DECIPHERING THE ROLE OF A MICROTUBULE SEVERING PROTEIN AND A PROTEIN KINASE IN CELL CYCLE AND CILIOGENESIS IN *CHLAMYDOMONAS REINHARDTII*

by

M. Qasim Rasi
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Name: **M. Qasim Rasi**

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Examining Committee:

Chair: **Dr. C. Krieger**
Professor of Kinesiology

Dr. Lynne Quarmby
Senior Supervisor; Professor of Molecular Biology and Biochemistry

---

Dr. Michel Leroux
Supervisor; Associate Professor of Molecular Biology and Biochemistry

---

Dr. Nick Harden
Supervisor; Associate Professor of Molecular Biology and Biochemistry

---

Dr. Christopher Beh
Internal Examiner; Associate Professor of Molecular Biology and Biochemistry

---

Dr. Stephen M. King
External Examiner; Professor of Biochemistry, Associate Director, Graduate Program in Molecular Biology and Biochemistry, University of Connecticut Health Center

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ABSTRACT

Many types of eukaryotic cells are ciliated – they project a microtubule-based structure extending from the cell surface called a cilium, which plays roles in cellular motility and sensing the environment. Cilia are anchored at the cell surface by microtubule-based structures called basal bodies or centrioles. In addition to nucleating cilia, centrioles act as spindle organizing centres during cell division. We are beginning to understand that several proteins are involved in simultaneously regulating cilia and centrioles with cell division. My research focused on the function of two proteins in the ciliated alga, *Chlamydomonas*. The first protein, katanin, severs microtubules and we predicted that it is required during the disassembly of cilia prior to mitosis. I used a genetic approach to repress expression of the gene encoding katanin in *Chlamydomonas* , and thereby demonstrated that katanin in fact severs microtubules at a distinct location between basal bodies and the cilium. The other gene that I studied encodes a cyclic GMP-dependent kinase type 2 (PKG2). Mutant cells deficient in PKG2 had either no cilia, or cilia of unequal length. I discovered that PKG2 is expressed in the cytoplasm as well as the flagella and may interact with other proteins to regulate ciliary length and structure. Taken together, this research identified novel mechanisms that help explain the coordinated regulation of cilia during the cell cycle, and a novel gene with roles in ciliary assembly was also identified.

Keywords: cilia; flagella; centrioles; basal body; Chlamydomonas

Subject Terms: cell biology; algae

To mom, dad, my sisters, my beautiful fiancée Mariam, Uncle Homy

and

William Lipsit: This would have been impossible without you!

"Pader Jan wa Madar jan, tashakor az hama lotf wa mehrabani-ha"

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LIST OF ABBREVIATIONS/ACRONYMS

KAT1	*Chlamydomonas* katanin p60
PKG2	*Chlamydomonas* cGMP-dependent protein kinase 2
KAT2	*Chlamydomonas* katanin2
EM	electron microscopy/micrograph
fa	*f*lagella *a*utotomy (deflagellation) mutant
fla	*fl*agellar *a*ssembly mutant (usually temperature-sensitive)
SOFA	Site of Flagellar Autotomy : Distal to the transition zone.
IFT	intraflagellar transport, a motility system within cilia/flagella

CHAPTER 1
GENERAL INTRODUCTION

1.1 Introduction

The quote "*Natura maxime Miranda in minimis*" (Nature is greatest in little things) first appeared in a publication by Francesco Redi (Redi, 1668), as he observed greatness in small insects and today as I write my thesis, I will try to describe and elaborate on the greatness I observed in small cellular structures known interchangeably as cilia and/or flagella.

Merriam-Webster dictionary defines a cilium as "*a minute short hair like process often forming part of a fringe; especially: one on a cell that is capable of lashing movement and serves especially in free unicellular organisms to produce locomotion or in higher forms a current fluid*". This out-dated definition serves well the early observations in *Paramecium*, *Tetrahymena*, *Vorticella* and many others, but it fails to point out the important discoveries of the 20th and 21st centuries, which recognize a cilium as a sensory organelle as well.

Almost all eukaryotic cells are ciliated with the exceptions of fungi and plants. The ubiquitous presence of cilia across all animal phyla supports the notion that cilia may play diverse and versatile cellular roles. In mammals, our ability to see, smell, and hear are tasks mediated by ciliary structures present on photoreceptors, olfactory neurons and auditory hair cells, respectively (reviewed by Veland *et al.* 2009). In *Chlamydomonas reinhardtii*, a bi-flagellated unicellular alga, cilia play an important role in motility and

mating (Harris, 2001). In *Caenorhabditis elegans*, a free living nematode, the ciliated neurons act purely as sensory structures and enable the organism to interact with its environment and make behavioural, physiological and developmental responses (Bae and Barr, 2008).

In this chapter, I will describe the structure of a cilium and how it is assembled and disassembled. From there, I will discuss the roles of cilia in cell cycle regulation and how perturbations in ciliary assembly and cilia/cell cycle regulation, could lead to a variety of human disorders. Finally, following this general introduction, I will introduce the specific details pertaining to my work on KAT1 and PKG2 in *Chlamydomonas reinhardtii*.

1.2 Basic Structure of a Cilium

Based on the literature, it appears that the earliest description of the structure of flagella was made in early 1800's by Ballowtiz (reviewed by Pickett-Heaps and Martin, 1993), whereby Ballowtiz noticed 'fibers' emerging from a flagella after it was allowed to undergo autolysis. Those fibers are now known to be microtubule bundles, which form the structural pillars of a cilium (Pickett-Heaps and Martin, 1993).

Structurally and functionally, there are two types of cilia – motile and immotile. A motile cilium generally contains a ring of nine outer doublet microtubules surrounding a central pair of singlet microtubules. This arrangement is also referred to as 9+2, where the 9 refers to the outer row of microtubules and 2 refers to the inner pair of microtubules. In contrast to a motile cilium, an immotile cilium only contains the outer row of doublet microtubules and lacks the inner central pair of singlet microtubules

(Figure 1-1). Exception to this generalization is observed in motile "6+0" of gregarine apicomplexan parasite *Lecudina tuzetae* (Schrevel and Besse , 1975), "9+0" centric diatom *Thalassiosira pseudonana* gametes (Manton *et al*., 1969), "9+0" mammalian embryo nodal cilia, and non-motile "9+2" kinocilium and stereocilium of mammalian inner ear (Dabdoub and Kelley, 2005).

The microtubule filaments, which constitute the bulk of the cilia, are made up of heterodimeric polymers of α and β tubulin. These polymeric structures assemble into an A-tubule and a B-tubule, which fuse to form the nine outer-doublet microtubules. Eight of the A-tubules of outer-doublet microtubules serve as a scaffold supporting inner and outer dynein arms (Figure 1-1). The remaining A-tubule lacks inner and outer dynein arms but comprises other specialized structures that distinguishes it from the rest of the A-tubules (Huang 1979; Huang, 1986; Dutcher, 1995; Satir and Christensen, 2007). The nine outer-doublet microtubules are connected to each other by nexin fibers, which appear in repeats of 100 nm along the length of a cilium (Witman *et al*., 1978). All of the A-tubules also scaffold radial spokes, which point radially towards the central pair MTs (Yang *et al*. 2006).

Structurally the outer microtubule doublets of a cilium contain 96 nm repeats each consisting of four outer arm dyneins, one of each I1, I2, I3 (a, b, c, d, e) inner arm dyneins, a dynein regulatory complex , two radial spokes and one nexin ring (Sakato and King, 2004) (Figure 1-2). The central microtubule pair has its own repeats and decoration (or projections). The inner microtubule, which contains the longer set of projections, is named C1 and the other C2. C1 projections (referred to as 1a, 1b, 1c, 1d) show 32 or 16

nm periodicity depending on the projection type and C2 repeats at 16nm intervals (Mitchell, 2003).

The mechanism of ciliary beating is based on a microtubule-sliding model, whereby, flagellar dyneins anchored on the A-tubule of one microtubule doublet will contact the B-tubule of the adjacent doublet and generate a forceful motion that would induce sliding of one tubule past the other (Satir, 1968; Brokaw, 1989). However, since the microtubule doublets are anchored at the base by the basal bodies as well connected to each other by nexin fibers, this sliding motion is converted into a ciliary bend (Sale, 1977). To induce a net ciliary bend, dynein arms are activated asymmetrically along the length of the flagella and this is proposed to be regulated by the asymmetry in the structure of central apparatus (Wargo and Smith, 2003).

