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Eukaryotic flagella: variations in form, function, and composition during evolution



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Abstract:	The microtubule axoneme is an iconic structure in eukaryotic cell biology and the defining structure in all eukaryotic flagella (or cilia). Flagella occur in taxa spanning the breadth of eukaryotic evolution indicating the organelle's origin predates the radiation of extant eukaryotes from a last common ancestor. During evolution the flagellar architecture has been subject to both elaboration and moderation. Even conservation of '9+2' architecture – the classic microtubule configuration seen in most axonemes – belies surprising variation in protein content. Classically considered as organelles of motility that support cell swimming or fast movement of material across a cell surface, it is now clear the functions of flagella are also far broader; for instance, the involvement of flagella in sensory perception and protein secretion is recently evident in both protists and animals. Here, we review and discuss in an evolutionary context recent advances in our understanding of flagellum function and composition.

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3 **Eukaryotic flagella: variations in form, function, and composition during**
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5 **evolution**
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8 Jonathan Moran, Paul G. McKean, and Michael L. Ginger
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22 ***Abstract***
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35 *evolutionary context recent advances in our understanding of flagellum function and*
36 *composition.*
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55 *Keywords: Chlamydomonas, centriole, cell biology, microtubules, Trypanosoma*
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Introduction

The origin of the eukaryotic flagellum (or cilium, terms referring to variants of essentially the same organelle) predates the radiation of known eukaryotes from a last common ancestor over a billion years ago (Roger and Simpson 2009). The presence of whip-like flagella or short, hair-like arrays of many cilia protruding from cell surfaces is a striking visual feature of many protists and animal cells (**figure 1**). Historically, distinguishing between a ‘flagellum’ versus a ‘cilium’ was often made on the basis of organelle length (flagella are typically longer than cilia) or organelle number (many, often >100, ‘cilia’ can be arrayed across a cell surface, whereas examples of eukaryotes with more than eight ‘flagella’ are rare), but the defining architectural feature in these organelles is always a microtubule axoneme.

Motility is the feature most classically associated with flagella and is often central to cell swimming, fast movement of material across a cell surface, cell feeding, and reproduction by eukaryotic organisms. Yet, eukaryotic flagella also function as sensory antennae and in the green alga *Chlamydomonas reinhardtii*, at least, as a secretory organelle, too. Our understanding of the flagellum as a secretory organelle is in its infancy (Wood et al. 2013), but then it was only fifteen or so years ago that the importance of flagella in the perception of extracellular stimuli, particularly during animal development, began to be widely realised (Goetz and Anderson 2010). Now there is widespread appreciation that a wide variety of genetic syndromes are underpinned by defects in cilium assembly or function, and that the underlying pathology is often a consequence of altered sensory perception. Moreover, in some individuals defects in flagellum assembly or function contribute to a pre-disposition to some chronic conditions, including cancer and obesity; again connections between signalling dysregulation and disease are becoming evident. In animals, sensory perception is more commonly associated with immotile flagella, often referred to as primary cilia; most animal

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3 cell-types are capable of building a single primary cilium in response to appropriate
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5 differentiation cues.
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8 Discussions of how motile cilia beat and connections between ciliary function and human
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10 disease are provided in other overviews in this special edition (Brown and Witman 2014,
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12 Satir et al. 2014). Here, we consider some of the ways in which the architecture and function
13
14 of the flagellum has been subject to surprising and potentially informative variation during
15
16 eukaryotic evolution.
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19 20 21 22 **Eukaryotic flagella - ancient organelles unrelated to bacterial flagella** 23

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25 Since the release in April 1996 of a nuclear genome sequence for the bakers' yeast
26
27 *Saccharomyces cerevisiae* (an aflagellate eukaryote), genetic blueprints for numerous
28
29 eukaryotes have steadily been released. Using the wealth of information provided in
30
31 annotated genome sequences, comparative analyses of protein-coding gene repertoires in taxa
32
33 spanning the breadth of eukaryotic evolution indicate the hallmark characteristics of
34
35 eukaryotic cell biology – a nucleus, endomembrane and vesicular trafficking systems,
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37 mitochondrial metabolism, a cytoskeleton based on actin and microtubules – were all
38
39 surprisingly well advanced in the last common ancestor of extant eukaryotes (Koumandou et
40
41 al. 2013). This includes the presence of a flagellum or flagella in that last common ancestor.
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43 From the outset, however, it is important to emphasize the architecture, composition, motility
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45 mechanism, and assembly of eukaryotic flagella are all fundamentally different from the non-
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47 homologous bacterial flagellum. Thus, (i) the number of proteins comprising a typical
48
49 eukaryotic flagellum is far greater than in the bacterial organelle (several hundred different
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51 proteins in the case of the eukaryotic organelle versus less than 30 in the case of prokaryotic
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53 flagella); (ii) rather than utilising a rotary motor that sits at the base of the flagellum,
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3 eukaryotic flagellar motility is dependent upon constrained, dynein-dependent microtubule
4 sliding and the application of applied force into flagellum bending; (iii)
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6 the bacterial flagellum is a self-assembling filament, whereas synthesis of the eukaryotic
7 flagellum is templated from a microtubule-based centriole to which flagellar proteins are first
8 targeted, and from which the axoneme extends. The centriole is also often referred to as a
9 flagellar basal body, although again the reader should not confuse this with the membrane-
10 embedded bacterial basal body, which includes the rotary motor that drives motility and the
11 pore through which flagellar proteins translocate in bacteria.
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21 In the iconic, textbook '9+2' configuration, axonemes are formed from a ring of nine outer-
22 doublet microtubules that surround two singlet central pair microtubules (**figure 2**). A
23 multitude of accessory structures decorate both the surface and the lumen of axonemal
24 microtubules, but the obvious features evident from electron microscopy that are key to
25 motility are the dynein ATPases, which are anchored to outer-doublet A tubules and, in most
26 motile flagella, the radial spokes and central pair projections, which link central pair with the
27 dynein ATPases and are essential for the regulated motility of flagella bearing these
28 structures. If one looks longitudinally along the length of the flagellum, then a conserved 96
29 nm periodicity to the organisation of radial spokes, central pair projections, and the outer- and
30 inner-arm dynein ATPases is evident. The dynein motor proteins form transient bridges
31 between adjacent outer-doublet microtubules resulting in microtubule sliding, but because
32 sliding is constrained by other connections between outer-doublet microtubules applied force
33 is converted into flagellar bending.
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50 51 52 53 **Origin of nine-fold symmetry** 54 55 56 57 58 59 60

