# Proteomic analysis of flagella from *Chlamydomonas* mutants lacking the central pair apparatus reveals loss of radial spoke head proteins

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**Abstract**

The flagellar axoneme is a complex protein-based machine capable of generating motile forces by coordinating the action of thousands of dynein motors. A key element of the axoneme is the central pair apparatus, consisting of a pair of microtubules surrounded by additional structures. In an effort to better understand the organization of the central pair, we used 2D-DIGE to identify proteins that are depleted from flagella isolated from two different *Chlamydomonas reinhardtii* mutants, *pf15* and *pf18*, that lack the central pair. The set of proteins contained almost no components of the central apparatus. We find that three proteins of the radial spoke head RSP1, RSP9, and RSP10, are depleted from flagella of mutants lacking the central apparatus. Two of the other proteins depleted from *pf15* and *pf18* flagella, FAP21 and FAP161, are missing from the genome of *Thalassiosira*, an organism that lacks a central pair and radial spokes, and RNAi of FAP21 in planaria shows that it has a role in ciliary motility. Based on the depletion of radial spoke head proteins from flagella lacking the central apparatus, we hypothesize that the central apparatus may play a role in scaffolding the assembly or retention of radial spokes and other axonemal structures.

## Introduction

The proper assembly of complex structures within cells is a challenging self-assembly problem. Flagella and cilia are among the most complex structures found in the cell. The internal structure of most motile cilia and flagella consists of nine parallel doublet microtubules arranged around a central pair of singlet microtubules (**Figure 1A**). Force for propulsion is provided by thousands of dynein motors, anchored in two rows along one side of each doublet and organized into multi-headed complexes called the inner and outer dynein arms (DiBella 2001; Yamamoto 2021), that can walk along the microtubule of the adjacent doublet.

The central pair apparatus is a complex sub-structure within the flagellum, which contains not only a pair of microtubules, C1 and C2, but also an elaborate set of projections (**Figure 1B**). Various projections from the central pair apparatus are responsible for different behaviors, for example, studies in sea urchin sperm and *Chlamydomonas* show that only those dynein arms located near one side of the central pair C1 microtubule are active, while the rest are inactive (Nakano et al., 2003; Wargo and Smith, 2003).

Radial spokes are protein complexes consisting of a stalk anchored on the outer doublet microtubules and extending a head inwards towards the central pair apparatus (Curry, 1993; Diener 2011; Poghosyan 2020). The projections on the central pair interact with the radial spoke head (Kohno et al., 2011), inducing a change in state on the radial spoke. The resulting signaling cascade is not well understood, but likely involves use of secondary messengers, especially calcium, signaling to the dynein regulatory complex located on the A tubule of the outer doublets, which in turn triggers

activation of a subset of dyneins (Heuser et al., 2009; Bower 2013; Oda 2015; Gui 2019). In order for all these interactions to occur in their prescribed manner, cells must properly assemble the proteins of the central pair apparatus in the proper structure, correctly aligned with the radial spokes.

Two mutants have been described in *Chlamydomonas*, *pf15* and *pf18*, which have paralyzed flagella and which lack the organized central pair apparatus structure (Warr 1966; Adams 1981; Witman 1978). The *pf15* strain carries a mutation in the p80 subunit of Katanin, a heterodimeric protein with microtubule-severing activity (Dymek 2004) while the identity of the PF18 gene is not known. Previous studies reported that the flagella of *pf18* cells fixed in situ retain a large amount of electron dense material within their center, where the central pair structure would typically be located (Adams et al., 1981). A similar electron dense core was reported in purified axonemes of *pf15* (Witman 1978). However, when axonemes are isolated from *pf18*, the dense material is lost from the center of the axoneme, suggesting that the proteins present in *pf18* flagella may have been removed during axoneme preparation (Zhao 2019). This fact was used to analyze the proteome of the central pair apparatus by comparing the proteins found in axonemes isolated from wild type versus *pf18* mutant cells (Zhao 2019).

Using a small panel of antibodies directed against selected central pair apparatus proteins, Lechtreck (2013) found that a subset of central pair proteins, normally associated with the C1 microtubule, are specifically retained in *pf15* and *p18* flagella even though they are missing from isolated *pf18* axonemes, while C2 associated proteins were missing from the flagella of the mutants as well as from the axonemes. This was an extremely interesting result, because it implies that central pair apparatus

C1 proteins can be imported into flagella, and placed in roughly the right location, but cannot be assembled into the proper arrangement in these mutants. Based on this study, we took an alternative, more systematic approach, in which we identified proteins depleted from isolated whole flagella from *pf15* and *p18* mutants, in the hopes of being able to divide up the central pair apparatus proteins into two groups, one that is retained in flagella and therefore likely to be associated with the C1 part of the complex, and another set depleted from the flagella and therefore likely to be associated specifically with the C2 microtubule. Contrary to our expectation, with a single exception, the proteins that we found to be depleted from *pf15* and *pf18* flagella did not correspond to any of the known central pair apparatus proteins, suggesting that most or all of the central pair apparatus protein components are still present in flagella of these mutants, presumably composing the disorganized electron dense core. Instead, we found that a specific set of radial spoke head proteins, RSP1, RSP9, and RSP10, were depleted in both *pf15* and *pf18* flagella, raising the possibility that a correctly organized central pair apparatus is required to scaffold the proper assembly of other axonemal structures such as the radial spokes. Our study also reveals FAP21 to be a protein involved in motility and whose presence correlates with the presence of the central pair apparatus.

## Results

### *Proteomic analysis of central apparatus mutants*

We isolated *Chlamydomonas* flagella, as detailed in Materials and Methods, by subjecting cells to pH shock, and isolating flagella using sucrose cushions to separate flagella from cell bodies. The efficiency of the pH shock and enrichment of flagella in the final preparation were confirmed visually using DIC microscopy, which also allowed for the verification of low amounts of cell body contamination of the preparation. As shown in **Figure 2**, flagella from WT, *pf15*, and *pf18 Chlamydomonas* strains were isolated and differentially labeled, then subjected to 2D DIGE (Viswanathan 2006), in which the proteins of the isolated flagella were separated using 2-dimensional gel electrophoresis after fluorescent labeling. This method provides a quantitative readout of protein differences between two samples by measuring the ratio of fluorescence intensity of each 2D gel spot in both wavelengths. The fold enrichment of individual spots on the gel was determined in pair-wise comparisons between the three different samples, and spots with a 1.5 fold change or more in either mutant relative to WT, were excised from the gel and their identity determined using mass spectrometry.

