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Radial Spokes—A Snapshot of the Motility Regulation, Assembly, and Evolution of Cilia and Flagella

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Propulsive forces generated by cilia and flagella are used in events that are critical for the thriving of diverse eukaryotic organisms in their environments. Despite distinctive strokes and regulations, the majority of them adopt the 9+2 axoneme that is believed to exist in the last eukaryotic common ancestor. Only a few outliers have opted for a simpler format that forsakes the signature radial spokes and the central pair apparatus, although both are unnecessary for force generation or rhythmicity. Extensive evidence has shown that they operate as an integral system for motility control. Recent studies have made remarkable progress on the radial spoke. This review will trace how the new structural, compositional, and evolutional insights pose significant implications on flagella biology and, conversely, ciliopathy.

The intricate radial spokes (RSs) and central pair apparatus (CP) in the 9+2 axoneme have attracted the interest of many. By all accounts, flagellar motility is contingent on their direct interactions, and thus they are often mentioned in the same context. Although motile cilia and flagella in some cell types or some organisms naturally lack both RSs and the CP, for the majority that possess the 9+2 axoneme, they prove to be critical for motility. Genetic defects afflicting either structure will result in a spectrum of dyskinesia, ranging from paralysis to jerking to a mixed population of motile and dysmotile cilia.

As mentioned in Loreng and Smith (2016), the CP is substantially more complex than the RS. The synchronized spinning of the CP with rhythmic beating in certain cilia (Omoto and Witman 1981) has especially fueled much imagination. In contrast, the RS is relatively simple. Nonetheless, the simplicity presented a distinctive opportunity for asking questions fundamental to flagella biology. Vigorous efforts in combination with brilliant ideas and new technologies in recent years have shed considerable insight on RSs from diverse organisms, including humans. Although many earlier RS studies used *Chlamydomonas*, emerging evidence showed species-specific differences in RSs and experimental advantages. This review will narrate the converging discoveries that unveil RSs' structures, molecular interactions, functions and divergence among different organisms, and the implications. The in-depth discussion of motility control mechanism, radial spoke proteins (RSPs) and RS mutants in *Chlamydo-*

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monas could be found in a previous review (Yang and Smith 2008).

MORPHOLOGY AND PERIODICITY OF RADIAL SPOKES

Physical attributes of axonemal components are integral to the mechanics of motile cilia and flagella. RSs were named after their radial pattern in the axoneme cross sections (Ishikawa 2016). Individual RSs rendered by conventional electron microscopy (EM) often were hard to discern or varied substantially. Recent cryo-electron tomography (ET) that bypassed fixations and metal decoration finally resolves the discrepancies. Interestingly, RSs from diverse organisms actually bear striking resemblances but indeed with distinctions. A side view of the *Trypanosome* axoneme (Koyfman et al. 2011) in which the CP is extruded (Fig. 1A) reveals the typical radial pattern of RSs, with the thinner spoke stalk docking to each outer doublet and the enlarged head projecting toward the CP. In a lateral view of *Chlamydomonas* axoneme (Fig. 1B), there are two RSs—RS1 and RS2—in each 96-nm repeat, contrary to three RSs for most organisms. The missing third RS—RS3—is replaced by a stand-in stub, RS3S (Pigino et al. 2011; Barber et al. 2012; Lin et al 2014). The distances among the three RSs adhere to a multiplication of 8 nm—the length of αβ-tubulins—namely 32, 24, and 40 nm.

As revealed by negative-stained splayed axoneme (Witman et al. 1978), RS1 and RS2 appear as a T shape, which, as the view rotates outward, turns into a Y shape with an enlarged head connected to a neck region by two arms (Fig. 1C). The top view shows spoke heads comprised of two asymmetric modules arranged in a twofold rotational symmetry (Fig. 1D), presumably each connected to the neck by one arm. At higher resolutions, the spoke head in humans (Fig. 1E) or sea urchins appeared smaller and more compact, lacking the tendril-like extensions in protist spoke heads. Notably, RS3 has a rather different morphology, with an asymmetric head (Fig. 1E) and a kinked stalk. Furthermore, RS3 seems unaffected in primary cilia dyskinesia (PCD) patients with defective spoke head proteins (Lin et al. 2014). The biochemical bases underlying the distinctive RS3 morphology are unknown.

