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HYPOTHESES

Insights & Perspectives

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Restriction of intraflagellar transport to some microtubule doublets: An opportunity for cilia diversification?

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Abstract

Cilia are unique eukaryotic organelles and exhibit remarkable conservation across evolution. Nevertheless, very different types of configurations are encountered, raising the question of their evolution. Cilia are constructed by intraflagellar transport (IFT), the movement of large protein complexes or trains that deliver cilia components to the distal tip for assembly. Recent data revealed that IFT trains are restricted to some but not all nine doublet microtubules in the protist *Trypanosoma brucei*. Here, we propose that restricted positioning of IFT trains could offer potent options for cilia to evolve towards more complex (addition of new structural elements like in spermatozoa) or simpler configuration (loss of some elements like in primary cilia), and therefore be a driver of cilia diversification. We present two hypotheses to explain how IFT trains could be restricted to some doublets, either by a triage process taking place at the basal body level or by the development of molecular differences between ciliary microtubules.

KEYWORDS
axoneme, cilia, eukaryotic evolution, flagella, intraflagellar transport, kinesin, microtubule

INTRODUCTION

Cilia are typical eukaryotic organelles usually composed of nine doublet microtubules called A and B, arranged in a cylindrical manner, defined as the axoneme, wrapped by a specific membrane. The A-tubule is composed of 13 protofilaments whereas the B-tubule usually contains 11 protofilaments and is fused to the A-tubule. Cilia are encountered on most cell types of the human body and can adopt at least three main configurations. First, the 9 + 2 disposition is characterised by the presence of a central pair of singlet microtubules and is the archetype of motile cilia, such as those found in epithelial cells of the respiratory tract (Figure 1A), the brain ventricles or fallopian tubes. Outer and inner dynein arms are attached to the A tubule of each doublet and these motors are responsible for ciliary beating. Their action is coordinated by the presence of a multitude of sub-structures such as radial spokes, central pair projections or the dynein regulatory complex that also links doublets between each other.[1] These cilia have a length of ~10–20 μm. A second, more complex configuration, is the 9 + 2 with extra-axonemal structures as encountered in the flagellum of spermatozoa. Several elaborate structures are found around the axoneme such as a ribbon of mitochondria and the outer dense fibres in the intermediate piece of the flagellum or the fibrous sheath in the principal piece. Nevertheless, the terminal piece only contains the axoneme. These flagella are longer, reaching 50–60 μm in humans.[2] The third type of cilia is the simpler 9 + 0 organisation found in many sensory cilia, including primary cilia (Figure 1C). These lack the central pair and all the other structures associated to beating but are highly enriched in sensory elements and function in perception of chemical, mechanical, or light signals.[3] Their structural organisation appears less stringent, with some or all doublets ending prematurely and only some of the A-microtubules extending further as singlet microtubules (Figure 1C). These cilia are often shorter, exhibiting a length of only a

Abbreviations: IFT, intraflagellar transport; PFR, paraflagellar rod.

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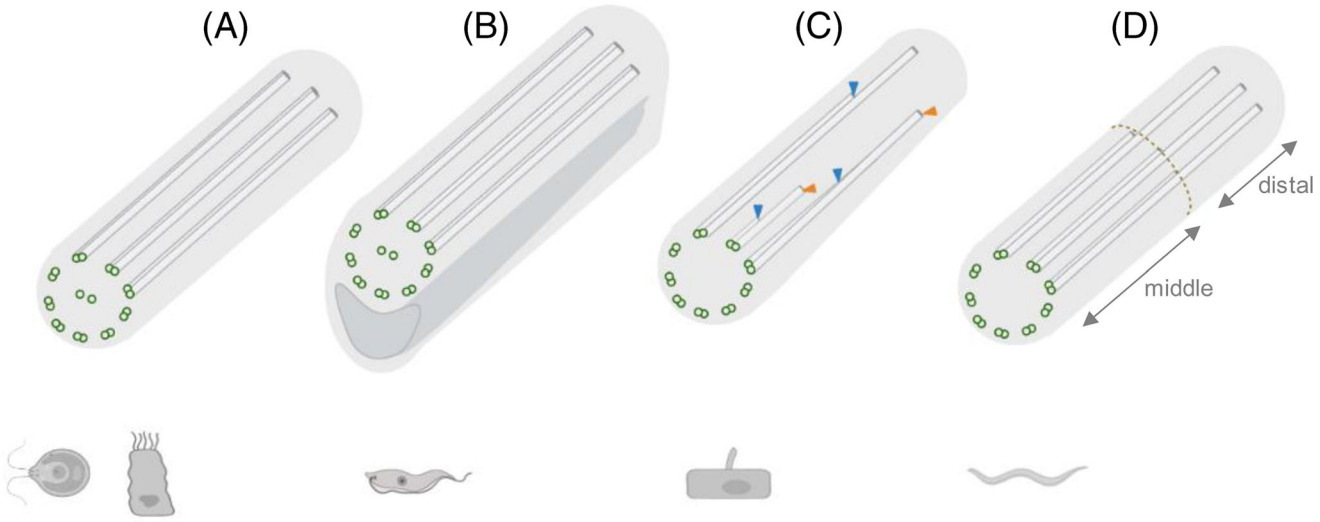

