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REVIEWS

Review

More than Microtubules: The Structure and Function of the Subpellicular Array in Trypanosomatids

Amy N. Sinclair[1] and Christopher L. de Graffenried[1,*]

The subpellicular microtubule array defines the wide range of cellular morphologies found in parasitic kinetoplastids (trypanosomatids). Morphological studies have characterized array organization, but little progress has been made towards identifying the molecular mechanisms that are responsible for array differentiation during the trypanosomatid life cycle, or the apparent stability and longevity of array microtubules. In this review, we outline what is known about the structure and biogenesis of the array, with emphasis on *Trypanosoma brucei, Trypanosoma cruzi,* and *Leishmania,* which cause life-threatening diseases in humans and livestock. We highlight unanswered questions about this remarkable cellular structure that merit new consideration in light of our recently improved understanding of how the 'tubulin code' influences microtubule dynamics to generate complex cellular structures.

The Anatomy of the Subpellicular Array

The subpellicular array is a single layer of microtubules that encompasses the cell body of **trypanosomatids** (see Glossary) and defines their shape (Figure 1A, Key Figure). It is conserved among essentially all free-living and parasitic **kinetoplastids** [1,2]. Several approaches have shown that the array microtubules in *T. brucei* are highly ordered, with their growing plus-ends oriented towards the cell posterior [3,4]. A microtubule-associated protein known as XMAP215 that binds microtubule plus-ends localizes to the posterior of the array in *Leishmania mexicana*, suggesting that the polarity of the array is conserved in other trypanosomatids [5]. The microtubules of the array follow a helical path as they traverse the cell body (Figure 1A) [2], and are separated by a uniform distance of 18–22 nm. Neighboring microtubules are crosslinked by regularly spaced fibrils at intervals of 12–30 nm (Figure 1B–D) [3,6,7]. The array microtubules are also attached to the plasma membrane by fibrils spaced at ~20 nm intervals (Figure 2A,B) [6].

The number of microtubules in the array varies between species and changes depending on the width of the cell body. There are over 100 microtubules in the array in thin-section transmission electron microscopy cross-sections that traverse the cell's widest point, which is typically where the nucleus is located [8,9]. The number of array microtubules decreases as the trypanosomatid cell body narrows towards the tapered posterior and anterior ends. It is not clear how many of the array microtubules span the entire length of the cell body. Transmission electron microscopy has shown that whenever a microtubule ends in the middle of the array, the microtubules on either side continue past it and become crosslinked to each other, maintaining the intermicrotubule distance (Figure 1D, asterisk) [3].

The stability and persistence of the array microtubules has not been established. The microtubules do not depolymerize when exposed to cold temperatures (termed 'cold-stable'), tolerate detergent extractions when prepared for whole-mount electron microscopy [2,10,11], and remain intact after treatment with microtubule-destabilizing drugs [12,13]. However, it is not clear if this apparent stability is due to the nature of the microtubules themselves or to their extensive crosslinking and organization. If these microtubules are persistent and long-lived, it is unclear how they are replenished or repaired over the lifetime of the cell, which is likely necessary due to the significant strains put on the array by parasite motility in the cases where the flagellum is attached to the cell surface (Figure 1A).

The continuity of the array is interrupted at specific points in the cell body. In *T. brucei* and *T. cruzi* **trypomastigotes** and **epimastigotes**, the flagellar pocket (FP) disrupts the array by forming an

Highlights

The microtubules of the subpellicular array in trypanosomatids are highly organized and appear remarkably stable, which differs from the dynamic instability of interphase microtubules found in mammalian systems.

Transitions between different parasite life-cycle stages require extensive rearrangement and remodeling of the microtubules of the array.

Post-translational modifications on tubulin alter microtubule dynamics and differentially recruit microtubule-associated proteins that are sensitive to these modifications.

Microtubule post-translational modifications are abundant in trypanosomatids, and new research suggests that they differ across the array. This may create a landscape for differential localization of microtubule-array associated proteins that locally alter array dynamics.

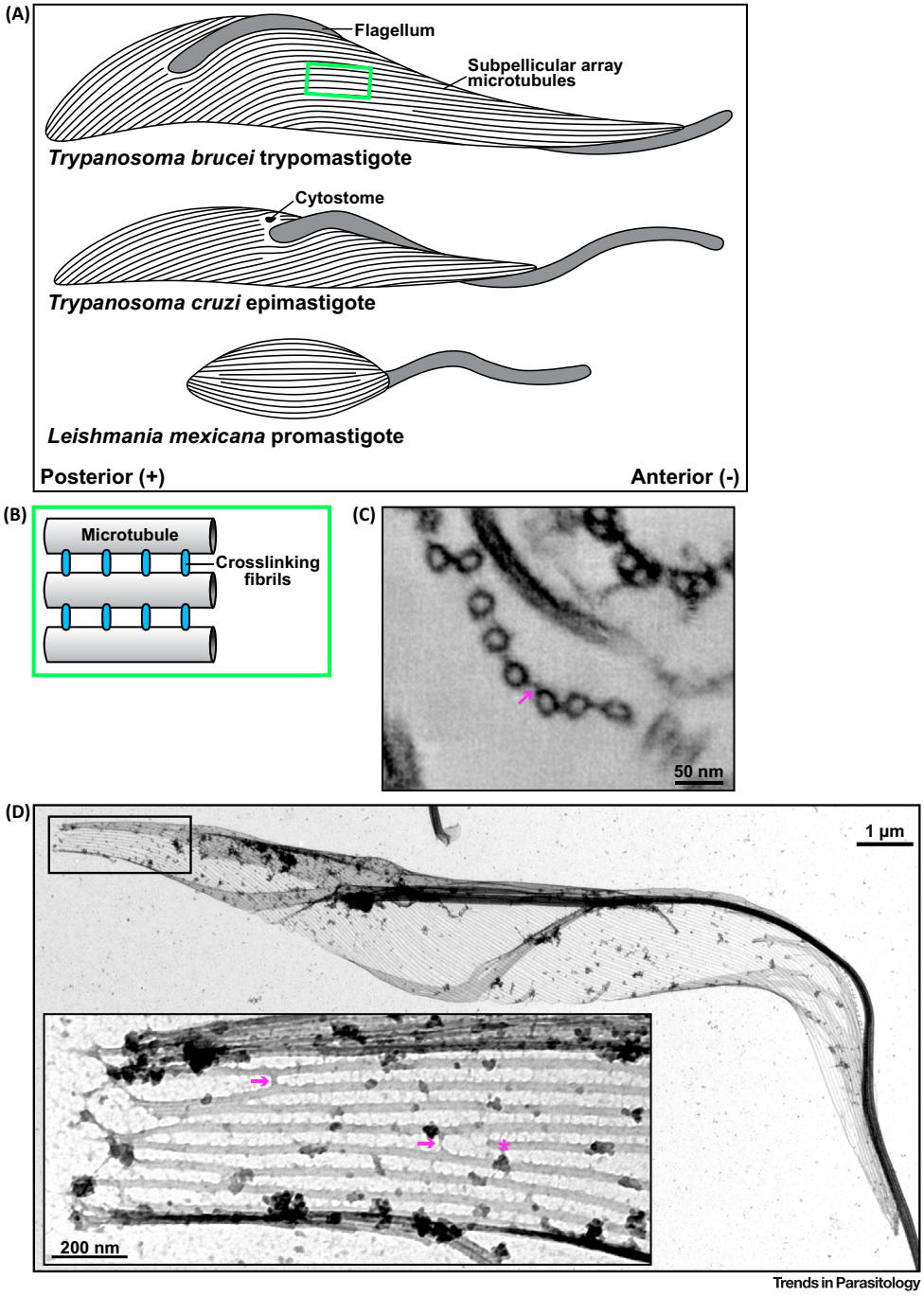
Recent studies have identified new microtubule-array associated proteins that are required to maintain cell shape and may be important in morphological changes during life-cycle transitions.