The molecular context at the base of each RS is also distinct, suggesting that the task of the three RSs are not identical. RS1 base is adjacent to the base of the two-headed inner dynein arm I1, whose base module contacts the MIA complex named after the deficiencies in *modifier of inner arm* (*mia*) mutants (King and Dutcher 1997; Yamamoto et al. 2013). *mia* mutants, albeit containing all I1 components, are slow swimmers deficient in phototaxis as I1 mutants (see the section Chemical Signaling for a detailed discussion). RS2 and RS3/RS3S are, respectively, adjacent to the front and back of the nexin–dynein regulatory complex (N-DRC) and perhaps the calmodulin- and spoke-associated complex (CSC) (Gardner et al. 1994; Dymek and Smith 2007; Heuser et al. 2009, 2012; Urbanska et al. 2015). This arrangement is consistent with distinct inner dynein anomalies in *mia* mutants (King and Dutcher 1997) and N-DRC mutants (Piperno et al. 1992). These precise structural relationships are predetermined by a complex coined as a molecular ruler that is, surprisingly, comprised of only two coiled-coil paralogous proteins (FAP59 and FAP172) (Oda et al. 2014a). Defects in their encoding genes in *Chlamydomonas* mutants (*pf7* and *pf8*) result in diminished inner dynein arms and N-DRC. Interestingly, the 32- to 64-nm RS periodicity is replaced by a 32-nm only periodicity. Lengthening these ruler proteins also alters the periodicity of RSs or their adjacent ensemble. It is interpreted that RS periodicity is founded on the molecular ruler that recruits N-DRC and inner dynein arms, which in turn inhibits the inherent ability of RSs in binding to outer doublets.

RADIAL SPOKE PROTEINS IN *CHLAMYDOMONAS*

The repertoire of RS mutants (Huang et al. 1981), the RSP 2D map (Piperno et al. 1981), nuclear transformation (Diener et al. 1990), and extraction of RS particles (Yang et al. 2001) set the stage for the comprehensive identifications of RSPs in *Chlamydomonas*, *Ciona* (Satouh et al.