The ability of a motile cilium to bend or beat requires a complex and co-ordinate network of interactions between the central apparatus, radial spokes, inner and outer dynein arms and other regulatory complexes such as DRC (Dynein Regulatory Complex), nexin filaments, and an assortment of kinases and phosphatases (Brokaw *et al*., 1982; Porter, 1996; Porter and Sale, 2000). Most of our current understanding of how a cilium beats or bends comes from studies of paralyzed *Chlamydomonas* flagellar mutants, as well as suppressors of these mutants (Huang *et al*. 1982). Mutations affecting radial spoke proteins and the central apparatus generally lead to paralyzed flagella. Suppressor screens were performed by introducing second-site mutations in radial spoke/central apparatus mutant backgrounds. Although radial spoke and central apparatus mutants were able to have their flagellar motility partially restored, it did not coincide with the appearance of the missing structures (Huang *et al*. 1982). This result paved the way to a

model whereby in the absence of radial spokes and/or central apparatus, the dynein arms are globally turned off along the length of the cilia and only in the presence of a second-site mutation, the global 'off' signal ceases to exist and the dynein arms are able to move (or slide) (Piperno *et al*. 1992; Piperno *et al*. 1996). This model is supported by the discovery that phosphorylation of IC138, a component of I1 (inner dynein specie f), correlates with the inability of dynein arms to move or slide. Western blots on extracted axonemes from paralyzed flagellar mutants or phototactic mutants reveal that IC138 is indeed hyperphosphorylated (Habermacher and Sale, 1997; King and Dutcher 1997; Hendrickson *et al.* 2004).

The *Chlamydomonas* axoneme is encapsulated in a sheath of membrane, which is an extension of the plasma membrane containing many unique features. These features include a sheath of surface carbohydrates (called glycocaylx), hair-like projections referred to as mastigonemes, and sexual agglutinins, which only appear in gametes (Ringo, 1967; Witman *et al*. 1972). The flagellar membrane also contains voltage-gated channels, calcium channels and calcium pumps, which may be required for transducing phototactic, photoshock and acid shock responses (Iomini *et al*. 2006; Quarmby, 2009). Only recently, a voltage dependent calcium channel (Cav2) was identified at the flagellar distal tip in *Chlamydomonas* (Fujiu *et al*. 2009). This channel appears to be important for mediating changes in the flagellar waveform, required during phototaxis (Fujiu *et al*. 2009). Another calcium channel that has been localized to the cilia and shown to have membrane localization is the mammalian homolog of polycystic kidney disease protein PKD2 (Huang *et al*. 2007). Little is known about the *Chlamydomonas* PKD2, but it is believed to be involved in regulating calcium signals during mating (Huang *et al*. 2007).

Similarly, studies of mammalian motile cilia have uncovered that signalling via membrane bound receptors and channels plays an important role in regulating the ciliary beat frequency and the waveform in mammals. For instance, Teilman *et al*. (2005 and 2006) have discovered that TRPV4 and progesterone receptors localize to the motile cilia lining the epithelia of the oviduct. Enhanced expression levels of ciliary membrane receptors coincide with Ca^{+2} and progesterone signalling during the estrous cycle, when the transport of ovulated oocytes occurs.

The *Chlamydomonas* flagellar membrane also contains a distinct array of particles identified by freeze-fracture transmission electron microscopy (TEM). These particles include those that run longitudinally along the length of the flagella and those that form the ciliary necklace and the ciliary bracelet (Weiss *et al*. 1977a, Weiss *et al*. 1977b). The longitudinally positioned particles are spaced within the membrane such that they are oriented over the axonemal outer-doublets (Snell, 1976). These particles could represent tether points between the membrane and the axoneme (Dentler, 1990). The ciliary necklace and bracelet appear at the base of the cilia. The ciliary necklace has been shown to be a common feature of most ciliates examined so far where as the ciliary bracelet appears to be a *Chlamydomonas* specific structure (Figure 1-3) (Gilula and Satir, 1972; Weiss, 1977a). The exact role of the ciliary necklace is not yet known, but it is believed that they may act as 'cross border' check posts (or diffusion barriers) between the cilia and the cell body (Rosenbaum and Witman, 2002). Studies into the localization of a protein involved in ciliary assembly (IFT52) show that it localizes at sites which mark the ciliary necklace (Deane *et al*. 2001). This suggests that the ciliary necklace also acts as docking sites for proteins sorting to the cilia.

The next question that one ponders is how a cilium is assembled into a functional organelle. The following section will discuss the process of ciliary assembly from a mechanistic perspective.

1.3 Ciliary Assembly

The basic architectural plan of a cilium is conserved across all eukaryotes (Satir *et al*. 2008). Events that define the early stages in ciliary assembly within the cytosol were described by Sorokin (1962). Using fibroblasts and smooth muscle cells, Sorokin showed that the process of assembly initially begins within the cytosol where a Golgi-derived vesicle fuses to the distal-end of a short microtubule-based cylindrical structure called the mother centriole (or basal body) (Figure 1-4). The vesicle starts to form a hollow cap over the mother centriole, within the lumen of which the axoneme starts to emerge from the mother centriole. As time progresses more vesicles fuse to form a membranous sheath encapsulating the growing axoneme. Finally, the membrane-adorned axoneme reaches the cell surface and ciliary membrane fuses with the plasma membrane. Although it is still unclear, it appears that during ciliary and plasma membrane fusion the mother centriole needs to dock to the plasma membrane via its distal appendages (Sorokin, 1968). The importance of distal appendages in docking the mother centriole and ciliary assembly came from the studies of ODF2 (Outer Dense Fiber 2, also called cenexin). ODF2 is a scaffolding protein that decorates the distal ends of the mother centrioles and complete knock down of ODF2 in murine tissue culture lines led to mother centrioles being unable to dock to the plasma membrane and assemble a cilium (Ishikawa *et al*. 2005).

After proper docking of the mother centriole and fusion of the ciliary membrane to the plasma membrane, the next stage of axonemal elongation requires the action of IFT (Figure 1-5A). Initially described by Kozminski *et al.* (1993), IFT involves particles/cargo (axonemal precursors) moving from the base of the cilia towards the tip of an assembling or previously assembled cilium (anterograde) and their return back to the base of the cilium carrying the ciliary turn-over products (Kozminski *et al*., 1993; Kozminski 1995). This bidirectional machinery is active within the spaces between the axoneme and the membrane matrix, and is driven by molecular motors (Figure 1-5B). The anterograde movement of IFT particles along the cilia requires kinesin-2 motor protein and the retrograde movement of IFT particles (returning to the base of cilium) requires the presence of a cytoplasmic dynein (Kozminski *et al.* 1995; Pazour *et al.*, 1999). The heterotrimeric kinesin-2 motor protein contains two motor subunits of 90 and 85 kDa, and a non-motor subunit of 100 kDa, called Kinesin-Associated Protein (KAP) (reviewed by Scholey, 2003). Although cilia contain more than one type of kinesins (Fox *et al.*, 1994) it appears that, kinesin-2 forms the core molecular motor of anterograde IFT, required for moving the cargo during ciliary assembly. Other kinesins found in the cilia may act in other capacities such as regulating the velocity of IFT in *C. elegans* (Evans *et al*. 2006), controlling ciliary length in *Leishmania major* (Blaineau *et al*., 2007) or beat frequency in *Chlamydomonas* (Yokoyama *et al*. 2004).

In *Chlamydomonas,* the two motor subunits of kinesin-2 are encoded by FLA10 and FLA8, where as the 100 kDa KAP subunit is encoded by FLA3 (Walther *et al*. 1994; Mueller *et al*. 2005). Data from the *Chlamydomonas* null and temperature sensitive *fla*10 mutants confirms the indispensability of FLA10 for ciliary assembly and more

importantly, the requirement of IFT for the maintenance of the fully assembled cilia (Kozminski *et al*., 1995; Iomini *et al*., 2001).

The retrograde IFT motor is an isoform of cytoplasmic dynein, an AAA (ATPase Associated with various cellular Activities) protein required for mobilization of the IFT particle back to the cytosol (Porter *et al*., 1996; Cole, 2003). Within a motile cilium, there are other classes of dyneins as well. The outer-arm dyneins (OAD) and inner-arm dyneins (IAD), are attached to the A-tubule of the outer doublet microtubule (MT) and interact transiently with the B-tubule MT (Sakato and King, 2004). This interaction of A-tubule dyneins OAD and IAD with B-tubule MTs confer motion to a cilium (Sakato and King, 2004).

The IFT particle, which is mobilized by the ciliary kinesin and cytoplasmic dynein along the length of the cilium, is itself a large multi-protein complex consisting of Complex A and Complex B (Cole *et al*. 1998). Complex A and B consist of at least 16 core polypeptides with an apparent molecular mass of ~550 kDa and ~ 750 kDa respectively (Cole, 1998; Cole, 2003). The most striking characteristic of IFT proteins studied so far, is that they are rich in motifs such as TPR (Tetratrico-Peptide Repeat), WD40 repeats and coiled-coiled domains, which are required for transient protein-protein interaction (Cole, 2003).

