A Structural Basis for How Motile Cilia Beat

PETER SATIR, THOMAS HEUSER, AND WINFIELD S. SALE

*The motile cilium is a mechanical wonder, a cellular nanomachine that produces a high-speed beat based on a cycle of bends that move along an axoneme made of 9+2 microtubules. The molecular motors, dyneins, power the ciliary beat. The dyneins are compacted into inner and outer dynein arms, whose activity is highly regulated to produce microtubule sliding and axonemal bending. The switch point hypothesis was developed long ago to account for how sliding in the presence of axonemal radial spoke–central pair interactions causes the ciliary beat. Since then, a new genetic, biochemical, and structural complexity has been discovered, in part, with *Chlamydomonas* mutants, with high-speed, high-resolution analysis of movement and with cryoelectron tomography. We stand poised on the brink of new discoveries relating to the molecular control of motility that extend and refine our understanding of the basic events underlying the switching of arm activity and of bend formation and propagation.*

Keywords: cilia, eukaryotic flagella, dynein, microtubules, axoneme, motility

Motile cilia are the earliest known cell organelle, discovered around 1647, when van Leeuwenhoek first saw ciliated protists through his microscope. Although the question of the mechanism of ciliary movement intrigued biologists from that time forward and was the topic of considerable speculation (Gray 1928), only with the beginnings of biological electron microscopy in the mid-twentieth century and the arrival of modern cell biology a few years later did a plausible model emerge. The key to the model was an understanding of the ciliary ultrastructure. The studies of several investigators (Manton 1953, Fawcett and Porter 1959, Afzelius 1959, Gibbons IR and Grimstone 1960, Gibbons IR 1961) established the universality of the 9+2 motile ciliary axoneme and its significant substructural elements, including radial spokes and the arms, which Gibbons IR and Rowe (1965) showed were the ciliary ATPases, which they named *dyneins*. The typical flexural movement of cilia and the undulatory movement of flagella beating is shown in figure 1. The figure was adapted from the results of Brokaw and Kamiya's (1987) quantitative analysis of forward and reverse swimming *Chlamydomonas*. Figure 2 shows an early electron micrograph of the 9+2 pattern of the gill cilia of the mussel, a standard experimental organism for early cilia studies (Warner and Satir 1974), and a diagram illustrating the axis of the 9+2 structure relative to the bending plane.

Ciliary beat form has been well studied. Although it could sometimes be highly variable, even on the same cell, the typical cilia beat is usually a short, apparently stiff effective stroke, in which the cilium points in the direction of force generation, and a flexible recovery stroke, in which the tip is more slowly drawn backward (figures 1a and 3), whereas typical sperm with 9+2 cilia tails (called *flagella*) beat with an undulatory motion (figure 1b). Both flexural and undulatory motion (figure 1) cause fluid flow, moving water or mucus along a ciliated surface (figure 1a) or propelling ciliated cells (figure 1b) through water at speeds up to about 1 millimeter per second, depending on parameters such as ciliary length and beat frequency. Almost all cilia operate in a low Reynolds number hydrodynamic regime, in which viscous forces are paramount and inertial forces can be neglected (Holwill 1974). In essence, the effective stroke in flexural motion (the open arrow in figure 1a) moves perpendicular to the cell surface, which induces a fluid velocity about double that of the recovery stroke, which moves more parallel to the surface. The hydromechanical aspects of ciliary motion have been extensively analyzed and modeled (e.g., Blake and Sleight 1974, Smith DJ et al. 2007).

In the context of 9+2 axonemal mechanisms, there are no outstanding differences between the organelles originally called *cilia* and those called *flagella*. Early models suggested that to produce a stroke, the nine doublet microtubules of

Figure 1. Bending patterns of ciliary (a) and flagellar (b) movement in *Chlamydomonas* captured by high-speed flash photography. The figures illustrate the flexural patterns of movement typical of epithelial cilia and the undulatory patterns of movement typical of eukaryotic flagellar bending. The grey arrows indicate the direction of cell movement. The black bar at the base of the cilia or flagella is 5 micrometers long and marks the site of ciliary or flagellar attachment to the cell. The open arrow in panel (a) indicates the direction of the forward ciliary bend. The open arrow in panel (b) indicates the direction of flagellar bend propagation. Adapted with permission from Brokaw and Kamiya (1987).

the 9+2 pattern contracted in sequence, and this seemed biophysically plausible, but with the growing understanding that muscle contraction depends on sliding actin and myosin filaments, people began to consider that ciliary contraction might also be caused by sliding. What elements would slide? When he first saw the arms, Afzelius (1959) proposed that they might cause the doublet microtubules to slide.