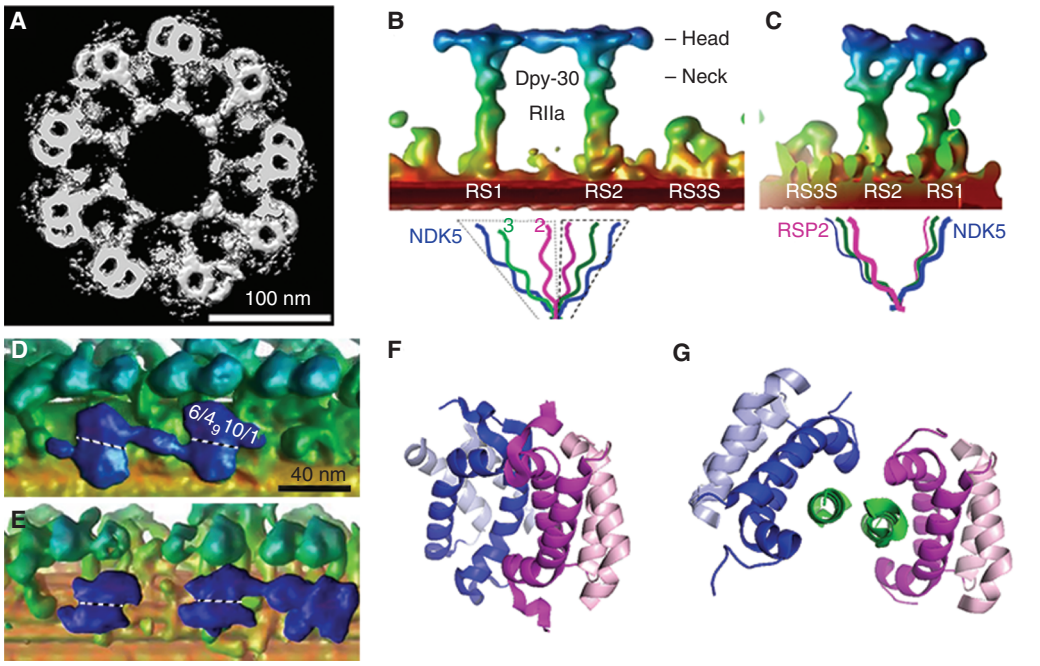

Figure 1. Structures of radial spokes. (*A–E*) Cryo-electron tomogram renditions of axonemes. (*A*) Cross section from *Trypanosome brucei* in which the central pair apparatus was extruded offered an unobstructed view of the entire radial spoke. The central pair apparatus is absent in this sample (electron microscopy [EM] Data Bank [EMDB] ID 5302; Koyfman et al. 2011). (*B*,*C*) Lateral view of the T-shaped twin radial spokes, RS1 and RS2, in one 96-nm repeat of *Chlamydomonas* flagella and a counterclockwise rotation view to reveal the bifurcation of the neck region (EMDB ID 1941; Pigino et al. 2011). The *bottom* panels depict predicted positions of the helices from RSP2, RSP3, and NDK5 (pink, green, and blue lines) that form the two arms at the neck and tether to two head modules. Spoke HSP40 at this position is not illustrated for clarity. (*D*,*E*) *Top* view of a 96-nm repeat in a *Chlamydomonas* flagellum (EMDB ID 5845; Oda et al. 2014b) and human respiratory cilium (EMDB ID 5950; Lin et al. 2014). Each spoke head has two modules arranged in rotary symmetry. Numbers in *D* indicate predicted locations of RSPs. Note humans have triplet RSs, with RS3 strikingly different from RS1 and RS2. The images were generated using the EM Navigator at the Protein Data Bank Japan (PDBj) (pdbj.org). (*F–G*) Crystallographic structures predicting the assembly process of the spokeneck region with Dpy-30 domains from RSP2 (pink ribbons) and NDK5 (blue ribbons) and amphipathic helices from a RSP3 dimer (green ribbons). (*F*) Crystallography of tetramers of the Dpy-30 domain that is comprised of two X helical bundles. The domain exists as a homodimer in solution. In crystals, staggering of the two dimers partially exposes the binding cleft for an amphipathic helix in their interacting partners. The polypeptides are from Dpy-30 protein. The illustration is generated from PDB ID 3G36 (Wang et al. 2009). This may account for the direct association of RSP2 and NDK5 human orthologs. (*G*) The face-to-face orientation of the two dimeric Dpy-30 domains binding to an amphipathic helix from Ash2 in the Set1-like histone methyltransferase complex. The illustration is generated from PDB ID 4RIQ (Tremblay et al. 2014). This may represent the conformational change as the RSP2/NDK5 complex associates with RSP3's amphipathic helix.

2005; Satouh and Inaba 2009), and other organisms. RSPs were first defined based on the missing 17 proteins in the spokeless axonemes of *Chlamydomonas* mutant, *pf14* (Huang et al. 1981). Extracted RS particles contain these 17 RSPs, tubulins, and at least six more polypeptides. Two are ubiquitous—calmodulin and LC8, which are present in multiple protein complexes—and therefore are not absent in *pf14*. Together, these 19 proteins reside in the Y-shaped RS complex. The rest are likely tethered to the RS base and coextracted when microtu-

bules are disrupted by the KI buffer. All *Chlamydomonas* RSPs have been identified except RSP13 (Yang et al. 2006). RSP13 may be orthologous to CMUB116 in *Ciona* RSs, a conserved protein with both a calmodulin-binding domain and a ubiquitin domain. There is no evidence yet that the content in RS1 and RS2 differs. Only those that were studied further will be discussed below.

ORGANIZATION OF RSPs

Based on partial deficiencies in a few *Chlamydomonas* RS mutants, the 17 RSPs were assigned to separate regions—five (RSP 4, 6, 1, 10, and 9) in the head, three (RSP2, HSP40, and RSP23) at the neck, and the rest at the stalk (Huang et al. 1981; Patel-King et al. 2004; Yang et al. 2006) (Fig. 1B–E). The comprehensive data showed that the largely symmetric RS1 and RS2 contained many homodimers and paired paralogs with an identical domain. For example, in the spoke head, RSP4 and 6 are paralogs expressed from duplicated genes (Curry et al. 1992), whereas RSP1 and 10 are homologs with membrane occupation and recognition nexus (MORN) repeats. Analysis of recombinant proteins and tagging indicate that each spoke head contains two copies of each protein that is inherently dimeric (Kohno et al. 2011; Oda et al. 2014b). Furthermore, the RIIa domains in RSP11 and RSP7, and the Dpy-30 domains in RSP2 and RSP23 (NDK5) in the stalk are dimerization and docking (DD) domains of striking similar tertiary structures (Sivadas et al. 2012). Finally, RSP3 that docks the RS to outer doublets also exists as a homodimer (Diener et al. 1993; Wirschell et al. 2008).

RIIa-containing RSPs showed the physiological relevance of the binding of RSP3 to the RIIa-containing RII in the cAMP-dependent protein kinase (PKA) (Gaillard et al. 2001) in an in vitro assay credited for the discoveries of many PKA-anchoring proteins (AKAPs). As such, RSP3 is often referred to as an AKAP. Deletion mutagenesis (Sivadas et al. 2012) and structural tagging (Oda et al. 2014b) confirmed that an RII-binding amphipathic helix in RSP3 binds to the RIIa domain in RSP7 and RSP11, which otherwise bears no other PKA signature features (Fig. 1B). This led to a prediction that some "AKAPs"—including RSP3 in *Chlamydomonas*—anchors proteins with a RIIa domain that tethers functional modules irrelevant to PKA, and the term of "AKAPs" should be reserved for those that anchor PKA holoenzyme in vivo (see detailed discussion in the section Evolution of Radial Spokes). In addition, RSP3 harbors another amphipathic helix that binds Dpy-30 domains in RSP2 and NDK5. Chemical cross-linking suggests further association of NDK5 with RSP1, and RSP2 with RSP4/RSP6 (Kohno et al. 2011).