This analysis provided a list of 57 proteins with differential abundance in the flagella of both mutants (**Table 1**), out of which 42 were depleted in both *pf15* and *pf18* relative to wild type, while 13 were enriched in both *pf15* and *pf18*. Two proteins (FAP155 and RAN1) behaved differently in the two mutants.

Out of the 42 proteins detected as being differentially depleted in both *pf15* and *pf18* mutant flagella, the majority (32) were known flagellar proteins. Eight proteins were clearly non-flagellar contamination, given that they are either cell wall or chloroplast

components, but all of these were among the differentially enriched, rather than depleted, proteins. The degree of enrichment or depletion was highly correlated between the two mutants (r=0.91), suggesting that even though the *pf15* and *pf18* mutations are in different genes, their effects on flagellar protein composition were similar.

Surprisingly, the most well-known central pair proteins, for example CPC1, Hydin, PF20/SPAG16, and PF16/SPAG6, were not included in our list. In fact, the only two CPC proteins identified were Hsp70 (Mitchell 2005), which is involved in many cellular processes, and DIP13, which was identified as part of the central pair apparatus by a proteomic analysis (Zhao 2019), but which also localizes extensively to the outer doublet microtubules (Pfannemschmid 1993). Thus, neither of the central pair proteins depleted in the mutant flagella proteomes localize exclusively, or even primarily, in this structure.

***Proteins depleted in central apparatus mutants***

Among the proteins depleted in *pf15* and *pf18* mutant flagella, three protein components of the radial spoke, RSP1, RSP9, and RSP10. Two of these proteins, RSP1 and RSP10, contain MORN domains. All three of these proteins are part of the radial spoke head (Yang 2006), and it has been speculated that these proteins in particular may take part in direct interactions with the central pair apparatus (Yang 2006). Consistent with this idea, structural studies show that RSP1 and RSP10 form protrusions from the spoke head (Grossman-Haham 2021).

In addition to the radial spoke head proteins, we also found that the *pf15* and *pf18*

flagella were depleted for Rib43a, a component of the protofilament ribbon (Norrander 2000; Ichikawa 2019); Fap52 and Fap106, two components of the inner junctional complex of the outer doublet (Owa 2019m Khalifa 2020); FAP85 and FAP115, two Microtubule Inner Proteins (MIPs) of the outer doublet (Kirima 2018; Li 2021); and thioredoxin, which is part of the outer dynein arm (Patel-King 1996).

Also depleted was phosphoglyceromutase (PGM), a glycolytic enzyme that is normally found in flagella. Although PGM is in the soluble membrane+matrix fraction (Mitchell 2005), the downstream glycolytic enzyme enolase is part of the central pair apparatus (Michell 2005), possibly suggesting that the central apparatus plays a role in retaining or organizing these enzymes.

***Comparative genomics***

As an alternative strategy to determine proteins that might depend on the central apparatus for their presence in flagella, we tested which of the proteins from Table S1 were represented in the genomes of other species with motile cilia, but absent from the genome of *Thalassiosira pseudonana*. *Thalassiosira* are well-studied marine diatoms, and this particular species was the first eukaryotic marine phytoplankton to be sequenced (Armbrust 2004). Importantly, *Thalassiosira* has flagella that lack central pairs, along with other flagellar structures that are responsible for regulating waveform, including the radial spokes and inner arm dyneins (Heath and Darley, 1972; Manton et al., 1970). This absence of the central pair makes it ideal for this type of comparison - genes for proteins that are lacking in *Thalassiosira* flagella are more likely to either be central pair components, or to rely on the central pair for their assembly into flagella.

Among the proteins we have identified as depleted from central apparatus mutant flagella in *Chlamydomonas*, we failed to find homologs in *Thalassiosira* for only 4: IDA4, FAP21, RSP9, and FAP161. IDA4 and RSP9 are already known as inner arm dynein and radial spoke proteins, respectively. The other two proteins, FAP161 and FAP21, have been identified in the flagellar proteome of *Chlamydomonas* (Pazour 2005), but no functional information is available about either protein. They are missing from the genomes of species that lack motile cilia but also from *Thalassiosira* (**Figure 3**).

***RNAi of FAP21 in Planaria***

The absence of FAP21 in *Thalassiosira*, which has motile flagella but lacks the standard machinery for coordinating flagellar motion via radial spokes and a central pair apparatus, suggested the possibility that this protein might be a component of the flagellum involved in regulating waveform or motility. The planarian flatworm *Schmidtea mediterranea* is a rapid and convenient platform in which to test candidate genes for a role in ciliary motility. The organism is covered in cilia used to drive motion over a surface, and RNAi is typically fairly robust and easily accomplished in Planaria via injection of double-stranded RNA (dsRNA) (Rompolas et al., 2010). We identified an ortholog of FAP21 in Schmidtea (gene mk4.003874.01.01) and generated constructs to produce dsRNA against this gene.

In Planaria injected with dsRNA corresponding to the homolog of FAP21, we were able to observe a significant motility defect (**Figure 4**). Microscopic analysis indicated that many of the cilia on the ventral surface of the worms subjected to RNAi

were still motile.  This was expected due to the phenotype we saw - complete paralysis of the cilia is known to result in an inching-worming behavior, which we did not observe.

## Discussion

### *Why weren't known central pair proteins detected?*

Given that our study analyzed proteins depleted from flagella lacking the central apparatus, the obvious expectation would have been that most of the proteins identified would be part of the central apparatus itself, but this was not observed. Our results instead support the idea that the electron dense material noted in the flagella of these mutants is composed of central pair proteins, that were transported to the correct sub-cellular location, but were then unable to assembly into a stable structure that could survive the process of axoneme isolation (Lechtreck 2013). The presence of this material in isolated flagella, which were used in our study, would result in little alteration in their levels when compared to wild type, such that our analysis of differential enrichment would not have detected them. Our results are thus consistent with a picture in which the mutants *pf15* and *pf18* are required for assembling central apparatus proteins into the correct structure, but are not required for the synthesis of these proteins or for their transport or retention in the flagellum. Our results differ from those reported by Lechtreck (2013) who found that C1 associated proteins were retained in *pf18* and *pf19* mutants. One possible difference is that our analysis looked for proteins retained in both *pf15* and *pf18* whereas Lechtreck focused on *pf18* and *pf19* individually. Differences could also simply reflect differences in the detection threshold for 2D-DIGE versus Western blotting. Further analysis will be required to establish with certainty the degree to which C1 versus C2 proteins are retained proteome-wide. In any case, our results are broadly consistent with the prior work of Lechtreck in the

sense that flagella from mutants lacking an organized central pair apparatus clearly contain a large number of the proteins that would normally compose this structure.