FIGURE 1 Major types of cilia. Cartoons representing the main types of cilia discussed in the text. Axonemes are viewed from the base to the tip (basal bodies and transition zone are not shown). The 9 + 2 configuration is characterised by the presence of a central pair and several sub-structures required for ciliary beating, as found in multiple motile cilia and flagella, such as in humans or in the green alga *Chlamydomonas* (A). More complex configurations include other structures in addition to the 9 + 2 axoneme. Here, the paraflagellar rod (PFR) of *T. brucei* has been represented as an example (B). Simplified architectures with a 9 + 0 configuration are encountered in primary cilia of (C) or in sensory neurons of *C. elegans* (D). For the sake of clarity, only three out of nine doublets are fully represented. Premature endings of A and B tubules of primary cilia are indicated with orange and blue arrowheads, respectively. In primary cilia, microtubules end at various places. In *C. elegans*, doublets are found in the middle segment and singlets in the distal segment as indicated

few microns, although some primary cilia of 20 μm have been described in culture.

These three main categories of cilia are encountered in many different living organisms and some of them constitute excellent models to study the three categories of cilia.[4] Three examples will be discussed for this review. First, the green alga *Chlamydomonas*, a leading model for the study of cilia assembly and function, possesses a typical 9 + 2 axoneme with all the elements required for beating (Figure 1A). Second, the protist *Trypanosoma brucei* assembles a 9 + 2 axoneme with an extra-axonemal structure called the paraflagellar rod (PFR) (Figure 1B). Finally, the nematode *Caenorhabditis elegans* exhibits various cilia with a 9 + 0 architecture involved in sensory perception. Doublets extend for a few microns and terminate at the distal region where only singlet microtubules are present (Figure 1D).

HOW TO ASSEMBLE DIFFERENT TYPES OF CILIA?

This raises the question as to how these different types of cilia emerged during evolution and how multicellular organisms proceed to assemble specific cilia shapes in dedicated cells. For this, it is essential to understand how cilia are constructed. The elongation of their microtubules takes place by the addition of new subunits at the distal end of the organelle.[5–7] However, ribosomes are absent from the ciliary compartment, so ciliary proteins are synthesised in the cell body. This means that the assembly site gets further and further away from the site of synthesis during microtubule elongation. This conundrum is solved by the existence of intraflagellar transport (IFT): protein complexes loaded with ciliary precursors are brought to the tip by the action of a kinesin motor (anterograde transport). Once the cargo is released and available for incorporation in the growing structure, complexes are recycled to the base thanks to the action of a dynein motor (retrograde transport) that was itself brought there as a cargo during anterograde transport.[8] These complexes were called trains since they move along microtubules that can be viewed as tracks and are dragged by molecular motors that can be compared to locomotives, with multiple copies of IFT complexes like a succession of coaches. IFT was discovered in *Chlamydomonas* in 1993[9] and turned out to be conserved in most ciliated organisms. Its inhibition prevents cilia assembly in all organisms investigated so far, from protists to animal cells.[8]

IFT has been proposed to play a crucial role in the generation of different types of cilia.[10] This can be achieved by at least two ways: the use of different molecular motors with unique specificities and the loading of different cargoes. The nematode *C. elegans* possesses different types of 9 + 0 cilia and is therefore a great model to investigate these differences. Amphid cilia are composed of an intermediate segment made of doublet microtubules and a distal segment with exclusively singlet microtubules. Two kinesin motors (one heterotrimeric, one dimeric) cooperate to build the intermediate segment whereas only the homodimeric kinesin can assemble the distal segment.[11] In other types of sensory cilia, the association of different cargoes to the IFT trains has been proposed to lead to the construction of more or less elaborate cilia.[12]