[1]Department of Molecular Microbiology and Immunology, Brown University, Providence, RI 02912, USA

*Correspondence: Christopher_degraffenried@Brown.edu

Key Figure

The Microtubules of the Subpellicular Array Are Extensively Crosslinked



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Glossary

Amastigote: cell morphology in which the kinetoplast is anterior to the nucleus, and the flagellum is nonmotile and does not protrude from the cell body.

Axoneme: an array of microtubules that run the length of the flagella, providing its shape and motility. Usually comprising nine microtubule doublets arranged in a ring, with a central doublet in motile flagella.

Basal body: a centriolar structure that nucleates the flagellum.

Cytokinesis: a late stage of cell division during which the duplicated organelles are partitioned into the two daughter cells, after which the daughter cells become separated.

Cytostome–cytopharynx complex: an endocytic compartment, present in certain kinetoplastids, that includes a set of specialized microtubules that extend into the cell body.

Epimastigote: cell morphology in which the kinetoplast is anterior to the nucleus, and the motile flagellum is attached along the cell body with a long cell-free overhang.

Flagellum attachment zone (FAZ): a series of junctional complexes that run along the cell body and attach the flagellum to the cell surface.

Kinetoplastid: a class of free-living and parasitic flagellated protozoa that contain their mitochondrial DNA in a specialized domain of the mitochondrion known as the kinetoplast.

Promastigote: cell morphology in which the kinetoplast is anterior to the nucleus, and the long, motile flagellum is unattached as it exits the cell body at the cell anterior.

Proventriculus: a muscular valve between the midgut and foregut in the tsetse fly that regulates the passage of food.

Trypanosomatids: a group of parasitic kinetoplastids, including *T. brucei*, *T. cruzi*, and *Leishmania*, which cause African trypanosomiasis, Chagas' disease, and leishmaniasis, respectively

Trypomastigote: cell morphology in which the kinetoplast is posterior to the nucleus, and the long, motile flagellum is attached to the cell body with little cell-free overhang.

invagination of the plasma membrane that serves as the flagellum exit site (Figure 1A) [14]. In these morphotypes, the **flagellum attachment zone** (**FAZ**) filament is situated between the microtubules of the array as it traverses the cell body (Figure 2A,B) [15]. *T. cruzi* has an additional interruption created by the **cytostome–cytopharynx complex** (Figure 1A) [11,16]. Four microtubules associated with the cytostome–cytopharynx complex also interrupt the array as it traverses a specialized membrane domain, known as the pre-oral ridge, that is located between the FP and the cytostome–cytopharynx complex. The FP and FAZ filament are also present in *Leishmania* **promastigotes**, but they do not interrupt the array; the flagellum is unattached after exiting the FP, which is located at the anterior cell tip in *Leishmania* cells [17]. In *T. brucei* and *Leishmania*, the FP membrane is the only plasma membrane domain that is not in close contact with the array, which makes it the primary site of endo- and exocytosis [14,17].

Tsetse flies: flies of the genus *Glossinia* that inhabit sub-Saharan Africa and are the insect vectors of *T. brucei*.

T. brucei, *T. cruzi*, and *Leishmania* have a conserved set of four microtubules called the microtubule quartet (MtQ) that originates near the **basal body** and wraps around the FP. In *T. brucei* and *T. cruzi* trypomastigotes and epimastigotes, the MtQ intercalates into the array at the point where the flagellum exits the pocket and lies adjacent to the FAZ filament (Figure 2A,B) [14,15]. The MtQ in *Leishmania* does not become part of the array and is present on the opposite side of the FP to where the FAZ filament resides [17]. The MtQ likely has an inverted polarity compared to the array microtubules as it is nucleated near the basal body and grows towards the cell anterior [14]. This arrangement creates a seam of antiparallel microtubules within the array in *T. brucei* and *T. cruzi* that may facilitate the targeting of protein complexes involved in **cytokinesis** and morphogenesis. The cytostome–cytopharynx microtubules in *T. cruzi* also create a seam that may be important for the formation of this organelle during cell division [16].

Morphogenesis and the Subpellicular Array

Differentiation Tunes Trypanosomatids for Survival in the Host

Trypanosomatids undergo a series of morphological changes as they cycle through their insect and mammalian hosts (Figure 3). Within the insect host, *T. brucei* and *T. cruzi* primarily adopt epimastigote and trypomastigote forms, which are characterized by tapered posterior arrays and attached flagella [18,19]. *Leishmania* is found in the promastigote form, which has a similar tapered shape but the flagellum is unattached after it exits the FP at the cell anterior [17]. Once transmitted to the mammalian host, *T. brucei* remains extracellular and circulates in the bloodstream or colonizes host tissues as trypomastigotes [20,21]. By contrast, both *T. cruzi* and *Leishmania* enter host cells and differentiate into **amastigote** forms after cell entry [8,18,22,23]. Amastigotes are ovoid-shaped with extremely short flagella that extend just past the FP.

These different trypanosomatid morphologies are tuned for survival in the environments in which they inhabit [24]. The trypomastigote morphology appears to be adapted for motion in crowded bloodstreams and tightly packed tissue compartments [20,25]. As the flagellum beats, the subpellicular array deforms, creating 'cellular waveforms' that facilitate swimming between blood cells and other

Figure 1. (A) Schematics of the arrangement of the subpellicular microtubules in a *Trypanosoma brucei* procyclic-form trypomastigote (top), a *Trypanosoma cruzi* epimastigote (middle), and a *Leishmania mexicana* promastigote (bottom). The growing plus-ends of the microtubules are oriented towards the cell posterior. In *T. brucei* and *T. cruzi* trypomastigotes and epimastigotes, the array microtubules bend around the flagellar pocket as the flagellum exits onto the cell surface. In *T. cruzi*, the array microtubules also curve around the cytostome, which is an endocytic organelle that forms an opening at the cell surface. In *Leishmania*, the array ends at the flagellar pocket located at the cell anterior. Based on [2,3,11,27,38]. (B) Schematic of the fibrils that crosslink the microtubules of the array together from the boxed region in (A). (C) Tomogram (z = 50 nm) of an extracted cytoskeleton of *T. brucei* in which the crosslinks between the array microtubules are visible (arrow). The microtubules of the array are separated from each other by a space of 18–22 nm. Image kindly contributed by Sue Vaughan and Timm Mohr. (D) Original transmission electron micrograph of a positively-stained procyclic-form *T. brucei* trypomastigote cytoskeleton. The cytoskeleton was critical-point dried and cleaved to remove half of the array. Inset. Higher magnification image of the cell posterior, in which the intermicrotubule crosslinks are clearly shown (arrows). Where a microtubule ends in the array (asterisk), the microtubules on either side of it continue and become crosslinked to each other.