### *A scaffolding function for the central apparatus*

The present study finds that when the central apparatus is absent, radial spoke head proteins, as well as proteins localizing to the protofilament ribbon and inner junctional complex are missing, suggesting that the central apparatus is playing a crucial role in supporting the assembly of other structures. We believe that these central apparatus interactions with other axonemal structures may represent a clear example of the general concept of one cellular structure scaffolding another.

We also note that the dependence can go the other way - it has been found that in mutations affecting radial spokes, the central pair apparatus can be affected (Castleman 2009; Jeanson 2015). Such complex interactions suggest that phenotypes resulting from mutations in one part of the axoneme can have effects that ramify to other parts, possibly contributing to the great pleiotropy seen in human ciliary disease mutations.

With respect to the radial spoke head proteins depleted in pf15 and pf18, it is interesting to note that RSP1 and RSP10 had previously been proposed to interact directly with the central pair apparatus, based on the presence of interaction motifs found in these proteins (Yang 2006).

## Materials and Methods

### *Chlamydomonas strains used in this study and culture conditions*

Strains cc125 (WT), cc1033 (*pf15)*, and cc1036 (*pf18)* were used in the 2D-DIGE analysis. All strains were obtained from the *Chlamydomonas* Center (www.chlamy.org). Strains were maintained on TAP plates, then were grown in liquid TAP media in constant light at 21C with agitation (5mL cultures in 18mm test tubes were grown on roller drums, 100-200mL cultures were grown on an orbital shaker running at 150rpm, and large 1L cultures were grown with gentle stirring and bubbling with house air).

### *Isolation of Flagella from Chlamydomonas*

1-3 small cultures of 120mL were started with half a loop's worth of cells from TAP plates, then were grown for 3 days as described above. 100mL of each of these cultures were used to start a 1L TAP culture in a 2L flask which was grown for 5 to 7 days. Cultures were checked visually for the presence of flagella. Each 1L culture was spun down in 1L bottles at 1500xg in a Beckman Coulter Avanti J-20XP centrifuge in a JLA 8.1000 rotor for 10 minutes (min) at 19-21C with a low brake. The cells were re-suspended in 150 to 200mL TAP media, transferred to a Corning 250mL centrifuge tube (430776) and then spun again at ~1000xg (2000rpm in a Beckman Coulter Allegra 6 with a GH3.8 rotor) for 10min at RT with no brake. Cells were re-suspending in 80mL TAP and transferred to a flask, where they were allowed to recover for 2 hours at RT in the light with gentle stirring and bubbling. These cultures were then pH shocked in the following way: a calibrated probe from a pH meter was inserted into the culture, at 0min 0.5M acetic acid was added until the pH of the culture was between 4.5 to 4.6 typically

within 30 seconds of first adding the solution. At 1.5min, 0.5M KOH was added until the culture reached a pH of between 7 and 7.2, again typically within 30 seconds, then 100 μL of Sigma protease inhibitor cocktail (P8340) and 100 μL of 100mM of EGTA, pH7.5 were added to the culture, which was then transferred to a 250mL tube and placed on ice. The efficiency of the pH shock was verified visually. The rest of the protocol was completed on ice. The culture was spun at ~1000xg (2000rpm GH3.8 rotor in an Allegra 6R centrifuge) for 10min at 4C with no brake. The supernatant was removed and transferred to a new tube, and the cell bodies were discarded. 50mL of 25% sucrose in TAP with red food coloring, to allowed it to be seen, was pipetted beneath the supernatant as an underlay, and the tube was spun at ~2500xg (3300rpm GH3.8 rotor) for 10min at 4C with no brake. The entire supernatant and the interface between the two layers was collected, transferred to a new tube, and the entire underlay process repeated. The entire supernatant and interface was again collected, transferred to a new tube, and placed on ice. 35mL of this supernatant at a time was transferred to a 50mL Nalgene polycarbonate centrifuge tube (3117-0500) and spun at ~16000xg (10000rpm in a HB-4 rotor in a Sorvall RC5C centrifuge) for 20min at 4C. The supernatant was discarded, and the next aliquot placed in the tube and spun. This was repeated until the entire sample was pelleted. The pellet was re-suspended in 1mL Applied Biomic's Cell Washing Buffer (10mM Tris-HCl, 5mM magnesium acetate, pH 8.0) and transferred to a 1.5mL microfuge tube. The quality of the isolation was assayed visually, checking for high concentrations of flagella with low amounts of cell body debris. The isolated flagella were pelleted one final time at ~20000xg (14000rpm

in an Eppendorf 5417C centrifuge) for 20min at 4C, the supernatant aspirated, and the pellet frozen in liquid nitrogen, then transferred to a -80C freezer.

### *2D DIGE and protein identification*

Sample labeling, 2D DIGE, image analysis, spot analysis using DeCyder software, excision from gel, and identification using mass spectrometry were all performed by Applied Biomics, Hayward, CA (http://www.appliedbiomics.com).

### *cDNA cloning*

FAP21 cDNA was obtained by RT-PCR on total RNA isolated using TRIzol (Invitrogen, 15596-026) according to the manufacturer's instructions. For Planaria, the RNA was isolated from the asexual strain, the cDNA sequences for our genes of interest amplified using the primers listed below, and the sequences were cloned into pPR-T4P, a modified pDONR-dT7 in which the gateway cloning site was replaced by a ligation-independent cloning site (kind gift from J. Rink). The primers for FAP21 were as follows:

Forward 5' CATTACCATCCCGGCCTAATCATGTGTTTGGAAC 3'

Reverse 5' CCAATTCTACCCGGCCAGTTAAAGCTGTCAGTAAA 3'

### *RNAi via dsRNA injection*

Sense and antisense DNA templates for *in vitro* transcription were obtained by amplifying sequences cloned into pPR-T4P using the following primer pairs:

*Sense* F: AACCCCTCAAGACCCGTTTAGA and R: GAATTGGGTACCGGGCCC;

*Antisense* F: CCACCGGTTCCATGGCTAGC and R: GAGGCCCCAAGGGGTTATGTG

dsRNA was synthesized using the T7 Ribomax Express RNAi system (Promega, Madison, WI). For RNAi experiments, 10 ~ 0.8-1cm long planarians were injected 3 consecutive days with 20-50ng dsRNA per injection using a Nanoject II injector (Drummond Scientific, Broomall, PA). The animals were then amputated from their head and tail at day 4. An additional injection was performed at day 11 and the flatworms were amputated again pre- and post-pharyngically at day 12. Phenotypes were assessed on regenerating heads, tails and trunks at day 21 (10 days after the second amputation).