IFT TRAIN PRESENCE AND POSITIONING IN FLAGELLA WITH AN EXTRA-AXONEMAL STRUCTURE

Here, we would like to propose that IFT presence and positioning could be a way to generate cilia diversity, either towards a more complex (addition of supplementary structures) or towards a simplified organisation. Stepanek and Pigino[13] have used correlative light and electron microscopy to investigate IFT train positioning in *Chlamydomonas*. These cells attach to a coverslip by their flagella and exhibit gliding motility. In these conditions, flagella lie flat on the support, offering the opportunity to examine long portions of axonemes and to look for IFT trains. Analysis of 15 axonemes revealed that IFT trains were found on eight out of nine doublet microtubules, with trains being under-represented on doublets close to the glass slide or to the exact opposite side of the axoneme (Pigino, personal communication). These trains are quite long (up to 700 nm for anterograde trains) and relatively wide (50 nm), and traffic at high speed (2–4 μm per second) and frequency (1–2 trains per second). Their presence would severely limit the possibility for the emergence of other elements, such as the extra-axonemal structures encountered in mammalian spermatozoa (Figure 1B).

Close examination of flagellum formation during mouse spermatogenesis revealed that IFT proteins are detected by immunofluorescence in all the early stages, that is, when the axoneme is constructed. However, IFT proteins are no longer present in more advanced stages, exactly at the time when extra-axonemal structures such as the fibrous sheath or the outer dense fibres begin to assemble.[14] IFT is therefore used to construct the axoneme, then somehow taken away to make way for the construction of the supplementary structures that wrap around the axoneme. The problem of IFT occupying the space around the axoneme mentioned above is therefore solved by temporal differences between IFT trafficking and axoneme construction first, and arrest of IFT and addition of novel structures next.

Could such a principle be applied to allow evolution of cilia towards a more complex organisation? Exciting insights are provided by the analysis of IFT train positioning in the flagellum of *T. brucei*. This organism is well known for being the parasite responsible for sleeping sickness in central Africa. It is transmitted by the bite of the tsetse fly, where it adopts different shapes to adapt to the changing environments encountered in various tissues.[15] It is also a powerful model for functional studies of cilia and flagella.[4] Like most members of the euglenoids, an extra-axonemal structure called the PFR is present alongside the axoneme.[16] It exhibits a lattice-like structure and is firmly attached to the axoneme via doublets 4–7 of the axoneme (Figure 1E, bottom image). This structure is composed of mostly unique cytoskeletal proteins, but it also contains well conserved proteins such as calmodulin or adenylate kinase.[17,18] It is assembled by addition of new subunits at the distal end of the growing structure, and in contrast to mammalian spermatozoa, its construction is virtually concomitant with that of the axoneme.[7,19]

IFT is very active in trypanosomes, with the injection of about one train per second in the anterograde direction and 2.5 in the retrograde direction in all the life cycle stages that could be investigated.[20,21] So, how are trains positioned to accommodate the PFR? Strikingly, 3D electron microscopy imaging revealed that IFT trains are only present on doublets 3–4 and 7–8.[22] As one could have predicted, no trains were detected on doublets 5–6 that are entirely covered by the PFR but surprisingly, trains are very rarely present on doublets 1, 2 and 9 where there is no PFR (Figure 2).

This result is significant as it means that IFT and extra-axonemal structures can cohabit, allowing the benefit of each for the good functioning of the flagellum: IFT is required for construction of the organelle[23] and maintenance of the correct distribution of several of its molecular components[24] whereas the PFR brings an essential contribution to motility.[25]

IFT POSITIONING AND THE EMERGENCE OF MORE COMPLEX CILIA AND FLAGELLA

In evolutionary terms, if we consider that the PFR was acquired after the emergence of the axoneme, two scenarios can be proposed (Figure 3). In the first one, novel protein(s) would attach to doublets 5 or/and 6 to permit the future addition of a new structure (Figure 3A). Their presence could have hindered the passage of IFT trains, resulting in their restriction to neighbouring doublets. In other words, the presence of new elements would force IFT trains to travel on other doublets. However, this interpretation does not explain why IFT trains are missing from doublets 1, 2 and 9 since there are no structural elements there. Nevertheless, the presence of the PFR could have other consequences. Cryofixation revealed that the flagellar membrane looks tightly adpressed to the axoneme at the level of doublets 1, 2 and 9, in contrast to the regions around doublets 3–4 and 7–8 that might be more amenable to the passage of IFT trains.[26]