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3 The barrel-like microtubule organizing center (MTOC) which templates any axoneme is
4 called a centriole or basal body, and is formed from a radially symmetrical arrangement of
5 nine triplet microtubules from which the outer-doublet microtubules of the axoneme extend.
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10 In some eukaryotes, centrioles are multi-functional serving also to organize assembly of the
11 mitotic spindle. The green alga *Chlamydomonas reinhardtii*, which since the 1950s has
12 served as the principal model for biologists studying flagellum assembly and function, is a
13 prime example: prior to cell division its flagellar basal bodies uncouple from their associated
14 axonemes and migrate to the nuclear periphery, defining opposite poles of the mitotic spindle
15 (Piasecki et al. 2008). Thus, to consider the origin of the flagellum compartment fully one
16 might consider (i) the origin of the centriole; (ii) the origin of nine-fold symmetry; (iii) the
17 evolution of an assembly pathway for flagellum biogenesis; (iv) the initial, and potentially the
18 subsequent, evolutionary advantage(s) offered by a flagellum or flagella.
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30 An early suggestion from the late Lynn Margulis that flagella evolved from an ancient
31 symbiotic relationship between a eukaryotic ancestor and a spirochete bacterium, or an idea,
32 based on experiments from the 1980s, that the centriole, like mitochondria and chloroplasts,
33 was an organelle that possessed its own genome have been dismissed. Yet, while the
34 autogenous origin of flagella is widely accepted, perhaps only speculation, rather than
35 answers, can ever be offered for the functional advantage conferred by the prototypical
36 flagellum. Even with respect to the centriole there is debate as to whether it perhaps evolved
37 from a single triplet blade (Marshall 2009). However, recent bioinformatics interrogation of
38 genome sequences suggests the structural complexity of the centriole is defined by only a
39 handful of conserved proteins (Carvalho-Santos et al. 2011). Central to the definition of nine-
40 fold triplet microtubule symmetry is SAS-6, a conserved protein present at the proximal end
41 of centrioles (**figure 3**). Structurally, SAS-6 has an N-terminal globular head domain and a C-
42 terminal coiled-coil domain that mediates protein dimerization. *In vitro*, SAS-6 dimers self-
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3 assemble into ring-like structures where the coiled-coil domains of nine SAS-6 dimers point
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5 outwards providing a hub-and-spoke arrangement similar in its dimensions to the cartwheel
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7 hub found at the proximal end of the immature (pro-) centrioles (Kitagawa et al. 2011). In the
8
9 absence of SAS-6 assembly of the hub-and-spoke cartwheel fails and although triplet
10
11 microtubule blades still assemble, nine-fold symmetry is lost (Nakazawa et al. 2007).
12
13 Curiously, an exception that proves the rule is the SAS-6 protein from the nematode
14
15 *Caenorhabditis elegans*, the organism from which SAS-6 was originally characterised. Here,
16
17 SAS-6 still defines a nine-fold symmetry, but assembles as a tubular, rather than radial, spiral
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19 illustrating how when even using conserved component parts there can be significant
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21 evolutionary variation in the mechanisms through which core flagellar structures assemble
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23 (Hilbert et al. 2013).
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30 31 **Axoneme assembly**

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33 Radiating out from the distal end of mature centrioles are transitional fibres to which
34
35 axonemal proteins are first recruited prior to transport into the flagellar compartment, and
36
37 thence to the distal tip of the flagellum where axoneme extension occurs (Deane et al. 2001).
38
39 Transitional fibres also denote the proximal end of the transition zone, the name given to the
40
41 most proximal region of a flagellum. The transition zone forms the boundary with the
42
43 cytoplasm of the cell body (*i.e.* flagella are spatially distinct compartments that unusually are
44
45 not entirely bounded by membrane) and a portal through which traffic of membrane and
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47 soluble proteins in and out of the flagellum is regulated (Czarnecki and Shah 2012).
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51 Movement of membrane and axonemal proteins along the flagellum utilizes a conserved
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53 intraflagellar transport (IFT) pathway. Since the discovery of IFT in *Chlamydomonas* just
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55 over twenty years ago (Kozminski et al. 1993), the IFT pathway has been studied in many
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3 flagellate eukaryotes and has featured prominently in the resurgence of interest in eukaryotic
4 flagella and realization of the importance of these organelles for human health. At a
5 molecular level, the IFT pathway involves three modular complexes, IFT-A, IFT-B, and the
6 BBSome, that collectively assemble from over 30 different proteins. Kinesin and dynein
7 motor proteins, respectively, are responsible for the forward (anterograde) and retrograde
8 movement of the IFT complexes and their cargo along the axoneme. Phylogenetic
9 reconstructions suggest IFT-A and the BBSome evolved via duplication of an IFT-B complex
10 that served as the proto-typical IFT apparatus, and that IFT-B shares a common ancestry, at
11 least in part, with protocoatome proteins COPI- α , β ', and ϵ (van Dam et al. 2013). The
12 protocoatome family of complexes are involved in vesicular trafficking within eukaryotic
13 cells and nuclear pore complex assembly. A relationship between IFT and protocoatome
14 proteins also provides a basis for understanding how the involvement of IFT in the vesicular
15 transport of some flagellar proteins to the centriole arose (Jekely and Arendt 2006), and
16 provides another example of how the complexity of flagellum assembly is likely to have
17 evolved from ancestral simplicity.
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40 **Initially an organelle of motility or sensory perception?**

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42 Motility is the characteristic most classically associated with eukaryotic flagella, but it does
43 not necessarily follow that it was the archetypal flagellar function. Indeed, as awareness
44 grows of how diverse flagellate protists use their flagella to interact with other cells and the
45 environment (Bloodgood 2010), it appears that it will be a rare exception to find a flagellum
46 that does not function in some capacity as a sensory antenna. Exploiting the flagellar
47 membrane as a surface from which to detect extracellular ligands and using a slender flagellar
48 antenna to concentrate (*i.e.* cluster) the protein players from signal transduction cascades
49 provides opportunities for cells (or organisms) to respond rapidly and sensitively to even
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3 small changes in the concentrations of stimulatory extracellular ligands. A compelling
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5 argument has been developed by Jékely and Arendt (2006) that suggests advantages offered
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7 from an enhanced capacity to sense and respond to environmental cues provided the driving
8
9 force for evolution of the proto-flagellum in an early eukaryotic organism. It is a theory that
10
11 also speaks to the evolution of the IFT system from other vesicular trafficking pathways
12
13 (Jékely and Arendt 2006) and is compatible with the suggestion that the proto-centriole
14
15 consisted of a single triplet blade, rather than a multi-triplet barrel. We will probably never
16
17 know why flagella evolved in eukaryotes. However, should a novel mode of motility have
18
19 provided the driving force for evolution of the organelle (the amoeboflagellate *Naegleria*
20
21 provides a prime example of how a flagellate cell can swim a hundred times faster than an
22
23 amoeboid cell can crawl), then this is also not inconsistent with a single triplet blade origin
24
25 for the centriole since it has been suggested that the application of flagellar membrane
26
27 adhesins to glide across a surface, rather than dynein-dependent microtubule sliding, was how
28
29 flagella first conferred cell motility. In extant flagellate protists, gliding-based motility is
30
31 common, and in *Chlamydomonas* gliding is also IFT-dependent (Shih et al. 2013),
32
33 underscoring the centrality of IFT to multiple aspects of flagellum assembly and function.
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42 **Conservation of the protein inventory in motile flagella**