***Imaging of Planaria***

Live images were acquired using a Stemi 2000C stereomicroscope equipped with an AxioCam MRc digital microscope camera (Carl Zeiss MicroImaging, Thornwood, NY). Planarian locomotion speed was determined using ImageJ software, by measuring the distance covered by a single worm between frames separated by known time intervals.

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**Table 1**. Proteins depleted from both pf15 and pf18, or enriched in both, or showing a mixed effect being depleted from one and enriched in the other.

| **Depleted** | | | | | |
|---|---|---|---|---|---|
| **pf15/WT** | **pf18/WT** | **name** | **flagellar location** | **Reference** | |
| -13.19 | -9.5 | DIP13 | central pair | Zhao 2019 | |
| -7.31 | -8.69 | TRXh1 | outer dynein arm | Patel-King 1996 | |
| -6.71 | -4.69 | RABB1 | | | |
| -5.8 | -2.08 | EF1A1 | | | |
| -5.77 | -9.67 | hypothetical | | | |
| -4.32 | -3.17 | FAP162 | flagellar proteome | Pazour 2005 | |
| -4.32 | -4.11 | HSP70A | central pair | Mitchell 2005 | |
| -4.18 | -3.97 | PGM1a | membrane matrix | Mitchell 2005 | |
| -4 | -3.88 | IDA4 | inner dynein arm | | |
| -3.91 | -7.35 | PGM1b | membrane matrix | Mitchell 2005 | |
| -3.48 | -5.05 | ICL1 | | | |
| -3.44 | -5.93 | Rib43a | PF ribbon of outer doublet | Norrander 2000 | |
| -2.97 | -5.26 | hypothetical | | | |
| -2.79 | -4.69 | FAP90/BUG13 | flagellar proteome | Pazour 2005 | |
| -2.74 | -3.83 | GDIC1 | | | |
| -2.73 | -3.57 | SIN3 | | | |
| -2.72 | -2.88 | FAP12 | flagellar proteome | Pazour 2005 | |
| -2.7 | -3.03 | FAP106 | flagellar proteome | Pazour 2005 | |
| -2.66 | -3.89 | FAP144 | flagellar proteome | Pazour 2005 | |
| -2.52 | -2.81 | TUA1 | outer doublet and CP | | |
| -2.44 | -5.13 | hypothetical | | | |
| -2.44 | -2.9 | IDA5 | inner dynein arm | | |
| -2.35 | -2.97 | FAP252 | flagellar proteome | Pazour 2005 | |
| -2.31 | -2.49 | TUA1 | outer doublet and CP | | |
| -2.28 | -3.49 | RSP10 | radial spoke | Yang 2006 | |
| -2.19 | -2.59 | FAP182 | flagellar proteome | Pazour 2005 | |
| -2.18 | -3.13 | FAP106 | inner junctional complex | Khalifa 2020 | |
| -2.11 | -5.41 | RIB43a | PF ribbon of outer doublet | Norrander 2000 | |
| -2.05 | -4.4 | TUA2 | outer doublet and CP | | 0 |
| -2 | -4.37 | FAP103 | flagellar proteome | Pazour 2005 | |
| -2 | -5.7 | MSD1 | | | |
| -1.92 | -2.39 | RSP1 | radial spoke | Yang 2006 | |
| -1.92 | -2.7 | TUB1 | outer doublet and CP | | 0 |

| | | | | |
|---|---|---|---|---|
| -1.82 | -2.25 | FAP115 | flagellar proteome | Pazour 2005 |
| -1.75 | -1.85 | RSP9 | radial spoke | Yang 2006 |
| -1.71 | -2.63 | RIB43a | PF ribbon of outer doublet | Norrander 2000 |
| -1.55 | -2.78 | FAP21/BUG15 | flagellar proteome | Pazour 2005 |
| -1.53 | -2.09 | DLC1 | outer dynein arm | |
| -1.5 | -1.78 | FAP52 | inner junctional complex | Owa 2019 |
| -1.5 | -2.92 | FAP85 | MIP of outer doublet | Kirima 2018 |
| -1.33 | -2.52 | ICL1 | | |
| -1.27 | -1.72 | FAP161 | flagellar proteome | Pazour 2005 |

**Enriched**

| pf15/WT | pf18/WT | name | flagellar location | Reference |
|---|---|---|---|---|
| 1.26 | 5.04 | hypothetical | | |
| 1.39 | 9.34 | CSP41b | | |
| 1.57 | 6.56 | FAP12 | flagellar proteome | Pazour 2005 |
| 2.25 | 4.9 | PRPL7 | | |
| 5.63 | 7.07 | PHC17 | cell wall | |
| 6.71 | 6.52 | FAP233 | flagellar proteome | Pazour 2005 |
| 7.13 | 7.26 | FAP233 | flagellar proteome | Pazour 2005 |
| 7.25 | 6.2 | PHC15 | | |
| 9.24 | 12.08 | hypothetical | | |
| 12.11 | 7.23 | PHC7 | cell wall | |
| 16.42 | 14.89 | PHC7 | cell wall | |
| 19.97 | 13.54 | PHC7 | cell wall | |
| 23.03 | 17.35 | PHC2 | cell wall | |

**Mixed**

| pf15/WT | pf18/WT | name | flagellar location | Reference |
|---|---|---|---|---|
| 1.06 | 3.73 | FAP155 | flagellar proteome | Pazour 2005 |
| 1.21 | -4.54 | RAN1 | | |

**References**

Adams GM, Huang B, Piperno G, Luck DJ. 1981. Central-pair microtubular complex of Chlamydomonas flagella: polypeptide composition as revealed by analysis of mutants. *J Cell Biol*. 91, 69-76.

Armbrust EV. 2004. The genome of the diatom Thalassiosira pseudonana: ecology, evolution, and metabolism. *Science* 306, 79-85.

Bower R, Tritschler D, Vanderwaal K, Perrone CA, Mueller J, Fox L, Sale WS, Porter ME. 2013. The N-DRC forms a conserved biochemical complex that maintains outer doublet alignment and limits microtubule sliding in motile axonemes. *Mol Biol Cell.* 24, 1134-52.