In the second model, IFT trains would *first* be restricted to some doublets, hence liberating space at their surface for the binding of proteins that could lead to the formation of new structures such as the PFR (Figure 3B). This proposal is very attractive because restricting IFT to a reduced number of doublets would still enable to benefit from IFT for the construction and maintenance of the axoneme, but it would open the door for molecular, structural and functional diversification at the level of the doublets where IFT trains do not traffic.

This makes sense for trypanosomes that are single-cell organisms whose flagellum is essential for motility, adhesion to host tissues, morphogenesis and possibly sensing. Their life cycle constrains them to very different environments: the gut and the salivary glands of tsetse flies, or the blood and the skin of mammals to cite just a few.[27,28] This requires a lot of cellular but also of flagellar plasticity. A striking example is found during the development in salivary glands where *T. brucei* attaches firmly to the epithelium to complete its maturation before becoming infective again for a mammalian host. This adhesion is essential since this stage does not express essential cell surface proteins for development in mammalian hosts, hence premature release in the saliva would be lethal upon feeding.[15] Adhesion takes place via the flagellum whose membrane extends exhaustively to wrap

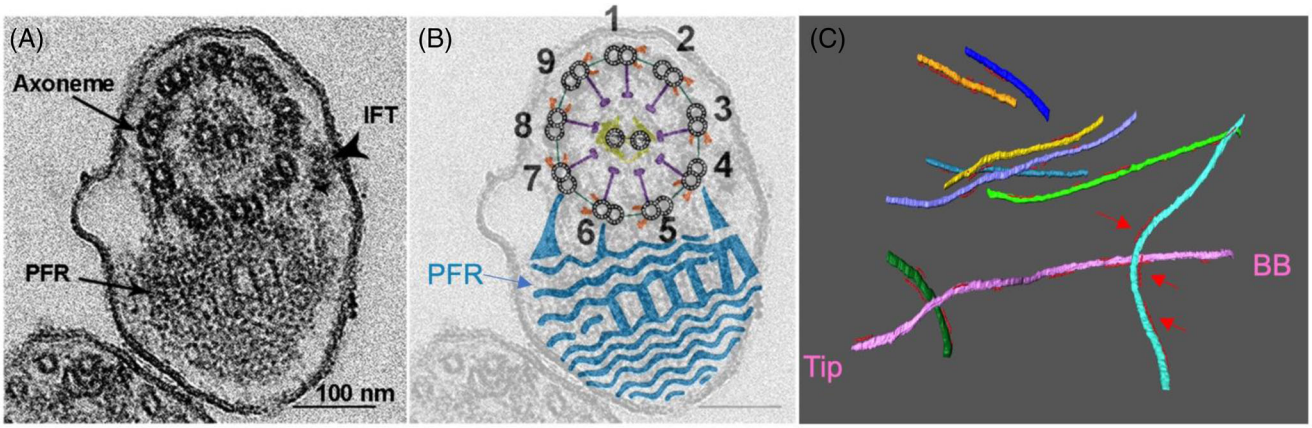

FIGURE 2 IFT trains are restricted to a subset of doublets in *T. brucei*. (A) A cross-section of the trypanosome flagellum reveals the presence of the PFR and of an IFT train positioned on doublet 4 (arrowhead). (B) The superimposed cartoon indicates microtubule doublets and their numbering, the PFR (blue) and highlights of typical axonemal structures such as dynein arms (orange), central pair projections (yellow), nexin links (green) and radial spokes (violet). (C) 3D electron microscopy using FIB-SEM analysis demonstrates the limited presence of IFT trains (red, indicated by red arrows on the sky blue flagellum) to only two opposite faces of the axoneme: doublets 3–4 and 7–8. BB, basal body; IFT, intraflagellar transport; PFR, paraflagellar rod

