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Regulation of ciliary motility: conserved protein kinases and phosphatases are targeted and anchored in the ciliary axoneme

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Abstract

Recent evidence has revealed that the dynein motors and highly conserved signaling proteins are localized within the ciliary 9 + 2 axoneme. One key mechanism for regulation of motility is phosphorylation. Here, we review diverse evidence, from multiple experimental organisms, that ciliary motility is regulated by phosphorylation / dephosphorylation of the dynein arms through kinases and phosphatases that are anchored immediately adjacent to their axonemal substrates.

1. Introduction

Understanding the mechanism of ciliary / flagellar movement is crucial: motile cilia play essential roles in human development, male and female fertility, protection and function of the airway and circulation of cerebral spinal fluid [1, 2]. Defects in ciliary motility lead to a variety of “ciliopathies” that can result in an especially wide range of diseases and syndromes including left-right pattern defects in early development, hydrocephaly, infertility and Primary Ciliary Dyskinesia (PCD) [3–9]. Furthermore, defective assembly of the “primary” cilium, an immotile, sensory and signal transduction organelle found on all differentiated cells, can lead to additional problems including failure in normal patterning, polycystic kidney disease, retinal degeneration, obesity, skeletal abnormalities and a growing list of syndromes [10–13]. Importantly, the cytoskeletal structure of the motile cilium, the axoneme, is a highly conserved 9 + 2 arrangement of microtubules, associated dynein motors and structures and signaling molecules which contribute to control of ciliary movement [14, 15]. Normal ciliary motility is a consequence of precisely controlled dynein

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motors and the mechanical and chemical interplay of axonemal structural and enzymatic components that regulate beating of the cilium. Thus, discovery of the conserved mechanisms that regulate the dynein motors is essential for understanding ciliary bending.

Here, we focus, specifically, on the key ciliary axonemal structures and conserved axonemal protein kinases and phosphatases that regulate ciliary movement. We review diverse evidence from multiple experimental systems revealing that ciliary motility is regulated by phosphorylation and that the key kinases and phosphatases are conserved, targeted to and anchored in the axoneme in positions to control motility [16]. We feature studies using the model genetic organism *Chlamydomonas*, that has not only revealed conserved genes that encode proteins essential for ciliary assembly and function, but that has also provided a very powerful experimental system for study of the mechanism and regulation of the dynein motors that control motility [17]. For example, taking advantage of isolated axonemes from informative *Chlamydomonas* ciliary mutants, a microtubule sliding assay has helped demonstrate that protein kinases and phosphatases in the axoneme control dynein activity through phosphorylation [16].

Also supporting regulation of ciliary motility by phosphorylation is the finding that kinases and phosphatases are a physical part of the axonemal structure [14, 18]. We focus on the pharmacological, biochemical and molecular approaches that revealed the protein phosphatases, PP1 and PP2A, and kinases, CK1 and PKA, are anchored in the axoneme and control movement [19–24]. We summarize how new proteomic analyses of cilia and axonemes have revealed additional conserved kinases, phosphatases, calcium-binding proteins and specific phosphoproteins in the motile cilia [25, 26] see also (<http://v3.ciliaproteome.org/cgi-bin/index.php>) and discuss opportunities for new research characterizing the role of kinases and phosphatases in regulation of motility.

2. Conserved axonemal structures and regulation of motility

Cilia, sometimes called flagella, are microtubule-based structures that are highly conserved in protein composition as well as structural organization [2, 9]. Indeed, recent studies have led to a revolution in our understanding of the diverse, vital roles of cilia and revealed the presence of cilia on nearly all differentiated cells in vertebrates [8, 11, 27–29]. In addition to motility, cilia display an extraordinary range of vital signaling roles required for normal control of the cell division cycle, sensory transduction required for vertebrate development and adult functions [10, 12, 30–32]. We focus on motile cilia and the 9+2 axoneme that not only localizes a number of distinct dynein motors required for motility, but also localizes ubiquitous kinases and phosphatases required for regulation of ciliary motility [14, 16, 19, 33]. As indicated in the Introduction, defects in either assembly or function of cilia can result in a wide spectrum of diseases, termed “ciliopathies” [8, 10, 34–38]. Despite the conservation, we are only beginning to understand how dyneins and ciliary motility are regulated.