Satir (1963) approached the problem by attempting to use the electron microscope to capture structural changes within the cilium as its beat form changed. The beat could be stopped by dropping an osmium tetroxide solution on a preparation beating with metachronal waves. Several investigators, beginning with Gelei (1925, cited in Satir 1963) had fixed the metachronal wave on protozoa, capturing the cilia in successive positions of a beat along a wavelength. Satir (1963, 1965) fixed the metachronal wave of the lateral cilia of a mussel, capturing the cilia pointing in different directions during a stroke through electron microscopy for the first time. Before fixation, the cilia had a beat frequency of approximately 17 hertz (Hz), so a beat occupied around 60 milliseconds (ms). Thirty-one individual cilia were measured along an 11-micrometer (μm) wavelength in the fixed preparation so that the cilia were captured with a phase difference of about 2 ms between adjacent cilia (Satir 1967).

Satir (1963, 1965) reasoned that, after such a quick fixation, examining the fine structure of the axoneme of cilia captured in different positions would show how contraction occurred. What to examine was not obvious, but then Roth and Shigenaka (1964) showed that, in certain axonemes, the tips did not retain the 9+2 pattern; some of the doublets became singlets. One could imagine that if some of the doublets contracted to bend the cilium in—say—the direction of the effective stroke, the doublets that contracted would become singlets and would disappear from the tips

before the doublets on the opposite side (that of the recovery stroke). Then, when the cilium was bending in the direction of the recovery stroke, just the opposite result would be seen: The doublets that contracted and disappeared would be those on the recovery stroke side, whereas those on the effective stroke side would persist.

To test this, one needed to unequivocally identify the axonemal doublets in a tip cross section. Fortunately, the lateral gill cilia had a special bridge between two doublets (figure 2), which seemed to be in the same position in every cross section and which seemingly identified the doublets in the direction of the effective stroke. It was assumed that the bridge was stable in these cilia and that the doublets identified were doublets 5 and 6, according to conventional numbering (figure 2). A further assumption was that

all doublets were of equal morphological length in a straight cilium before the bending occurred.

An initial demonstration (Satir 1965) showed that the doublets at the tips of effective pointing and those of recovery pointing cilia disappeared in different sequences, with doublets 4–6 persisting in the former after doublets 9, 1, and 2 (1 is adjacent to 9 at the end of the sequence) ended and with doublets 9, 1, and 2 persisting in the latter after 4–6 had ended (figure 3). This result was not compatible with the simple contractile model as it was stated but was, in fact, the complete opposite of what was expected. However, it could be explained if, in order to accommodate a bend, the doublets slid past each other without changing in length. Although it was controversial at the time, this became the original evidence for the sliding microtubule model of ciliary motility.

The model was strengthened in an important way with Brokaw's (1965) demonstration that bends on sea urchin and other flagella were circular arcs, whereas the unbent regions were ruler straight. This analysis was applied to the mussel gill cilia in different beat positions and shown to be correct (Satir 1967). The simplified geometry of ciliary bending permitted easy calculation of the amount of tip displacement (Δl_n) that would be generated by a bend ($\Sigma\alpha$) in the effective and recovery pointing cilia—that is,

$$\Delta l_n = d_n \Sigma\alpha,$$

where d_n is the effective diameter of the axoneme to doublet n and $\Sigma\alpha$ is the sum of the bends on the cilium, measured in radians. For doublets 1 and 5, which are on opposite sides of the axoneme, d_n is simply the axonemal diameter, about 0.2 μm , and the value of Δl expected at the tip is 3.5 nanometers (nm) per degree ($^\circ$) of bend. The bend that

Figure 2. Transverse section of motile cilium. (a) An electron micrograph of a single cilium from the lateral cilia of the clam gill viewed from inside the cell out toward the distal end of the cilium. Doublet 1 is located at the 12 o'clock position, and the other outer doublet microtubules are numbered clockwise in the direction in which the dynein arms point. Also illustrated is the 5–6 bridge. The lateral cilia beat in a plane defined by doublet 1 and the 5–6 bridge, and in these cilia, the effective or forward bend direction is toward the 5–6 bridge. Source: Reprinted with permission from Warner and Satir (1974). **(b)** Diagram illustrating the main features of the 9 + 2 axoneme from motile metazoan cilia, including the outer doublet and central pair microtubules, the outer and inner dynein arms, the central pair projections, the 5–6 bridge, the radial spokes, and the dynein regulatory complex and nexin links.

forms at the beginning of the effective stroke is about 100°, giving a maximum predicted displacement of about 100 nm between adjacent doublets.