around microvilli of the insect epithelium and anchors via electron-dense plaques resembling hemi-desmosomes.[15] Remarkably, these plaques are found on the side of the axoneme opposite to the PFR, between doublets 8–9 and 1–3, that is, where IFT trains are not present (Figure 4). This means that the shape of the flagellum can be drastically modified while still enabling IFT. Experimental evidence revealed that IFT was still active in this developmental stage, as shown by monitoring parasites expressing a fluorescent IFT protein over the course of infection.[21] Moreover, IFT could even contribute to parasite attachment by delivering the large amount of material that is required for the formation of these adhesion plaques, by participating to membrane expansion or by transporting signalling molecules. So far, this is the only life cycle stage known where such an adhesion to host tissues is encountered, suggesting that trypanosomes need to recognise the appropriate tissue before developing these membrane extensions.

How to explain the absence of IFT from doublets 1, 2 and 9 (where there is no PFR)? We propose that these doublets are used by other molecular motors that could be involved in different functions, for example, the trafficking of sensory elements proposed above. Proteomic analyses of purified flagella have detected the presence of several kinesin motors whose function remains elusive.[29–31] Possibly some of them use the microtubule doublets not occupied by IFT trains to transport different sets of cargoes. An intriguing case is a member of the kinesin 9 family termed KIF9B that localises to the axoneme and to the basal body area. It turned out to be essential for proper assembly of the PFR.[32] Resolving its exact localisation on microtubule doublets could provide a potential answer to this question. However, it will require electron microscopy or super-resolution imaging, which is not easily applicable for a cylindrical structure. Nevertheless, the recent development of expansion microscopy for trypanosomes[33] opens new possibilities for precise localisation studies.

IFT POSITIONING AND THE EMERGENCE OF SIMPLER CILIA

We have seen that restricting IFT to some doublets could permit the complexification of cilia and flagella, by allowing the acquisition of new molecular or structural elements. We propose that it could also contribute to *simplify* cilia structure. Correlative light and electron microscopy was used to position IFT trains in the flagella of *Chlamydomonas*. It revealed that anterograde trains travel on the B-tubule and retrograde trains on the A-tubule.[13] This precise distribution explains why trains do not collide. However, this elegant model cannot apply to cilia that contain only singlet microtubules in their distal part, such as primary cilia[34,35] or *C. elegans* sensory cilia.[36] Since the B-tubule is missing, how do anterograde trains make it to the tip of the structure? Cryo-electron microscopy analysis revealed that anterograde trains are present on the A-tubule, at least in the distal part of primary cilia in MDCK cells.[35] Since retrograde trains are supposed to travel on the A-tubule, how could collisions be avoided?

We would like to propose a new explanation: restriction of IFT trafficking to eight (or possibly less) of the nine doublets would allow the loss of the B-tubule of this 'doublet' where IFT is not taking place anymore (Figure 5A). Since retrograde trains normally travel on the A-tubule, we propose that this singlet could be used by dynein motors (Figure 5A). In the next step, another B tubule could be lost, giving the opportunity to the kinesin motor to walk on the remaining A tubule. Once at the tip, the IFT material would be returned to the base on the A tubule already devoted to by retrograde transport (Figure 5A). In that theory, an ultra-simplified system could function with only two singlet microtubules, one dedicated to anterograde transport and one to retrograde transport, leaving the other microtubules available to other functions. They could also degenerate as frequently observed in primary cilia.