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44 How well does visual conservation of the iconic '9+2' microtubule ultrastructure align with
45
46 the conservation of proteins that are incorporated into the '9+2' microtubule scaffold? Initial
47
48 proteomic analyses of flagellum composition involved the analysis by two-dimensional gel
49
50 electrophoresis of detergent-extracted flagella isolated from *Chlamydomonas* and indicated
51
52 250-300 different proteins adorn the outer-doublet and central pair microtubules, thereby
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54 contributing to the characteristic axonemal sub-architecture of dynein ATPases, radial
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56 spokes, and central pair projections.
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3 Results from the early proteomic studies were reported in late 1970s and early 1980s
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5 (reviewed in (Luck 1984). 20-25 years on from those studies, and with nuclear genome
6
7 sequences of model flagellates at hand, mass spectrometry-based proteomics of detergent-
8
9 extracted flagella provided a candidate list of proteins not dissimilar in number to initial
10
11 estimates (Broadhead et al. 2006, Pazour et al. 2005). Proteomics of intact flagella (*i.e.*
12
13 combining axoneme, matrix, and flagellar membrane fractions), moreover, combined with
14
15 results from genome comparisons between eukaryotes that build flagella with those that do
16
17 not indicate that the number of proteins typically contributing to flagellum architecture and
18
19 function is between 600 and 700 (Li et al. 2004, Pazour et al. 2005). Both comparative
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21 genomics and proteomics approaches have their limitations: for instance, the former cannot
22
23 easily be used to identify flagellar protein kinases, phosphatases or other types of protein
24
25 belonging to gene families where the functionality provided by the protein product is also
26
27 used for core processes within the cell body of any eukaryote, and for the latter likely
28
29 candidature of flagellar components identified by proteomics is always a function of the
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31 purity of biochemically fractionated material.
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37 Flagellar candidacy of proteins identified by either *in silico* comparisons or proteomics is
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39 steadily being confirmed by experimental analyses of location and function. Unsurprisingly,
40
41 given the importance of the structural conduit that extends from the central pair microtubules
42
43 to both inner- and outer-dynein arms, flagellar proteomes are enriched with proteins
44
45 containing domains that commonly function in mediating protein-protein interactions (*e.g.*
46
47 WD and tetratricopeptide (TPR) repeats), as well as pfam domains associated with signal
48
49 transduction cascades (Li et al. 2004, Pazour et al. 2005). What is surprising though are the
50
51 number of candidate axonemal proteins in *Chlamydomonas*, *Trypanosoma brucei*, and the
52
53 ciliate *Tetrahymena thermophila* (around a third of proteins in each flagellate) for which
54
55 homologs in other organisms are not evident from sequence comparisons (Broadhead et al.
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3 2006). Whilst these findings could be viewed as pointing towards lineage-specific novelties,
4
5 it is important to remember structural studies of many protein classes indicate that primary
6
7 sequence similarities between proteins sharing common ancestry diverge long before loss of
8
9 shared protein folds. Indeed, comparisons of the major protein components from the
10
11 eukaryotic and prokaryotic cytoskeletons (*i.e.* tubulin versus FtsZ and actin versus MreB
12
13 family proteins) provide a prime illustration of this phenomenon (Wickstead and Gull 2011).
14
15 Structural studies are therefore needed to gauge how closely conservation of ‘9+2’
16
17 architecture across the breadth of evolution aligns with conservation of individual protein
18
19 components. However, there are also very clear examples of variation in the evolutionary
20
21 distribution of flagellar proteins with experimentally verified or, on a basis of their domain
22
23 architecture, predicted regulatory functions.
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31 **Species-specific variation of regulatory networks**

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33 Changes in intraflagellar Ca^{2+} and cyclic nucleotide concentrations are ubiquitous regulators
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35 of motility, providing second messenger mediation of switches between ciliary- and flagellar-
36
37 style waveforms and waveform reversal. A balance between the rates of synthesis and
38
39 degradation regulates the steady-state cyclic nucleotide concentrations, whereas *trans*-
40
41 membrane fluxes effect changes in intraflagellar Ca^{2+} concentration. The ecology of different
42
43 flagellate cells influences the ways in which these ubiquitous second messengers regulate
44
45 motility. For instance, in the freshwater- and soil-dwelling alga *Chlamydomonas*,
46
47 intraflagellar Ca^{2+} concentrations underpin swimming behaviour in response to the alga’s
48
49 perception of changes in light intensity. In *Chlamydomonas*, the photo-response is all the
50
51 more interesting because although the micro-compartmentalization of second messengers is
52
53 key to dynamic amplification of signalling cascades, the phototactic response of
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55 *Chlamydomonas* requires transmission of signals initially detected at an eyespot located at an
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3 equatorial position on the cell body, relative to the polar location of the flagella. This
4
5 illustrates how holistic regulation of dynamic, transient changes to swimming patterns can
6
7 extend far beyond the confines of the flagellar compartment. Photosynthetic activity is the
8
9 physiological process in phototrophs most obviously influenced by changes in light intensity.
10
11 The complexity of an ill-understood interplay between light-dependent and light-influenced
12
13 processes in *Chlamydomonas* that takes place across different organelles (chloroplast and
14
15 flagella) is further illustrated by the observation that the duration of the alga's photoresponse
16
17 is influenced by photosystem I activity and NADPH:NADP turnover in the chloroplast
18
19 (Wakabayashi and King 2006).
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22
23 A more ubiquitous example of a recently characterised, but a still poorly understood
24
25 regulatory network in flagella is ATP homeostasis. Movement of the IFT machinery, dynein-
26
27 dependent sliding of outer-doublet microtubules, and cAMP synthesis are all energy-
28
29 consuming processes. Yet, the evidence from proteomic datasets and biochemical studies for
30
31 diverse ATP re-generating pathways operating within flagella of different protists during the
32
33 2000's still came as a surprise (Ginger et al. 2008). It indicates organelle assembly and
34
35 motility cannot always rely on the diffusion of ATP from the cytosol. The regulation of
36
37 dynein ATPase activity by ADP emphasizes further a requirement for intraflagellar
38
39 nucleotide homeostasis although the environmental parameters under which flagellate protists
40
41 need to make use of their intraflagellar ATP-regenerating systems is still not known.
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46 Gene duplication and gene fusion provide a molecular basis through which species-specific
47
48 elaboration of regulatory networks can occur, and again work with *Chlamydomonas* provides
49
50 examples for each mechanism. Lineage-specific duplication of dynein heavy chains gives rise
51
52 to differential localisation of individual isoforms along the flagellum (Yagi et al. 2009). The
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54 functional significance of proximal- and distal-specific dynein arms is not yet clear but points
55
56 to a species-restricted, rather than a more widely distributed, regulatory network.
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3 *Chlamydomonas* RSP2, a conserved radial spoke protein, provides a likely example of gene
4 fusion. In most flagellate eukaryotes, RSP2 is a small protein (~120 amino acids) principally
5 comprising of a Dpy-30 domain which forms a helix-loop-helix structure and is essential for
6 radial spoke assembly. Yet, in *Chlamydomonas*, an additional C-terminal domain binds the
7 ubiquitous Ca²⁺-binding protein calmodulin, but is required only to support the normal helical
8 swimming trajectory of *Chlamydomonas* under bright illumination (Gopal et al. 2012). In
9 summary, as biologists mine through flagella proteome inventories it appears likely that
10 structural approaches will be required to resolve just how many axonemal proteins are
11 actually conserved across evolution, although examples of lineage-specific tinkering with
12 regulatory proteins are also emerging.
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28 **Variation on a grander scale: extra-axonemal structures**