Taken together, these observations support a model that one RSP3 dimer serves as a scaffold to anchor the rest of the RSPs. The conserved region of RSP3 likely defines the RS length that is strictly conserved (Gaillard et al. 2001). Its amino-terminal region docks RSs to the outer doublets, whereas its carboxyl terminus directs toward the spoke head (Sivadas et al. 2012; Oda et al. 2014b). This orientation positions the RIIa-containing RSP7 and RSP11 toward the outer doublets and the two Dpy-30-containing RSP2 and NDK5 toward the spoke head (Fig. 1B). As such, the carboxy-terminal helices in RSP2, RSP3, and NDK5 may form the arms that bifurcate at the neck and secure two spoke head modules directionally (Fig. 1B,C, bottom panel). For the spoke head, we designate that RSP6 is peripheral to RSP4, and RSP1 is peripheral to RSP10 (Fig. 1D), because both RSP6 and RSP1 are not required for spoke head assembly in *Chlamydomonas* (Wei et al. 2010; X Zhu, unpubl.); and RSP10 could be chemically cross-linked into a homodimer (Kohno et al. 2011). RSP9 is required for spoke head assembly but there is insufficient data to predict its location.

EVOLUTION OF RADIAL SPOKES

In spite of the similar Y-shaped morphology, RSs diverged evolutionarily—toward simplicity—perhaps accelerated by gene duplication, redundancy, and the versatility of DD domains. Consistent with partial redundancy of spoke head proteins, the Basic Local Alignment Search Tool (BLAST) search indicates that RSP1 and

its predicted partner, NDK5, are absent in *Tetrahymena* and other ciliates, whereas *Ciona intestinalis* as well as sea urchins only have one gene for RSP4/6 (Table 1) (P Yang, unpubl.). This is substantiated by the presence of only one RSP4/6 and one MORN protein in purified *Ciona* RSs (Satouh and Inaba 2009). This may be also true for humans, although humans have genes for RSP4, 6, 1, and 10. The abundance of the transcripts (Kott et al. 2013) or express sequence tags (ESTs) for RSP1 (RSPH1) and RSPH10 are opposite in airway and testis. Similarly, RSPH4 transcripts are far more abundant than RSPH6 transcripts. This raises the possibility that the spoke head in human cilia may contain homodimers of RSPH4 and RSPH1, forsaking RSPH6 and RSPH10, whereas RSPH1 is absent in sperm flagella. As such, the notion that certain defective RS genes will not affect fertility and thus will be more prevalent (Onoufriadis et al. 2014) should be entertained.

Simplification also reflects in the sizes of RSP1, 2, 3, and NDK5 in the head/neck region (Table 1). These proteins from *Tetrahymena* and *Chlamydomonas* have a long carboxy-terminal extension that is absent in their metazoan counterparts. For instance, contrary to *Chlamydomonas* RSP2's 738 aa residues, human RSP2 counterparts—DYDC1 and DYDC2—are only ~170-aa long, encompassing only the region required for rhythmic beating, including the Dpy-30 domain for docking to RSP3, and the flanking helices perhaps for forming the bifurcated arms that interact with RSP4/6 (Fig. 1B,C) (Gopal et al. 2012). As the extensions are predicted to be near the spoke head, absence of the extension is consistent with metazoans' smaller, compact spoke head. Although these carboxy-terminal tails are dispensable, RSP2s and NDK5s have calmodulin-binding motifs. *Chlamydomonas* cells lacking RSP2's tail cannot steer properly under bright light (Gopal et al. 2012). This suggests that the calmodulin-binding extension acts on the spoke head region (Fig. 1B,C) to coordinate flagellar motility as this photosynthetic organism navigates illuminated environments. Such measures apparently are unnecessary for most organisms.

The RS epitomizes the versatility of DD domains and their amphipathic helix partners (Gopal et al. 2012; Sivadas et al. 2012). The DD domains bring to molecular complexes

Table 1. Divergence of radial spoke proteins (RSPs) in the head and neck region of four representative species, as shown by their sizes and selective absence

	Species			
RSP	C. r.	T. t.	C. i.	H. s.
RSP4	465	463 486	527	717
RSP6	459	493	N/D	612
RSP1	814	N/D	300	309
RSP10	216	227 221	N/D	870
RSP9	269	296 463	276	276
RSP2 (DYDC1,2)	738	628	169	177
RSP23 (NDK5)	586	N/D	257	212
RSP3	516	691 950 764	336	380 (^{N}160)

RSPs in the head region are shaded in gray. C.r., *Chlamydomonas reinhardtii*; T. t., *Tetrahymena thermophile*; C. i., *Ciona intestinalis*; H. s., *Homo sapiens*. The polypeptides were identified based on a BLAST search against *Chlamydomonas* RSPs. Those from C. r. and C. i. were also confirmed by protein biochemistry of isolated RS particles (Yang et al. 2006; Satouh and Inaba 2009). Note that some RSPs in the protists are far larger, while human RSP3 has an additional 160-aa fragment at the amino terminus (^{N}160) (Jivan et al. 2009). N/D, Not detectable in the genome or RS proteome.

