, but rather that *Tb*FOPL deficiency caused a specific defect in PFR assembly. PFR assembly apparently initiates in some *Tb*FOPL RNAi-induced cells but then subsequently fails: PFR material accumulates as large unstructured deposits within or at the end of the flagellum in a majority of cells (approx. 96% of flagella 48 h post-RNAi induction in which PFR2 could be detected by immunofluorescence using monoclonal antibody L8C4; at 24 h post-RNAi induction, approximately 60% of flagella showed abnormal accumulation of PFR2). Basal body-localized *Tb*FOPL could play a critical, direct role in the import of PFR-specific cargo into the flagellum.
(i.e. although major components such as PFR1, PFR2 and FLAM3 are imported into the flagellum, it is possible that not all PFR components are imported thereby compromising intraflagellar assembly of the PFR lattice). In this context, there is precedent for selective transport of axonemal subcomplexes and/or roles for cytoplasmic chaperones or other accessory assembly proteins in pre-assembly of a variety of proteins associated with distinct axonemal substructures (e.g. dynein arms) prior to subcomplex import into the flagellum [51–54]. Indeed, in immunofluorescence experiments using monoclonal antibody ROD1, which recognizes an antigen from the outer or most distal region of the PFR lattice, signal intensity was severely reduced on some cells or non-existent in others (data not shown); this was reminiscent of similar immunofluorescence experiments in PFR-deficient snl- mutants [49].

Alternatively, the domain architecture of TbFOPL is not typically reflective of either an enzyme or a chaperone. Rather, its architecture and available experimental evidence are more consistent with roles in scaffolding or mediation of protein–protein interaction. For instance, in mammalian cells, FOP recruits the centrosomal protein CEP19, which in turn interacts with the GTPase RABL2, and it is RABL2 that regulates IFT-B function and thereby cilium assembly [55]. Similarly, centrosome-localized FOP is required for anchoring microtubules to subcellular structures and localization of the centrosomal protein EB1, a plus-end microtubule-binding protein that has critical functions in regulating + end microtubule dynamics [56]. Notwithstanding the possibility that the filament-like PFR lattice could be self-assembling rather than chaperone- or accessory protein-dependent, TbFOPL could feasibly play a role in recruiting another protein or proteins that act in PFR assembly and/or intraflagellar PFR attachment. At this point, our data are consistent with a direct or an indirect role for TbFOPL in PFR assembly.

Presently, a final possibility to consider regarding how TbFOPL influences PFR assembly is a possible dependency and/or interaction between TbFOPL and TbKIF9B, a trypanosomatid-specific, basal body- and axoneme-localized kinesin required for normal PFR construction [57]. Although there is similarity between the RNAi phenotypes of TbFOPL and TbKIF9B, the KIF9B phenotype is unique in that in a majority of cells, a PFR forms in patches along the length of the flagellum; in other cells, a PFR is either absent or accumulates in a single patch. Whether it is basal body- and/or axoneme-localized KIF9B that is required for normal PFR construction is not known [57]. Probing for potential interaction between these proteins represents one avenue with which to move forwards to understand the mechanism by which TbFOPL defines PFR formation.

Additional to understanding how a trilaminar PFR lattice assembles, there is also the specification of attachment of the PFR proximal region to outer doublets 4–7 of the axoneme. Cues that define the asymmetric attachment of PFR to axoneme

Figure 5. TEM of flagella following induction of TbFOPL RNAi. (a) Transverse thin section through the flagellum of a TbFOPL RNAi non-induced cell shows normal arrangement of axoneme and PFR. (b) The absence of PFR assembly following TbFOPL RNAi; dynein ATPases, radial spokes and central pair (CP) projections are all present. (c) Massive accumulation of unstructured PFR material in association with an axoneme where dynein ATPases, radial spokes and CP projections are present. (d) A rare example of loss of axoneme integrity following TbFOPL RNAi. Scale bars indicate 100 nm.
at a position several microns distant from the basal body are unknown. Although structural asymmetries exist within basal/probasal bodies (e.g., [40,58,59]), it is difficult to understand how a centriolar-located protein such as TbFOPL could influence the asymmetric attachment of the PFR to the axoneme. We note, however, that in the ciliate *Tetrahymena*, an FOP1-like protein (and polyglutamylated tubulin) is asymmetrically distributed around the basal body; it is proposed asymmetric distribution of the FOP1-like protein and polyglutamylated tubulin may stabilize basal bodies against mechanical forces generated during ciliary beating [40]. From our current DeltaVision imaging, we have been unable to detect asymmetric localization of TbFOPL or polyglutamylated tubulin (using the anti-polyglutamylation monoclonal antibody GT335) at the *T. brucei* basal body, but nevertheless, the *Tetrahymena* example raises the possibility that asymmetric distribution of basal body-localized proteins, such as FOP, could, either directly or via post-translational modification of axonemal microtubules, affect PFR attachment.