The equation could be tested by serial sectioning of the cilia tips of bent cilia in the fixed metachronal wave and measuring Δl directly. Each section was approximately 100 nm thick. Within measurement error, both for effective and recovery pointing cilia, the measured tip displacement

matched the predicted value (figure 3; Satir 1968). Moreover, several different patterns were seen at the tips, which correspond to snapshots of cilia with different total bends along the axoneme during a fixed beat. The tip patterns and the extent of doublet displacements were later confirmed by unrolling isolated ciliary axonemes of *Tetrahymena* (Sale and Satir 1976).

A major objection to this analysis is that fixation could cause relaxation of the contracted microtubules back to their original lengths. It could be argued that this is unlikely because fixation is so fast and because multiple changes along the axoneme—to be discussed in a moment—are not consistent with relaxation. The issue was settled in an unequivocal way by Summers and Gibbons (1971), who used dark field microscopy to directly demonstrate sliding between outer doublet microtubules.

By the early 1970s, it became clear that if the ciliary membrane was carefully removed with a mild detergent (such as Triton X-100) treatment, the resultant naked axoneme could be reactivated to beat by the addition of adenosine triphosphate (ATP). Gibbons BH and Gibbons (1972) showed that, for sea urchin sperm, the reactivated beat was identical to the beat of living sperm, which meant that virtually the entire mechanism of bend production and control resided in the axoneme, a conclusion confirmed in many later experiments. The membrane kept the ATP concentration and various ions at the required level for motility. It also and provided signals such as cAMP (cyclic adenosine monophosphate) or calcium ions (Ca^{2+}), which acted on proteins in the axoneme to influence ciliary behavior.

Summers and Gibbons (1971) used this preparation of axonemes but digested them briefly with trypsin before adding ATP. A transmission electron microscopy analysis showed that the radial spoke integrity was affected by the trypsin. Instead of reactivating the beating, these axonemes now slid apart, eventually telescoping to eight or nine times the length of the original axoneme. This result supported the conclusion that the basic interaction in ciliary motility was doublet sliding, powered by dynein arm activity, such that the arms on any axonemal doublet (n) would produce sliding of the adjacent doublet ($n + 1$). Evidently, dynein arms could be activated along the length of every doublet, and almost every doublet in an axoneme was capable of sliding. Furthermore, the difference between sliding and bending was thought to be due to the integrity of the radial spokes and interdoublet links.

So, there are two systems within the axoneme necessary for ciliary motility: (1) a trypsin-insensitive force-generating system based on dynein-microtubule interactions capable of generating unconstrained, isotropic sliding of sometimes over 10 μm per doublet without systematic bend formation or propagation and (2) a trypsin-sensitive control system, depending on spoke–central sheath and other interactions, where a sliding maximum about 0.1 μm per doublet (i.e., between adjacent doublets) is strictly coupled to systematic bend formation and propagation at high frequency

Figure 3. Diagrams illustrating (a) the phases of ciliary bending and (b) the geometry of a sliding microtubule model for ciliary bending. The details of each diagram are discussed in the text and illustrate the geometry of microtubule sliding for effective and reverse bends for microtubules anchored in the basal body and free to slide at the distal axoneme. The model is based on the discovery that microtubules are inextensible and that the bends are in the form of circular arcs. Source: Reprinted with permission from Satir (1968).

(up to 100 Hz). Brokaw (1989, 1991) provided direct confirmation and a critical, quantitative analysis of doublet sliding during bend generation and propagation in undulatory motion. The relative motion of 40-nm gold beads bound to the exposed outer doublet microtubules sperm flagellar axonemes during ATP-reactivated swimming is consistent with a sliding model of doublet interactions. Vernon and Woolley (2002) were able to see sliding at the tip consistent with a switch point model in mammalian sperm.

The waveform and direction of a bend progression are epiphenomena dependent on the timing of sliding, switching, and control events within the axoneme and are subject to change. A major demonstration of this conclusion is the change from forward swimming flexural motion to undulatory motion leading to backward swimming and back again

in *Chlamydomonas* flagella showing an avoidance reaction (compare ciliary, figure 1a, with flagellar motion, figure 1b). This change can be demonstrated with demembranated, ATP-reactivated *Chlamydomonas* cell models or flagellar apparatuses through the addition or removal of Ca^{2+} from the reactivation solution (Hyams and Borisy 1978, Kamiya and Witman 1984).