Castleman VH, et al. 2009. Mutations in radial spoke head protein genes RSPH9 and RSPH4A cause primary ciliary dyskinesia with central-microtubular-pair abnormalities. *Am J Hum Genet*. 84, 197-209.

Curry AM, Rosenbaum JL. 1993. Flagellar radial spoke: a model molecular genetic system for studying organelle assembly. *Cell Motil Cytoskeleton*. 24, 224-32

DiBella LM, King SM. 2001. Dynein motors of the Chlamydomonas flagellum. *Int Rev Cytol.* 210, 227-68.

Diener DR, Yang P, Geimer S, Cole DG, Sale WS, Rosenbaum JL. 2011. Sequential assembly of flagellar radial spokes. *Cytoskeleton* 68, 389-400.

Dymek EE, Lefebvre PA, Smith EF. 2004. PF15p is the Chlamydomonas homologue of the Katanin p80 subunit and is required for assembly of flagellar central microtubules. *Eukaryotic Cell*. 3, 870-879.

Fowkes ME, Mitchell DR. 1998. The role of preassembled cytoplasmic complexes in assembly of flagellar dynein subunits. *Mol. Biol. Cell* 9, 2337-2347.

Grossman-Haham I , Coudray N, Yu Z , Wang F, Zhang N, Bhabha G, Vale RD. 2021. Structure of the radial spoke head and insights into its role in mechanoregulation of ciliary beating. *Nat Struct Mol Biol* 28, 20-28.

Gui L, Song K, Tritschler D, Bower R, Yan S, Dai A, Augspurger K, Sakizadeh J, Grzemska M, Ni T, Porter ME, Nicastro D. 2019. Scaffold subunits support associated subunit assembly in the Chlamydomonas ciliary nexin-dynein regulatory complex. *Proc Natl Acad Sci U S A.* 116, 23152-23162.

Heath IB, Darley WM. 1972. Observations on the ultrastructure of the male gametes of biddulphia levis ehr. *J. Phycol*. 8:51-59.

Heuser T, Raytchev M, Krell J, Porter ME, Nicastro D. 2009. The dynein regulatory complex is the nexin link and a major regulatory node in cilia and flagella. *J Cell Biol.* 187, 921-933.

Ichikawa M, Khalifa AAZ, Kubo S, Dai D, Basu K, Maghrebi MAF, Vargas J, Bui KH. 2019. Tubulin lattice in cilia is in a stressed form regulated by microtubule inner proteins. *Proc Natl Acad Sci U S A.* 116,19930-19938.

Jeanson L, et al. 2015. RSPH3 mutations cause primary ciliary dyskinesia with central complex defects and a near absence of radial spokes. *Am. J. Hum. Genet.* 97, 153-62.

Kirima J, Oiwa K. 2018. Flagellar-associated Protein FAP85 Is a Microtubule Inner Protein That Stabilizes Microtubules. *Cell Struct. Funct*. 43, 1-14.

Kohno T, Wakabayashi K, Diener DR, Rosenbaum JL, Kamiya R. 2011. Subunit interactions within the Chlamydomonas flagellar spokehead. *Cytoskeleton*. 68, 237-246.

Lechtreck KF, Witman GM. 2007. Chlamydomonas reinhardtii hydin is a central pair protein required for flagellar motility. *J Cell Biol*. 176, 473-482.

Lechtreck KF, Gould TJ, Witman GB. 2013. Flagellar central pair assembly in Chlamydomonas reinhardtii. *Cilia* 2, 15.

Li S, Fernandez JJ, Fabritius AS, Agard DA, Winey M. 2021. Electron cryo-tomography structure of axonemal doublet microtubule from Tetrahymena thermophila. *Life Sci. Alliance* 5, e202101225

Maddison DR, Schulz KS. (eds.) 2007. *The Tree of Life Web Project*. http://tolweb.org.

Manton I, Kowallik K, von Stosch HA. 1970. Observations on the fine structure and development of the spindle at mitosis and meiosis in a marine centric diatom (Lithodesmium undulatum). 3. The later stages of meiosis I in male gametogenesis. *J Cell Sci.* 6, 131-157.

Mastronarde DN, O'Toole ET, McDonald KL, McIntosh JR, Porter ME. 1992. Arrangement of inner dynein arms in wild-type and mutant flagella of Chlamydomonas. *J. Cell Biol*. 118, 1145-1162.

Merchant SS, et al. 2007. The Chlamydomonas genome reveals the evolution of key animal and plant functions. *Science*. 318, 245-250.

Mitchell BF, Pedersen LB, Feely M, Rosenbaum JL, Mitchell DR. 2005. ATP production in Chlamydomonas reinhardtii flagella by glycolytic enzymes. *Mol Biol Cell*. 16, 4509-4518.

Mitchell DR, Nakatsugawa M. 2004. Bend propagation drives central pair rotation in Chlamydomonas reinhardtii flagella. *J Cell Biol*. 166, 709-715.

Nakano I, Kobayashi T, Yoshimura M, Shingyoji C. 2003. Central-pair-linked regulation of microtubule sliding by calcium in flagellar axonemes. *J. Cell Science.* 116, 1627-1636.

Norrander JM, deCathelineau AM, Brown JA, Porter ME, Linck RW. 2000. The Rib43a protein is associated with forming the specialized protofilament ribbons of flagellar microtubules in Chlamydomonas. *Mol Biol Cell*. 11, 201-15.

Oda T, Yanagisawa H, Kikkawa M. 2015. Detailed structural and biochemical characterization of the nexin-dynein regulatory complex. *Mol Biol Cell*. 26, 294-304.

Omoto CK, Brokaw CJ. 1985. Bending patterns of Chlamydomonas flagella: II. Calcium effects on reactivated Chlamydomonas flagella. *Cell Motil*. 5, 53-60.

Omoto CK, Gibbons IR, Kamiya R, Shingyoji C, Takahashi K, Witman GM. 1999. Rotation of the central pair microtubules in eukaryotic flagella. *Mol Biol Cell*. 10, 1-4.

Owa M, Uchihashi T, Yanagisawa HA, Yamano T, Iguchi H, Fukuzawa H, Wakabayashi KI, Ando T, Kikkawa M. 2019. Inner lumen proteins stabilize doublet microtubules in cilia and flagella. *Nat. Commun*. 10, 1143.

Patel-King RS, Benashki SE, Harrison A, King SM. 1996. Two functional thioredoxins containing redox-sensitive vicinal dithiols from the Chlamydomonas outer dynein arm. *J. Biol. Chem*. 271, 6283-91.