Motile cilia contain a bundle of nine outer doublet microtubules and two central microtubules called the 9+2 axoneme (Fig. 1). The ciliary axoneme originates from the basal body which provides a template for microtubule growth and is required for assembly of all cilia, anchoring the 9+2 axoneme to the apical end of the cell [39–42]. The central pair and the nine outer doublet microtubules are continuous throughout the length of the axoneme. Each outer doublet appears to be associated with the adjacent outer doublet by the dynein arms and structures called nexins recently determined to be part of the dynein regulatory complex – (DRC) [43]. The 9+2 structural configuration is highly conserved among all ciliated eukaryotes [25, 44–46] and the basis of this conservation appears to be related to the conservation of basal body organization.

Ultrastructural analyses have revealed axonemal structures in fine detail (Fig. 1). When viewed along the longitudinal axis, the complexity of the axonemal structures is appreciated in greater detail (Fig. 2). Each component associated with the outer doublet microtubule is organized in regular, repeating 96-nm units that are best illustrated using cryoEM tomography of intact axonemes [43, 47–52] which revealed unprecedented fine detail in axonemal morphology. In each 96-nm axonemal repeat, there are four outer arm dyneins that repeat at 24 nm and a surprisingly complex collection of at least seven different inner dynein arm structures, including the inner dynein arm called “I1 dynein” thought to regulate bending by a mechanism that includes phosphorylation and associated kinases and phosphatases [14, 19]. Although the 96-nm repeat organization is highly conserved, the molecular basis that establishes this organization is unknown. Understanding the basis for the 96 nm repeat structure is a high priority since it not only establishes the precise location of dynein motors, radial spokes and DRC, it must also provide the foundation for targeting and anchoring of the protein kinases, phosphatases and calmodulin complexes localized to the outer doublet microtubule (Fig. 3).

The central pair and radial spokes are essential for normal control of motility. The central pair apparatus is composed of the “C1” and “C2” microtubules and associated structures [47, 50, 51, 53–60]. The asymmetric structure of the central pair is founded on the distinct composition of components associated with the C1 and C2 microtubules including ubiquitous and conserved kinases and phosphatases, such as PKA (and associated A-kinase anchoring proteins-AKAPs), PP1, as well as calmodulin-containing complexes important for calcium-based regulatory pathways (Fig. 3; [60–63]). The asymmetric nature of the central pair apparatus is a fundamental feature for control of ciliary movement; in some motile cilia, the central pair rotates, directing signals through the radial spokes to specific outer doublets on one side of the axoneme (Fig. 1). Signaling proteins and complexes, localized to the outer doublets (Fig. 3), relay the signals from the central pair-radial spokes to the dynein motors, ultimately impacting dynein motor function [14, 19, 60]. The signaling molecules localized to the outer doublets include kinases (CK1 and PKA), phosphatases (PP1 and PP2A) and the DRC—an axonemal structure also important for control of dynein activity [33]

The radial spokes are T-shaped structures anchored to the A microtubule of the outer doublets that project towards the central pair. There are two radial spokes, termed S1 and S2, in each 96-nm repeat (spoke S1 is most proximal). Radial spokes are a highly conserved collection of at least 23 proteins found in all ciliated organisms and required for motility and regulation of the ciliary dyneins [64–70]. One radial spoke protein (RSP3) anchors the radial spokes to the A-microtubule [71, 72]. Moreover, RSP3 has been identified as an AKAP [22, 23]. Therefore, as discussed below, one predicted function of RSP3 is to anchor the protein kinase, PKA, in the axoneme, in position to regulate the kinase activity by physically localizing it near its substrate including the dynein motors [23]. The radial spokes also contain calmodulin as part of their structure [69, 73] and interact with a novel calmodulin complex located at the base of the radial spokes on the outer doublet microtubule (Fig. 3; [62]).