Sale and Satir (1977) extended the Summers and Gibbons (1971) experiments with a transmission electron microscopy analysis of sliding axonemes of *Tetrahymena*. After sliding, the overlap between adjacent doublets could be studied. They found that doublet $n + 1$ was always positioned tipward of doublet n in such overlaps. They concluded that the dynein arms had a uniform polarity, always walking the doublet to which they were permanently attached toward the ciliary base. With the advent of systems to study microtubule polarity, the ciliary base was defined as the doublet's negative end, and axonemal dyneins in aggregate were therefore a minus end motor. Tests of isolated outer dynein arms and many inner dynein arm components in *in vitro* assays confirmed this conclusion (Vale and Toyoshima 1988).

The consequence of a uniform polarity of active sliding means that, during ciliary beating, only some doublets or portions of the doublets have active arms at any one time. When doublet $n + 1$ is moving baseward of doublet n , the movement is passive and the arms of doublet n are, overall, inactive. Careful examination of mussel gill cilia showed that, during the effective stroke, the dou-

blets on about half of the axoneme generated active sliding, whereas the opposite half's sliding was passive, and during the recovery stroke, the halves reversed (Satir and Matsuoka 1989). During a ciliary beat, sliding activity switches between the two halves of the axoneme defined by a line that runs between the central pair and that bisects the bridge and doublet 1 (figure 2). This hypothesis is the *switch point model* of ciliary beat (see also Morita and Shingyoji 2004, Hayashi and Shingyoji 2008).

The fixation of the metachronal wave permits one to follow the development of a bend at electron-microscope resolution. If spoke or interdoublet attachment to the central pair is at least partly responsible for bending, as the Summers and Gibbons (1971) experiments suggested, sliding should cause any attached spoke or link to be displaced by the amount

of sliding. When a bend originates at the ciliary base, the amount of sliding increases as the bend develops, and the spokes tilt, but neither the spokes nor the links continuously stretch as sliding continues to increase; instead, more spokes become involved along the axoneme as the bend grows.

Warner and Satir (1974) considered how spoke tilt might change as the bend grows along the axoneme. Within the bend as it grows, all spokes of each 96-nm spoke repeat group tilt to a maximum of 33° from normal. Because the spoke angles do not lie on radii through the center of the bend curvature and because sliding accumulates in the bend, the spokes must be attached to the sheath projections around the central pair when the bending occurs. The spokes are aligned with the two rows of projections along each of the central microtubules that form the central sheath. The projections repeat and form a vernier with the radial spokes in the precise ratio of six projection repeats to one spoke group repeat. As a basal bend develops, new distal spoke groups are added in quantal fashion.

The spoke tilt within an outer doublet microtubule 96-nm repeat unit remains constant as the bend grows to a maximum curvature, then as the bend passes, the spokes detach as the axoneme straightens. In straight regions of the axoneme, either proximal or distal to a bend, the relative position of the spoke groups between any two doublets remains constant for the length of that region, which means that sliding only takes place in regions of bending. In straight regions of the axoneme, almost all of the spokes of each group are normal (90°) both to their doublet of origin and to the central microtubules. All of these spokes must be considered, functionally, as detached from the sheath projections. The observed radial spoke configurations strongly imply that there is a precise cycle of spoke detachment–reattachment to the central sheath, which, we conclude, forms the main part of the mechanism converting active interdoublet sliding into local bending.

Although bending requires dynein arm activity, the locus of arm activity along the axoneme is still unknown (see below and Lin et al. 2014). It may be that arm activity along a doublet only occurs at the position of the bend. Nor is it known how many cycles of dynein arm activity are necessary to produce a 96-nm unit of bend on one doublet. *In vitro*, a single dynein is capable of causing sliding, but axonemal outer arm dyneins have a low duty phase:cycle ratio, which implies that, for smooth continuous motility during bend development, multiple dyneins acting out of phase must be involved. (In this article, we have not focused on dyneins' force-generating cycle; for excellent reviews, see Sakakibara and Oiwa 2011, Kikkawa 2013, and Roberts et al. 2013). A reasonable assumption is that, during bend development, every dynein—both inner and outer—within a spoke group on an active doublet potentially cycles once.

Shingyoji and colleagues (1977) performed an illuminating experiment. They attached a demembranated straight sea urchin axoneme to a microneedle and added a small amount of ATP iontophoretically to the middle of the axoneme.