Pazour GJ, Agrin N, Leszyk J, Witman GM. 2005. Proteomic analysis of a eukaryotic cilium. *J Cell Biol*. 170, 103-113.

Pfannenschmid F , Wimmer VC, Rios RM, Geimer S, Kröckel U, Leiherer A, Haller K, Nemcová Y, Mages W. 1993. Chlamydomonas DIP13 and human NA14: a new class of proteins associated with microtubule structures is involved in cell division. *J. Cell Sci*. 116, 1449-62.

Poghosyan E, Iacovache I, Faltova L, Leitner A, Yang P, Diener DR, Aebersold R, Zuber B, Ishikawa T. 2020. The structure and symmetry of the radial spoke protein complex in Chlamydomonas flagella. *J Cell Sci*. 133, jcs245233

Porter ME, Power J, Dutcher DK. 1992. Extragenic suppressors of paralyzed flagellar mutations in Chlamydomonas reinhardtii identify loci that alter the inner dynein arms. *J Cell Biol*. 118, 1163-1176.

Porter ME, Knott JA, Gardner LC, Mitchell DR, Dutcher SK. 1994. Mutations in the sup-pf1 locus of Chlamydomonas reinhardtii identify a regulatory domain in the beta-dynein heavy chain. *J. Cell Biol*. 126, 1495-507.

Rompolas P, Patel-King RS, King SM. 2010. An outer arm Dynein conformational switch is required for metachronal synchrony of motile cilia in planaria. *Mol Biol Cell*. 21, 3669-3679.

Smith EF. 2002. Regulation of flagellar dynein by calcium and a role for an axonemal calmodulin and calmodulin-dependent kinase. *Mol Biol Cell*. 13, 3303-3313.

Stolc V, Samanta MP, Tongprasit W, Marshall WF. 2005. Genome-wide transcriptional analysis of flagellar regeneration in Chlamydomonas reinhardtii identifies orthologs of ciliary disease genes. *Proc Natl Acad Sci USA*. 102, 3703-3707.

Viswanathan S, Unlu M, Minden JS. 2006. Two-dimensional difference gel electrophoresis. *Nat. Protoc.* 1, 1351-8.

Wargo MJ, Smith EF. 2003. Asymmetry of the central apparatus defines the location of active microtubule sliding in Chlamydomonas flagella. *Proc Natl Acad Sci USA*. 100, 137-142.

Warr JR, McVittie A, Randall J, Hopkins JM. 1966. Genetic control of flagellar structure in Chlamydomonas reinhardtii. *Genet. Res*. 7, 335-351.

Witman GB, Plummer J, Sander G. 1978. Chlamydomonas flagellar mutants lacking radial spokes and central tubules. Structure, composition, and function of specific axonemal components. *J. Cell Biol*. 76, 729–747.

Yamamoto R, Hwang J, Ishikawa T, Kon T, Sale WS. 2021. Composition and function of ciliary inner-dynein-arm subunits studied in Chlamydomonas reinhardtii. *Cytoskeleton* 78, 77-96.

Yang P, Diener DR, Rosenbaum JL, Sale WS. 2001. Localization of calmodulin and dynein light chain LC8 in flagellar radial spokes. *J Cell Biol*. 153, 1315-1326.

Yang P, Yang C, Sale WS. 2004. Flagellar radial spoke protein 2 is a calmodulin binding protein required for motility in Chlamydomonas reinhardtii. *Eukaryotic Cell*. 3, 72-81.

## Figure Legends

**Figure 1. The structure of a flagellum.** Microtubules (blue circles) make up the backbone of the structure. (**A**) Cross section of a flagellum, showing nine outer doublets arranged around the central pair microtubules, with radial spokes projecting inward. (**B**) Magnified view of the central pair, consisting of the C1 and C2 tubules with their associated projections (1a-2c, purple) and sheath (light blue); together with a single outer doublet with its radial spoke (green) and inner (ida) and outer (oda) dynein arms (red).

**Figure 2. Gel images from 2D-DIGE experiment.** (**A**) Images of the individual channels of WT and *pf15*, as well as an image of the overlay of the two channels together. (**B**) Equivalent images to (A), but for WT and *pf18*. (**C**) Images showing the comparisons for WT:*pf15* and WT:*pf18*, with circles indicating the spots picked for analysis. Estimated molecular weights (kDa) are indicated by the blue lines on the gel, and the pH range for isoelectric focusing (pH) by a red line at the bottom of the gel.

**Figure 3. FAP 21 and FAP161 are present only in organisms which have motile cilia with central pairs.**

This figure is adapted from the Tree of Life project, and is based on the relationships between the different species. The presence or absence of the FAP homologs in each species is indicated with the presence or absence of an orange box. The diagrams on the left column illustrate the axoneme configuration in each species, with an empty box

denoting species that lack cilia and flagella entirely. FAP161 and FAP21 appear only in organisms with motile cilia containing central pairs, and are absent in organisms lacking this structure. Rib72 is included as a control; it is a structural component of the A-tubule of the outer doublets. It therefore can be found in all organisms with cilia, but is absent from organisms that do not have cilia or flagella.

**Figure 4. Knock-down of FAP21 impairs cilia-driven motility in Planaria.**

RNAi knock-down of FAP21 in *S. mediterranea* results in impaired movement of the flatworm. Their motility is reduced by 20% when compared to control worms. This data represents the quantification of two separate experiments, denoted xpt1 and xpt2, with the analysis of all collected data represented in the 'total' columns.