The DRC is a part of a mechano-chemical pathway that regulates dynein activity and acts as the nexin interdoublet linker (Fig. 2; [43, 74–80]). Given its location, one prediction is that the DRC plays a role in mediating signals between spoke S2 and the dynein motors. The nature of such signals and the exact function of each of the DRC subunit are only just beginning to be defined [33, 77, 78, 80–84]. Challenges include identification and functional characterization of individual DRC subunits and testing the idea that the DRC, in part, localizes kinases and phosphatases required for regulation of dynein activity.

The axoneme bears at least eight different axonemal dynein motors, each localized to a unique position in the 96 nm repeat and apparently responsible for distinct features of ciliary movement (Fig. 3; [85]). Most generally, the dynein arm structures are categorized in two rows – the outer dynein arms (ODA) and the inner dynein arms (IDA; Fig. 1 and 2). The outer and inner rows of dyneins are structurally and functionally distinct: the outer dynein arms are homogenous in composition and structure, whereas, the inner dynein arms are complex, composed of at least seven distinct dynein isoforms each localized in a fixed pattern in the 96-nm repeat (Fig 2). Studies in several experimental systems indicate ciliary beat frequency is regulated by the ODA and that beat frequency can be regulated by phosphorylation [18]. In addition, phosphorylation plays a role in control of the inner dynein arms and regulation of axonemal bending. In this article, we feature the inner arm dynein called I1 dynein: data has revealed I1 dynein displays unusual properties and is required for normal control of the size and shape of the ciliary/ flagellar bend (*i.e.* the amplitude and the degree of curvature of flagellar bending) [19, 86]. Genetic and biochemical analysis reveals that the key I1 dynein regulatory phospho-protein is IC138 [21, 87–90]. Notably, the key kinases and phosphatases that control I1 dynein are physically localized to the axoneme. Therefore, we argue these enzymes must be relatively abundant, targeted and anchored near I1 dynein, regularly repeating every 96 nm on the doublet microtubules (Fig. 3). Alternatively, and of equal interest, the kinases and phosphatases that regulate IC138 may be localized to subsets of doublet microtubules. This result would be quite important since it would further address a functional axis of the axoneme (defined in Fig. 1) important for modulation of bending movement.

3. A “sliding microtubule-switching” model for oscillatory ciliary bending

Before proceeding to a discussion of the axonemal kinases and phosphatases and how they might regulate motility, it is important to describe a working model for ciliary bending (Fig 1 and 4). Seminal work, using multiple experimental systems including isolated motile axonemes, has revealed that the basis for ciliary motility lies in controlled dynein-driven microtubule sliding- a “sliding microtubule” model for ciliary bending [91–96]. In addition, it was determined that the dynein motors generate force in one direction (minus-end direction) relative to microtubule polarity [97, 98]. Thus, since all dyneins are minus-end motors, this observation directly led to a “switching” model for alternating effective (principal/forward) and recovery (reverse) bending: dyneins on one side of a structural and functional axis (Fig. 1) of the axoneme are active for bending in one direction. Dynein activity and associated microtubule sliding are regulated, in part, by a phospho-regulatory mechanism (described below) that includes the central pair and radial spoke structures. One model indicates that the central pair and its associated appendages acts as a distributor [60, 99], selectively stimulating specific radial spokes to alter dynein activity on specific outer doublets.