Because the axoneme was straight and its ends in rigor, when the dynein arms became activated, they produced equal and opposite bends. Presumably, according to the switch point hypothesis, activity along half of the axoneme (doublets 1–5; figure 2) at one region is balanced counteracting activity of the opposite half (doublets 6–9 and 1; figure 2) further along the axoneme. Bending results in resistances to sliding, and mechanical strain produced by arm activity on the axoneme at one position can transmit the strain along the axoneme, probably via spokes and central pair attachments to activate countervailing arm activity in doublets at a more distant position—exactly what is needed for undulatory wave progression in the switch point hypothesis. Brokaw's (1985) curvature control model presents a simplified view of the transmission, which remains to be completely specified. In normal ciliary motility, the control of such transmission is very complex, which permits a wide variety of bending waves of different magnitudes and timing to progress along the axoneme. Moreover, the transmission of arm activity must occur at quite high speeds to permit the completion of a full beat cycle along a 10–15- μ m-long axoneme in less than 10 milliseconds in some instances. Additional questions about ciliary oscillation and bending are discussed in the excellent recent reviews in Brokaw (2009) and Lindemann and Lesich (2010).

Structural and biochemical complexity illuminates the unsolved problems of the switch point model

The switch point model was developed at a time when electron microscope resolution and axonemal biochemistry were in their infancies. It was assumed that outer and inner arm dyneins were mirror imaged identical molecules, that the sheath around the central pair was functionally symmetrical so that the central pair microtubules were essentially identical, and that axonemal biochemistry was essentially simple. None of these assumptions proved to be correct. To extend the model to account for motility required new structural, biochemical, and genetic approaches.

Importantly, the asymmetry in central pair organization necessarily means that, at any moment in the bending cycle, individual central pair projections interact with a radial spoke from a single unique outer doublet microtubule (Smith EF and Yang 2004). Therefore, one model is that the central pair controls the ciliary waveform by selectively regulating dynein activity, at any point in the beat cycle, on a single outer doublet microtubule or a subset of them. Furthermore, in addition to the asymmetry of the central pair apparatus common to motile cilia, in many organisms, including *Chlamydomonas*, the central pair rotates as the cilium bends (Smith EF and Yang 2004). Therefore, in these cases, the central pair is thought of as a distributor, and with rotation during the ciliary beat cycle, the central pair is thought to systematically interact with different radial spokes projecting from different outer doublet microtubules. Presumably, this leads to systematic activation of dynein motors at different phases of beating. However, the precise

role of central pair rotation in cilia in certain organisms is unresolved (Mitchell and Nakatsugawa 2004).

Asymmetry in the central pair apparatus also implies a role in the selective control of forward and reverse bending and the switch point discussed above and required for alternating ciliary bends. For example, in the comparison of movement between wild type and mutant *Chlamydomonas* flagella, Brokaw and colleagues (1982) determined that the central pair–radial spoke system is important for converting symmetric forward and reverse bending to the asymmetric bends that are required for effective flexural ciliary motion and normal physiological forces. More specifically, one of the functions of the radial spokes appears to be the relative inhibition of development of the reverse bend relative to the curvature of the forward bend (Brokaw et al. 1982).

The *hydin* mutation in *Chlamydomonas* and in the mouse has revealed a role for the central pair–radial spoke system in the control of switching between forward and reverse bends (Lehtreck and Witman 2007, Lehtreck et al. 2008). Remarkably, mutation in the *HYDIN* GENE results in a large defect in ciliary bending, but a structural analysis reveals only a very small defect in the axonemal structure. In both *Chlamydomonas* and the mouse, a single central pair projection is the only missing structural component in the mutant axonemes. In both *Chlamydomonas* and the mouse, the *hydin* mutation results in a relative hesitation or stalling between the forward and reverse bends. Therefore, the simplest interpretation is that the *hydin* mutation affects a switch point mechanism required for switching between forward and reverse bends (see the commentary by Smith EF 2007). Together, the data indicate the central pair–radial spoke system plays a central role in switching between and in the control of forward and reverse bends—that is, which doublets have active arms when a bend forms and progresses—and in the precise control of the curvature in each bend. However, these models require further direct testing.

***Chlamydomonas* mutants and structural analysis reveal functional specialization of many conserved axonemal dynein motors and regulators of motility**

Chlamydomonas mutants have been particularly informative, yielding insight into the composition and structure of the axoneme, the regulation of motility, the role of different dynein motors, and the assembly and length regulation of the cilium (Avasthi and Marshall 2012). Here, we focus on how *Chlamydomonas* has furthered our understanding of the mechanism and regulation of ciliary bending and, particularly, how it has contributed to knowledge of the general roles of the outer and inner dynein arms for the control of beat frequency and waveform.