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31 Assembly of extra-axonemal structures occurs in a handful of flagellate eukaryotes, although
32 insight into their function(s) and composition has relied on recent advances in reverse genetic
33 and molecular approaches. Among protists, the lattice-like paraflagellar rod (PFR) seen in
34 euglenozoan protists provides a well-studied, elegant example of an extra-axonemal structure.
35 The Euglenozoa are a cosmopolitan group of unicellular flagellates, descended from a
36 divergent, possibly even early-branching eukaryotic lineage, and include the parasitic
37 trypanosomatid family (Tekle et al. 2009). Trypanosomatid species belonging to the genera
38 *Trypanosoma* and *Leishmania* are responsible for several of the so-called 'neglected tropical
39 diseases'. Composed of two major proteins PFR1 and PFR2 and ~30 proteins of lesser
40 abundance, the PFR is a complex lattice of different filamentous structures that is attached to
41 and runs alongside the axoneme (Portman and Gull 2010). It is also a multifunctional
42 structure required for (i) motility, although it is difficult to distinguish whether motility is
43 influenced more by the intrinsic biophysical properties of PFR structure or because a PFR
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3 provides the platform into which regulatory enzymes are anchored; (ii) parasite attachment to
4 epithelial surfaces within the insect vectors responsible for parasite transmission; and (iii)
5 stable attachment of the flagellum to plasma membrane, which in some trypanosomatids is
6 critical for normal cell morphogenesis (Ginger et al. 2013, Portman and Gull 2010).
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12 Accessory structures are also found alongside the axoneme in mammalian sperm. In contrast
13 to protists, and indeed other animal cells, where flagella represent *bona fide* organelles
14 isolated from the rest of the cell body by the transition zone, mammalian sperm are better
15 considered as a flagellate cell-type almost devoid of cytosol. Regarding the presence of
16 accessory structures, however, an outer dense fibre is paired with each outer doublet
17 microtubule along the length of the axoneme and a fibrous sheath surrounds the axoneme
18 along the principal piece of the sperm tail (which accounts for ~3/4 of the flagellum length)
19 (Eddy et al. 2003). Like the PFR, the fibrous sheath also acts as a scaffold into which
20 enzymes assemble, most notably several glycolytic enzymes. Transgenic mice lacking the
21 fibrous sheath-bound isoform of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the
22 sole isoform of GAPDH found in sperm, complete spermatogenesis and build the fibrous
23 sheath, but are infertile and exhibit severe motility defects, consistent with the idea that
24 glycolytic flux provides the ATP necessary for sperm swimming (Miki et al. 2004). At least
25 one fibrous sheath-bound glycolytic enzyme, enolase, however, is necessary for assembly of
26 the accessory structure (Nakamura et al. 2013), as is the major sheath component AKAP4,
27 which like the sheath components AKAP3 and TAKAP-80 serves as a protein-interaction
28 partner for cAMP-dependent protein kinase A (Eddy et al. 2003, Miki et al. 2002). Thus, in
29 very evolutionarily distant eukaryotes extra-axonemal structures are built in order to
30 accommodate enzymes and regulatory proteins associated with energy homeostasis and
31 signal transduction cascades. In the case of the fibrous sheath, scaffold integrity is dependent
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3 upon the regulatory proteins that define its function; it is not yet clear if this is the case for the
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5 PFR.
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10 **Moderation of axoneme structure**