Mutations in any of the complex B proteins lead to defects in ciliary assembly where the cilia are either absent or severely short, whereas mutations in complex A proteins yield variable phenotypes depending on cell type studied (reviewed by Blacque *et al.* 2008). For example, in *C. elegans* IFT140 (Che-11; a component of complex A) mutations lead to shortened cilia with accumulation of complex B proteins at the ciliary

tip (Cole, 2003; Qin *et al*., 2001). This observation leads to an interesting prediction that in *C.elegans,* the complex B subunit of IFT is responsible for anterograde IFT and the complex A subunit of IFT is only required for retrograde IFT (Blacque *et al.* 2008).

In *Chlamydomonas*, mutations in IFT140 lead to complete loss of the cilia (Cole, 2003). This seemingly different observation between *Chlamydomonas* and *C. elegans* has been resolved by the identification of a homodimeric kinesin-2 (OSM3) in *C. elegans*, which associates with IFT complex B (Collet *et al*., 1998; Pan *et al*., 2006). Thus, if there were any mutations in complex A (in *C. elegans*) that would disrupt its association with kinesin-2, OSM3 can still mobilize IFT particles to the tip. So far, in *Chlamydomonas*, no known homodimeric kinesin-2 has been identified to associate with complex B. Therefore, the only motor that drives anterograde IFT in *Chlamydomonas* is the kinesin-2, which associates with complex A and hence any mutations that would disrupt kinesin-2 association with complex A could lead to a cilia-less phenotype similar to that of complex B mutations (See Figure 1-6).

The presence of an assembled cilium is a temporal clue that marks the stationary or G_o phase of most ciliated cells (Tucker *et al.* 1979). It has been observed that soon after exit from the stationary phase and prior to entry into mitosis, the cilia gets disassembled (Reider, 1979; Wheatly, 1996). This mode of disassembly is referred to as resorption and is dependent on IFT. The following section will discuss the process of ciliary disassembly.

1.4 Ciliary Disassembly

Almost all ciliated eukaryotes disassemble their cilia prior to entry into mitosis, and soon after exit from mitosis, they reassemble their cilia (Tucker *et al.* 1979). This dynamic process of assembly and disassembly is due in part to the requirement of basal bodies or centrioles to also serve as mitotic-spindle organizing centres during cellular division (Wheatley *et al*., 1996; Quarmby and Parker, 2005). It is important to note that not all ciliated cells disassemble their cilia prior to entry into the cell cycle. For example, during the final stages of butterfly spermatogenesis, the centrioles can simultaneously act as spindle organizing centres and basal bodies (Wilson, 1928). Exception to this is found in *Tetrahymena* and *Paramecium* where in cells undergoing division, the cilia remain intact. This is due to the fact that there are roughly about 750 and 4000 basal bodies, respectively and they do not act as spindle organizing centres (Pearsen and Winey, 2009).

During flagellar shortening or resorption, retrograde IFT dismantles the cilia from the tip of their cilia and this is followed by a concomitant decrease in ciliary assembly (Marshall, 2001). Flagellar disassembly can also be achieved by flagellar amputation induced by physical or chemical stimuli (Blum 1971; Lewin and Lee 1985). This form of flagellar loss is commonly referred to as deflagellation and occurs at a unique site distal to the flagellar transition zone also known as the SOFA (Site Of Flagellar Autotomy) (Quarmby, 2004; Mahjoub *et al*. 2004) (Figure 1-7).

Following deflagellation of the *Chlamydomonas* cells, flagellar regeneration begins and flagella are re-assembled to full length within 1 to 2 hours (Rosenbaum *et al*. 1967). Flagellar regeneration is concomitant with an increased level of tubulin mRNA, synthesis of new flagellar proteins and up regulation of many other flagellar genes

(Rosenbaum *et al*. 1967; Lefebvre *et al*. 1980; Pazour *et al*. 2005). How exactly genes are upregulated in response to deflagellation is unknown but it is believed that the absence of a cilium triggers gene expression (reviewed by Quarmby, 2008).

A genetic screen for mutants unable to deflagellate identified multiple alleles at three loci: *FA1*, *FA2* and *ADF1* (Finst *et al*. 1998). *FA1* codes a 175 kDa protein with little or no homology to any other known protein and is predicted to be a scaffolding protein localizing to the SOFA (Finst *et al*. 2000; Parker and Quarmby, Unpublished data). The *FA2* gene encodes a NIMA-related kinase and localizes to the SOFA (Mahjoub *et al*. 2002). The *adf*1 (*a*cid *def*lagellation 1) mutants are unable to deflagellate as well, but unlike *fa*1 and *fa*2, *adf*1 mutants can lose their flagella only when the cells are permeabilized with a non-ionic detergent in the presence of calcium (Finst *et al*. 1998). Although ADF1 gene has not been cloned yet, but the ability of *adf1* mutants to deflagellate when their membranes are permeabilized in the presence of calcium predicts that ADF1 could be a membrane-bound Ca^{+2} channel, calcium sensor or an acid-sensor.

Calcium has long been identified as a key inducer of the severing signal (reviewed by Quarmby, 2008). The first direct implication of Ca^{+2} as the flagellar excision signal came from the studies of Lohret *et al*. 1998, where addition of 1μM Ca^{+2} to purified FBBC (Flagellar Basal Body Complex) induced severing of the respective axonemes from the basal body. This also suggested that the downstream targets of Ca^{+2} which mediate flagellar severing, are contained within the flagellar basal body complex.

Severing of flagella by the deflagellation machinery requires the disentanglement of nine outer-doublet microtubules (Quarmby, 2008). Initially it was believed that deflagellation is induced by the contraction of stellate fibers (or transition zone fibers) in

response to Ca^{+2} binding to a calmodulin-like *Chlamydomonas* protein (centrin), which is found to mark the stellate fibers (Sanders and Salisbury, 1989, 1994). To date, the role of centrin in deflagellation is unclear but the role of stellate fibers in torsion-mediated deflagellation model has been challenged by data from a *vfl2* mutant. The *vfl2* mutant (*V*ariable *F*lagellar *L*ength) is devoid of regular stellate fiber arrangement within the transition zone and instead contains a poorly organized electron dense topology (Jarvik and Suhan, 1991). This is caused by a missense mutation within the coding sequence of centrin where a glutamic acid is converted to a lysine (Taillon, 1992). Despite a loss in regular stellate fiber arrangement, the *vfl*2 mutant deflagellates normally (Quarmby, 2004). Therefore, torsional strain model could not explain why cells deflagellate (Quarmby, 2004; Quarmby, 2008).

Deflagellation requires severing of microtubule bundles at the base of *Chlamydomonas* flagella; therefore, a microtubule severing protein, katanin was proposed to be involved as well (Lohret *et al*. 1998; Quarmby and Lohret, 1999). Katanin – an AAA (ATPase Associated with various cellular Activities) is a 60 kDa, microtubule-stimulated and microtubule-severing ATPase (Vale, 1991; McNally and Vale, 1993; McNally and Thomas, 1998) involved in severing of centrosomal as well as non-centrosomal microtubules (McNally *et al*., 1996; McNally and Thomas 1998; Ahmad *et al*. 1999). As reviewed in Quarmby (2004), three lines of evidence hinted at the possibility of katanin being involved in deflagellation. First, purified sea urchin katanin could sever microtubules of the axoneme *in vitro* (Lohret *et al*., 1998). Second, pan-specific katanin antibodies could inhibit Ca^{+2}-induced severing of the axonemes in FBBC preparations (Lohret *et al*., 1998). Third, immunogold EM studies, revealed the presence

of katanin at the proximal and distal ends of the transition zone as well as the basal bodies (Lohret *et al*., 1999). Although definitive evidence is missing to demonstrate the role of katanin in deflagellation, my current model for acid-induced, calcium-mediated deflagellation predicts ADF1-dependent activation of FA1 may recruit FA2 and katanin to the site of severing.

Another protein that may play a role in deflagellation is a *Chlamydomonas* aurora-like kinase (CALK), which undergoes changes in phosphorylation state in response to deflagellation (Pan *et al*. 2004). RNA interference mediated repression of CALK expression as well as inhibition of CALK activity by kinase inhibitor staurosporine blocks acid-induced deflagellation (Pan *et al*., 2004). Although data presented by Pan *et al*., implicates CALK as a candidate protein in the deflagellation pathway, more work needs to be done to determine: (1) if the RNAi knock down was indeed specific to CALK (V4: 192550), and not 4 other predicted aurora-like kinases in the *Chlamydomonas* genome (V4: ALK2 (145741), ALK3 (131531), ALK4 (196061), ALK5 (103830)) ; (2) if staurosporine-mediated inhibition was specific to CALK and not Fa2 (or other kinases); (3) and, if the phenotype of CALK-RNAi was indeed due to knock down of CALK expression and not insertional mutagenesis of a *FA* gene.