their tethered effector moieties—for cNMP signaling in the case of RII in PKA, for calcium-sensing such as RSP7, for nucleoside metabolism such as NDK5, and for assembly such as DYDC2 and RSP11. The tethered moieties could further duplicate or combine, as in *Chlamydomonas*' RSP2 and NDK5. Mammals particularly have multiple genes that encode RIIa-containing proteins, most of which are enriched in testis. But they share little semblance to each other, RSP7, or RSP11 except the RIIa domain (e.g., Newell et al. 2008). The divergence of DD domain proteins may allow airway and testis to build slightly different RSs and perhaps CPs that also contains an RIIa-binding protein (Gaillard et al. 2001; Rao et al. 2016). Some of them bind to "AKAPs" in the fibrous sheath of sperm flagella (e.g., Fiedler et al. 2012). As such, bona fide RIIa partners of RSP3 and any AKAPs may differ in each cell type, and should be determined empirically. Although RSP3 does not bind PKA's RII in *Chlamydomonas*, it potentially could in animals' motile cilia or flagella as a bona fide AKAP. Different RIIa partners may explain opposite effects of PKA on *Chlamydomonas* flagella and other cilia and flagella (Hasegawa et al. 1987). It is worthwhile to point out that RSP3 indeed possesses a key feature of AKAPs, the scaffold of molecular complexes, to integrate proteins of diverse functions for flagellar motility control. Although *Chlamydomonas* RSP3 is not a typical PKA-anchoring AKAP, it still anchors calcium-sensing molecules perhaps for calcium-related motility control and structural proteins for transducing mechanical feedback.

RS ASSEMBLY

RSPs are assembled into RSs in two phases. Fractionations of cell body extracts and the flagellar membrane matrix suggest that the cell body only makes partially furbished RSs that sediment as 12S particles in the sucrose gradient (Qin et al. 2004). After delivery into flagella by intraflagellar transport (IFT), 12S precursors are converted into 20S mature RSs. The direct interaction of human orthologs of NDK5 and RSP2 at the neck (e.g., Rual et al. 2005) suggests that they assemble first, perhaps mediated by the Dpy-30 domain that was crystallized as two partially staggered dimeric dimers (Fig. 1F). Interactions with amphipathic helices in the RSP3 dimer during the assembly of the 12S RS precursors might reposition the two sets of dimers into a face-to face orientation (Fig. 1G). This structure-based interpretation is concordant with the detection of RS subparticles in the RSP3 mutant (Diener et al. 2011).

The distinct sedimentation coefficients of 12S and 20S RS particles are more likely caused by disparateness in shapes than masses. While the Y-shaped mature RSs are about 32 nm by 42 nm, the 12S RS is a Γ-shape of 20 nm by 28 nm in negative-stained EM (Diener et al. 2011). The precursors lack the two strictly conserved small dimeric proteins—HSP40 and LC8—that are involved in the conformational change during RS assembly. Most organisms have a multitude of HSP40 genes. Typically, HSP40s cooperate with other chaperones to mediate protein folding, whereas some may act solitarily. Flagella that lack HSP40 jerk incessantly although the other RSPs appear normal (Yang et al. 2008). EM revealed irregular tilt or a lower bifurcated neck of RSs. These observations support a model that the V-shaped dimeric HSP40, transported differently from 12S precursors, bind to the polypeptides at the neck region at the flagellar tip where final assembly occurs (Johnson and Rosenbaum 1992). LC8 dimer, present in numerous protein complexes including multiheaded dyneins and RSs, typically brings two polypeptides in a complex together. But in RSs, a stack of LC8 dimers bring together the amino terminus of an RSP3 dimer in RS precursors to form a rod at the RS base, promoting RSP3's phosphorylation and docking to the outer doublets (Yang et al. 2009; Gupta et al. 2012).

RSP3 phosphorylation is tightly linked to RS docking. RSP3 is hypophosphorylated in RS precursors and in mutants with reduced RSs, such as *fla14* that lacks LC8 and *pf27* (Yang and Yang 2006; Gupta et al. 2012), suggesting that the RSs in *pf27*, as in *fla14*, are not assembled via a normal process that involves phosphorylation. The *PF27* gene has not been identified but likely

encodes a protein outside of flagella (Huang et al. 1981). Interestingly, RSs in *pf27* preferentially distribute toward the base of flagella (Alford et al. 2013). It is proposed that *PF27* is an adaptor linking spoke precursors to IFT.