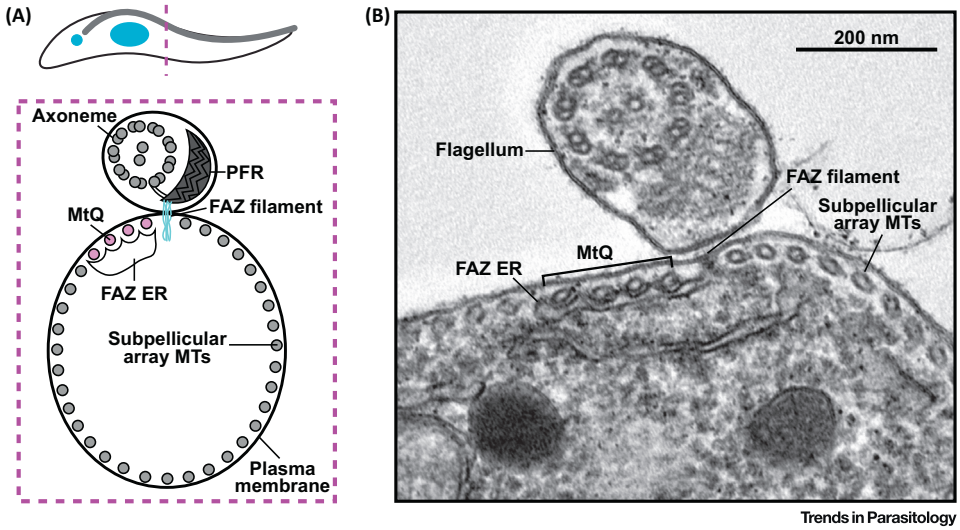

Figure 2. The Microtubules of the Array Are Tightly Associated with the Plasma Membrane.
(A) Schematic of a trypomastigote cross-section, looking from posterior to anterior. The array microtubules are located just 10 nm from the plasma membrane. The microtubule quartet (MtQ) has the opposite polarity to the other array microtubules. The flagellum attachment zone (FAZ) filament interrupts the array and attaches the flagellum to the cell surface. Abbreviation: PFR, para-flagellar rod. (B) Thin-section transmission electron micrograph of a *Trypanosoma brucei* cell in the same orientation as the schematic in (A). Kindly contributed by Sue Vaughan and Timm Mohr.

biological barriers [26,27]. Within the mammalian hosts, motility is important for immune evasion; the high velocity reached by *T. brucei* trypomastigotes in blood (more than 20 μm/s) may create a hydrodynamic drag that concentrates host antibodies at the FP for rapid uptake and endocytosis [28]. Epimastigote and promastigote morphologies may be primed for attachment to epithelial layers in the insect host, as more of the flagellum is free from the cell body to associate with epithelium and other structures of the insect, such as microvilli and chitin mouthparts, or in the case of *T. cruzi*, the waxy cuticle of the hindgut [18,19]. The epimastigote and promastigote morphologies may facilitate cell division while attached to a substrate. Likewise, the amastigote morphology may be adapted to maximize cell numbers in the crowded environment of the host cell, while minimizing the total amount of surface exposed to host cell factors [23]. Moreover, the lack of an extended flagellum in amastigotes may protect the host cell from damage produced by flagellar beating, which would enhance amastigote replication [22].

The Subpellicular Array Is Extensively Remodeled in Morphological Transitions

The array undergoes changes as the parasites transition into different morphologies during the trypanosomatid life cycle. Two primary mechanisms, which are not mutually exclusive, have been shown to alter array morphology: the direct remodeling of array microtubules, and specialized asymmetric cell divisions that result in changes in the shape of the array.

In *T. brucei*, direct microtubule remodeling is responsible for the differentiation of mammalian bloodstream-form trypomastigotes into the insect vector **tsetse fly**-resident procyclic trypomastigotes [29]. In bloodstream-form trypomastigotes, the kinetoplast is in close proximity to the posterior of the array. During differentiation into procyclic forms, the posterior array lengthens through the extension of pre-existing microtubules or the insertion of new microtubules at the array posterior, which repositions the kinetoplast to a more central location of the cell body to create the procyclic morphology.

The posterior section of the array in procyclic cells lengthens as they migrate towards the **proventriculus** of the tsetse fly, producing what are known as mesocyclic trypomastigotes [30,31]. In the proventriculus, the nucleus of mesocyclic cells migrates towards the cell posterior as they undergo an

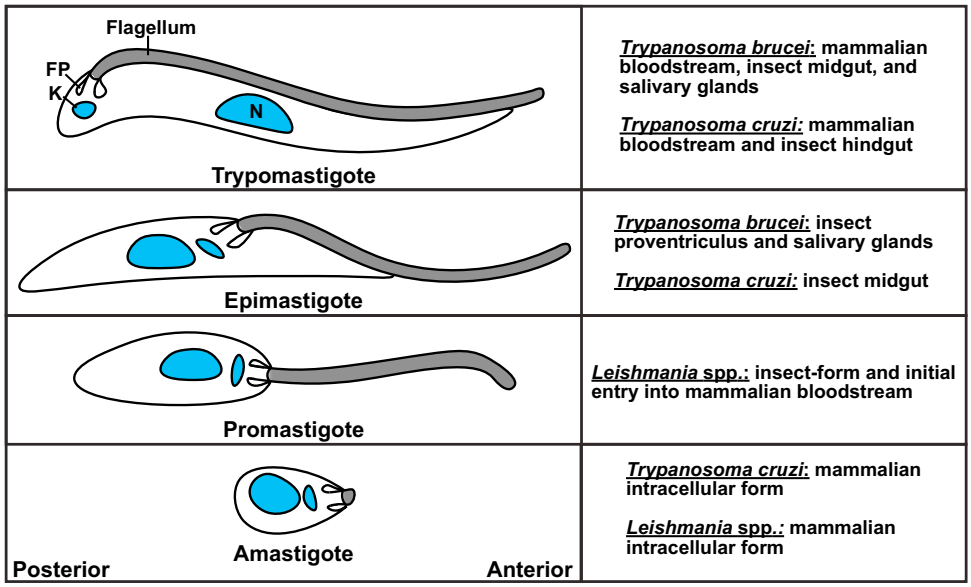

Figure 3. The Primary Morphologies of Human-infectious Trypanosomatid Parasites.
Left. Schematic of cell structure and topological order of single-copy organelles in each morphology. The trypomastigote form is characterized by the posterior localization of the mitochondrial DNA aggregate, called the kinetoplast (K), to that of the nucleus (N). An attached flagellum exits from the flagellar pocket (FP) near the cell posterior. In the epimastigote form, the kinetoplast is anterior to the nucleus. While the flagellum is still attached to the cell surface, it also has a long, cell-free overhang. The promastigote form has a similar arrangement of DNA-containing organelles as the epimastigote, but the flagellum is unattached after exiting the flagellar pocket at the cell anterior. The kinetoplast is also positioned anterior to the nucleus in the smaller, more-spherical amastigote form. The flagellum is short and nonmotile, and barely protrudes from the flagellar pocket. Right. A list of the life-cycle stages in which the human-infectious trypanosomatids adopt these morphologies.