Predictably, when the direction of bending changes or “switches”, there is a switch in activity where the previously inactive dyneins, on one side of the axis, are activated, and the formerly active dyneins on the opposite side, are turned off [100, 101]. The precise mechanism for sensing the end point of each bend direction is not understood, but clearly for oscillatory bending, dynein motors must be regulated. Detailed experimental evidence indicates the basic oscillatory movement and “switches” in bend direction are inherent properties of the dynein motors involving microtubule curvature and a mechanical feedback control [102, 103]. The switching sensor is not known, but Patel-King and King (2009) propose a model in which the outer dynein arm plays a role as a sensor of microtubule curvature [104]. Thus, the phospho-regulatory mechanism discussed in this chapter is not responsible for control of the basic oscillatory bending in ciliary axonemes, a mechanism that does not appear to require any form of alternating posttranslational modification.

Rather, the phospho-regulatory mechanism discussed here is superimposed on the basic dynein – driven mechanism to modulate beat frequency or the size and shape of the bend, parameters we will refer to as ciliary waveform.

4. Kinases targeted and localized to the 9+2 axoneme

PKA, PKG and A-kinase anchoring proteins (AKAPs)

Both direct and indirect approaches have revealed that PKA and PKG are associated with and regulate axonemal motility by phosphorylation [18]. This includes a wide range of studies indicating that, for example, sperm motility is activated or altered by cyclic nucleotides [105–112]. However, only a few studies have focused on the kinases directly localized to the axoneme for control of motility. In most cases studied to date, the upstream regulator of phosphorylation is calcium [18, 63, 113–115].

The focus on kinases associated with the axoneme began with biochemical and physiological assays using isolated ciliary axonemes or detergent extracted “models” [116, 117]. For example, classical studies using ciliary axonemes or detergent extracted *Tetrahymena* and *Paramecium* cells revealed that cAMP and cGMP alter ATP –induced ciliary beating [114, 118, 119]. Consistent with the functional and pharmacological analysis, PKA has been isolated and characterized from axonemes [120] and may alter dynein activity in cilia from *Paramecium* [121]. Additional biochemical and functional evidence indicated one of the axonemal substrates is a 29-kDa outer dynein arm light chain [122–124] and that phosphorylation leads to increased ciliary beat frequency consistent with the known role of the outer dynein arms for control of beat frequency. In studies of mammalian respiratory airways, cGMP and PKG have been shown to modulate ciliary beat frequency [115, 125–128], while, in *Paramecium*, cGMP alters the direction of ciliary beating and PKG–dependent phosphorylation of ciliary targets is required for normal ciliary motility [114, 129, 130]. Furthermore, in studies of *Corbicula fluminea*, a freshwater clam, sperm motility is reduced upon the addition of a pharmacological inhibitor of PKG [131].

Similar studies with ciliary axonemes from mammalian epithelia also reveal that outer dynein arm-mediated changes in beat frequency correlate with increased cyclic nucleotide second messengers [18, 127, 132–135] and that the ciliary axoneme bears AKAPs (see below [136]), PKA and PKG. However, to date, the regulatory phosphoprotein substrates are not defined with certainty in the mammalian axonemes, but the substrates must be part of a regulatory pathway that mediates outer dynein arm activity for control of ciliary beat frequency and could include dynein subunits such as the 29 kDa subunit cited above or the outer arm dynein heavy chains [137]. Since these studies make use of the intact, isolated 9+2 axoneme, predictably the kinases are targeted and anchored in the axoneme near the substrates by specialized scaffold proteins such as the AKAPs (discussed below).

Genetic and *in vitro* studies using isolated axonemes from *Chlamydomonas* have also revealed that inner arm dynein activity is regulated by phosphorylation and that the kinases are located in the axoneme. Based upon functional assays and the use of the PKA inhibitor, PKI, one of the kinases is PKA [138], and, as discussed further below, another axonemal kinase that controls inner arm dynein phosphorylation is CK1 [21, 24]. Genetic and biochemical evidence reveals that the dynein target is I1 dynein and its regulatory subunit IC138 (Fig. 5) and much experimentation has been devoted to their study [21, 87–90, 139, 140]. Notably, this mechanism was revealed in axonemes defective in control of kinase activity. Mutations that result in failure of assembly of the central pair or radial spokes result in ciliary paralysis. Part of the reason for paralysis is a failure in normal control of the axonemal kinases that appear to become constitutively active in these mutants leading to a global inhibition of the dynein motors.