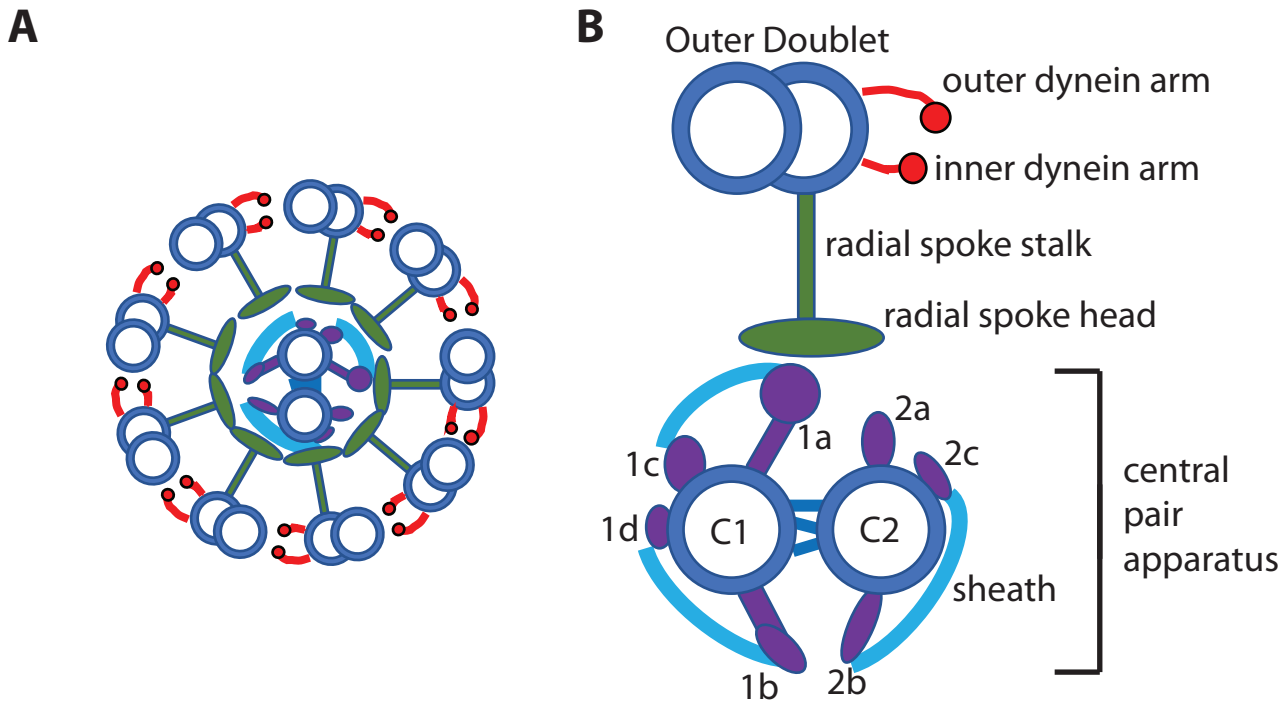
A
B
Outer Doublet
outer dynein arm
inner dynein arm
radial spoke stalk
radial spoke head
1a
1b
1c
1d
2a
2b
2c
C1
C2
sheath
central
pair
apparatus

**Figure 1**

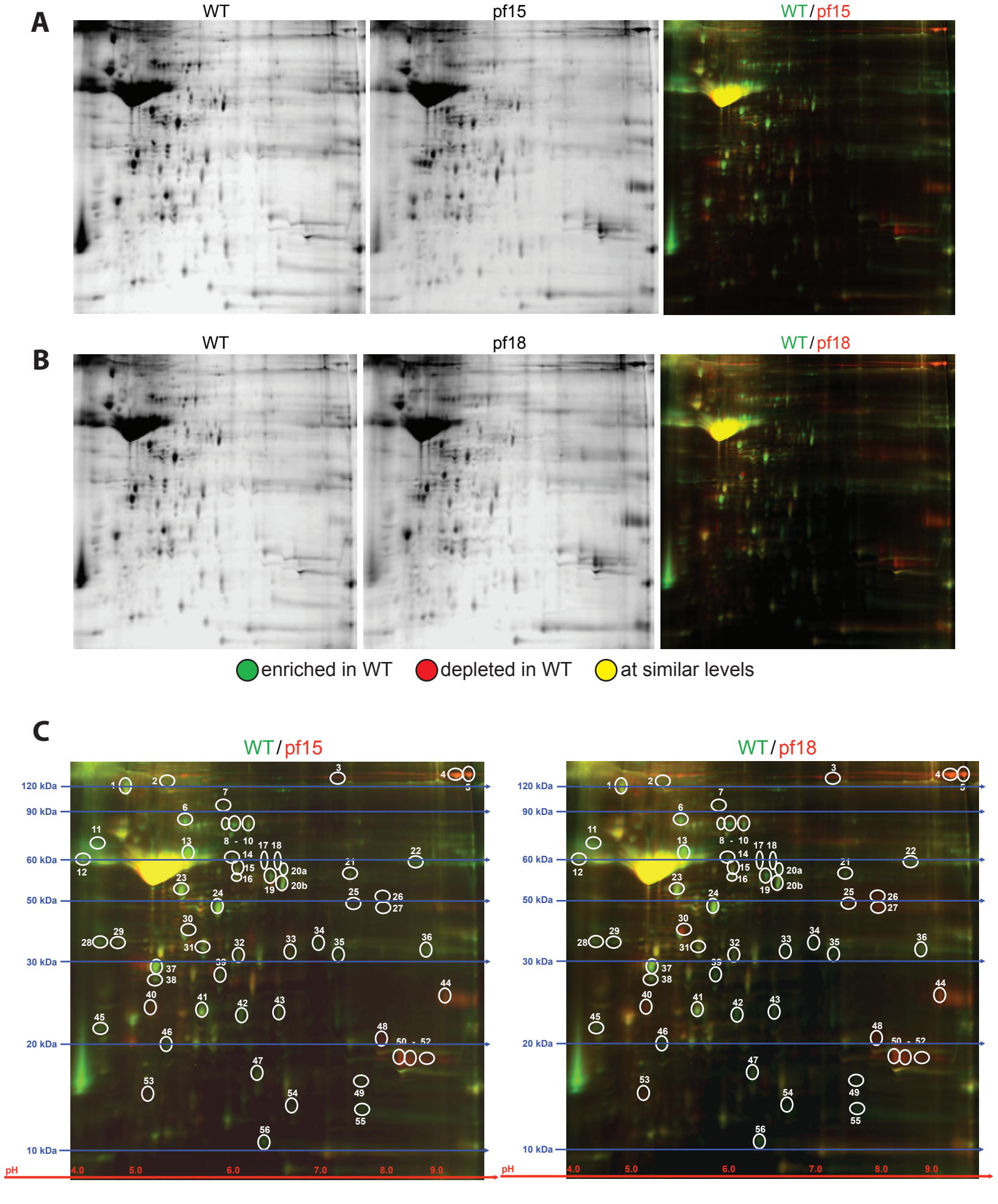
A
WT
pf15
WT / pf15
B
WT
pf18
WT / pf18
enriched in WT
depleted in WT
at similar levels
C
WT / pf15
WT / pf18
120 kDa
90 kDa
60 kDa
50 kDa
30 kDa
20 kDa
10 kDa
pH
4.0
5.0
6.0
7.0
8.0
9.0