As stated earlier, FA2 is a member of the NIMA-related kinases required for deflagellation in *Chlamydomonas* (Mahjoub *et al*., 2002). Mutations in the *FA2* gene not only lead to defects in ciliary severing but also to a cell cycle delay at the G2/M transition (Mahjoub *et al*., 2002). Therefore, we recently suggested that there is a link between deflagellation and the cell cycle. This observation is supported by our large-scale mutagenesis screen to uncover mutants in the deflagellation pathway. In our experiment,

we uncovered multiple alleles of only three genomic loci (Finst *et al*. 1998). Perhaps this is due to cell cycle roles of deflagellation genes, mutations of which could be lethal. The role of CALK and katanin in deflagellation further supports the overlap between deflagellation and the cell cycle, since both proteins are also predicted to have cell cycle roles (Pan *et al*., 2004; Quarmby, 2008).

1.5 Cross Talk between Cilia and Cell Cycle

The signalling connection between cilia and the cell cycle was originally hypothesized by Tucker *et al.,* (1979), where they showed that the presence or absence of a cilium in mouse fibroblasts (3T3) is linked to cell cycle-induced DNA replication. It is now well established that many other signalling pathways, that play a role in the cell cycle also localize to the cilia, including Hedgehog (Hh), Wnt and Platelet-drived growth factor alpha (PDGF-α) (reviewed by Christensen *et al*., 2008).

An intimate relationship between cilia and cell cycle comes from the studies of mammalian inversin (Yokoyama *et al*., 1993). Mice mutant in *inversin (inv)* suffer from *situs* abnormalities and severe kidney and pancreatic cysts (Morgan *et al*., 1998). A GFP-tagged Inv protein was expressed in ciliated cell lines and shown to localize to cilia (Morgan *et al.*, 2002). This localization appeared to be dynamic. In interphase, inversin was mostly ciliary. In early prophase, inversin appeared to localize to the centrosomes and in metaphase and anaphase it appeared to localize to the spindle poles. In cells undergoing division, inversin appeared to localize at the mid section of cytokinetic bridges, commonly referred as the midbody (Morgan *et al*., 2002, Nurnberger *et al*., 2004). Investigation into understanding the interaction network of inversin revealed that it interacts with calmodulin (Morgan *et al*. 2002a), Apc2 (Anaphase promoting complex

#2), nephrocystin, β-catenin and N-cadherin (Morgan *et al*. 2002; reviewed by Benzing *et al*. 2007). The interaction with Apc2 supports the premise that inversin may have a cell cycle role (Morgan *et al*., 2002). Apc2 is an important component of APC responsible for ubiquitin-mediated degradation of cell-cycle proteins during metaphase and the mitosis-to-G1 transition (Glotzer *et al*. 1991; Zachariae and Nasmyth, 1999 and Eley *et al*. 2004). Thus, the involvement of a ciliary protein during cell cycle strengthens the notion that perhaps cilia regulate the cell cycle rather than just sequester the basal bodies (or the mother centrioles). Another example to support this notion comes from the studies of IFT27 and IFT88 (Qin *et al*., 2007; Robert *et al*., 2007). IFT27 is a Rab-like GTPase identified in *Chlamydomonas*. RNA interference-mediated knock down of IFT27 lead to several cytokinesis defects as well as retarded cell growth in *Chlamydomonas* (Qin *et al*., 2007). Over-expression of IFT88 in HeLa cells caused a dramatic arrest at the G1-to-S transition. RNAi-mediated knock down of IFT88 lead to an accelerated cell cycle, mediated via the retinoblastoma tumor suppressor pathway (Robert *et al*. 2007).

Research from our lab into the role of ciliary proteins in cell cycle regulation has shown that members of the NIMA-related kinases, FA2 and CNK2 localize to *Chlamydomonas* cilia and have a role in the G2/M transition and commitment size, respectively (Mahjoub *et al*., 2002; Mahjoub *et al*., 2004; Bradley and Quarmby, 2005). Mutations affecting the *FA2* gene are associated with the inability of *Chlamydomonas* cells to sever their cilia and exhibit a cell cycle delay at the G2/M transition (Mahjoub *et al*., 2002). Over-expression and knock-down of CNK2 showed cells that normally can assess their optimal size for initiating cell division (commitment size) were unable to do so when CNK2 levels were altered (Bradley and Quarmby, 2005). For example, when

CNK2 was knocked down, cells had a larger cell volume and longer than normal flagella. Interestingly, over-expression of CNK2 led to cells with a smaller cell volume and shorter than normal flagellar length (Note: cell volume provides an indication of possible roles is often used as a proxy to determine cell-cycle roles of a protein in *Chlamydomonas*) (Bradley and Quarmby, 2005).

In mammals, mutations in Nek1 and Nek8 have been identified to be causal for cystic kidneys in two mouse models of polycystic kidney disease (Upadhya *et al*., 2000; Liu *et al*. 2002). Mutations affecting both Nek1 and Nek8 lead to ciliary defects and causes cyst formation in mouse renal tubules. Nek1 interacts with important cell-cycle proteins such as KIF3A (Lin *et al*., 2003) and tuberin (Kleymenova *et al*., 2001), as well as those in the DNA damage repair pathway (Surpili *et al*., 2003). Although little is known about Nek8, it is believed to be important for expression and localization of polycystin-2, a protein implicated in Autosomal Dominant Polycystic Kidney Disease (ADPKD) (Sohara *et al*., 2008; Burtey S. *et al*, 2008)

In summary, it is now well established that communication networks exist between cilia and cell cycle. It is not clear if cilia regulate the cell cycle (or vice versa) but deciphering the signalling networks that exist between cilia and cell cycle can help us better understand cell proliferation and differentiation.

1.6 Cilia and Human Disease

The inter-dependence of previously well-studied pathways such as Hedgehog, Wnt, and PDFG on cilia, and the implication of many other cell cycle and signalling proteins for various aspects of ciliogenesis, has recently opened new avenues of

discovery in understanding cilia-related diseases collectively named ciliopathies (reviewed by Christensen, 2007). In humans, complete loss-of-cilia mutants have not been identified but certain mutations in the components of ciliary assembly and function have been identified which leads to a wide range of human diseases such as Primary Cilia Dyskinesia, Polycystic Kidney Disease, Nephronophthisis, Meckel-Gruber syndrome, Alstrom syndrome, Joubert syndrome, Kartagener syndrome, Bardet-Biedl syndrome and Orafaciodigital syndrome (reviewed by Inglis *et al*., 2006). Most of our current understanding of ciliopathies comes from the initial discovery of the ORPK mouse (Oak Ridge Polycystic Kidney) which has an insertion in the gene encoding IFT88/Polaris (Moyer *et al*., 1994; Pazour *et al*., 2000). Although, ORPK mice were originally described as a model system for studying human polycystic kidney disease (Moyer *et al*., 1994), histological sectioning of the mice have revealed that aside from cystic kidneys it also has cysts in the liver and pancreas, as well as skeletal abnormalities, hydrocephalus, neuroblast migration defects, cerebral hypoplasia, sterility and hair follicle defects (reviewed by Lehman *et al*., 2008). The IFT88 'syndrome' seems to originate as a result of the hypomorphic *IFT88* allele in ORPK mice, which renders cells unable to assemble a functional motile and/or sensory cilium (Pazour *et al.*, 2000; Lehman *et al*. 2008). Orthologs of mammalian IFT88 in *Chlamydomonas* and *C. elegans* (Osm-5: Qin H., *et al*. 2001) are integral components of the IFT complex B.

Another example, which further highlights the role of cilia in human disorders, comes from the studies of HEF1, aurora A, and HDAC6. HEF1 is regarded as a prometastatic factor because elevated expression of HEF1 is associated with melanomas (O'Neill, 2007). A downstream target of HEF1 has been identified to be the mitotic-

checkpoint protein Aurora A (Pugacheva *et al*., 2007). In naturally occurring tumors, high levels of Aurora A correspond to increased instances of centrosomal duplication, cytokinesis defects, and aneuploidy (Anand *et al*., 2003; Gritsko *et al*., 2003). Moreover, ectopic expression of either HEF1 and/or Aurora A in mammalian cell lines result in tumerogenic perturbations (Minn *et al*. 2005), and microinjection of active Aurora A protein is always associated with ciliary resorption (Pugacheva *et al*., 2007). HDAC6 has been shown to serve as a direct substrate for Aurora A phosphorylation and is involved in ciliary disassembly (Hubbert *et al*., 2002; Pugacheva *et al*., 2007). Aside from being a target for Aurora A, HDAC6 has recently been shown to interact with BBS10 – a 10 kDa Bardet-Biedl Syndrome protein (Loktev *et al*., 2008). What this interaction means in a larger context is still under investigation, but it certainly adds another body of evidence to the emerging role of ciliary proteins in human disorders.