Initially and most generally, the axonemal dyneins are referred to as the outer and inner dynein arms, but, in reality, there are many different, conserved dynein motors that are highly localized in the axoneme and that serve special purposes for the control of movement (King and Kamiya 2009). The best understood axonemal dynein is the outer

dynein arm. The outer dynein arms are biochemically very complex: each is composed of two or three distinct dynein ATPases and at least 16 different subunits. They are assembled individually in rows on doublet numbers 2–9 in *Chlamydomonas* axonemes and repeat at a regular 24-nm period along each outer doublet (see figure 4; Goodenough and Heuser 1982). Along most of the axoneme, each outer dynein arm is structurally—and, usually, biochemically—identical to its neighbor. Mutations in the genes that encode the structural proteins of the outer dynein arm or associated factors involved in outer dynein arm targeting and assembly can result in a failure to assemble all or a part of the outer dynein arm and, consequent, in defective motility (King and Kamiya 2009).

Consistent with the pioneering studies of Gibbons BH and Gibbons (1973), the most notable consequence of failure in assembly of the outer dynein arm is the reduced ciliary beat frequency (Brokaw and Kamiya 1987, Brokaw 1994, Kamiya 2002). The outer dynein arm and ciliary beat frequency can be regulated by phosphorylation and changes in calcium (Christensen et al. 2001, King and Kamiya 2009, King 2010). In the ciliates *Paramecium* and *Tetrahymena*, an increase in cAMP around the axoneme produces faster swimming by phosphorylating a small outer dynein arm–associated protein (Christensen et al. 2001). *In vitro*, the phosphorylated outer dynein arm causes faster sliding of microtubules, which implies that less time is taken to produce the same amount of sliding throughout a beat cycle—that is, that the beat frequency is faster. Diverse evidence also indicates the outer dynein arms respond to mechanical perturbation of the axoneme (Hayashibe et al. 1997). A mechanical feedback is probably important for switching between active and inactive states and required for forward and reverse bending. Consistent with a mechanical feedback control, as was discussed above (Shingyoji et al. 1977), bending of the axoneme can activate dynein-driven microtubule sliding (Morita and Shingyoji 2004, Hayashi and Shingyoji 2008). In addition, these data may also indirectly address an important model for the mechanical control of bending, called the *geometric clutch hypothesis*, which envisions changes in interdoublet distances corresponding to arm activity and bend production related to distortion of the axoneme during bending (for a full discussion, see Lindemann 2011). The predicted distortion in the axoneme during bending has been observed through electron microscopy (Lindemann and Mitchell 2007).

The inner dynein arms are much more complex than the outer dynein arms (for reviews, see Kamiya 2002, King and Kamiya 2009). Along an axonemal 96-nm repeat on a doublet microtubule (figure 4, which has four identical outer dynein arms) the inner dynein arms include at least seven different dyneins, which are distinct in composition and location (see Bui et al. 2012). The inner dynein arms were characterized through biochemical fractionation of dynein components and through electron microscopy of axonemes missing subsets of the inner dynein arms (King and Kamiya

Figure 4. The *Chlamydomonas* axoneme ultrastructure. Cryoelectron tomography slices show (a) a longitudinal, (b) a three-dimensional view, and (c) a cross-sectional view of a *Chlamydomonas* axoneme. The red boxes highlight one 96-nanometer (nm) axonemal repeat unit in each view. (d, e) Isosurface renderings and (f, g) a simplified schematic show an averaged 96-nm axonemal repeat in (d, f) longitudinal and (e, g) cross-sectional orientation. The cross-sectional slice is taken close to radial spoke 2, viewing from the proximal to the distal end. Key axonemal structures are highlighted: the A- and B-tubules (A_t , B_t), the nexin-dynein regulatory complex (N-DRC), radial spokes (RS1, RS2), the calmodulin and spoke associated complex (CSC), and the inner and outer dynein arms (IA, OA). The inner arm dyneins include the I1 complex (dynein f α and β) and dyneins a – g . Source: Adapted with permission from Heuser and colleagues (2012a, 2012b).

2009). In *Chlamydomonas* axonemes, which lack a subset of inner dynein arms, the ciliary waveform is altered, phototaxis is disrupted, and the swimming speed of the cells is slowed. A direct analysis of ciliary beating through high-speed video has confirmed that a failure in assembly of any of the individual inner dynein arms results in an altered ciliary waveform, a parameter that is essential to effective ciliary beating and physiology (Brokaw and Kamiya 1987).

The precise role of each inner dynein arm is not yet understood. However, one of the two-headed inner dynein arms, called *I1 dynein*, is thought to be particularly important for the control of axonemal bending. I1 dynein activity is regulated by kinases and phosphatases located in the axoneme (for a review, see Wirschell et al. 2011). Changes in I1 activity can be measured through changes in microtubule sliding velocity, but it is not yet known how changes in velocity corresponds to changes in bending. One possibility is that an increase in sliding velocity produced by the inner dynein arms without a change in beat frequency would correspond to an increase in bend magnitude. In addition, I1

dynein may regulate bending through control of the activity of other dyneins, including the outer dynein arms and the single-headed inner dynein arms (Kotani et al. 2007, Yamamoto et al. 2013).