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13 Aside from extra-axonemal structures, variations on a canonical '9+2' microtubule
14
15 architecture are also known. For instance, in many insect spermatozoa outer-doublet
16
17 microtubules have an associated singlet microtubule (*i.e.* a '9+9+2' axoneme) (Dallai et al.
18
19 2006), whereas in spermatozoa from some arachnids three singlet central microtubules are
20
21 seen (a '9+3' axoneme) (Michalik et al. 2003). In marine picoplankton of the genus
22
23 *Micromonas* only a short 9+2 axoneme of ~1 μm length is built, but the central pair then
24
25 extends for ~4 μm beyond the termination of the outer-doublet microtubules (Omoto and
26
27 Witman 1981). However, variations on '9+2' architecture also occur via moderation of this
28
29 iconic architecture. For instance, in moss, land plants that do produce flagellate sperm, the
30
31 '9+2' axoneme is thought to lack outer dynein arms (Merchant et al. 2007). In flagellate
32
33 gametes of the centric diatoms *Milosira* and *Thalassiosira*, radial spokes, central pair
34
35 microtubules and inner dynein arms are absent (Idei et al. 2013), and in nodal cilia, which
36
37 establish left-right asymmetry during the development of mammalian embryos, the central
38
39 pair and radial spokes are also absent. More extreme is the change in underlying axonemal
40
41 symmetry as seen, for example, in '6+0' and '3+0' axonemes built by male gametes of
42
43 gregarine protists (Prensier et al. 2008, Schrevel and Besse 1975). Loss of the central pair
44
45 microtubules and radial spokes often yields a helical waveform, rather than the ciliary or
46
47 flagellar waveforms seen in flagella with a '9+2' axoneme. Yet, moderation without loss of a
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49 canonical '9+2' architecture is also evident in evolution, and malarial parasites provide a
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51 prime example.
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3 Globally, one of the leading causes of human mortality is malaria; the species responsible for
4
5 much of the morbidity and mortality associated with the disease is *Plasmodium falciparum*.
6
7 Malarial parasites undergo an obligatory sexual cycle during their life cycle that begins with
8
9 gametocytogenesis in the host and culminates with fertilization within the mid-gut of a
10
11 mosquito vector. Flagellum biogenesis in the male gamete is unusual not least because IFT-
12
13 independent assembly of the axoneme occurs in the cytosol and the flagellum is produced
14
15 from a rapid exflagellation (Sinden et al. 1976). Male gamete maturation, including axoneme
16
17 assembly and exflagellation takes places within fifteen minutes following the perception of
18
19 environmental cues within the mosquito mid-gut, with axoneme assembly taking as little as
20
21 three minutes to occur. Unsurprisingly, axoneme assembly is error prone, but then again,
22
23 fertilization is also completed soon after ingestion of an infected bloodmeal and the male
24
25 gamete is only motile for up to 1 hour following exflagellation. Glycolysis as the major, if not
26
27 sole, source of ATP to support motility is again evident (Sinden et al. 2010), but given the
28
29 ongoing challenge of elucidating mechanisms through which flagellar motility is regulated, a
30
31 very significant outcome from comparative genomic and proteomic analyses is the indication
32
33 that very few conserved flagellar proteins are present in *Plasmodium* species (Broadhead et
34
35 al. 2006, Sinden et al. 2010). This suggests assembly of a stripped-down '9+2' axoneme.
36
37 From a systems biology perspective, *Plasmodium* parasites therefore provide a genetically
38
39 tractable system from which to decipher a minimal regulatory system supporting motility of a
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41 flagellum with a '9+2' axoneme.
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47
48 Moderation of axoneme ultrastructure is even evident within axonemes built by the model
49
50 flagellate *Chlamydomonas*. Thus, in most flagellates, three radial spokes are present with 96
51
52 nm periodicity per outer-doublet microtubule along the length of the axoneme, but for more
53
54 than thirty years it has been known that in *Chlamydomonas* only two spokes are present per
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56 96 nm. The reason for this difference was unknown. In the last decade, the rise of cryo-
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3 electron tomography as an experimental tool in cell biology has facilitated high resolution
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5 views of axoneme ultrastructure and led to the identification of new multi-protein complexes,
6
7 which provide seemingly unbroken connectivity to the regulatory conduit that links outer and
8
9 inner dynein ATPases with the central pair projections. Recently, electron tomographic views
10
11 of *Chlamydomonas* axonemes provided an explanation for the enigma of missing radial spoke
12
13 3. Thus, in *Chlamydomonas*, in place of the third radial spoke there is instead a stump into
14
15 which a third spoke could be anchored (Pigino et al. 2011). The parsimonious explanation for
16
17 this observation is that degeneration of radial spoke 3 has occurred during evolution of
18
19 flagellate green algae. Moreover, high resolution views of radial spoke architecture in another
20
21 flagellate protist, *Tetrahymena thermophila*, which builds cilia possessing three radial spokes,
22
23 indicates radial spoke 3 is likely to differ in its protein composition relative to spokes 1 and 2
24
25 (Pigino et al. 2011). To our knowledge, no examples of conserved axonemal proteins that are
26
27 unexpectedly missing from *Chlamydomonas* have been reported. So, in the same way
28
29 extensive moderation of malarial parasite '9+2' axonemes potentially informs on a minimal
30
31 network for regulation of flagellar motility, further study of recently revealed differences in
32
33 spoke composition and organisation in *Chlamydomonas*, relative to other flagellates, is likely
34
35 to provide greater mechanistic understanding of how radial spokes 1, 2, and 3 each impact
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37 upon dynein activity, and thence waveform regulation.
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46 **'Ciliary' proteins in other guises**