asymmetric division that produces two epimastigote daughter cells – one long, which is thought to be terminal, and the other short, which goes on to colonize the salivary glands [19,32]. The transition from a trypomastigote to an epimastigote morphotype requires an extensive repositioning of single-copy organelles, resulting in a cell where the kinetoplast, FP, and associated flagellum are anterior to the nucleus, along with an extended flagellar overhang (Figure 3). These repositioning events appear to be mediated by delayed growth of the new flagella and an altered division plane in the subpellicular array [19,32]. It is unclear how the new division plane is selected to achieve such an asymmetric partitioning of daughter cells, although RNAi depletion of certain FAZ components generates epimastigote-like cells [33–35].

In *T. cruzi*, the transition from the trypomastigote to the amastigote form in the mammalian host is also accomplished by an extremely asymmetric division event that leads to the expulsion of the trypomastigote flagellum and positions the kinetoplast anterior to the nucleus [36]. After the asymmetric division event, it is not known how the array is altered to become more spherical in the amastigote. There are more microtubules in the cross-sections of *T. cruzi* amastigotes than at any point in the cell body of trypomastigotes, while the intermicrotubule distance is consistent between these two life-cycle stages [9]. This suggests that additional microtubules are intercalated into the array to create the spherical amastigote shape.

In *Leishmania*, the transition between promastigote and mammalian-resident amastigote forms does not require significant rearrangement of the kinetoplast and nucleus. Amastigotes are ovoid with a

short, nonmotile flagellum, but the single-copy organelles are retained in the same topological order (Figure 3). It has been shown that *Leishmania* remodels the long promastigote flagellum to the short amastigote flagellum through two pathways: a specialized asymmetric division event in which one daughter cell forms a short and nonmotile flagellum, or through the direct remodeling and shortening of the existing flagellum [37].

It is unclear how *Leishmania* promastigotes remodel their subpellicular array to become more spherical in the amastigote form. *In vitro*, *Leishmania* promastigote cells first elongate their cell body before undergoing a drastic reduction in length while doubling their width just prior to cytokinesis [38]. Rapid depolymerization of array microtubules at the cell posterior, along with the simultaneous insertion of new microtubules at the cell anterior, could decrease cell length while increasing cell width in the promastigote division cycle; a similar mechanism could be employed in the promastigote to amastigote differentiation. Another potential transition mechanism has been suggested by cross-sections of promastigote and paramastigote forms of *Herpetomonas samuelpessoi*. The helical pitch of array microtubules is tighter in the more-rounded *H. samuelpessoi* paramastigote morphology, demonstrating the capability of the array to increase microtubule torsion to accommodate additional cell volume [39]. However, little is known about array ultrastructure and microtubule dynamics during the amastigote differentiation of *T. cruzi* and *Leishmania*, and even less about their return transition into trypomastigotes and promastigotes, respectively. This is an important area of investigation as more genetic tools are developed in these parasites.

It is likely that the choice of differentiation mechanism employed during each life cycle transition reflects the extent of cytoskeletal remodeling taking place. Smaller changes that do not alter the topological order of single-copy organelles are mediated by microtubule growth or remodeling, while topological reordering requires a division event. These mechanisms illustrate that even though the array is made up of microtubules that are extensively crosslinked and appear highly persistent, it is not a static structure.

Array Replication

Array replication has been most thoroughly studied in *T. brucei*, where the addition of new microtubules to the array appears to be cell-cycle regulated [3]. New microtubules nucleate from the sides of existing ones, and extant microtubules are elongated from both the plus and minus ends as detected by an antibody against tyrosinated α-tubulin, which is indicative of microtubule growth [3]. After the nucleation and growth of a new flagellum, new microtubules are inserted between the old and new flagella as the width of the cell increases (Figure 4) [40]. Once the growth of the new flagellum reaches a 'stop point' along the old, the cell posterior rapidly elongates [41]. Additionally, short new microtubules are inserted throughout the array during cell division. It is unknown if these short microtubules are elongated or depolymerized, but they are no longer present once cell division is complete [3]. The array microtubules do not appear to originate or nucleate from one specific point during replication [42], although many microtubule minus ends are concentrated at the cell anterior in trypomastigotes and epimastigotes, and at the exit of the FP in promastigotes.

As the array microtubules do not depolymerize during cell division, trypanosomatids have developed a unique cytokinetic mechanism that ensures the fidelity of array duplication and segregation [3,40]. In *T. brucei*, *T. cruzi*, and *Leishmania*, cytokinesis initiates at the cell anterior and progresses along the array's helical path towards the cell posterior (Figure 4). The microtubules are then partitioned into two discrete arrays by sorting and bundling (Figure 4A–C) [40]. In *T. brucei*, it has been shown that the inheritance of the array is semiconservative, providing each daughter cell with a chimeric structure comprising both old and new microtubules [3]. As the array does not appear to depolymerize in *T. cruzi* and *Leishmania* during cell division [38,43,44], it is likely that inheritance in these organisms is also semiconservative.

Considering that the microtubule array persists throughout cell division, and must be expanded and duplicated in this state, trypanosomatid cytokinesis is likely to be microtubule-directed and