In mammals, the hedgehog-signalling pathway is associated with early embryonic patterning and positioning of neurons in the neural tube and in adults, maintaining stem cell and/or progenitor cell populations (Reviewed by Wong and Reiter, 2009). Hh-mediated signalling includes hedgehog proteins such as Sonic, Indian and Desert Hedgehog. Hh binds to a ciliary receptor called Patched to relieve suppression of Smoothen, a seven trans-membrane protein (Hooper *et al*., 2005). Binding of Hh to Patched causes translocation of Patched from cilia to the cell body and concomitant localization of Smoothen to the cilia (Rohatgi *et al*., 2007). Localization of Smoothen to the cilia leads to inactivation of differential proteosomal processing of the Gli family of transcriptional factors, a step necessary for Gli2/3 activation. Once activated, Gli2/3 induces transcription of target genes such as cyclins (reviewed by Jenkins, 2009). The

role of hedgehog signalling in the transcription of cell-cycle proteins such as the cyclins, and the localization of hedgehog signalling to the cilia, further highlights the importance of cilia and cell cycle cross talk, perturbations of which could lead to tumorigenesis.

Within the last decade, *Chlamydomonas* has come forward as an organism of choice to study many of the ciliary proteins, some of which are orthologs of those involved in ciliopathies. For example, Juvenile myoclonic epilepsy (also known as Janz syndrome or JME) is a common form of epilepsy. In 2004, Suzuki *et al*., found that *EFHC1* is the gene mutated in JME, and over-expression studies of EFHC1 in hippocampal primary neurons induced apoptosis. Subsequently, over-expression constructs containing mutations in EFHC1 introduced into neurons prevented apoptosis, indicating that these mutations inactivated EFHC1. Suzuki *et al*., concluded that EFHC1 is involved in a mechanism of 'neuronal clean up', and patients with JME suffer from epilepsy due to increased neuron density. *Chlamydomonas* RIB72 is a homolog of mammalian EFHC1 (Patel-King *et al*., 2002). RIB72 is a flagellar protein and localizes to the outer-doublet microtubules. Although the role of RIB72 is unclear, but it is believed to be involved in inter-doublet linkage that hold the nine outer doublet microtubules (King, 2006). Ikeda *et al*. (2005) examined the tissue distribution of EFHC1 and found that it is mostly expressed in ciliated tissues such as testis, trachea, and oviduct, and at much lower levels in the brain. Suzuki *et al*. (2008) re-examined their work and found that the antibody used in the study was not specific and with new antibodies against EFHC1, they showed that EFHC1 localizes to the motile cilia lining the ventricles of the brain and that JME is probably a ciliapathy.

Primary cilia dyskinesia (PCD) is an autosomally inherited disorder of immotile cilia affecting the cilia lining the respiratory tract, male fertility, and left-right symmetry (*situs inversus*) (Badano *et al*., 2006). Afzelius (1976) determined that individuals suffering from PCD had a mutation in the gene encoding the dynein arms. Although the identity of the gene responsible remained illusive, Pennarun *et al*. 1999 used conserved sequences of *Chlamydomonas* dynein intermediate chain IC1(King and Witman, 1990; King *et al*., 1991) to clone the homologous human *DNAI1* and show that PCD individuals have mutations in *DNAI1*.

Hydin is a protein involved in a human disorder that is also represented by another *Chlamydomonas* protein. Mutations in the hydin gene have been found to be causal for hydrocephalus, causing abnormal accumulation of cerebrospinal fluid in the brain ventricles (Davy and Robinson, 2003). Based on the proteomic data of Pazour *et al*. (2005), Lechtreck and Witman (2007) showed that a close homologue of hydin localizes to the flagellar central pair in *Chlamydomonas*. Further analysis by RNA interference revealed that the knock down of Hydin leads to the loss of C2b projections of the central apparatus, and these cells were partially defective in flagellar beating. Lechtrek *et al*. (2008) extended this work by examining hydin mutant mice and showed that these mutants lack the C2b projections of the central apparatus, and that the ciliary beat of the brain epithelial cells had similar defects as those observed in hydin-RNAi in *Chlamydomonas*.

The few examples presented here into the role and identification of *Chlamydomonas* ciliary homologues involved in human diseases, offer a segue for the next section of my thesis, where I introduce a previously well-studied family of cyclic

GMP-dependent kinases. Based on my work in *Chlamydomonas*, I discuss how one type of cyclic GMP-dependent kinase may have a role in ciliary assembly.

1.7 Cyclic GMP-dependent Protein Kinase

Cyclic GMP (cGMP) is a second messenger molecule involved in modulation of phosphodiesterases (PDE), cGMP-gated cation channels, and activation of cGMP-dependent protein kinases (cGKs) (reviewed by Kots *et al*., 2009). Cellular cGMP production is catalyzed by guanylyl cyclases (GC) from GTP (Guanosine-5'-Triphosphate). Signals that regulate GCs depend on their cellular localization, whether they are either membrane bound (mGC) or soluble cytosolic (sGC) (Roelofs *et al*., 2002). sGCs are heterodimeric proteins, with one alpha and one heme-binding beta subunit. The heme-binding domain of sGC enables it to act downstream of nitric oxide signalling in its Fe(II)-bound form (Poulos, 2006). The mGCs have an extracellular ligand-binding domain, a short trans-membrane region and an intracellular domain. Membrane bound GCs are activated by natruretic peptides (peptide hormones) and have been implicated in cell differentiation, retinal phototransduction, and olfaction (reviewed by Kuhn, 2009).

Phylogenetic analysis of membrane bound and cytosolic forms of guanylyl cyclases have revealed that GCs are present in most eukaryotes with the exception of Fungi (Schaap, 2005). The only prokaryote known to have a predicted guanylyl cyclase is a marine, phototrophic and heterotrophic cyanobacterium *Synechocystis* (Morton 2004). A direct consequence of GC-mediated cGMP production is activation of cGMP-dependent protein kinases. In mammals, there are two types of cGMP-dependent protein kinases and they are referred to as cGKI and cGKII (or PKG1/PKG2, PrKG1/PrKG2). Type I cGKs are cytosolic and soluble, where as type II are anchored to the plasma membrane via an N-terminal myristoylation motif (reviewed by Hoffman *et al*., 2009). Despite structural similarities, cGKI and cGKII are different in their expression pattern and biological function. CGKI are expressed in smooth muscles, the hippocampus, and platelets, where as cGKII are expressed in kidneys, lungs, bones, intestine, and certain neuronal cells (Hoffman *et al*., 2009). Although much is known about cGKI, the cellular roles of cGKII are still unclear. In one of the few physiological studies of cGKII, researchers have shown that membrane-bound cGKII inhibits an Na^+/H^+ exchanger (NHE3), in an oppossum proximal tubule cell line (Cha *et al*., 2005). In the intestine, cGKII is involved with the trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) (Goline-Bisello *et al*., 2005). Mice mutant in cGKII (*pkg2*) exhibit dwarfism and current research has identified transcriptional factor sox9 and cilia/cell cycle regulator Glycogen Synthase Kinase β (GSK β) as downstream substrates of cGKII in chondrocytes (Chikuda *et al*., 2004; Kawasaki *et al*., 2008).

In *Chlamydomonas*, there are over 50 genes that code for guanylyl cyclases (Merchant *et al.*, 2007) and three genes that encode cyclic GMP-dependent protein kinase (PKG) type II. CrPKG (V2: C_50062, v3:181974) localize to the ciliary membrane matrix and is required for flagellar adhesion during early stages of *Chlamydomonas* mating (Wang *et al*. 2006). We recently reported the cloning of PKG2 (V2: C_740056, V3:153242) that has a role in ciliary assembly (Chapter 3). The third PKG (V2: C_60149, V3: 131695) has not been studied yet, but is one of the most abundant proteins found in the flagellar proteome (Pazour *et al*. 2005). The emergence of guanylyl cyclases in eukaryotes, the role of cGMP-signalling in *C. elegans* ciliated neurons and mammalian sensory organelles of olfaction and vision, as well as our work on PKG2, suggest the possibility that the phenotypes associated with mice *pkg*2 mutants may be derived from ciliary defects.