In general, we do not yet know the function of each of the axonemal single-headed dyneins. However, an analysis of a mutant called *ida9* has revealed that one of the inner dynein arms, dynein *c*, is required for ciliary movement in a viscous fluid (Yagi et al. 2005). In addition, powerful screens have revealed new mutants that regulate inner dynein arm activity (Kamiya et al. 1991, Kamiya 2002). For example, screens have revealed the enzymes responsible for polyglutamylation and that this posttranslational modification of tubulin is crucial for the activity in a subset of the inner dynein arms (Kubo et al. 2010).

The central pair and radial spokes are required for normal ciliary motility and the control the dynein motors. In *Chlamydomonas*, a failure in assembly of the central pair or radial spokes results either in ciliary paralysis (Witman et al. 1978, Smith EF and

Yang 2004) or in greatly altered and unproductive bending movement. Together, the central pair apparatus and the radial spokes operate by both mechanical and chemical signaling to ultimately control axonemal dynein activity (Smith EF and Yang 2004). The mechanism of interaction between the central pair and the radial spoke was confirmed in recent experimental studies involving *Chlamydomonas* (Oda et al. 2014). They showed that the addition of nonspecific proteins to the radial spoke head could suppress paralysis of a paralyzed central pair mutant that was missing part of the central pair projections. The simplest interpretation of this result is that the added proteins permitted a physical interaction between the spoke head and the projections that is required to activate axonemal dyneins.

Transmission of signals from the central pair and the radial spokes to the dynein motors. The analysis of *Chlamydomonas* mutants has also revealed conserved axonemal components that transmit signals from the central pair and radial spoke structures to the dynein motors. Most notable are the calmodulin and spoke associated complex (CSC; Dymek et al. 2011, Heuser et al. 2012a) and the DRC; see the next section for insights from recent cryoelectron tomography (cryo-ET) analyses of the axoneme (for reviews, see Heuser et al. 2009, Porter 2012) and of the Mia-I1 dynein complex (Yamamoto et al. 2013). The CSC and the DRC play roles in the control of the dynein motors: They are both ideally located to link the radial spokes to the outer doublets and the dynein motors. An important current goal is to determine how calcium and the calmodulin complexes, located in the central apparatus and the CSC, operate to control axonemal dyneins. The I1 dynein and the associated Mia complex (Yamamoto et al. 2013) may also play a role similar to that of the DRC in the regulation of or resistance to dynein-driven microtubule sliding and in the control of axonemal bending.

Suppressor mutations in *Chlamydomonas* and the DRC. Major insight for understanding the control of axonemal dyneins resulted from the classic genetic studies of Huang and colleagues (1982). A genetic screen revealed new genes that suppressed paralysis (i.e., rescue motility) in radial spoke or central pair mutants so that the mutants once again became motile. The suppressor mutants included new mutations in the dynein motor heavy chains that restored movement without the recovery of the radial spoke and without central pair defects. These results indicate that, in the absence of the radial spokes and the central pair, dynein motor activity is inhibited throughout the axoneme, but it can be restored by any molecular change that permits the dynein to become active without input from the spoke-central pair system.

The same genetic suppressor screen (Huang et al. 1982) revealed another regulatory complex, which was named the DRC by Piperno in 1994. Subsequent studies further defined the DRC protein subunits (for a review, see Porter 2012). A major recent advance, made possible by the increased resolution of cryo-ET, is the discovery that the DRC is also the

nexin interdoublet link. Therefore, the DRC structure is now called the *nexin-dynein regulatory complex* (N-DRC; Heuser et al. 2009). Recent studies have also shown a biochemical-structural interaction between the N-DRC and the outer dynein arm intermediate chains, physically linking the outer dynein arm and the N-DRC (Oda et al. 2013). Therefore, the N-DRC appears to play roles in the regulation of the dynein motors and serves as an interdoublet link such that it likely aligns outer doublets to ensure efficient interactions between the dynein motors and the B-tubule of the adjacent doublet microtubule (Bower et al. 2013). To accommodate the sliding of doublet microtubules, all N-DRC-outer dynein arm interdoublet links on the sliding doublet must, at some point, break their connection with the adjacent B-microtubule (Holwill and Satir 1990). In a final understanding of the switch point model, it will be important to know the manner in which the N-DRC links are regulated and the timing of breakage and reformation for the active and passively sliding halves of the axoneme.