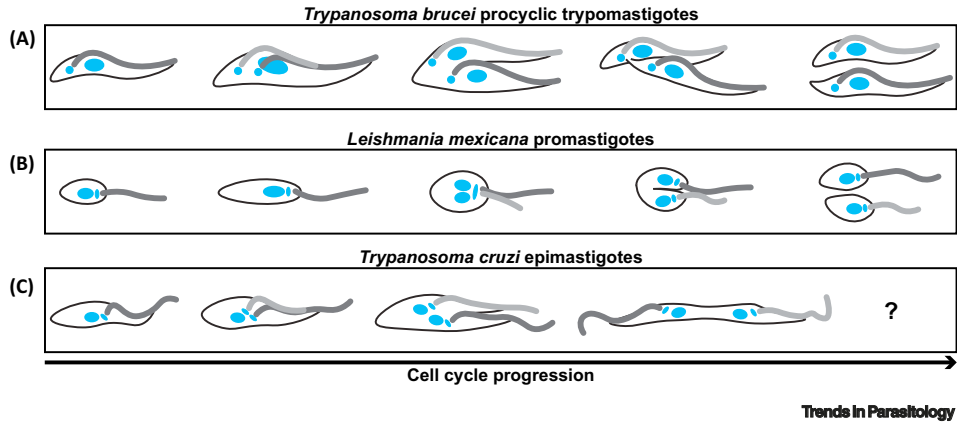

Figure 4. Cell Division in Trypanosomatid Parasites.
(A) In a *Trypanosoma brucei* trypomastigote, the kinetoplast replicates prior to the nucleus. The array enlarges to accommodate the increase in cell volume, after which cytokinesis initiates at the cell anterior and progresses towards the cell posterior. Microtubule segregation and bundling separates the subpellicular array, leading to abscission. The division is asymmetric, producing daughter cells with either a rounded or pointed posterior. The old and new flagellum are indicated in dark and light gray, respectively. Based on [40]. (B) The cell body of a *Leishmania mexicana* promastigote lengthens before undergoing a dramatic reduction in cell length while increasing in cell width during replication. The nucleus replicates prior to the kinetoplast. Cytokinesis also proceeds towards the cell posterior. Based on [38]. (C) In a *Trypanosoma cruzi* epimastigote, the kinetoplast replicates prior to the nucleus, after which cytokinesis initiates at the cell anterior. The daughter cells are attached end-to-end in the final stage of cytokinesis, but it is currently unknown how *T. cruzi* epimastigotes resolve the array to complete abscission. Based on [44].

dominated by processes that sort and remodel microtubules. In procyclic *T. brucei*, cytokinesis does not appear to rely on a conventional actomyosin ring, and the parasite lacks the nonmuscle myosin II required for ring constriction [45]. However, microtubule-severing enzymes, such as spastin and katanin, are required for cytokinesis in *T. brucei* [46,47]. Recently, a kinetoplastid-specific orphan kinesin has also been identified in *T. brucei* that is required for array segregation and daughter-cell separation in the final stages of cytokinesis [47,48]. These proteins are present in *T. cruzi* and *Leishmania* [35,48], which suggests a conserved cytokinetic mechanism in trypanosomatids, although their function has yet to be tested in these organisms.

Tubulin in Trypanosomatids

Tubulin Expression

The unique properties of the subpellicular array microtubules may be reflected in the expression and regulation of trypanosomatid tubulin. Most eukaryotes have multiple copies of α- and β-tubulin genes throughout their genome. In trypanosomatids, these genes are linked together in clustered repeats. In both *T. brucei* and *T. cruzi*, alternating α- and β-tubulin repeats are grouped together within the genome, ranging from 13 to 17 tandem copies [49,50]. *Leishmania* tubulin genes are also clustered together in upwards of 15 copies, but have separate monotypic arrays of α- or β-tubulin located on different chromosomes [51,52]. In *T. cruzi* and *Leishmania*, there also appears to be an additional repeat of α- and β-tubulin outside of the main cluster, but it is not known if these are differentially regulated [50,53]. Phylogenetic analysis has shown that the alternating arrays are the ancestral genome architecture, as they are present in nonparasitic kinetoplastids. As kinetoplastids transcribe their genome polycistronically [54], the repeating array structure ensures high expression levels, which is likely required for the maintenance and assembly of the subpellicular array microtubules.

Trypanosomatids tune tubulin expression as they transition into different life-cycle stages [55–57]. When *T. cruzi* and *Leishmania* differentiate from motile to nonmotile forms, tubulin expression decreases with the loss of their extended flagella and differentiation into the amastigote form. Likewise, tubulin biosynthesis is upregulated as *T. cruzi* and *Leishmania* initiate new flagellum growth and elongate their arrays in their return transitions to trypomastigotes and promastigotes, respectively.

Many eukaryotes encode a variety of α- and β-tubulin isotypes that have different polymerization dynamics and can be differentially expressed [58]. These tubulin isoforms differ primarily in the sequence of their carboxy-terminal tails, which are charged and disordered [59]. There is no clear evidence that trypanosomatids contain α- or β-tubulin isoforms [52,60,61]. An interesting exception to this is in *Leishmania*, where it has been demonstrated that specific β-tubulin genes are differentially regulated as cells transition into infective metacyclic promastigotes and amastigotes [62,63]. However, it is unclear if these are distinct isotypes.

Trypanosomatids encode a single copy of γ-tubulin in their genome. In metazoan cells, γ-tubulin complexes are responsible for nucleating microtubules at organizing centers, such as centrosomes [64]. In the subpellicular array, γ-tubulin localizes at the cell anterior where many microtubule minus ends are located, as well as discrete spots at the minus ends of individual microtubules within the array [65]. A γ-tubulin complex has been identified in *T. brucei* (γ-TuSC) but does not appear to have a role in subpellicular array biogenesis [66]. The function of γ-tubulin in the array is unknown, although it is important for the assembly of a subset of flagellar **axoneme** microtubules [67].

The trypanosomatid genomes also contain copies of δ-, ε-, and ζ-tubulin [68,69]. These do not appear to be a part of the α/β tandem arrays. Although the localization and function of these tubulins are unknown in trypanosomatids, in *Chlamydomonas* and animal cells, δ-/ε-/ζ-tubulin form a 'module' that is involved in basal body and centriolar structure and orientation [70,71].

Tubulin has been purified from *Crithidia*, *Leishmania*, and *T. brucei* [72,73,120]; interestingly, this tubulin has a tendency to form ribbon and sheet-like structures instead of tubules. Tubulin purified from procyclic *T. brucei* polymerizes into tubes in the presence of high magnesium and GTP concentrations. However, the biophysical characteristics of trypanosomatid tubulin – such as its stability and flexural rigidity – are unknown. It is possible that the tubulin produced by these organisms has unique biophysical properties that are tuned to its specialized use, considering the highly organized and seemingly persistent nature of the array microtubules. *T. cruzi* and *Leishmania* also have a small number of cell-cycle regulated cytoplasmic microtubules [74]. It is not known if these cytoplasmic microtubules exhibit dynamic instability like in mammalian cells, so their nature is an open question.

Post-translational Modifications

Tubulin post-translational modifications comprise the tubulin 'code', which modifies microtubule dynamics and alters microtubule-associated protein (MAP) binding and behavior [75]. The disordered C-terminal tails of tubulin are the most common site of post-translational modifications and MAP interactions. Interestingly, different post-translational modifications and MAPs are enriched in different tubulin structures in *T. brucei* and *T. cruzi*, which suggests that they play a key role in cytoskeletal regulation [76–78].

The majority of known tubulin post-translational modifications are present in trypanosomatids (Figure 5A). These modifications have been identified with specific antibodies or mass spectrometry; the enzymes involved in the modification pathways are not yet well described in trypanosomatids. To date, acetylation [12,79,80], detyrosination/tyrosination [80–85], and phosphorylation [83–86] have been detected in *T. brucei, T. cruzi,* and *Leishmania*. Polyglutamylation has been described in *T. brucei* and *Leishmania* [87,88], although polyglycylation does not appear to be present. We discuss these modifications and their potential functions below.