Chlamydomonas mutants that are defective in I1 dynein or exhibit hyperphosphorylated IC138 display abnormal flagellar waveform, defective dynein – driven microtubule sliding and failure in cellular phototaxis [19]. As indicated in Figure 5, there appears to be a tight correlation between IC138 phosphorylation and regulation of microtubule sliding: inhibition of microtubule sliding correlates with phosphorylation of IC138; active or rescued microtubule sliding correlates with de-phosphorylation of IC138. We do not yet know how phosphorylation alters dynein activity. Much research in several labs is now focused on the downstream kinases and phosphatases that regulate IC138 and I1 dynein. One of the kinases is CK1 (see below), and to date, the only mechanism for localizing PKA in the axoneme is through AKAPs [141–143].

AKAPs are proteins that interact with the regulatory subunits of PKA and confer sub-cellular localization [134, 135] and have been identified in the axoneme [22, 23, 136]. Based on biochemical analysis, the *Chlamydomonas* flagellar axoneme has at least two AKAPs: one located in the central pair apparatus (AKAP240) and the other located at the base of the radial spokes (RSP3) (Fig. 3; [22]). To further test the hypothesis that RSP3 is an AKAP and responsible for regulation of flagellar motility, an RSP3 gene containing a mutation in the PKA-binding domain was transformed into a null mutant of RSP3 (*pfl4*) and the motility of the transformants was analyzed [23]. The results indicated that disruption of the PKA-binding domain results in failure of motility in a manner that is consistent with a role for PKA, and precise localization of PKA, in control of flagellar motility. RSP3 is a highly conserved protein of all motile cilia [72]: thus, predictably, the RSP3 AKAP plays a conserved regulatory role in all cilia and flagella. Further tests of these ideas now require the identification and characterization of PKA in *Chlamydomonas* flagella and analysis of RSP3 in cilia from other organisms. Moreover, these AKAPs are predicted to localize PKA in the axonemal structure, placing PKA in position near its substrates, including IC138 of I1 dynein.

An axonemal CK1 regulates dynein – driven microtubule sliding

Experimental analysis of isolated *Chlamydomonas* ciliary axonemes has implicated the protein kinase, CK1, in regulation of dynein-driven motility [21, 24]. CK1 is a highly conserved serine/threonine kinase that has multiple cellular functions including regulation of the cell cycle, apoptosis, motility, organelle transport, circadian rhythms and regulation of developmental pathways such as the *Wnt* pathway [144–146]. CK1 kinases are monomeric enzymes with constitutively active enzymatic activity, utilizing ATP as an exclusive phosphate donor (139). Several CK1 isoforms have been described in yeast and mammalian systems. They share common signature domains including the ATP- and substrate-binding domains, the catalytic triad, nuclear localization signal and a kinesin-homology domain [146].

Using CK1-specific inhibitors, it was shown that the characteristic slow-sliding velocities of paralyzed radial spoke mutant *Chlamydomonas* axonemes were restored to wild-type levels and that IC138 phosphorylation / dephosphorylation correlates with control of microtubule sliding [21, 24]. *In vitro* assays using recombinant CK1 proteins demonstrate that an enzymatically functional CK1 is required to inhibit I1 dynein-dependent microtubule sliding [24]. CK1 is located in the flagellar axoneme in *Chlamydomonas* [21, 24, 25] where it functions as a downstream component of the central pair-radial spoke mechanism and together with I1 dynein, controls flagellar waveform.

Several functions of CK1 involve interaction with the cytoskeleton, presumably for localization of CK1 near its substrates [145, 147–150]. However the molecular mechanisms for targeting CK1 within the cell are not well understood. It is likely that CK1 is targeted to the outer doublet microtubules by a type of CK1-anchoring protein (CKAP) analogous to

AKAPs. This model could define a basis for regulating CK1 activity and thereby ciliary motility. Identification of such a putative CKAP could define a general class of proteins that localize CK1 in the cell to direct and regulate CK1 function.