Insights from cryo-ET. A new level of resolution of the axoneme has been achieved through cryo-ET. These data have already had a major impact on the understanding of ciliary movement. The basic methodology has been reviewed (e.g., Nicastro 2009, Bui and Ishikawa 2013), and methods that now merge structural localization with cryo-ET have been described (Oda and Kikkawa 2013). Briefly, the process involves the rapid freezing of live cells or isolated axonemes, followed by low-dose electron microscopy of the structures in the frozen native state, data collection, computation-based tomography, and image averaging, which produce high-resolution images in three dimensions. Combined with very informative structural mutants in *Chlamydomonas*, a very-high-resolution picture of axonemal structure has emerged. The new structural information is summarized in figure 4, including examples of electron tomograms (figure 4a–4c), averages and 3D isosurface renderings (figure 4d, 4e), and summary schematic diagrams of a single doublet microtubule in longitudinal and cross section (figure 4f, 4g).

The cryo-ET approach offers numerous advantages: high resolution, approaching and exceeding 3 nm, revealing the detailed substructure of each axonemal component; pristine preservation by rapid freezing; and visualization of structures in 3D in intact organelles. The notable features in figure 4 include a definition of the longitudinal 96-nm axonemal repeat (figure 4 D and E), which is probably the basic unit of activity along the axoneme and the substructure of the outer dynein arms, including the resolution of the globular motor domains (figure 4d–4g). Also illustrated are the locations and substructure of each inner dynein arm (I1/f dynein and dyneins a–g; Bui et al. 2012, Heuser et al. 2012b, Lin et al. 2014), the N-DRC (Heuser et al. 2009), the CSC (Dymek et al. 2011, Heuser et al. 2012a), and the radial spokes (Pigino et al. 2011, Barber et al. 2012, Oda et al. 2014). In addition, cryo-ET has revealed the substructure of the central pair apparatus (Carbajal-Gonzalez et al. 2013, Oda et al. 2014).

In a relatively short time, these structural advances have contributed new understanding of the structural basis of ciliary bending. For example, cryo-ET has also revealed physical links among the outer dynein arms, the inner dynein arms, and the N-DRC that could explain the coordination of activity among the structures (e.g., Oda et al. 2013). This result begins to address a major question—that of how the activity of the outer and inner dynein arms is coordinated (Kamiya 2002). Along with other biophysical and structural studies, cryo-ET has also revealed a structural basis for axonemal dyneins' power stroke (Lin et al. 2014). The promise of these new studies is that these structural advances will define the structural changes associated with ciliary bending and will directly test models for ciliary bend formation and bend propagation. For example, the structural analysis of rapidly frozen live sea urchin sperm axonemes reveals that the structural state of the dynein motors on the doublet located on the inside of the bend differs from the state of the dyneins located on the doublets on the opposite side of the axoneme, on the outside of the bend (see supplemental figure 1 in Lin et al. 2014). These data appear to support a switch point model for axonemal bending, possibly revealing “on” and “off” states of the dyneins (see Brokaw, 2009). Predictably, taking advantage of cryo-ET and preservation through freezing, further analysis of the sea urchin sperm axonemes, *Chlamydomonas* mutants, and cilia preserved in metachronal beat stages will provide a comprehensive picture of axonemal and dynein structural changes associated with bend initiation and bend propagation.

Conclusions

We are poised at a new stage in the understanding of the movement of cilia in molecular detail hardly envisioned as possible when the sliding model and the switch point hypothesis were first proposed. The basic hypothesis, broadly speaking, seems to have withstood the tests of time. There are still important basic questions to be resolved, such as how the dynamic links among the structures within the axoneme correspond to the exact development and progression of a bend (Brokaw 2009). New opportunities include defining the function of tubulin posttranslational modifications for the regulation of dynein activity (e.g., Kubo et al. 2010). Because of a combination of new genetic and structural techniques, as was illustrated by the ingenious use of *Chlamydomonas* mutants and cryo-ET (e.g., Oda et al., 2014), which have led to a new understanding of the complexity of the motility mechanism, we are in a position to answer many of the remaining questions in a meaningful way.

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Peter Satir (peter.satir@einstein.yu.edu) is affiliated with the Department of Anatomy and Structural Biology at Albert Einstein College of Medicine, in New York, New York. Thomas Heuser is affiliated with the Electron Microscopy Facility, in the Campus Science Support Facilities of the Campus Vienna Biocenter, in Vienna, Austria. Winfield S. Sale is affiliated with the Department of Cell Biology at Emory University, in Atlanta, Georgia.