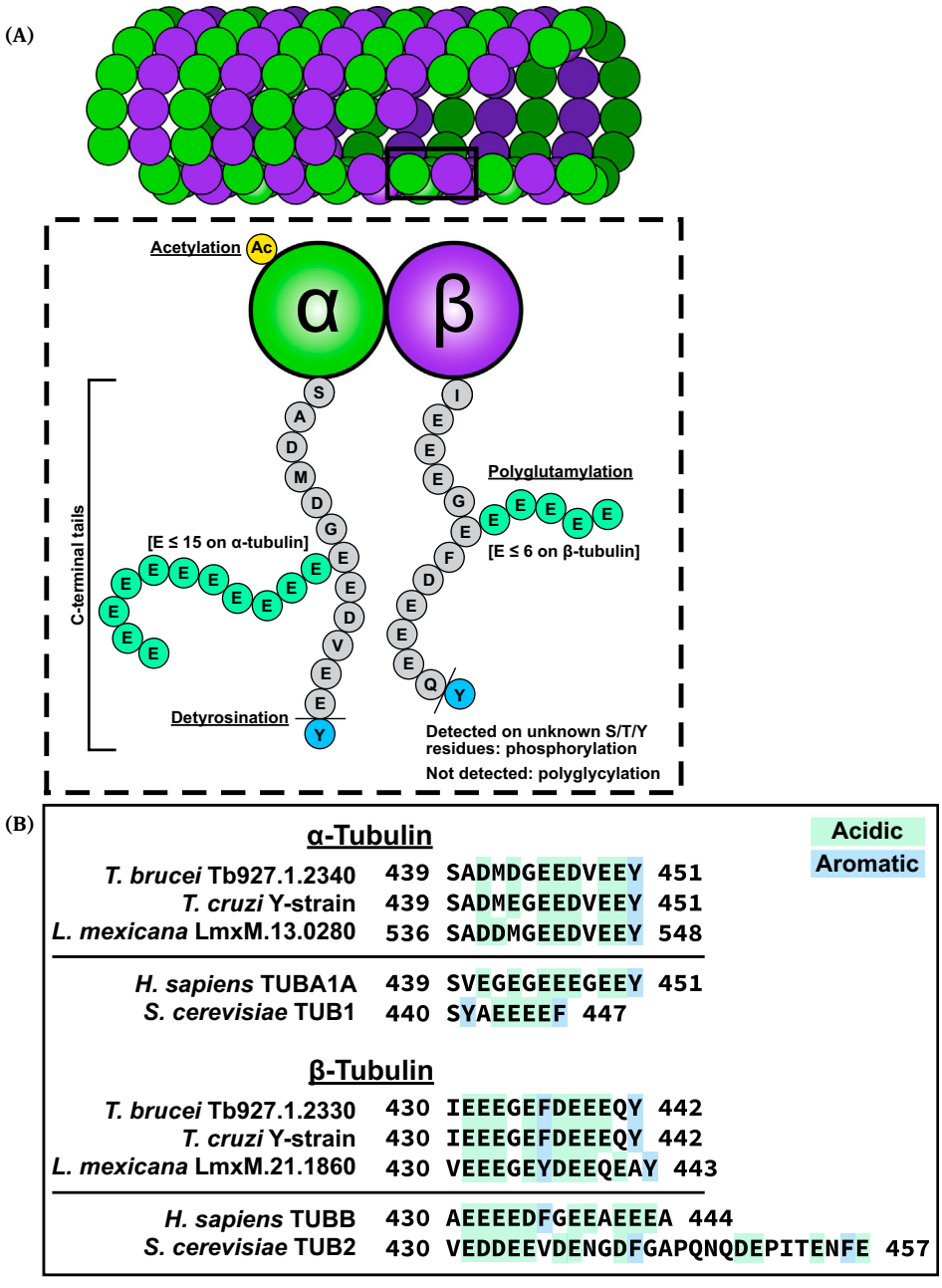

Figure 5. Microtubule Modifications Found in the Trypanosomatid Subpellicular Array.

(A) Top. A microtubule is a hollow tube made up of tubulin protofilaments, which are comprised of α- and β-tubulin heterodimers (green and purple, respectively. Bottom. Both α- and β-tubulin subunits have long, intrinsically disordered and highly charged C-terminal tails that face the cytoplasm, which are the most common site of post-translational modifications. Trypanosomatid microtubules are highly glutamylated on both α- and β-tubulin subunits. The α- and β-tubulin subunits also undergo detyrosination in trypanosomatids, in which the terminal tyrosine residue is cleaved. Unlike most tubulin post-translational modifications, acetylation occurs at an inward-facing site on the α-tubulin subunit. The amino acid sequence of the tails in this schematic is from

(See figure legend at the bottom of the next page.)

Acetylated α-tubulin, which is commonly associated with long-lived and stable microtubules, occurs on the microtubule lumen-facing side of the α-tubulin subunit [75]. It is found in the subpellicular array as well as the flagellum of *T. brucei*, *T. cruzi*, and *Leishmania* [12,79,80]. α-Tubulin is acetylated after its incorporation into microtubule polymers [61]. The acetylation of α-tubulin appears to weaken inter-protofilament interactions within the microtubule, which increases microtubule flexibility and protects against mechanical stress [89]. As flagellar beating has been shown to deform the subpellicular array and the cell body in *T. brucei* [26,27], acetylation may protect the microtubules in the array from damage and catastrophe. Reduction in α-tubulin acetylation levels impairs cleavage furrow formation and ingression in bloodstream-form *T. brucei*, although it is unclear by what mechanism [90].

When α-tubulin is synthesized and polymerized into microtubules, it typically contains a terminal tyrosine residue on its cytoplasmic tail [91]. Tyrosinated α-tubulin is often used as a marker for newly synthesized microtubules, as the terminal tyrosine is removed by a tubulin carboxypeptidase over time [3,91,92]. Soluble α-tubulin can be retyrosinated by a tubulin tyrosine ligase, which creates the detyrosination–tyrosination cycle. This cycle is present in *T. brucei* [3,81,87], and likely in *T. cruzi* and *Leishmania* as well, based on the presence of tyrosinated α-tubulin by antibody staining [80,82]. The genomes of *T. brucei*, *T. cruzi*, and *Leishmania* contain many putative tubulin tyrosine ligases, as well as the newly identified tubulin carboxypeptidase Vasohibin (VASH1/SVBP) [88,93,94]. Tyrosinated α-tubulin is found at locations within the array that are undergoing microtubule growth, such as the cell posterior and the growing flagellum tip during cell division [3,40,79]. Newly divided cells elongate and remodel their posterior arrays, which are heavily tyrosinated [40]. During cellular replication in *T. brucei*, the short microtubules that invade between the existing microtubules to enlarge the array are tyrosinated along their entirety [3]. The function of tyrosination is unclear, although in other systems particular kinesins specifically recognize and interact with tyrosinated tubulin [95]. Interestingly, in *T. brucei*, *T. cruzi*, and *Leishmania*, β-tubulin is also synthesized with a terminal tyrosine residue on its C-terminal tail, which is not found in mammalian or yeast systems (Figure 5B) [91,93]. In *T. brucei*, it has been shown that this tyrosine can be cleaved [87], which suggests that a detyrosination–tyrosination cycle also exists for β-tubulin in trypanosomatids. The function of the β-tubulin terminal tyrosine, if any, has yet to be investigated.