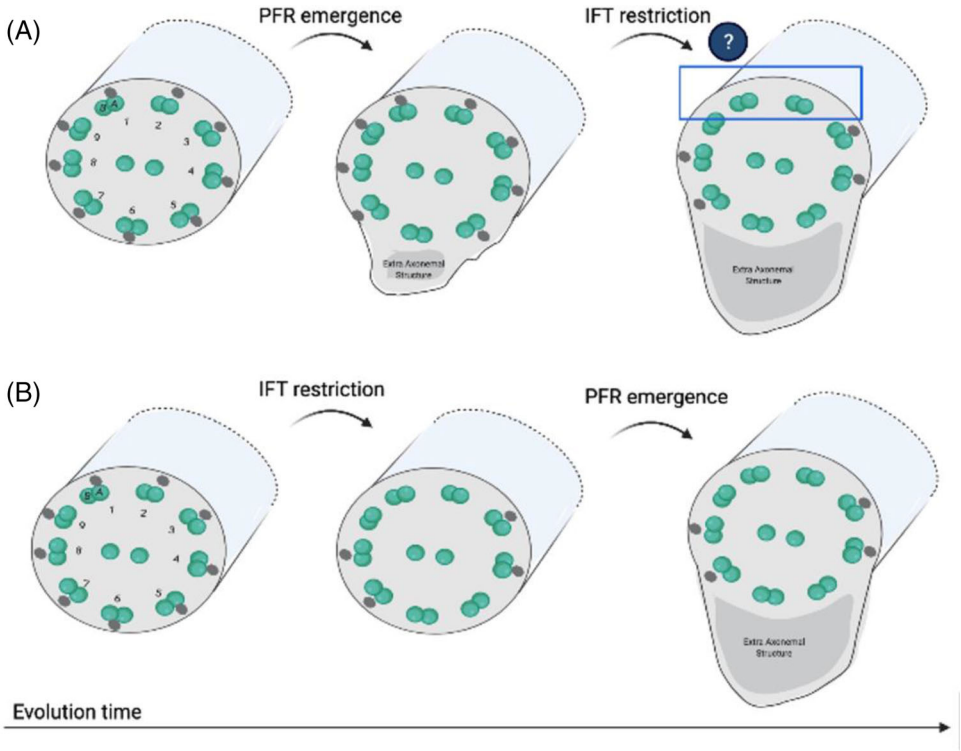

FIGURE 3 Possible models explaining the relationship between IFT train positioning and the acquisition of the PFR. (A) The emergence of the PFR would prevent trains from accessing doublets hidden by this extra-axonemal structure. This model however does not explain why trains are missing from doublets 1, 2 and 9 (highlighted by the blue rectangle and the question mark). (B) IFT trains would first be restricted to some doublets, hence allowing the emergence of the PFR. Doublets 1, 2 and 9 could be used for other functions (see text for details). IFT, intraflagellar transport; PFR, paraflagellar rod

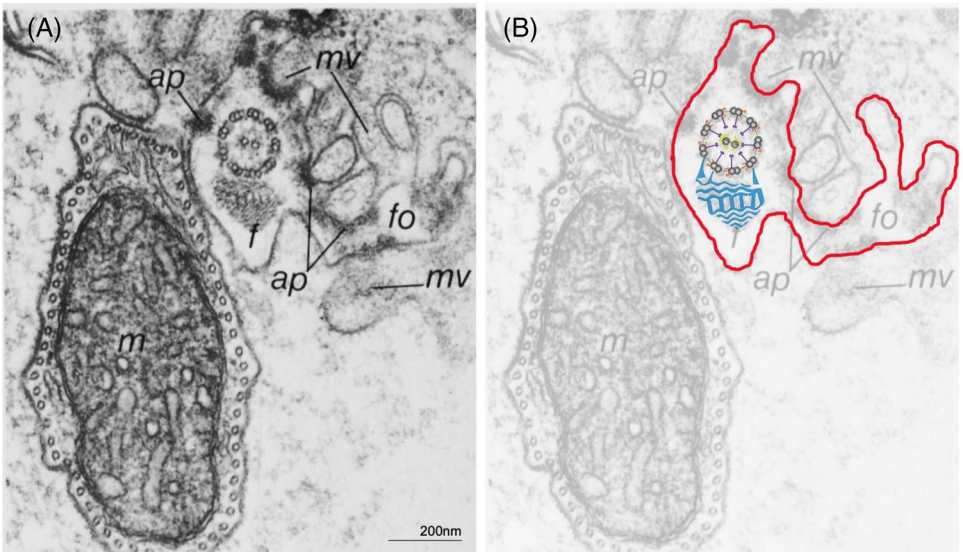

FIGURE 4 The trypanosome flagellum attaches to host epithelium on its face where IFT does not take place. (A) Cross-section of a *T. brucei* cell attached to the salivary gland of an infected tsetse fly. The large mitochondrion (m) and the flagellum (f) are easily recognisable. (B) The extensions of the flagellar membrane or fo are highlighted in red. They spread around mv of the salivary gland epithelial cells and appear to be connected thanks to ap, electron-dense structures resembling hemi-desmosomes. These plaques are detected around most doublets, leaving the possibility for active IFT on doublets 4 and 7. Credit: [15]. ap, attachment plaques; fo, flagellum outgrowths; IFT, intraflagellar transport; mv, microvilli