RS OUTER DOUBLET CONNECTIONS

Much progress has been made in elucidating the distinct structural linkages of the three RSs. Three proteins (CaM-IP2, IP3, and IP4) cosediment with the 20S RS fraction but were not abundant enough for identification. Instead, they were identified in the pull-down of the anticalmodulin antibody. Knockdown of CSC proteins impaired RS2, RS3S, N-DRC, and a single-headed dynein, e (Dymek et al. 2011; Heuser et al. 2012), suggesting that the CSC is among the base of RS2 and RS3, N-DRC, and the nearby dynein. Consistent with this, the antibody for a CSC protein inhibits microtubule sliding velocity as the reduced microtubule sliding velocity of *pf14* axonemes. And yet the seemingly normal CSC in *pf14* axonemes indicates that the CSC assembly is independent of the rest of RSs (Dymek and Smith 2007).

A pull-down of the spoke stalk recovered the CSC and another protein that associated with RS2, FAP206 (Gupta et al. 2012). FAP206 is reduced in *pf14* axonemes (Lin et al. 2011). Knockout of *Tetrahymena* FAP206 also impaired RS2 specifically, and indicated that FAP206 linked the proximal end of RS2 base to the single-headed dynein, c (Vasudevan et al. 2015). Therefore, FAP206 and the CSC appear to link the base of RS2 to different single-headed dyneins aligned in tandem. The biochemical evidence for direct coupling among RS1, the MIA complex, and I1 has not yet emerged.

THE SPECTRUM OF MOTILITY ANOMALIES OF RS MUTANTS

It is important to note that not all RS mutants are immotile. Although *Chlamydomonas* RS mutants are known for paralyzed flagella, they actually show a spectrum of motility anomalies, depending on the defective RSP, the nature of mutations, and, surprisingly, the culture conditions. They are paralyzed if RS-CP contacts are absent because they lack the entire RS, the spoke head, or spoke head and neck. Flagella lacking spoke HSP40 jerk constantly. In contrast, the paralysis of the spoke head mutant *pf26* (RSP6) is conditional. In the healthy log phase cultures at room temperature, the motility and the other spoke head proteins appear normal (Huang et al. 1981; Wei et al. 2010). However, in stationary phase cultures, *pf26* cells are largely paralyzed, lacking nearly all spoke head proteins in flagella. In an intermediate condition, a culture will contain swimmers, immotile cells, and cells with jerky (uncoordinated) flagella. Therefore, although RSP26 is dispensable in the laboratory, it is presumably needed in *Chlamydomonas*' natural environment in which the temperature and nutrient availability are expected to fluctuate. Similar motility phenotypes are shown by *pf25* that lacks RSP11 (Yang and Yang 2006) and partially rescued RS mutants (Gupta et al. 2012).

Similar scenarios were noted in other species. *Tetrahymena* FAP206 knockout could swim despite diminished RS2. Its velocity is reduced, attributed to altered waveform, likely because of the deficit of inner dynein arms (Vasudevan et al. 2015) and uncoordinated beat as expected of minor RS deficits. Unexpectedly, cilia of human RS PCD patients beat rhythmically with helical waveform—amid asynchronous beating ones in some cases—despite lacking spoke heads in RS1 and RS2 or most of RSs (Burgoyne et al. 2014; Jeanson et al. 2015). The helical movement is attributed to the presence of RS3, because *Chlamydomonas* flagella with same genetic defect and lacking RS3 are immotile. However, one should bear in mind that natural 9+0 cilia and flagella generate rhythmic beating with helical waveform without any RS (Nonaka et al. 2002). Therefore, phenotype polymorphism, cell conditions, and evolutionary divergence should be considered in phenotyping RS mutants.

ROLES OF RSS

It will be easier to appreciate RSs if one considers the 9+2 cilia and flagella as reversible biological nanomachines.

Generation of Planar Waveform

This is not absolute. The 9+0 motile cilia is rare, such as that found in the node or certain eel and horseshoe crab sperm (Gibbons et al. 1985; Ishijima et al. 1988). As such, they connote an inferior perception. However, these sperm swim rapidly with a 9+0 flagellum of a high beat frequency. Importantly, the typical waveform is helical, similar to the movement of the respiratory cilia of RS PCD patients (e.g., Castleman et al. 2009; Jeanson et al. 2015) and reactivated axonemes of paralyzed *Chlamydomonas* RS or CP mutants under altered conditions (e.g., Yagi and Kamiya 2000; and see Loreng and Smith 2016).

In contrast, 9+2 cilia usually display planar waveform of a large amplitude, but retain the ability to generate helical waveform. For *Chlamydomonas*, power strokes are largely planar, but recovery strokes are helical. The planar waveform with large amplitude appears to be crucial for respiratory cilia to generate sufficient propulsive forces, because the helical movement of cilia in RS PCD patients does not offer sufficient mucociliary clearance despite a normal beat frequency. An exception is the 9+2 axoneme of *Trypanosome* that generates 3D bihelical movements, perhaps because of the paraflagellar rod, a cytoskeletal system unique to kinoplastid parasites (Portman and Gull 2010; Koyfman et al. 2011). Interestingly, helical and planar waveforms of both 9+0 and 9+2 flagella could be switched by changes in physical properties of the aqueous environment, such as surface tension and viscosity (Ishijima et al. 1988; Woolley and Vernon 2001). Therefore, nine outer doublets are inherently switchable to various waveforms.