**Figure 2**

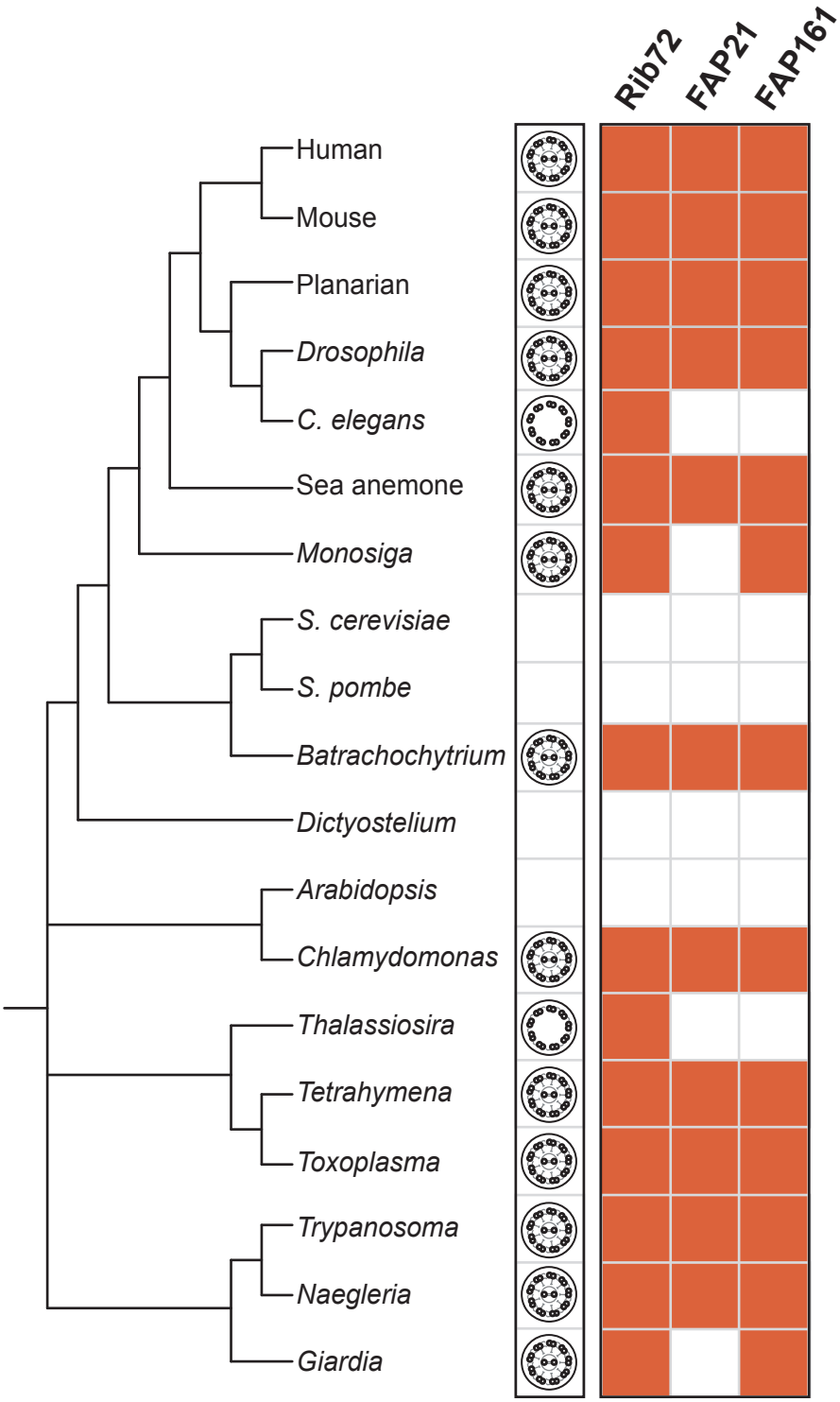
Rib72
FAP21
FAP161
Human
Mouse
Planarian
Drosophila
C. elegans
Sea anemone
Monosiga
S. cerevisiae
S. pombe
Batrachochytrium
Dictyostelium
Arabidopsis
Chlamydomonas
Thalassiosira
Tetrahymena
Toxoplasma
Trypanosoma
Naegleria
Giardia

**Figure 3**

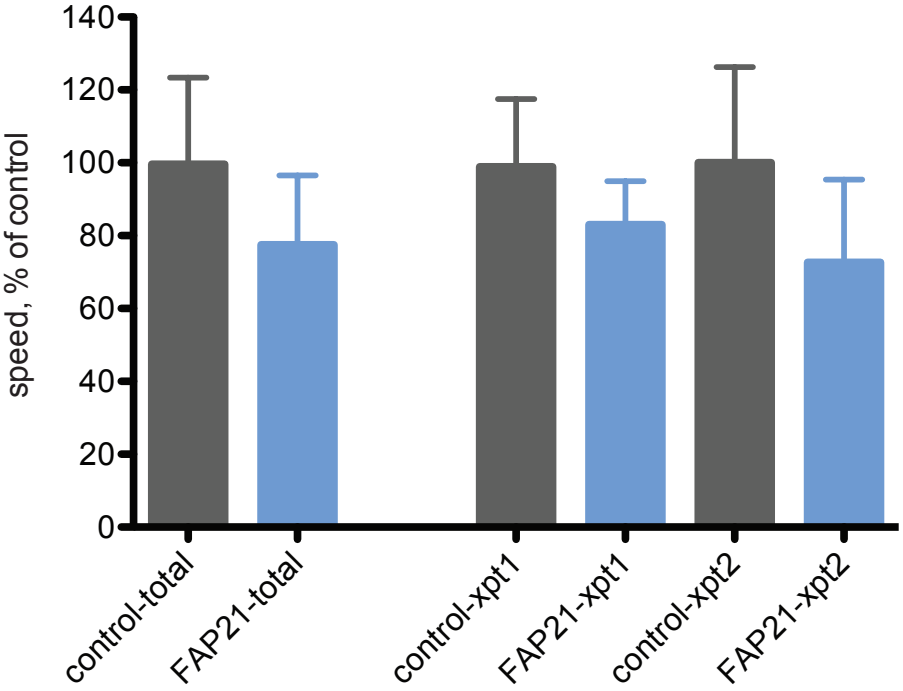
speed, % of control
140
120
100
80
60
40
20
0
control-total
FAP21-total
control-xpt1
FAP21-xpt1
control-xpt2
FAP21-xpt2

**Figure 4**