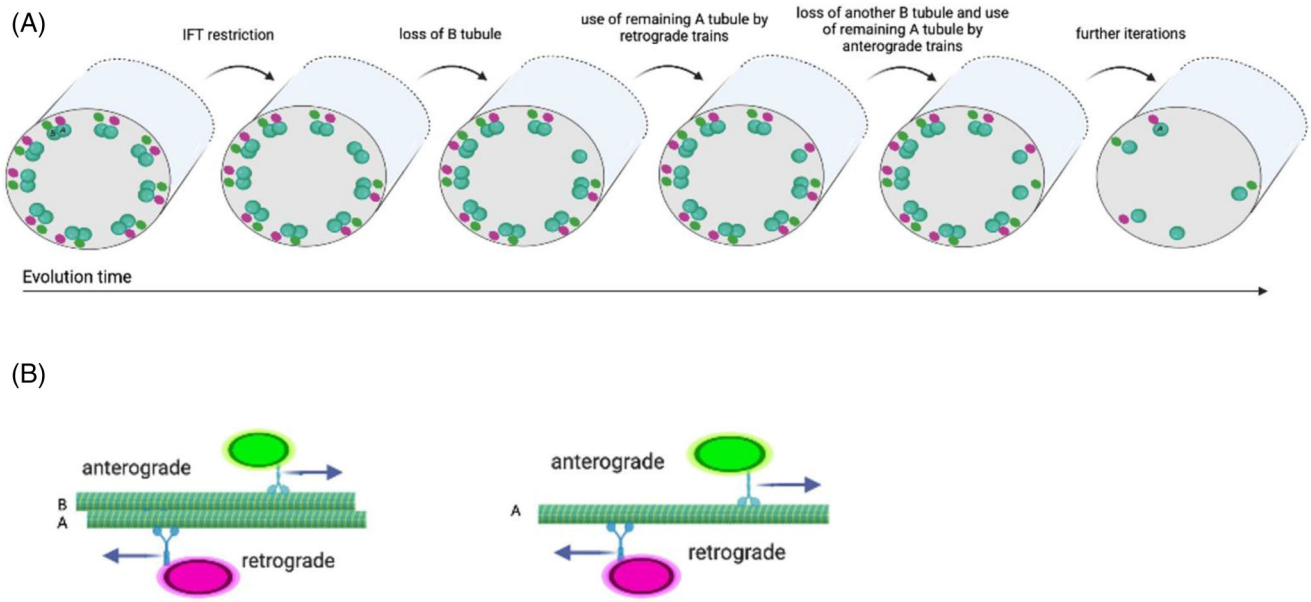

FIGURE 5 Possible models for bidirectional intraflagellar transport (IFT) trafficking in cilia composed of only A tubules. (A) Restriction of IFT to some doublets allows loss of the B tubule of at least one doublet. The remaining A tubule can be used for retrograde transport by trains returning from neighbouring doublets. In the next step, loss of the B tubule from another doublet would force anterograde trains to use the remaining A tubule. Retrograde transport would then use the flanking A tubule already devoted to this function. This scenario could be repeated several times to lead to cilia functioning only with singlets ("further reiterations"). In principle, a couple of singlets would be sufficient to ensure anterograde and retrograde transport. (B) In motile cilia of *Chlamydomonas*, anterograde (green) and retrograde (pink) trains travel on the B and A tubule, respectively, hence avoiding each other. In primary cilia, anterograde and retrograde trains could use different portions of the remaining A tubule to avoid collision

Nevertheless, other models could be considered. The A-tubule of most sensory cilia does not bear neither dynein arms nor radial spokes, meaning that its whole circumference could be available for IFT trains. Anterograde and retrograde trains could therefore traffic on different portions of the remaining A-tubule, hence avoiding collisions and ensuring efficient trafficking (Figure 5B). However, IFT trains have so far only been reported in proximity to the ciliary membrane[13,22,35,37] and it is not known if they could function at other positions around the microtubule. Exhaustive characterisation of IFT trains by electron microscopy has only been achieved in a handful of organisms so far,[22,37] so precious information could be gained by further investigation in different types of cilia, either from various types of mammalian cells or from different model organisms.

HOW TO RESTRICT IFT TRAINS TO SOME DOUBLETS?