47 **(a) Plants**

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49 The most extreme form of axoneme moderation is, of course, loss of the ability to build a
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51 flagellum. Secondary loss of flagella has occurred on multiple occasions during eukaryotic
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53 evolution, most obviously during evolution of the fungi, red algae, land plants, and many
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3 amoebae. Recently constructed phylogenies indicate the secondary loss of flagella among
4
5 plant and fungal lineages is surprisingly complex, and has occurred repeatedly during the
6
7 evolution of these taxonomically diverse groups (Hodges et al. 2012, James et al. 2006). Loss
8
9 of the capacity to build a flagellum offers an opportunity to ask the question of what happens
10
11 to the several hundred genes encoding proteins required for assembly and function of a
12
13 typical motile flagellum. Are they all lost from the genome or do alternative functions remain
14
15 or evolve for the protein products encoded by some? With publicly available nuclear genome
16
17 sequences accumulating for diverse aflagellate eukaryotes it is possible to begin to answer
18
19 this question.
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22
23 One recent genomic analysis led to the identification in angiosperms (land plants that possess
24
25 neither flagella nor centrioles) of a small cohort of genes (~20) for which homologs are
26
27 otherwise normally only found in flagellate eukaryotes (Hodges et al. 2011). Closer analysis
28
29 of these genes suggests only one (a homolog of axonemal protein BUG22) owes its origin to
30
31 a gene duplication event and subsequent divergence of the duplicates prior to loss of a
32
33 capacity to build flagella, and thence the degeneration of flagellum-associated genes, in an
34
35 angiosperm ancestor. Did the remaining 'flagellum-associated' genes retained in *Arabidopsis*
36
37 *thaliana*, *Oryza sativa*, and *Populus trichocarpa* (the angiosperms analysed by Hodges et al.
38
39 2011) gain alternative, non-ciliary functions *prior to* or *following* the loss of cilia in a
40
41 common ancestor? Intriguingly, high expression of some of the retained genes in pollen
42
43 suggests these gene products still have gamete-associated function(s) and the experimental
44
45 characterization of others indicates they are required for microtubule-associated cytoskeletal
46
47 processes (Hodges et al. 2011).
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52
53 The list of experimentally studied proteins includes a likely protein phosphatase 2A
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55 regulatory sub-unit encoded by the TONNEAU2/FASS gene and a phragmoplast-localised
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57 protein kinase, FUSED. The former is required for organization of the cortical cytoskeleton,
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3 and is thus a determinant of cell morphology (Camilleri et al. 2002, Wright et al. 2009).
4
5 Regarding the latter, the phragmoplast is a plant cell-specific microtubule array formed
6
7 during mitosis and FUSED is required for cytokinesis (Oh et al. 2005).
8
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10 A protein not reported as a candidate ciliary protein conserved in angiosperms, but is
11
12 nonetheless clearly homologous to a protein associated with cilium assembly in flagellate
13
14 eukaryotes is TONNEAU1. TONNEAU1 is most closely related to a centrosomal protein
15
16 from animals called FOP, and failure to identify TONNEAU1 as a ciliary protein conserved
17
18 in angiosperms perhaps merely serves to highlight the common limitation of using automated
19
20 pipelines to identify candidate orthologous proteins (*i.e.* proteins encoded by genes that are
21
22 separated only by speciation) that are divergent at the level of their primary amino acid
23
24 sequence. It suggests further flagellum-associated proteins may await discovery in land plants
25
26 that do not build flagella. TONNEAU1 is required for normal cell morphogenesis: it
27
28 orchestrates organization of the cortical microtubule cytoskeleton, interacts with the classic
29
30 MTOC protein centrin, and has most recently been detected in a protein complex containing
31
32 TONNEAU2 (Azimzadeh et al. 2008, Spinner et al. 2010, Spinner et al. 2013). In other work,
33
34 a screen for TONNEAU1-interacting proteins identified a novel family of 34 proteins that
35
36 share in common six short sequence motifs; conservation of some of these motifs, including
37
38 their order of appearance, is evident in another animal centrosomal protein, CEP350
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40 (Drevensek et al. 2012). Collectively, recently published data are therefore rapidly revealing
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42 previously hidden evolutionary commonalities between the centrosome in animal cells and
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44 MTOCs of the plant cortical cytoskeleton.
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49
50 It is plausible that not all of the 'ciliary' proteins retained in angiosperms function in
51
52 cytoskeletal processes. Then, the question would be how any gain of new function(s)
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54 occurred. Here, we provide two alternative views. First, if we consider another obvious
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56 example of multi-cellularity among eukaryotes, the animals, then their phenotypic complexity
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3 is not explained simply by an increase in the number of protein-coding genes present in the
4
5 genome, relative to near unicellular relatives. Instead, multiple mechanisms contribute to the
6
7 generation of protein diversity including alternative splicing; in the case of the human
8
9 genome over 90% of protein-coding genes are alternative spliced, often with tissue-specific
10
11 dependency, resulting in protein isoforms that differ markedly with regard to function(s)
12
13 and/or intracellular localisation(s) (Wang et al. 2008). In plants, alternative splicing has also
14
15 emerged as an important mechanism for generating protein diversity (Reddy et al. 2013).
16
17 Thus, alternative splicing could have readily provided the mechanism by which the function
18
19 or localization of some ancestrally ciliary proteins changed in an angiosperm ancestor;
20
21 following loss of the ability to build flagella during land plant evolution, alternatively spliced
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23 gene products conferring significant fitness benefits would provide a selective pressure for
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25 retention of a 'ciliary' gene.
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30 Alternatively, retention and neo-functionalization in plants of proteins otherwise associated
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32 with ciliary function can also be explained by the concept of protein moonlighting. Thus, a
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34 classic reductionist view is that individual proteins typically have a single function, but
35
36 examples of proteins fulfilling multiple, often very different, roles inside cells are becoming
37
38 increasingly common (Copley 2012). The concept of moonlighting is different from the
39
40 functional diversity that results from alternative splicing in that it describes the functionality
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42 of an individual polypeptide in multiple, distinct cellular processes. For moonlighters,
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44 functionality in different processes may occur as a consequence of conformational change
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46 induced by post-translational modification or utilise different regions of protein surface. For
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48 instance, moonlighting functions were first observed for abundant, soluble enzymes, such as
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50 those involved in the glycolytic pathway, where the active site used for catalysis represents
51
52 only a small part of a protein surface. The prevalence of protein moonlighting is now such
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54 that it is no longer considered an oddity; indeed some consider that protein multi-
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3 functionality is likely to contribute very significantly to cellular and organismal complexity
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5 (Copley 2012). If any ciliary proteins had assumed ‘moonlighting’ functions in an
6
7 angiosperm ancestor, then fitness benefits conferred by some of those moonlighting activities
8
9 could again explain the retention of genes encoding typically ‘ciliary’ proteins in land plants
10
11 such as *Arabidopsis*.
12

13 14 15 **(b) Apicomplexan parasites**

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17 We mentioned earlier the variations on typical ‘9+2’ axoneme architecture seen in malarial
18
19 and gregarine parasites. These protists belong to the phylum Apicomplexa. Over 5000
20
21 apicomplexan species have been described. Some exist as cysts in the environment and others
22
23 undergo an obligatory sexual stage in order to complete often complex life cycles, but the
24
25 apicomplexans are more generally thought of as a family of obligate intracellular parasites.
26
27 They are thought to have evolved from free-living marine algae; among their closest extant
28
29 relations are the recently discovered chromerid algae from the Australian Great Barrier Reef
30
31 and the predatory colpodellids (Kuvardina et al. 2002, Moore et al. 2008). As with many
32
33 other eukaryotic lineages some apicomplexans have secondarily lost the ability to build a
34
35 flagellum, but as a group the apicomplexans owe their name to the unique, polarity-defining
36
37 MTOC called the ‘apical polar ring’ from which sub-pellicular microtubules that define cell
38
39 shape radiate. Structures homologous to the apical polar ring are evident in the chromerids
40
41 and colpodellids, and recent studies suggest a flagellum-based origin for a structure that in
42
43 the Apicomplexa is involved in cell invasion, as well as the definition of cell shape (Portman
44
45 and Slapeta 2014).
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51 In some apicomplexans, such as *Toxoplasma gondii*, an extremely successful parasite of
52
53 vertebrates globally and opportunistic pathogen of the immunocompromised, a spiral-like
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55 arrangement of tubulin sheets folded in the shape of a cone, and known as the ‘conoid’ is also
56
57 associated with the apical polar ring (**figure 4**). Genetic tractability and easy visualization by
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3 microscopy make *Toxoplasma* a well-used model for many general studies of apicomplexan
4 cell biology. In the animal host, extracellular *Toxoplasma* parasites extend and retract the
5 conoid through the apical polar ring, a process thought to contribute mechanically to the
6 process of host cell invasion (Gubbels and Duraisingh 2012). Recently, it has been observed
7 that following duplication of the parasite centrosome, which is associated with the nucleus, a
8 fibrous connection extends from the duplicated centrosome to the apical polar ring providing
9 a hard-wired link that helps orchestrate daughter cell budding. With regard to a possible
10 connection with eukaryotic flagella, essential components of this fibrous connection appear
11 homologous to proteins that form the rootlet fibres which link the flagellar basal bodies in
12 *Chlamydomonas* (Francia et al. 2012).
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16 Further evidence of a possible ancient relationship between the flagellar cytoskeleton and the
17 cytoskeletal apparatus that facilitates cell invasion by *Toxoplasma* comes from the
18 localisation of SAS6-like proteins to the conoid in *Toxoplasma* and to proximal end of the
19 axoneme in trypanosomes (potentially to the basal plate from which the central pair
20 microtubules are nucleated) (de Leon et al. 2013). Thus, the N-terminal region of SAS6, a
21 protein integral to nine-fold symmetry of the centriole, provides the conserved domain
22 annotated in the pfam database (pfam_B_2528) and is the feature also shared with SAS6-like
23 proteins. Despite the large evolutionary distance between trypanosomes and apicomplexans
24 (which spans major eukaryotic groups (Tekle et al. 2009)), and even though SAS-like
25 orthologs are absent from animals and their nearest flagellate unicellular relatives (de Leon et
26 al. 2013), the flagellar localization of SAS6-like protein in trypanosomes raises a possibility
27 that during apicomplexan evolution flagellar SAS6-like protein found a novel function within
28 the conoid structure as this unusual cytoskeletal structure evolved. If the view of a flagellum-
29 based origin for the apical polar ring is correct, then not only does it provide an unexpected
30 example of co-opting flagellar proteins into an alternative structure but it also provides an
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3 intriguing comparison with retention and function of genes encoding ‘ciliary’ proteins in
4
5 acentriolar plants, or indeed any other eukaryotes.
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7