We envisage that the RS-CP system is a device for swift reordering of the activation sequence of motors in the nine outer doublets. The resulting high-frequency beating of planar waveform is hydrodynamically favorable in most circumstances. But such movement may not be appropriate for a select few. The low-frequency helical beat of 9+0 nodal cilia may be just right for establishing a gradient of morphogens that direct the development of left–right asymmetry (Nonaka et al. 2002; Shinohara et al. 2015). By the same token, 3D-bihelical movement is what *Trypanosome* adopt to navigate in the viscous bloodstream.

Coordination of Molecular Motors by Mechanical Feedback

Diverse evidence suggests that the RS-CP system overrides the inherent helical movement of the outer doublet bundle by serving as a venue for mechanical feedback, perhaps of forces or tensions. Although *Chlamydomonas* RS or CP mutants have paralyzed flagella, their axonemes are able to vibrate in reactivation conditions, suggesting that dynein motors are active but are not coordinated (Yagi et al. 1994). An additional mutation in outer dynein or N-DRC partially rescues the paralysis (e.g., Huang et al. 1982; Porter et al. 1994; Rupp et al. 1996). This leads to the prediction that the RS-CP system coordinates the activation of dynein motors via N-DRC—to generate planar waveform in most cases.

Generation and propagation of a planar waveform are a result of alternate sliding of opposing subsets of outer doublets at any particular moment. The selection appears determined by the CP with asymmetric projections (see Loreng and Smith 2016). The geometric clutch model (Lindemann and Lesich 2015) offers an explicit explanation of mechanical feedback. It posits that the CP and RSs, which contact intermittently during each beat cycle (Warner and Satir 1974), transmit transverse forces developed from flagellar bend, which in turn differentially alter the distances among doublets confined by the base, tip, and N-DRC links. Like the action of an automobile clutch, increasing and reducing interdoublet distances would, respectively, disengage and engage distinct subsets of dynein motors, leading to bend propagation and rhythmic beating. This theory is concordant with elliptic versus circular cross sections of motile and immotile axonemes, and oscillation of axoneme diameters during rhythmic beating (Warner 1978; Sakakibara et al. 2004; Lindemann and Mitchell 2007; Yang et al. 2008).

Mechanical feedback is also supported by perturbations that paralyze motile flagella or rescue the paralyzed. For example, simply removing HSP40 at a spoke neck resulted in jerky flagella with uncoordinated bend initiation, propagation, and switching, presumably because of weakening of RS rigidity necessary for force transmission (Yang et al. 2008). Conversely, forced bending could jolt paralyzed flagella to beat rhythmically for a brief period (Hayashibe et al. 1997). Finally, adding a large tag to the spoke head rescued paralyzed flagella of a CP mutant, probably complementing a missing CP projection to restore structural contact necessary for mechanical signaling (Oda et al. 2014b). Suppressor mutations may alter N-DRC or motors as to bypasses the defective mechanical feedback, or return toward the 9+0 machinery.

Chemical Signaling

The RS-CP system is integral to the modulation of cilia and flagella motility by calcium, calmodulin, cyclic nucleotides, and phosphorylation. Consistent with this, the calcium-dependent waveform changes appeared missing in 9+0 sperm flagella (Gibbons et al. 1985). Contrary to the expected universal principle of mechanical feedback, the mechanisms of chemical signaling may differ substantially for *Chlamydomonas*, which use two relatively short flagella to steer, and for sperm's long flagella, which are activated by Wnt signaling (Koch et al. 2015).

The biflagellate *Chlamydomonas* is known to show two calcium-dependent responses: a waveform switch when encountering barriers or stimulated by intense white light (photoshock), and phototaxis in an environment with differential light intensity (Yang and Smith 2008). The RS-CP system is proposed to be involved in the former because of the symmetric waveform of the suppressor of RS mutants (Brokaw et al. 1982). While this is likely, calcium signaling pathways are far more complicated given more than 80 proteins in algal flagella are capable of binding calcium or calmodulin (Pazour et al. 2005), including multiple ones residing in the structure corridor from the CP to dynein motors (see Loreng and Smith 2016). Indeed, the mutant lacking the calmodulin-binding region in RSP2 is capable of normal swimming and both light-induced responses, but cannot steer under bright light (Gopal et al. 2012). It is likely that motility control is via concerted actions of multiple calcium sensors, rather than a handful of calcium switches. RSs may be involved in this via the RSPs with calcium- and calmodulin-binding motifs. If calmodulin binding of *Tetrahymena* RSP4 and RSP6 (Ueno et al. 2006) proves physiologically relevant, these two conserved molecules are appealing candidates for universal major calcium switch.