5. Axonemal Phosphatases

Biochemical and pharmacological analyses in diverse experimental systems have revealed that protein phosphatases are also localized to the cilium [20, 25, 151–159]. In addition, the role of PKA and CK1 in regulation of microtubule sliding in axonemes from *Chlamydomonas* was first defined by taking advantage of mutant cells defective in normal control of the kinases and resulting in constitutively active axonemal kinase activity [21, 60, 87, 138, 160]. A consequence of the mutations and the constitutively active axonemal kinases was paralyzed flagellar axonemes and inhibition of dynein. Consistent with this interpretation, and illustrated in Figure 5, kinase inhibitors “rescued” microtubule sliding in isolated axonemes [21, 138]. Therefore, since protein kinase inhibitors rescued microtubule sliding in isolated axonemes, predictably the axonemes must contain protein phosphatases required for de-phosphorylation of phospho-proteins and increased microtubule sliding [20, 160, 161].

Consistent with this prediction, Habermacher and Sale (1996), determined that phosphatase inhibitors, including microcystin LR and okadaic acid, when applied before or with the kinase inhibitors, block kinase inhibitor-dependent rescue of microtubule sliding [161]. Subsequent biochemical, molecular and proteomic analyses revealed the axoneme contains several phosphatases including the highly conserved Protein Phosphatase 1 (PP1) and Protein Phosphatase 2A (PP2A) [25, 162–164]. PP1 is a highly conserved serine/threonine phosphatase comprising one catalytic subunit and a range of regulatory mechanisms [165–167]. PP1 has been shown to be involved in various cellular processes such as mitosis, meiosis, transcription, apoptosis, cell cycle progression, and cell division [168, 169]. PP1 was shown to exist in flagella using microcystin-sepharose affinity purification [20]. Analyses of flagella from *Chlamydomonas* mutants revealed that PP1 is primarily, but not exclusively, anchored in the central pair apparatus, and is associated with the C1 microtubule [20]. Thus, PP1 is thought to be part of the central pair-radial spoke signaling mechanism that controls axonemal dynein activity. A small fraction of PP1 is also present on the outer doublet microtubules, possibly in close association with the dyneins.

PP2A is a highly conserved serine/threonine phosphatase important for normal development, cell cycle, and other many cell processes [169]. The PP2A heterodimeric core enzyme comprises one scaffold subunit (A-subunit) and one catalytic subunit (C-subunit), while the PP2A heterotrimeric holoenzyme comprises the A- and C- heterodimer and any one of a family of regulatory subunits called B - subunits (also known as B55 or PR55), B' (B56 or PR61), B'' (PR48/PR72/PR130), or B''' (PR93/PR110). By using microcystin-sepharose affinity purification, a scaffold subunit and a catalytic subunit of PP2A were identified in *Chlamydomonas* flagellar axonemes [20]. In *Chlamydomonas*, all three subunits (Scaffold, Regulatory, and Catalytic) of PP2A have been identified biochemically or in the flagellar proteome [20, 25]. Moreover, analysis of structural mutants revealed the axonemal PP2A is localized to the outer doublet microtubules, presumably in position to regulate the dynein motors.

Based on proteomic (see below) and biochemical analyses, additional protein phosphatases have been identified in ciliary and flagellar fractions, including recent analysis of primary cilia [151–153, 155, 164, 170]. In many cases, the phosphatases are localized to the membrane – matrix fractions, where they likely play important roles in control of ion channels, ATPases or receptors [156, 159]. Consistent with these ideas, in *Paramecium*,

PP1 is involved in a sustained backward swimming response to depolarizing stimuli, suggesting PP1 functions on the ciliary/flagellar membrane [157]. However, to date, other than PP1 and PP2A, we do not have an idea for the functional roles of other axonemal phosphatases. The mechanism for anchoring of such a diverse pool of phosphatases in the axoneme is largely unknown; presumably, they are anchored by specialized proteins similar to the axonemal kinases or by the same kinase-anchoring proteins [171–173]. In summary, PP1 is primarily localized to the central pair, possibly acting upstream in a pathway that regulates the ciliary dynein motors and PP2A and a small fraction of PP1 are localized to the outer doublet microtubules, presumably near the dynein substrates, as downstream phosphatases (Fig. 3).