We find the possible nuclear localization of TbFOPL::myc in early mitotic cells intriguing. Promoter-driven expression of TbFOPL::myc occurs throughout the cell cycle but nuclear acquisition of TbFOPL::myc is cell cycle dependent. We are therefore inclined to believe recruitment to the nucleus (and potentially to the MTOCs nucleating the mitotic spindle) is genuine. Although no discrete structures, such as centrosomes or
spindle pole bodies (seen in yeast cells), have been observed in *T. brucei*, distinct ring-like structures, that appear to nucleate spindle microtubules, can be visualized by electron microscopy (reviewed in [60]). Our observation of nuclear recruitment of *Tb*FOPL indicates that in addition to a critical basal body-related function, the protein may also be required to establish the acentriolar MTOCs responsible for spindle microtubule nucleation. This observation potentially provides a parallel with the acentriolar MTOC localization of another FOP family protein, TONNEAU1, in plants [13]. We have not observed nuclear recruitment of FOR20 and *Tb*RP2 (i.e. other trypanosome TOF-LisH proteins) even after over-expression (data not shown), and so recruitment of *Tb*FOPL to a nuclear MTOC does not appear to be a general feature of TOF-LisH targeting in trypanosomatids. In common with many unicellular eukaryotes, *T. brucei* undergoes a closed mitosis [61] and proteins involved in mitosis must be transported across the nuclear envelope. However, any possibility *Tb*FOPL is involved in MTOC function other than at basal bodies should be balanced with the observation that, like other known trypanosomatid basal body proteins, a *Tb*FOPL orthologue is absent from *Perkinsela*, an acentriolar, aflagellate basal kinetoplastid that is an endosymbiont within *Paramoeba* isolates [62]. This could imply that *Tb*FOPL is either not essential for spindle assembly and/or function in trypanosomes or that mitosis in *Perkinsela* occurs independently of FOPL.

In this, and previous work [4,24], we have investigated all four TOF-LisH motif-containing proteins expressed in *T. brucei*. All four proteins localize to the mature basal body. *Tb*FOR20 additionally locates to the probasal body
and TbFOPL potentially to mitotic spindle poles. TbFOPL, TbOFD1 and TbRFP2 have distinct flagellum assembly-related functions, but no apparent phenotype is detected in FOR20-depleted cells. We have previously shown that RNAi-mediated knockdown of TbRFP2 affects flagellum assembly and it is proposed that TbRFP2 acts as a GTPase activating protein with a role in protein trafficking; human RP2 acts as a GAP for the small GTPase ARL3 [63]. In T. brucei cells depleted for OFD1, the short flagellum phenotype generated suggests that IFT-mediated transport may be compromised in these cells, consistent with a proposed IFT-related role for mammalian OFD1. By contrast, the protein we have identified as being most similar to mammalian FOP appears to have a distinctive phenotype relating to PFR assembly, but without affecting axoneme assembly. Studying FOP-like function in trypanosomes affords a unique opportunity to study assembly of extra-axonal structures. Finally, although the PFR is unique to trypanosomes and evolutionary close relatives, extra-axonal structures are observed in flagella in a diversity of flagellated eukaryotes, including Gauda intestinalis, Gymnodinium aureolum and other dinoflagellates. There are also unusual MTOCs, some of which are thought to have a flagellar origin (e.g. the apical polar ring of apicomplexans and their near relatives [64–67]). Determination of whether FOP-related or other FOP family proteins play roles in the assembly or function of these cytoskeletal structures offers intriguing possibilities for future research.

5. Notes added in revision

Reference to TrypTag.org, the genome-wide project to localize every protein-coding gene-product in T. brucei [68], gives an indication of basal body localization for N-terminally mNeon-Green-tagged TbFOPL, but there are currently no localization data available for TbOFD1. mNeonGreen::TKIF9B localization does not mirror precisely the published localization: an additional, cell-cycle-stage-specific flagellar tip signal is reported alongside localization to pro- and mature basal bodies. This potentially adds further complexity to understanding the mechanistic basis for KIF9B-dependent PFR assembly [57] or any hypothetical interaction or dependency between KIF9B and FOPL.

Data accessibility. Datasets supporting this article are provided in the electronic supplementary material.

Authors’ contributions. P.G.M. and M.L.G. designed research. J.H. generated mutants and performed light microscopy, with M.A. providing additional contribution. K.T. and S.V. analysed mutant cells by SEM and TEM, J.H., M.L.G. and P.G.M. wrote the manuscript.

Competing interests. We declare we have no competing interests.

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