Both the flagellar and subpellicular array microtubules are glutamylated (up to 15 residues on α-tubulin, and six on β-tubulin in *T. brucei*) [87,88]. The posterior portion of the array, which grows extensively during cell cycle and differentiation events, is preferentially polyglutamylated in *T. brucei* trypomastigotes and *Leishmania major* promastigotes [88]. Glutamylation is important for cytokinesis in procyclic *T. brucei*, as decreasing glutamylation levels by depleting glutamylases cause cytokinetic defects [88]. In mammalian systems, polyglutamylation of up to 10 residues on β-tubulin increases the binding affinity of the microtubule-severing enzyme spastin [96]. Recent work showing that microtubule severing by spastin and katanin can amplify microtubule number and generate microtubule arrays through their activity [97] may provide a mechanism for the production of the short microtubules found in dividing trypanosomes. Microtubule severing by katanin is required for cleavage furrow ingression in *T. brucei*, while spastin is necessary for the final abscission event [47].

Tubulin phosphorylation has also been detected in trypanosomatids, but the function of this modification is not known. The tubulin of arsenite-resistant *Leishmania* is hyperphosphorylated [83,86]. In *T. cruzi*, tubulin is dephosphorylated upon parasite interaction with host extracellular matrix proteins [84]. These results suggest that tubulin phosphorylation can be regulated by signaling cascades initiated by external stimuli.

Figure 5. *Continued*
Trypanosoma brucei. (B) The amino acid sequences of the cytoplasmic C-terminal tails of α- and β-tubulin from *Trypanosoma brucei*, *Trypanosoma cruzi* (Y-strain), and *Leishmania mexicana*. The human and yeast sequences are listed beneath for comparison, and acidic and aromatic charges are indicated. Sequences obtained from [93,119].

These findings indicate that there is a landscape of post-translational modifications across the array that could be used to control the differential recruitment of MAPs and motors at different times in the cell cycle. This landscape could be driven by levels of microtubule tyrosination. Newly polymerized microtubules are enriched with tyrosinated tubulin [98]. The detyrosination of α-tubulin preferentially occurs on the microtubule polymer, while retyrosination only occurs on free, soluble tubulin. This substrate specificity creates a gradient of tyrosination along the length of individual microtubules, which has been documented in *T. brucei* [3]. This gradient may act as a rheostat to recruit specific MAPs or modification enzymes that are sensitive to levels of tyrosination. In this fashion, the tubulin code could tune the biophysical properties of array microtubules to account for the local curvature of the cell body, or control how the force generated by flagellar beating is transformed into cell motility.

Subpellicular Array Regulation

Microtubule-Array Associated Proteins

In vitro, single microtubules are inherently dynamic and cycle through states of polymerization and depolymerization called 'rescues' and 'catastrophes', respectively. Cells regulate this dynamic instability through the activity of tip-binding proteins, molecular motors, and MAPs [75]. MAPs can stabilize and bundle microtubules into persistent structures. Few MAPs have been identified in trypanosomatids to date, and little is known about how they function to build, maintain, and remodel the array.

In Table 1 we have listed the proteins currently known to associate with the subpellicular array. The vast majority of these proteins have been identified in *T. brucei* procyclic forms; it is unknown if their functions are conserved in *T. cruzi* or *Leishmania*, although many of these proteins are found in all three species. Although these proteins are broadly referred to in the literature as MAPs, few of them have been shown to bind directly to microtubules or tubulin *in vitro*, and so caution must be used when applying this term.

Microtubule-array associated proteins were initially identified through the extraction and solubilization of the array, followed by fractionation and isolation of individual proteins. Some of these proteins, such as a 52 kDa and a 33 kDa protein, are able to form crosslinks on mammalian microtubules *in vitro* with a similar periodicity as found in the subpellicular array [99,100]. This suggests that the fibrils that link microtubules in the array are made up of proteins that bind in a specific orientation along microtubules, thus creating the regular spacing seen in electron micrographs.

Most microtubule-array associated proteins identified thus far have been described as microtubule crosslinkers or stabilizers. The intermicrotubule crosslinks themselves appear to be composed of a complex set of proteins that differ in composition across the array. The proteins CAP15 and CAP17, which bundle and stabilize microtubules when expressed ectopically in mammalian cells, localize to the anterior portion of the array [101]. The recently identified protein PAVE1 preferentially localizes to the posterior array [48], while TbAIR9 is found across the entirety of the array [102]. This distribution pattern suggests that different MAPs are recruited to different parts of the array, perhaps to maintain array shape and facilitate morphogenesis.

In *T. brucei*, many microtubule-array associated proteins are found as paralogous pairs within the genome and are preferentially expressed in either the bloodstream or procyclic form of the cell. CAP15 is expressed in bloodstream forms, while CAP17 is expressed in procyclics [101]. This pattern is the same for CAP5.5 and CAP5.5V [103], as well as CAP51 and CAP51V [104]. The localization and function of these related proteins is similar in both life-cycle stages. The reason for encoding two paralogous proteins with the same function is unclear. Although *T. cruzi* and *L. mexicana* possess homologs of CAP5.5 and CAP51, they do not appear to encode their paralog (Table 1) [24,93]. Whether unique paralogous pairs of microtubule-array associated proteins exist in *T. cruzi* or *Leishmania* has yet to be determined. However, the example of paralogs in *T. brucei* presents the possibility that trypanosomatids may differentially regulate microtubule-array associated proteins during

Microtubule-array associated protein	Accession	Organism	Localization	Conservation	Description/ purported function	Phenotype	Refs
p41	Unknown; isolated from *Trypanosoma brucei* cytoskeletal extractions	*T. brucei*	Unknown	Unknown	Has a covalently-bound fatty acid	N/A	[111]
52 kDa protein	Unknown; purified from *T. brucei* microtubule fractionation	*T. brucei*	Unknown	Unknown	Crosslinks microtubules *in vitro*	N/A	[99]
COP33	Unknown; purified from *Crithidia* microtubule fractionation	*Crithidia*	Entire subpellicular array	Unknown	Crosslinks microtubules *in vitro*	N/A	[100,112]
COP61	Unknown; purified from *Crithidia* microtubule fractionation	*Crithidia*	Unclear	Unknown	Unknown	N/A	[100,112]
p15	Unknown; purified from *T. brucei* microtubule fractionation	*T. brucei*	Entire subpellicular array	Unknown	Bundles microtubules and copolymerizes with tubulin *in vitro*	N/A	[113]
WCB	Tb927.7.3550	*T. brucei*	Membrane-facing side of entire subpellicular array	Found in *Leishmania* and *T. cruzi*	Crosslinks the plasma membrane to the subpellicular array	RNAi shortens cells and causes microtubule disorganization and membrane blebs in procyclic forms	[114]
MARP-1 (p320)	Tb927.10.10360	*T. brucei*	Entire subpellicular array	Found in *Leishmania* and *T. cruzi*	Contains 38 repeats which consist of a microtubule-binding domain; binds microtubules in mammalian cells	N/A	[111,115]
MARP-2	Unknown; identified by polyclonal antiserum against a *T. brucei* phage library	*T. brucei*	Unknown	Unknown	Closely related to MARP-1; binds microtubules in mammalian cells with a non-repeat containing C-terminal domain	N/A	[116]
Gb4	Tb927.9.2520	*T. brucei*	Microtubule tips at the posterior subpellicular array	Found in *Leishmania* and *T. cruzi*	Large and repetitive	N/A	[117]