8 **(c) other aflagellate eukaryotes**

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10 How many proteins with known or inferred flagellum functions will turn out to have
11
12 homologs (or orthologs) in other eukaryotes that do not build flagella (*i.e.* most fungi, red
13
14 algae, and a wide variety of heterotrophic protists) is really a topic for future investigations,
15
16 although some intriguing observations have been recorded. For instance, miniature green alga
17
18 belonging to the genus *Ostreococcus* are abundant oceanic picoplankton abundant and are
19
20 believed to lack the capacity to build flagella; yet around forty proteins associated with
21
22 flagellum function are still encoded within the nuclear genomes of different *Ostreococcus*
23
24 species (Merchant et al. 2007). In contrast, using the same pipeline as for their identification
25
26 of ‘ciliary profile’ proteins in land plants, Hodges et al. (2011) predict that the number of
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28 ‘flagellar’ proteins in fungi, at least, will be lower than in aflagellate land plants.
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36 **Closing summary**

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38 It is approaching 350 years since cilia or flagella were first described by Antoni van
39
40 Leeuwenhoek as the ‘little legs’ on the protists he saw in rainwater using a homemade
41
42 microscope (Leeuwenhoek 1677), a little over a 100 years since a sensory function was first
43
44 suggested for the primary cilium (Zimmermann 1898), just over 60 years since the iconic
45
46 ‘9+2’ arrangement of axonemal microtubules was first predicted by Irène Manton and Bryan
47
48 Clarke from their analysis by electron microscopy of zoospore ultrastructure in *Sphagnum*
49
50 peat moss (Manton and Clarke 1952), and 50 years since Peter Satir and Ian Gibbons began
51
52 to develop the dynein-dependent sliding filament model as the basis for flagellar and ciliary
53
54 motility (Satir et al. 2014). It is during the last 30 or so years that molecular bases
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3 underpinning the diversity in form and function of eukaryotic flagella, and their importance
4
5 to human health have become apparent. As with other aspects of modern cell biology, our
6
7 understanding of flagellum origins, function, and variation, and the ensuing paradigm
8
9 revisions, have been hugely informed by advances in molecular biology and DNA
10
11 techniques. Indeed, it is only recently apparent that the versatility of eukaryotic flagella as
12
13 organelles of motility and sensory perception is exploited across the breadth of eukaryotic
14
15 evolution (Bloodgood 2010, Brown and Witman 2014). Further surprises regarding flagellar
16
17 (or ciliary) versatility continue to be revealed; for instance, the immunological synapse – a
18
19 specialised surface membrane region formed when cytotoxic T lymphocytes recognise target
20
21 cells and from which cytotoxic granules are secreted – has very recently been proposed to
22
23 represent a highly modified cilium (de la Roche et al. 2013). With the new view of the
24
25 flagellum as a secretory organelle (Wood et al. 2013), it might come as no surprise if opinion
26
27 as to whether the eukaryotic flagellum ancestrally arose as either a sensory or motile
28
29 organelle is called into question.
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Figure Legends

Figure 1. Diversity of cilia and flagella. (a) Cilia or flagella project as whip-like structures from the cell surface; a cross-section through a flagellum reveals the arrangement of outer-doublet (arrowheads) and central pair (asterisk) microtubules in a motile axoneme. (b) motile cilia lining the human respiratory tract. (c) Human sperm. (d) *Chlamydomonas* sp., a biflagellate green alga and principal model system for many studies of flagellar biology. (e) The voracious predator *Didinium* sp. devouring its favoured prey *Paramecium*; note the two bands of cilia that support the motility of this ciliate. (f-g) In other ciliates, e.g. *Paramecium caudatum*, cilia are arrayed across the cell surface. Cilia are also present along the oral groove and are used to waft food into the oral cavity (arrowhead). In the image shown in (g) the ciliates are on a mat of false-coloured filamentous *Oscillatoria* cyanobacteria. (h) The intestinal parasite and possibly ancient eukaryote *Giardia lamblia*; its basal bodies are located deep in the cytoplasm and the axonemes of eight flagella traverse through the cytosol before emerging from different exit points on the cell surface. (i) The uniflagellate human sleeping sickness parasite *Trypanosoma brucei* shown amongst red blood cells. All images used with permission from Encyclopaedia Britannica ImageQuest.

Figure 2. Axoneme ultrastructure. (a) Cartoon schematic of a transverse section through a typical '9+2' microtubule axoneme with key architectural features highlighted. (b) A transmission electron micrograph of a transverse section through a detergent-extracted axoneme isolated from human respiratory epithelium; image averaging through 110 doublet microtubules allows the individual protofilaments of outer-doublet microtubules to be seen. (c) Electron tomographic averaging of the 96 nm periodicity in axonemes from the ciliate *Tetrahymena thermophila*. Images in (b) are reproduced with permission from Figure 1B and