Independent approaches strongly suggest that RSs also regulate dynein motors via phosphorylation—of the inner dynein arm, I1. While RS mutant flagella are paralyzed, their axoneme can undergo interdoublet sliding albeit with a reduced velocity (Witman et al. 1978; Smith and Sale 1992). The inhibition stems from phosphorylation mediated by kinases such as PKA and CK1 (Howard et al. 1994; Yang and Sale 2000) when RSs are defective, and could be antagonized by calcium-independent phosphatases (Habermacher and Sale 1996). The key substrate of these enzymes is the intermediate chain IC138 at I1 base, near RS1 (Habermacher and Sale 1997). IC138 hyperphosphorylation as well as defects in I1 and the MIA complex correlate with reduced sliding velocity (Yamamoto et al. 2013) and, interestingly, ineffective phototaxis (King and Dutcher 1997; Okita et al. 2005). Therefore, I1 is a key effector that enables the calcium-dependent dominance of one flagellum for turning the trajectory during phototaxis. RS may operate to suppress IC138 phosphorylation that may otherwise hinder such responses.

Maintenance of 9+2 Axoneme Structural Stability

This role is not as well known as motility control, and is less obvious in *Chlamydomonas* than in some other organisms. First of all, RSs nudge the CP to its central location (e.g., Sivadas et al. 2012). The CP shifts from the central location

when RSs are defective. In addition, RSs enhance the stability of the CP and the entire axoneme. Cilia EMs from RS PCD patients often show an array of structural anomalies such as two CPs and transposition—the replacement of the CP by an outer doublet (e.g., Castleman et al. 2009; Kott et al. 2013). Cilia tomography suggests that with defective RSs, the CP actually is assembled but tends to disassemble, leading to transposition (Burgoyne et al. 2014). *Tetrahymena* axonemes with diminished RS2 are also susceptible to deformation (Vasudevan et al. 2015). Eel 9+0 axoneme is exceptionally fragile when the membrane is removed (Gibbons et al. 1985). Conversely, the perceived structural stability of spokeless axonemes in *Chlamydomonas* RS mutant may be caused by multiple factors (LeDizet and Piperno 1995; Kubo et al. 2015). Nonetheless, the structural instability surfaces as short flagella of double mutants lacking both RSs and the CP, likely caused by an increasing disassembly rate.

While all major axonemal complexes may confer stability to the axoneme that is built on inherently unstable microtubules (Kubo et al. 2015), we take the liberty to speculate that the RS-CP system is particularly so by filling up the hollow center, and by the transient contact that dissipates forces and tensions, which the axoneme endures as it repetitively sweeps through fluid of relatively high viscosity. This may explain the nearly identical dimensions of RSs and thus the diameter of 9+2 axonemes across all sources. Interestingly, the ratio of the dimensions of the CP and RS versus the CP in cross sections is strikingly similar to the golden ratio (φ). Perhaps the 9+2 axonemal platform of the last common ancestor of eukaryotes is as good as it gets. Much of the subsequent changes are all but individual preferences.

FUTURE DIRECTIONS

Much about RSs is now known, but key questions remain. First and foremost is how the RS-CP system is involved in calcium/calmodulin-signaled motility changes and, second, is regarding phosphorylation. It is still unclear how RS defects enhance phosphorylation of dynein mediated by PKA and CK1 that also regulate critical events occurring at the basal body area in diverse organisms (Briscoe and Therond 2013). Although *Chlamydomonas*, like plants, do not have the typical PKA tetramer common in animals, PKA activity is indisputable (Hasegawa et al 1987; Howard et al. 1994; Yang and Sale 2000). Identification of PKA in this organism and elucidation of how anchored PKA and CK1 phosphorylate IC138 is central to elucidate RS-mediated regulation. Related, but not identical, to this are the questions about how RSPs become phosphorylated and whether the phosphorylation is a consequence or a cause of RS assembly. The third is RS assembly, likely via spoke-unique mechanisms because of drastic differences between spoke precursor and mature RSs, the multiple chaperone-like spoke subunits, and subunits of a low stoichiometry (Piperno et al. 1981). The fourth is characterization of RS3. Aside from satisfying curiosity, the findings may lead to new insight of RS evolution—how *Chlamydomonas* managed to lose RS3, and what are the expense and benefits to retain a distinct RS3. Last, but not least, is to elucidate RSs of mammals and phenotype RS PCD patients. With the advance of technologies and reagents (Lin et al. 2014; Frommer et al. 2015), the challenges inherent to these systems (Papon et al. 2010) will be overcome and the findings will complement the limitations of simple model systems.

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