6. Proteomic studies—and future opportunities

Several studies have recently focused on identifying all ciliary proteins. These studies include proteomic analyses of isolated motile and sensory cilia and ciliary fractions (axonemes, membranes and matrix proteins) as well as comparative genomic approaches (<http://v3.ciliaproteome.org/cgi-bin/index.php>) [25, 44, 45, 162–164, 174–177]. These approaches contributed to a revolution in our understanding of cilia, revealing conserved genes that, when defective, result in diseases and syndromes in the human. For example, the genomic analysis of Li et al., (2004) contributed to discovery of a link between cilia and Bardet-Biedel Syndrome [178] and helped lead investigators to discovery of the “BBSome” and mechanisms for trafficking ciliary membrane proteins [179–181].

The comparative genomic approaches omitted the conserved kinases and phosphatases that function in cilia because they also have non-ciliary roles within the cell. The proteomic studies, using tandem mass spectrometry coupled with interrogation of available genome databases, have identified several classes of signaling proteins including kinases, phosphatases, calcium-binding proteins, AKAPs and others [25]. A comprehensive proteomic analysis of the *Chlamydomonas* cilium and ciliary fractions identified 20 kinases, 11 phosphatases, 9 GTP-binding proteins, 27 EF hand calcium-binding proteins, 5 ion channels, 1 IP3 receptor and 2 14-3-3 proteins indicating that the *Chlamydomonas* cilium is rich in signaling proteins and that motile cilia and flagella also function as sensory/signaling organelles. The challenge is to determine the functional role of each ciliary component. In addition, genes predicted to encode candidate cAMP and cGMP dependent protein kinases were identified. These studies have provided the foundation for characterization of specific signaling proteins and their role in regulation of ciliary motility.

Clearly, reversible phosphorylation is a critical feature of control of ciliary movement. Both biochemical and proteomic analyses demonstrate that the axoneme houses a large repertoire of phosphoproteins [26, 68]. Using ³²P labeling of axonemal proteins and 2D SDS-PAGE analysis, more than 80 axonemal phosphoproteins were identified *in vivo* and at steady state, five of which are known radial spoke stalk components [68], providing a preview that the radial spoke is an exceptionally complex axonemal signaling structure [70]. Given the large number of axonemal kinases, phosphatases and phosphoproteins, and the important role of phosphorylation in ciliary function, it is critical to identify the exact substrates in the phosphoregulatory pathways. To begin to address this question, Boesger and colleagues took a proteomics approach to identify ciliary phosphoproteins [26]. Using isolated cilia from *Chlamydomonas* and immobilized metal affinity chromatography, this study identified 141 phosphopeptides with 126 phosphorylation sites belonging to 32 ciliary proteins including structural and motor proteins, kinases, EF hand proteins, FAPs (Flagellar Associated Protein —proteins identified in the *Chlamydomonas* flagellar proteome (25)), and many uncharacterized ciliary proteins. Importantly, phosphorylation sites were determined in IC138 and radial spoke protein 17 (RSP17). This important study has provided a new and

important database of candidate substrates with specific phosphorylation sites which can be tested for their role in motility. One opportunity is to couple this sensitive method for identifying the phospho-proteins in the axoneme with a useful selection of *Chlamydomonas* mutant axonemes defective in control of kinase or phosphatase activities.