If IFT trains are first restricted to specific doublets, what could be the molecular mechanism? We can propose two different models, relying either on a triage at the base or on specific molecular identity for each track.[38] In the first model, the base of the axoneme could function as a sorting platform, exactly like in a train station, sending trains to the right tracks. Immunogold or super-resolution immunofluorescence have shown that IFT proteins are concentrated all around the transition fibres.[39,40] This would require molecular differences, which do not look obvious in an organelle exhibiting a nine-fold symmetry. Intriguingly, electron microscopy revealed that basal bodies are often associated with various structures such as rootlets, fibres or basal feet whose positioning is very precise and break the apparent symmetry of the organelle.[41] This implies molecular differences between the triplet, what starts to be supported by molecular evidence. A combination of super-resolution microscopy and immuno-electron microscopy revealed an asymmetric distribution of the FOP1 protein at basal bodies of the ciliate *Tetrahymena*.[42] Such a defined molecular information could impact on train positioning. Very recently, a combination of cryo-electron microscopy and expansion microscopy data revealed that IFT trains are assembled around the basal body in *Chlamydomonas*.[43] These trains could form exclusively along some triplets of the basal body, according to the associated structural and molecular features mentioned above. Once the kinesin motor gets in action, its processivity would ensure that the trains travel on the doublet where it was assembled.

In the second model, doublets could have their own molecular identity, which would make them suitable or not for IFT.[38] Even if IFT precursors are found all around the basal body, the kinesin motor could only either bind or walk on some doublets and hence trains would traffic exclusively on these. Inter-doublet molecular differences have been reported in *Chlamydomonas*[44,45] and in *T. brucei*.[46,47] Molecular differences have not been reported in primary cilia, but the fact that microtubules doublets or even singlets do not end at the same position suggests some potential for individualisation.[34,35]. The same could be

said to explain the fact that anterograde and retrograde trains use only the B-tubule or the A-tubule respectively.[13]

What kind of difference(s) could exist and how could it facilitate (or restrict) access to IFT trains? In *T. brucei*, cryo-electron microscopy analysis of microtubule doublets indicated structural differences between some groups of doublets, but this was only reported for the face of the doublets directed towards the centre of the axoneme.[] Cryo electron tomography with volta phase plate reveals novel structural foundations of the 96-nm axonemal repeat in the pathogen *T. brucei*, so where IFT trains have not been detected. However, differences do not need to be structural and could be more discrete. A favourite hypothesis is the tubulin code: the C-terminal tail of alpha- and beta-tubulin is extensively modified in cilia and flagella with the addition of glutamate or glycine residues that can alter biochemical properties of the microtubules. Since these tails are at the surface of microtubules, they are strong candidates to modulate interaction with molecular motors.[49] In vitro experiments have shown that both motor velocity and processivity can be significantly altered according to the glutamylation profile. Since the tail of tubulins is not structured,[50] differences between microtubules are unlikely to be detected by cryo-electron microscopy.[13] This model is supported by immunogold studies using antibodies detecting glutamylated tubulin glutamylation and showing a very pronounced enrichment on the B-tubule of isolated flagella from the green alga *Spermatozopsis similis*.[51] Furthermore, some doublets of the flagellum of mouse spermatozoa show up to three-fold more staining than others with an antibody detecting long poly-glutamate chains,[52] providing evidence for molecular differences between axonemal doublets.

CONCLUSION

Cilia can adopt many different configurations, including within the same organism. Mechanisms governing these differences remain elusive, although some secrets slowly start to be unveiled. Several studies in *C. elegans* have suggested that fine tuning of IFT could be involved in shaping different categories of cilia, with evidence for the contribution of distinct kinesin motors or the availability of different cargoes.[10,12] Here, we propose a novel possible explanation, with the restriction of IFT trafficking to some doublets (or singlet) ciliary microtubules that could offer the opportunity for either an increased structural complexity or a simplified architecture. Challenging this model will require the identification of the molecular causes underpinning restricted IFT trafficking, such as observed in *T. brucei*. The tubulin code is a promising lead, but its complexity will have to be untangled.[53,54] The recent rapid progress in imaging, both at the level of cryo-electron microscopy and of super-resolution and expansion microscopy are providing the tools, needed to get to the heart of these complex organelles. It is often said that the development of multicellular organisms recapitulates evolution history. Responses will come from studies in multiple models, from single-cell organisms (that display enormous ciliary diversity) to multicellular species such as nematodes, flies, frogs, worms or mammals.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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