1.8 *Chlamydomonas* as a Model System

In my research as a doctoral student, I have made use of *Chlamydomonas reinhardtii* as a model organism to answer questions related to the roles of katanin in the cilia / cell-cycle connection and the roles of PKG2 in ciliary assembly. *Chlamydomonas* is haploid unicellular green algae and has long served as an excellent model system in studies of photosynthesis (Grossman, 2004), flagellar biogenesis and function (Rosenbaum *et al*., 1999), phototaxis (Witman, 1993) and circadian rhythm (Wagner and Mittag, 2009). Recent technological advances have enabled *Chlamydomonas* researchers to have access to publicly available sequenced chloroplast, mitochondrial and nuclear genomes (Merchant *et al*., 2007). Furthermore, proteomic databases of the flagella,

mitochondria, eyespot, *Chlamydomonas* BAC and EST libraries as well as microarray chips have added to the value of *Chlamydomonas* as an organism of choice for research.

Some of the fundamental processes inherent to ciliary assembly and disassembly have come from the pioneering work of *Chlamydomonas* researchers and this is made possible by the ease of combining biochemical and genetic methods to study flagella in *Chlamydomonas*. Through out evolution, the structure and function of cilia have been conserved and as a result, many of the *Chlamydomonas* flagellar proteins have human homologues (Pazour *et al*., 2005). Therefore, to study the functions of human ciliary proteins and how some of them lead to human diseases, *Chlamydomonas* can serve as an excellent model system to decipher and understand the basic biology of these proteins.

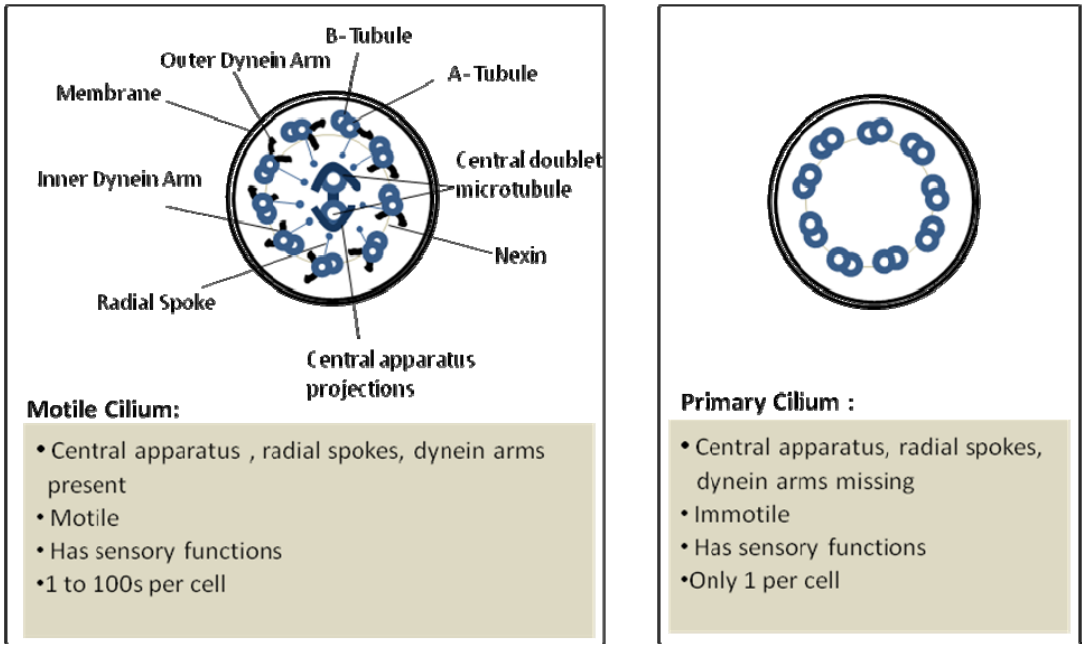
1.9 Questions

In my thesis, I will first revisit the role of katanin, which our laboratory cloned and implicated in deflagellation in *Chlamydomonas reinhardtii* (Lohret *et al*., 1998; Lohret *et al.*, 1999). Data from other laboratories working on katanin in other model systems have shown that katanin plays important cell-cycle roles. We asked whether katanin has a dual role in the cell cycle and deflagellation in *Chlamydomonas.* In Chapter 2 of this thesis, I present my work on katanin and show that katanin indeed has a role in the cell cycle and deflagellation in *Chlamydomonas*. Furthermore, I show that there is an additional site of ciliary severing; One involved in stress-induced deflagellation and the other needed during basal body disengagement prior to the entry into mitosis.

As we were investigating the dual role of katanin, I serendipitously stumbled upon a *Chlamydomonas* mutant, which displayed defects in ciliary assembly. I identified the mutant to have an insertion in the second exon of a gene coding for Cyclic GMP-dependent protein Kinase II (PKG2). In Chapter 3 of this thesis, I present my work on the cloning and characterization of PKG2, which is the first cyclic GMP-dependent protein kinase identified to have a direct role in ciliary assembly. With the emerging trend of identifying important protein families and their implication in various aspects of ciliogenesis, my work on cyclic GMP-dependent protein kinases offers new insights into a protein family that has been the subject of intense research and therapeutic drug discovery, but has never been studied in the context of ciliogenesis.

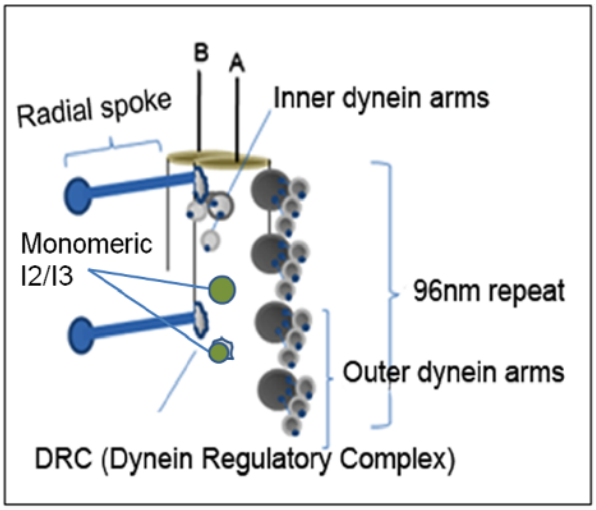
1.10 Figures

Figure 1-1. Cross-section of a motile cilium and an immotile primary cilium.



Based on data from multiple sources, especially Pazour *et al*., 2005; Satir *et al.*, 2008

Figure 1-2. Diagram showing 96 nm of the flagellar axoneme

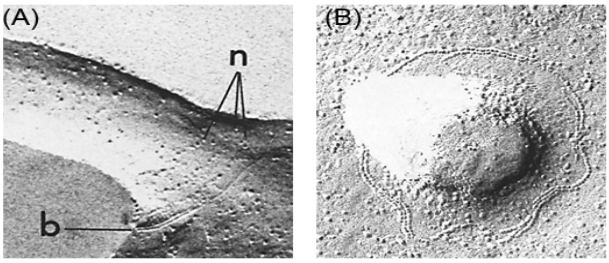


Shown in the diagram, the repeat contains outer arm dyneins, one dynein regulatory complex, two radial spokes and one of each different kinds of inner dynein arm (only I1 or f-specie shown).

B-tubule and A-tubule, marked as B and A, respectively.

Based on data from multiple sources, especially King and Kamiya, 2008; Wirschell *et al*., 2008)

Figure 1-3.Flagellar necklace and bracelet

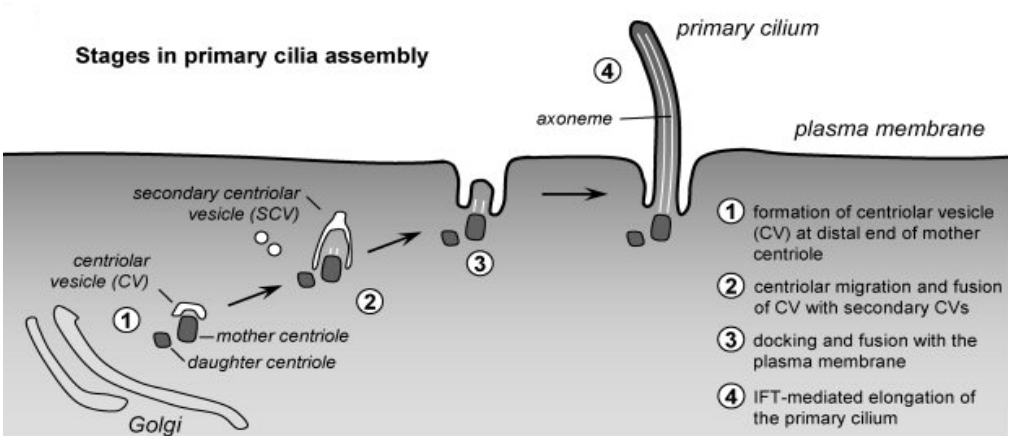


(A). Flagellar necklace (n) and bracelet (b) in freeze-fractured TEM of *Chlamydomonas* plasma membrane.

