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# A post-assembly structural modification to the lumen of flagellar microtubule doublets

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The most common form of the motile eukaryotic flagellum contains a 9 + 2 axoneme, consisting of nine outer doublet microtubules and a central pair of single microtubules. The flagellum of the African trypanosome *Trypanosoma brucei* fits this pattern, but in addition possesses a paraflagellar rod (PFR) connected to the axoneme at doublets 4 through 7. The PFR acts as a structural platform for metabolic enzymes and is essential for motility (for review, see [1]). Here we describe a novel intra-lumenal microtubule structure, which we term the ponticulus, that bridges the B-tubule lumen in all 9 outer doublet microtubules of the 9 + 2 axoneme. We show that ponticuli are not incorporated into the axoneme during new flagellum assembly, but instead are a post-assembly modification.

By tilting many axoneme sections in the transmission electron microscope, we confirmed that a bridge-like structure is present in the B-tubule of all nine outer doublets but is never present in the central pair microtubules (Figure 1A and inset). We have termed this structure a ponticulus (little bridge). Ponticuli are present in both tsetse-form and bloodstream trypanosomes and in axonemes of *Leishmania* and *Crithidia*.

We note references in the literature to electron densities or specific structures in the lumen of axonemal microtubules in other eukaryotes. Early descriptions of trypanosomes commented on microtubule structures that were interpreted inappropriately as either extensions into the PFR [2] or additional tubules [3]. In *Chlamydomonas* a 'beak-like' projection [4] is described as projecting from the B-tubule lumenal wall of some, but not all, outer doublets. Other electron-dense structures have been described in the A-tubule lumen [5–7]. Thus, although many observations have been made, often in passing, of microtubule lumenal structures in axonemes, the variation in presence has often hindered a sustained study and explanation.

Ponticuli were not easily observed in tannic-acid-stained B-tubules and are clearly distinguished from the very apparent tubulin protofilaments of the A- and B-tubule walls (Figure 1B). Overlaying a Markham-analysed tannic-acid-stained and -unstained axoneme (Figure 1B,C) established that the ponticulus forms a bridge between protofilament numbers A3 and B7 (using the numbering system in Figure 1D). In *T. brucei* tannic-acid-stained axonemes the A-tubule is composed of 13 protofilaments, whilst the B-tubule has 10 protofilaments plus an additional small linking structure between protofilaments A1 and B1 (Figure 1B, arrow).

We never observed a variation in the number of B-tubules containing ponticuli within a given cross-section of an axoneme. However, a specific count revealed 7 out of 43 axonemes did not contain ponticuli (see Figure S1A,B in Supplemental Data, published with this article online). Three likely explanations exist; firstly, ponticuli are sensitive to depolymerisation under some conditions; secondly, ponticuli are only present at certain points; or thirdly, a variation in ponticuli occurs with flagellum age. The first explanation is unlikely since all of the ponticuli would need to be destabilised (or not) in all doublets. We addressed the second explanation by studying the presence of ponticuli along the flagellum (Figure S1). Neither the triplet nor doublet microtubules of the basal body contain ponticuli (Figure S1D,E). However, as soon as the 9 + 2 configuration occurs, and hence when the PFR is added, ponticuli are present (Figure S1F,G). Given that the trypanosome cell is thicker at the region of flagellum exit (close to the posterior end of the cell) than it is towards the anterior end of the cell (Figure S2A), examination of different cell profiles shows clearly that ponticuli extend along the entire flagellum length (Figure S2B,C).

In *T. brucei* a specific orientation allows one to distinguish between new and old flagella in the cell cycle, such that when viewed from the posterior end of the cell the new flagellum is always on the

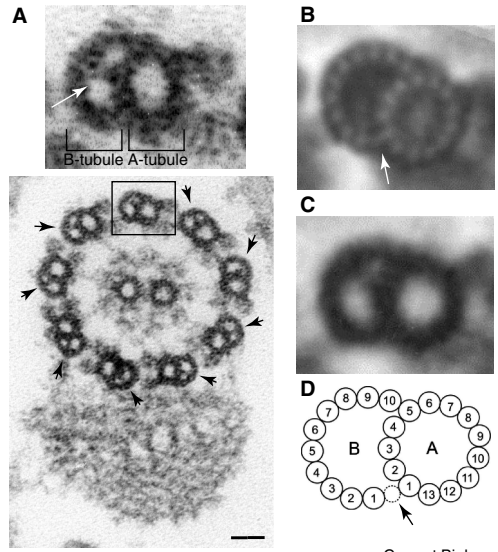


Figure 1. Location of the ponticulus.