Important questions for the future include (1) how does phosphorylation of dynein subunits alter dynein activity? (2) How do the kinases and phosphatases, and yet to be determined substrates located in the axoneme, modulate the dynein motors? (3) How do signals, initiated in the central pair and transmitted through the radial spokes, affect dynein activity? (4) What is the link between calcium signaling and axonemal protein phosphorylation? These questions, and others, will be best addressed using a combination of model organisms and useful, informative functional assays for ciliary motility.

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Figure 1. Cross section of ciliary and axonemal structure

A cross-section through a *Chlamydomonas* cilium shows the ciliary membrane which surrounds the internal axoneme structure. The axoneme is composed of a specific “9 + 2” arrangement of microtubules; 9 outer doublet microtubules, each comprised of an A- and B-tubule, surrounding a pair of internal microtubules called the central pair (CP; C1 and C2). The central pair has associated structures (grey), which transiently contact the radial spokes (RS) to generate signals which regulate the inner and outer dynein arm motors (ODA and IDA). Connecting the outer doublets are structures including the DRC-nexin link and the 5–6 bridge. The line through the cilium depicts a structural and functional ciliary axis. Dyneins on one side of the axis (on the orange outer doublets) are active for bending in one direction. When the direction of bending changes, the location of dynein activity switches; *i.e.* the active dyneins (on the orange outer doublets) become inactive and the dyneins on the opposite side of the axis (on the blue outer doublets) become active.

Figure 2. Predicted location of axonemal signaling proteins

The diagram depicts a cross-section view of the central pair, one radial spoke and one outer doublet microtubule. The outer and inner dynein arms (ODA and IDA respectively) are anchored to the outer doublet microtubules. Regulatory kinases and phosphatases include PP1 (located primarily in the central pair with a small amount on the outer doublets, possibly near the ODA), PP2A (located on the outer doublets, possibly near I1 dynein), CK1 (located on the outer doublets, possibly near I1 dynein), and PKA (predictably located at the base of the radial spoke and in the central pair by the RSP3-AKAP and AKAP240 respectively). Also shown are the predicted locations of calcium-sensitive regulatory complexes that contain calmodulin.

Figure 3. Schematic of a longitudinal view of the 96-nm repeat

A longitudinal view of an outer doublet microtubule with the dynein motors (ODA and IDA), radial spokes (S1 and S2), and the DRC-nexin link. These structures are arranged in a precise manner which forms a 96-nm structural element that repeats along the entire length of the axoneme. Within each 96-nm repeat, there are four ODA, two RS, one DRC-nexin link (DRC), and one copy each of several different types of IDA motors. The IDA shown in red is called I1 dynein (also known as dynein-f). This particular motor is regulated by a phosphorylation by a mechano-chemical mechanism that involves the central pair and radial spoke structures.

Figure 4. Dynein-driven microtubule sliding in cilia and flagella

The dynein arms attach to the A-tubules of the outer doublets and the motor heads transiently interact with the B-tubules of the adjacent outer doublets. Movement of the motor domains in the minus end direction toward the base of the cilium (white arrows on the red dynein heads) causes the A-tubule of one doublet to slide toward the base of the adjacent B-tubule pushing that outer doublet tipward (black arrow). Because both outer doublets are anchored at the minus end by the basal body and connected along the length by the DRC-nexin links, this sliding movement forces the outer doublets to bend, resulting in a ciliary bend.

Figure 5. Model for regulation of I1 dynein by phosphorylation

I1 dynein activity is regulated by phosphorylation of IC138. When phosphorylated, I1 dynein is inactive; upon dephosphorylation, I1 dynein is active. The kinases and phosphatases involved were identified based on pharmacological data using inhibitors and include CK1, PKA, PP1 and PP2A. The central pair (CP) and radial spokes (RS) function, in part, to regulate the kinases *in vivo*; when mutated, the kinases are constitutively active, resulting in uniform phosphorylation of I1 dynein and global inactivation of dynein activity. This inactivation can be relieved, *in vitro*, by the addition of kinase inhibitors. Consequently, *in vitro* inhibition of phosphatase activity prevents dynein activation.