(B). Flagellar bracelet in a *Chlamydomonas* cell, which has lost its flagella.

Images from Weiss *et al.,* 1972 (Image copyright of respective authors and reproduced here under Creative Commons License from the Journal of Cell Biology and Rockefeller University Press).

Figure 1-4. Stages in the formation of a primary cilium

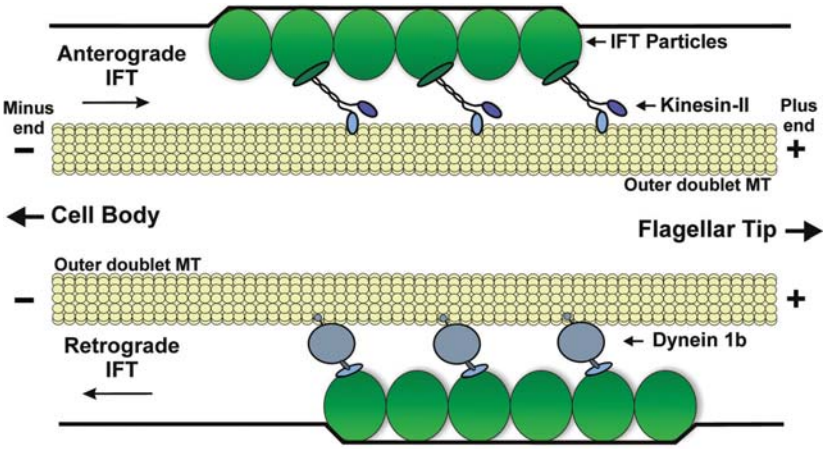


Above diagram illustrates the different stages of ciliary assembly. In (1) a Golgi-derived vesicle fuses to the distal end of the mother centriole. In (2) other vesicles fuse to the initial vesicle and an invagination appears in the lumen of vesicles. As depicted in the diagram, axonemal structures also starts to appear distal to the mother centriole. In (3) the membrane bound axoneme reaches the plasma membrane and the two membranes fuse, forming the ciliary necklace. In (4), IFT mediated axonemal elongation marks the final stages of building the primary cilium.

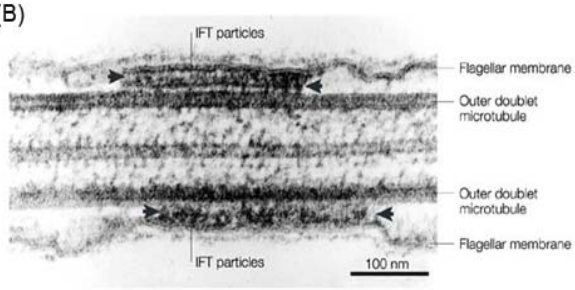
Diagram from Pedersen *et al*., 2008 (Image copyright of respective authors, and reproduced here under Creative Commons License from Developmental Dynamics).

Figure 1-5. Intraflagellar Transport

(A)



(B)

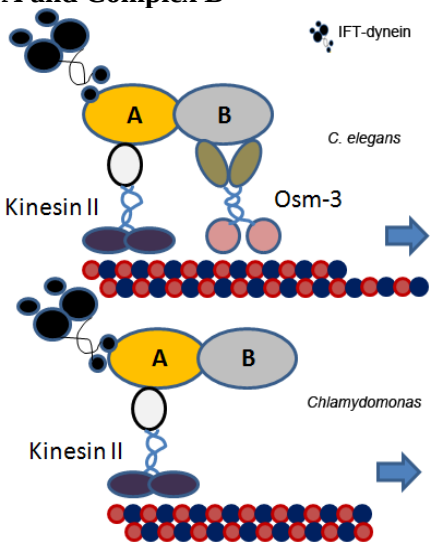


(A). Kinesin and dynein mediated anterograde and retrograde movement of particles in the spaces between the membrane matrix and the axoneme of the flagellum. The particles move along the B-tubule of the flagella. Diagram © Cole, 2003. Image used within the terms and conditions of Creative Commons License - Traffic (A Blackwell Munksgaard Journal) and is copy right of the respective author.

(B). EM of a longitudinal section of Chlamydomonas flagella showing IFT particles.

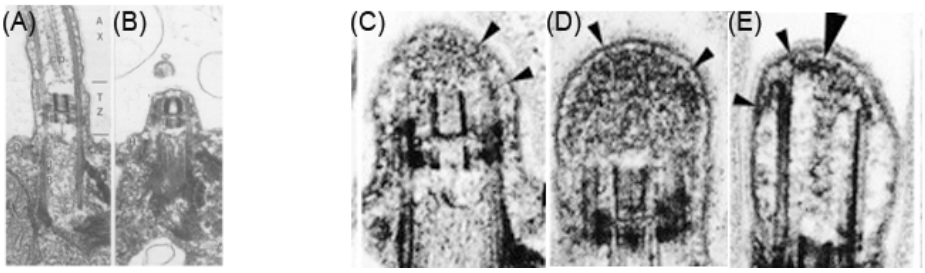
Kozminski *et al*., 1995; Rosenbaum and Witman, 2002. (Image copyright their respective authors, and reproduced here under Creative Commons License from the Journal of Cell Biology and Rockefeller University Press).

Figure 1-6. IFT Complex A and Complex B



Simplified diagram showing molecular motors mobilizing IFT complex A and B in *C. elegans* and *Chlamydomonas.* Based on many sources especially Blacque *et al*., (2008).

Figure 1-7. Flagellar Disassembly and Assembly



(A). Electron micrograph of *Chlamydomonas* flagella prior to pH shock. (Ax: Axoneme, TZ: Transition zone).

(B). Electron micrograph of the axoneme and the basal body immediately after pH shock (deflagellation). Transition zone is defined by the presence of 'H' and distal to the transition zone is the site of deflagellation. (Image from Sanders and Salisbury, 1989).

(C to E). *Chlamydomonas* flagellar assembly after pH shock. Accumulation of electron dense particles distal to the transition zone marks the presence of IFT particles (arrowheads). (D) to (E) shows the gradual increase in the flagellar length. (Images from Dentler, 2005)

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CHAPTER 2
KATANIN KNOCKDOWN SUPPORTS A ROLE FOR MICROTUBULE SEVERING IN RELEASE OF BASAL BODIES PRIOR TO MITOSIS IN *CHLAMYDOMONAS*

M. Qasim Rasi[1], Jeremy D.K. Parker[1], Jessica L. Feldman[2], Wallace F. Marshall[2] and Lynne M. Quarmby[1]

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[1]Department of Molecular Biology & Biochemistry, Simon Fraser University, Canada
[2]Department of Biochemistry & Biophysics, University of California San Francisco, USA

Author Contributions:

MQR designed pEZ, assayed for katanin knock-down by various means, including screening colonies, mRNA determinations, antibody purification, Western blotting and immunoflurescence. JDKP backcrossed the original RNAi isolates, and assisted in cell cycle-related experiments, including synchrony and immunofluorescence; also co-wrote the manuscript. JLF and WFM contributed conceptually to the idea of flagella remaining attached to cells during mitosis and provided Figure 4E. LMQ contributed intellectually to all experiments and co-wrote the manuscript.

2.1 Abstract

Katanin is a microtubule-severing protein that participates in the regulation of cell cycle progression and in ciliary disassembly, but its precise role is not known for either activity. Our data suggest that in *Chlamydomonas*, katanin severs doublet microtubules at the proximal end of the flagellar transition zone, allowing disengagement of the basal body from the flagellum prior to mitosis. Using an RNAi approach we have discovered that severe knock-down of the p60 subunit of katanin, KAT1, is achieved only in cells

that also carry secondary mutations that disrupt ciliogenesis. Importantly, we observed that cells in the process of cell cycle-induced flagellar resorption sever the flagella from the basal bodies before resorption is complete and we find that this process is defective in KAT1 knock-down cells.

2.2 Introduction

Almost all eukaryotic cells are ciliated (note that in some cells, such as *Chlamydomonas*, cilia have historically been called flagella, but eukaryotic flagella are in fact cilia). Although cells with highly specialized cilia, such as respiratory or olfactory epithelial cells or sperm, do not re-enter the mitotic cell cycle, most ciliated cells do divide. With rare exception, there is a tightly orchestrated sequence involving loss of cilia prior to mitosis, and then, shortly after exit from mitosis, ciliogenesis occurs (Reider *et al.*, 1979). This coordination is thought to reflect the alternating roles of centrioles, as the basal bodies, which nucleate cilia and as the organizing foci of mitotic spindle poles (Quarmby and Parker, 2005). The regulatory relationship between cilia and cell cycle progression has not been elucidated, but it has been proposed that one function of primary cilia is to sense environmental signals pertinent to cellular decisions about division, differentiation and apoptosis (Pan and Snell, 2007; Marshall and Nonaka, 2006).