(A) The ponticulus intersects the lumen of the B-tubule of all nine outer doublets in the 9 + 2 axoneme (arrows). Inset shows outer doublet marking the A- and B-tubules and the ponticulus (arrow). (B) Markham rotation of outer doublet stained with tannic acid to highlight the arrangement of protofilaments. (C) Markham rotation of doublet not stained with tannic acid. (D) By overlaying (B) and (C) we established that the ponticulus is closely associated with protofilament numbers A3 and B7. This numbering system was developed in collaboration with K. Downing and formally excludes the linker structure (arrow). Scale bar, 30 nm.

left-hand side [8]. Examination of dividing cells provided the explanation for the absence of ponticuli in some axonemes since the old flagellum contains ponticuli (Figure 2A), but the new flagellum of the pair does not (n = 18 pairs; Figure 2B). Thus ponticuli are not built when the axonemal doublets are assembled and must appear later by a post-assembly mechanism. How and when they are added to the lumen of the B-tubules will require identification of molecular components and analysis of their addition using antibodies.

Is a post-assembly process compatible with observations of other intra-lumenal structures in other systems? In *Chlamydomonas* the intra-microtubule 'beak-like' projections referred to earlier are only present in the B-tubules of outer doublets 1, 5 and 6 [4]. Although limited to this subset of doublets, these structures have some resemblance to the ponticuli that we define here. The 'beak-like' projections of *Chlamydomonas* are reported to be limited to the proximal half of the axoneme. This conclusion was drawn from observations of serial sections at the proximal end and random sections [9]. A possibility worth considering is that the 'beak-like' projections also represent a post-assembly modification given that flagella are resorbed and grown anew during division in *Chlamydomonas.* In addition a post-assembly modification may also explain the variability observed in other studies [10].

Questions arise concerning the transport and accessibility of ponticuli components to the B-tubule lumen after assembly of the axonemal microtubules. Could components be assembled and transported along the lumen of the microtubule (and if so, from which end) or inserted through fenestrations between the tubulin subunits? In each case how could such an assembly be coordinated both spatially and temporally after the microtubule is assembled? How is the distinction made between microtubules within the axoneme and the basal body (which does not contain ponticuli)?

In its general form, the intraflagellar transport (IFT) system has, at present, been considered mainly as a mechanism for moving large cargo on the surface of microtubules. These cargo particles are larger than the diameter of the microtubule lumen. Hypothetically, IFT particles could deposit components along the outside of the microtubule, for entry into the lumen through the microtubule wall or could deposit components at the distal end for post-assembly insertion into the microtubule lumen. If polarised assembly of the extended ponticulus complex occurs, then we suggest that the existence and operation of an intra-lumenal microtubule transport system should be considered.

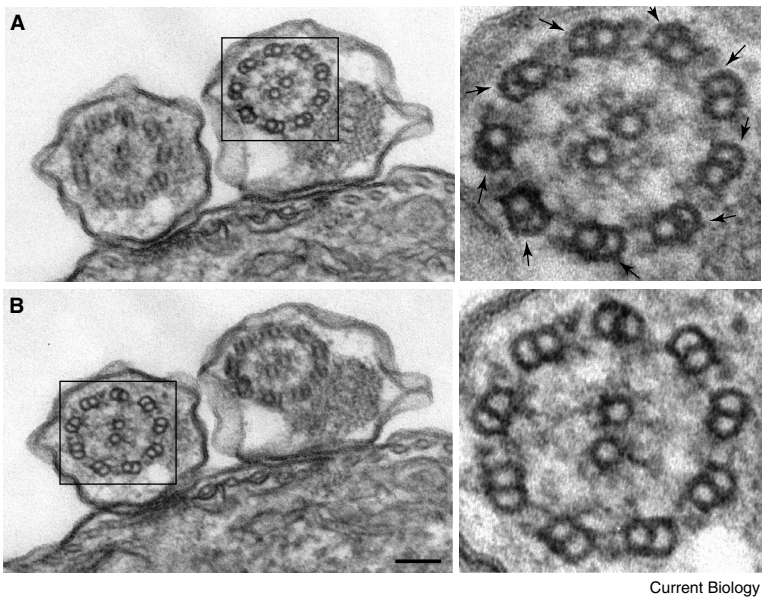


Figure 2. The ponticulus is a post-assembly modification to outer doublet microtubules.
(A) The old flagellum contains the ponticulus in all 9 outer doublets. (B) The new flagellum does not contain the ponticulus in any of the outer doublets despite the presence of the 9 + 2 axoneme. Scale bar, 100 nm.

### Supplemental Data

Supplemental Data including experimental procedures are available at http://www.current-biology.com/cgi/content/full/16/12/R449/DC1/

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