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3 *1D* in O'Toole et al. 2012; image in (c) reproduced with permission from Figure 6D in
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10 **Figure 3.** SAS-6 and nine-fold radial symmetry of the centriole. (a) A transmission electron
11 micrograph of a longitudinal section through the basal body (centriole) and proximal region
12 of a *Chlamydomonas* flagellum – stacks of cartwheel structures that define the proximal pole
13 of the centriole are arrowed. (b) A transmission electron micrograph of a transverse section
14 through the proximal pole of the *Chlamydomonas* basal body showing the 'hub and spoke'
15 arrangement of the cartwheel structure. (c) Structural model showing the assembly of nine
16 SAS-6 homodimers into a nine-fold symmetrical cartwheel structure. (d) Cartoon showing the
17 SAS-6-based cartwheel within the context of the triplet microtubules of the basal
18 body/centriole. Images in (a) and (b) are reproduced from Figure 2C in Nakazawa et al.
19 2007 ; images in (c) and (d) are reproduced from Figures 5B and 7, respectively in Kitagawa
20 et al. 2011 under the terms of a Creative Common Attribution 3.0 unported licence.
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38 **Figure 4.** Apical polar ring – flagellum connections? (a) Cartoons of *Toxoplasma gondii*
39 tachyzoites (which invade and replicate within host cells) and flagellate micro-gametes – the
40 centriole-containing centrosome is located close to the nucleus distant from the apical polar
41 ring (APR); the closed conoid (CC) is topped by preconoidal rings (PCR), and the rhoptries
42 are secretory organelles that release their contents during cell invasion. Note the very
43 different morphologies of tachyzoites versus biflagellate micro-gametes, and an absence of
44 the APR and associated structures in the latter. (b) *Toxoplasma* tachyzoites expressing SAS-6
45 protein fused to yellow fluorescent protein, grown within human fibroblasts, and decorated
46 for immunofluorescence microscopy with anti-sera recognizing SAS-6 like protein: SAS-6 is
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3 *found only within the centrioles and SAS-6 like protein is present only at the apical end of the*
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5 *cell; the central position of the nucleus is also shown. Images in (a) and (b) are reproduced*
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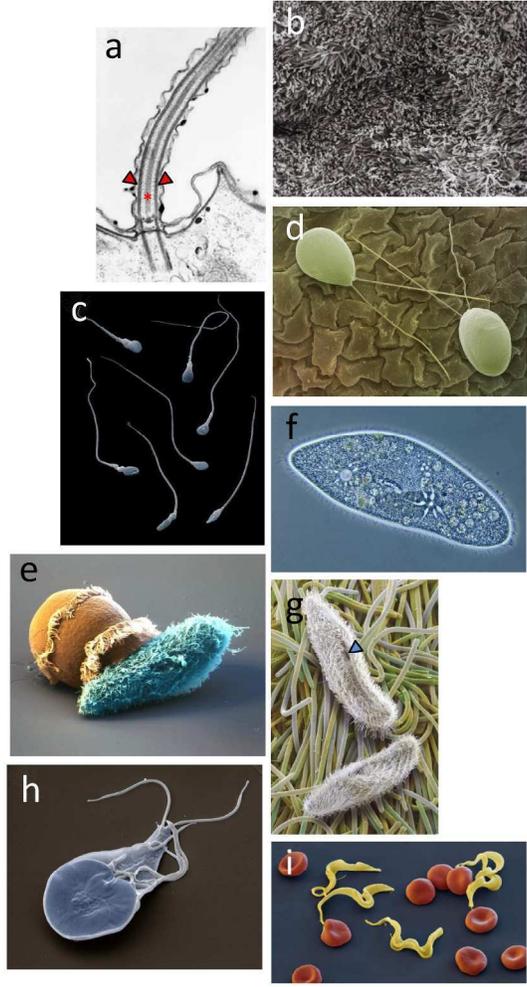


Figure 1

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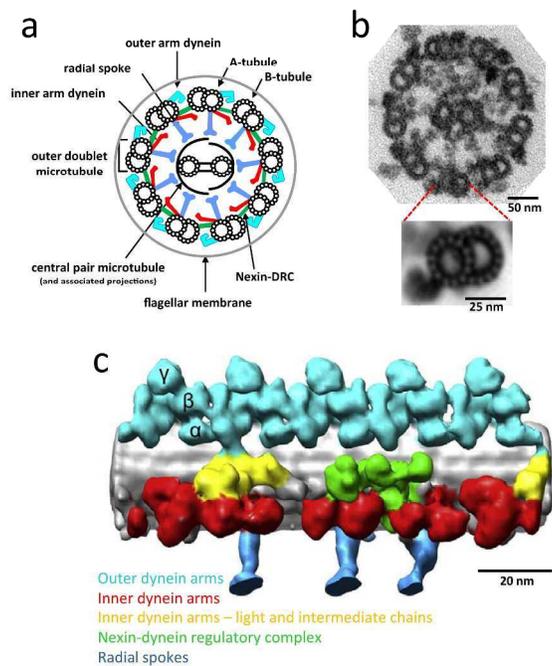


Figure 2

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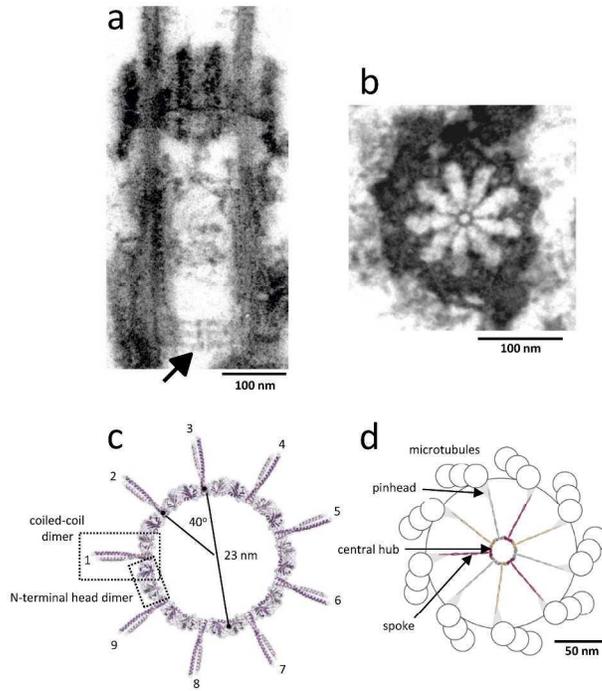


Figure 3

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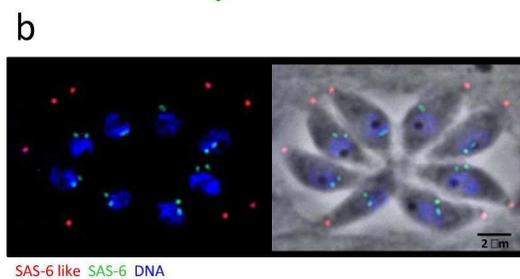
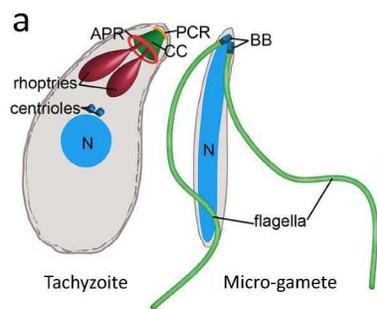


Figure 4

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