Table 1. Microtubule-Array Associated Proteins in Trypanosomatids

(Continued on next page)

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Microtubule-array associated protein	Accession	Organism	Localization	Conservation	Description/ purported function	Phenotype	Refs
I/6 autoantigen	Tb927.7.3440	*T. brucei*	Entire subpellicular array	Found in *Leishmania* and *T. cruzi*	Alternatively spliced; may crosslink microtubules	N/A	[118]
CAP15/17	Tb927.11.11980/ Tb927.11.16200	*T. brucei*	Anterior subpellicular array	Found only in *T. brucei*	Bundles and stabilizes microtubules in mammalian cells; CAP15 is preferentially expressed in bloodstream forms; CAP17 is preferentially expressed in procyclic forms	Overexpression of CAP15 or CAP17 in procyclic cells causes cytokinetic defects	[101]
CAP5.5/5.5V	Tb927.4.3950/ Tb927.8.8330	*T. brucei*	Entire subpellicular array	CAP5.5 found in *Leishmania* and *T. cruzi*	CAP5.5 is preferentially expressed in procyclic forms; CAP5.5V is preferentially expressed in bloodstream forms	RNAi causes cytokinetic defects in both procyclic and bloodstream forms	[103]
TbAIR9	Tb927.11.17000	*T. brucei*	Entire subpellicular array	Found in *Leishmania* and *T. cruzi*	Also found in plants where it localizes to interphase microtubules and the phragmoplast during division	RNAi causes elongated posterior arrays, nuclear mispositioning, and cytokinetic defects in procyclic forms	[102]
XMAP215	Tb927.6.3090	*T. brucei*	Positive growing ends of microtubules throughout the subpellicular array	Found in *Leishmania* and *T. cruzi*	Well known (+)-end tip binding protein in mammalian systems	Unknown	[40]
EB1	Tb927.9.2760	*T. brucei*	Positive growing ends of microtubules throughout the subpellicular array	Found in *T. cruzi*	Well known (+)-end tip binding protein in mammalian systems	Unknown	[42]
CAP51/51V	Tb927.7.2640/ Tb927.7.2650	*T. brucei*	Entire subpellicular array	CAP51 found in *Leishmania* and *T. cruzi*	CAP51 is preferentially expressed in procyclic forms; CAP51V is preferentially expressed in bloodstream forms	RNAi results in multiple rows of microtubules in the subpellicular array and causes cytokinetic defects in both procyclic and bloodstream forms	[104]
PAVE1	Tb927.8.2030	*T. brucei*	Posterior subpellicular array	Found in *Leishmania* and *T. cruzi*	Predicted coiled-coil	RNAi truncates the subpellicular array at the cell posterior and causes cytokinetic defects in procyclic forms	[48]

Table 1. Continued

morphogenesis to tune array dynamics in specific life-cycle stages. Identification of new proteins in *T. cruzi* and *Leishmania* will be of special interest, as these organisms have both extra- and intracellular life-cycle stages with vastly different array morphologies.

The microtubules of the array must be organized into a planar orientation. This is exemplified by CAP5.5/5.5V and CAP51/51V, which are required to maintain the array as a single layer of microtubules in *T. brucei*. When depleted by RNAi, multiple layers of microtubules accumulate underneath the plasma membrane [103,104]. Additionally, the attachment of microtubules to the plasma membrane must also be maintained for correct array organization. The protein WCB, which localizes to the plasma-membrane-facing surface of microtubules in extracted cytoskeletons, is one of the proteins that mediates this stable connection [105]. WCB depletion disrupts array spacing and causes the plasma membrane to bleb across the cell surface; cells divide aberrantly or cease to divide altogether. These results demonstrate the importance of intermicrotubule and microtubule–plasma membrane linkages in both array organization and plasma-membrane integrity, which appear critical for cell division.

Outstanding Questions

How are the microtubules of the array partitioned during cytokinesis?

How are microtubules nucleated in the array? How are they inserted?

How is the array repaired during the lifetime of the cell?

Do MAPs promote microtubule flexibility or change the degree of microtubule torsion?

How are MAPs, motors, and enzymes targeted to specific locations in the array?

How does the array deform to accommodate the flagellar pocket and endocytic structures?

How conserved are MAPs and their functions across trypanosomatid species? Are the arrays regulated similarly?

Are the biophysical characteristics of mammalian tubulin conserved in trypanosomatids?

Molecular Motors and Enzymes

Molecular motors have also been implicated in array biogenesis and remodeling. *Leishmania major* and *T. brucei* genomes encode over 40 kinesins [106]. These include kinesins from well characterized families as well as ungrouped kinetoplastid-specific orphan kinesins. Many of the orphan kinesins characterized thus far have roles in regulating the subpellicular array. In procyclic *T. brucei,* TbKIN-C is found at the extreme posterior of the array and is required to maintain cell shape. Depletion of TbKIN-C results in cells with highly-extended 'nozzle' posteriors [107]. TbKIN-D interacts with TbKIN-C and has a similar localization at the posterior tip. Strikingly, TbKIN-D depletion results in the accumulation of multiple layers of microtubules underneath the plasma membrane, similar to the CAP5.5/5.5V and CAP51/51V depletion phenotypes. This suggests that TbKIN-D has a role in remodeling or inserting microtubules into the existing array [108]. KLIF, a newly identified orphan kinesin, is required in the final stages of cytokinesis in *T. brucei*. Cells depleted of KLIF stall mid-way through cleavage furrow ingression [48]. RNAi against katanin in procyclic *T. brucei* also stalls the cleavage furrow in a similar manner [47]. Interestingly, stalled cleavage furrows were first seen in *T. cruzi* after cells were treated with taxol, which stabilizes microtubules [109]. These results suggest that microtubules in the array are remodeled by kinesins and microtubule-severing enzymes, which have specific functions in maintaining cell shape or segregating the array during cytokinesis. Biophysical characterization of these motors and enzymes is required to determine their mechanism of action and how they localize and function within the context of the array.

Concluding Remarks

Although the subpellicular array appears static and rigid in electron micrographs, these are snapshots in time. The array may be more flexible than previously thought; it shows a high degree of plasticity in its ability to bend into 'cellular waveforms' during motility [26,27] as well as remodel into different life-cycle morphologies. How the array is able to accommodate these shape changes is an open field of research (see Outstanding Questions). The new resource of TrypTag (www.tryptag.org), which is determining and reporting the localization of every protein in the *T. brucei* genome, will facilitate the identification of previously uncharacterized array-localized proteins that may be important to the biology of the microtubule array [110]. Research into array dynamics is imperative to understanding trypanosomatid life-cycle differentiation and survival in the host, and will have far-reaching implications on microtubule regulation in general cell biology.

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