There are two distinct ways that cells can lose their cilia: by deciliation or by resorption. Deciliation, also known as deflagellation, refers to the shedding of cilia into the environment (reviewed by Quarmby, 2004). Most (if not all) ciliated cells deciliate in response to chemical or physical stress. When cilia are resorbed in advance of mitosis, both axonemal and membrane components are retained by the cell. Although these two processes appear to be distinct in both function and mechanism, we have previously

shown that they share common aspects of regulation and that resorption likely involves activity at the base, in addition to the well-established disassembly, which occurs at the ciliary tip (Marshall and Rosenbaum, 2001; Parker and Quarmby, 2003). We have previously proposed that elements of the deciliation machinery might play roles in resorption.

Deflagellation involves the precise severing of the axoneme at a specific site between the axoneme proper and the flagellar transition zone, known as the SOFA (Site of Flagellar Autotomy; Mahjoub *et al.*, 2004). The microtubule-severing ATPase, katanin, has been implicated as the protein responsible for the breakage of the outer doublet microtubules during deflagellation, but the evidence has been indirect (Lohret *et al.*, 1998; Lohret *et al.*, 1999). Purified sea urchin katanin is capable of breaking axonemes, antibodies against katanin inhibit calcium-activated axonemal severing in vitro, and immuno-gold labeling with an anti-katanin antibody places katanin at the site of action. However, a screen for deflagellation mutants in *Chlamydomonas* yielded multiple alleles of each of three genes, *FA1*, *FA2*, and *ADF1*, but not katanin (e.g. Finst *et al.*, 1998). The failure to isolate katanin mutants potentially indicated that either katanin plays no role in deflagellation or it plays an additional role that is essential for survival of the cell. Consistent with the latter possibility, it is well-established in other systems that katanin plays a role in cell cycle progression (Buster *et al.*, 2002; Toyo-Oka *et al.*, 2005; McNally *et al.*, 2006; Zhang *et al.*, 2007).

Based on the idea that cells with partial loss of katanin, but still retaining enough katanin to survive, might reveal hypomorphic phenotypes, we have taken an RNAi approach to reduce levels of expression of the catalytic p60 subunit of katanin in the

haploid unicellular alga, *Chlamydomonas reinhardtii*. Katanin knock-down strains were recovered infrequently, and only in the context of additional mutations which affected flagellar assembly. We went on to show that there is a second site of axonemal severing proximal to the transition zone (the SOFA is distal) and that severing occurs at this site prior to complete pre-mitotic resorption of flagella. Experiments with a cell wall-less strain revealed that cells deficient in katanin fail to release resorbing flagella from the basal bodies. Taken together, our data suggest that one important role of katanin is separation of the basal body from ciliary remnants prior to functional reassignment of the basal bodies to the spindle poles.

2.3 Materials and Methods

Strains, culture conditions and nuclear transformations

Chlamydomonas strains 137c, *cw2*, *bld1*, and *ift88-1* were obtained from the *Chlamydomonas* Genetics Center (Durham, NC). *fa2-3* mutants were previously generated in our lab (Finst *et al.*, 1998). All cells were maintained on TAP (Tris-Acetate-Phosphate) medium plates (Harris, 1989) supplemented with 1.5% agar, under constant illumination at 22^{o}C. Nuclear transformation was by the glass bead method (Kindle *et al.*, 1989); recipient strains other than *cw2* were treated with gametic lytic enzyme (Harris, 1989) prior to transformation. All *par* and *ble* transformants were grown on 1.5% TAP plates supplemented with paromomycin (15µg/ml; Sigma-Aldrich, Oakville, ON) or Zeocin (30µg/ml; Invitrogen, Burlington, ON).

For experiments involving mitotic cells, partial cell cycle synchrony was achieved by growing cells for ~2 days in liquid TAP, followed by starvation in minimal medium (MI) in foil-wrapped flasks for 24-30 hours (Umen and Goodenough, 2001). Cells were

then resuspended in fresh TAP in unwrapped flasks, and aliquots fixed at several timepoints starting 12 hours after the shift to light. The exact timing and degree of synchrony is dependent upon culture conditions, but typically at 12 hours ~50% of cells were in division (as judged by the presence of furrows, unhatched cells, or phospho-histone H3 immunofluorescence).

Molecular constructs

Nuclear transformation of *Chlamydomonas* is accomplished by non-homologous insertion of the exogenous DNA into the genome, a process that can frequently involve deletions of the transforming and genomic DNA. In order to maximize the frequency with which selected colonies are competent to express the transgene, we constructed transformation vectors with different selectable markers at each end (pEZ-KAT1-RNAi; see Figure 1A). Briefly, pEZ-KAT1-RNAi is derived from pGenD (Fischer and Rochaix, 2001), pSI103 (Sizova *et al.*, 2001) and pSP124S (Lumbreras *et al.*, 1998). The *aphVIII* gene, along with its promoter and 3'UTR (the "par cassette"), was amplified from pSI103 using primers Spe-par(+) and Spe-par(-) and cloned into the unique *Spe*I site in pGenD, while the *ble* cassette was removed from pSP124S by restriction digestion and ligated into a *Hin*dIII site in pGenD. The PsaD-derived promoter elements from pGenD drive expression of a cDNA hairpin such that transcription will result in double-stranded RNA. The hairpin was derived from the 5' end of the katanin cDNA (*KAT1*, *Chlamydomonas* gene c_80022; base pairs 114 to 800, then 114-552 in reverse orientation). This same hairpin targeting *KAT1* was also inserted into the pNI-537 vector (Rohr *et al.*, 2004; generously provided by H. Certutti). This region of the cDNA is outside of the coding region for the AAA domain, and has no significant similarity to the most similar genes in

the *Chlamydomonas* genome (*KAT2*, C_620017, 46% identity to KAT1 and a predicted spastin-like protein, *Chlamydomonas* gene model e_gwW.1.632.1, 39% identity to *KAT1*). Primers used are in Table 1.

Assessment of mRNA levels

To assess relative transcript levels for our initial KAT1-RNAi experiments, we adopted a competitive PCR-based approach (Wang *et al.*, 1989). Experimental cDNA pools were normalized to equal concentrations, and then independently mixed with increasing concentrations of a competitor that differs in sequence and length from the target sequence, but which shares with the target the primer binding sites used for amplifying the target (or competitor) sequences. Primers K311A(+) and K1440-64R(-) give a product of 300 bp from the KAT1 cDNA. To make the competitor (pGEM-T-mimic), we performed low-stringency PCR using those same primers on *Chlamydomonas* genomic DNA to obtain a product of 400 bp, followed by a high-stringency second round of PCR using the same primers. The resulting product was cloned into pGEM-T Easy (Promega, Madison, WI). As a control, experimental cDNA pools were also assayed using the gene for ribosomal subunit RPS14 at the *CPH1* locus using the "CRY1" primers described by Kathir *et al.* (2003).

Total *Chlamydomonas* RNA, mRNA and cDNA were obtained as previously described (Mahjoub *et al.*, 2002). Varying concentrations of pGEM-T-mimic were mixed with the cDNA pool from pEZ-KAT1-RNAi cells as the substrate for PCR reactions. Quantification of PCR bands was performed with ImageQuant (GE Healthcare). For RT-PCR of genes putatively disrupted by insertion of pEZ-KAT1-RNAi, cDNA pools were

tested with control primers against centrin; primers specific to *IFT88* and *PKG2* are shown in Table 1.

Real Time Quantitative PCR

Chlamydomonas cDNA pools from wild-type (137c), KAT1-RNAi;*ift88-1* and KAT1-RNAi;*bld1-1* cells were prepared as described above. Primers specific to *KAT1* (Qcp60+/Qcp60-, Table 1) were initially used to PCR-amplify a 227 bp fragment from the 5' end of the *KAT1* cDNA cloned earlier into pEZ-KAT1-RNAi. Conditions that would yield a specific band corresponding to KAT1 were empirically determined. Next, Qcp60+/Qcp60- were used to quantitatively amplify target sequence in a 50 µl reaction mix containing 500 ng of cDNA pool (IQ SyberGreen Supermix; Bio-Rad, Mississauga, ON). The annealing and elongation steps of the amplification cycle were performed at 58^{o}C and 72^{o}C respectively. PCR was run for 44 cycles. Gene expression was standardized to the expression levels of ribosomal subunit RSP14. The MiniOpticon RT-PCR system (Bio-Rad) was used to monitor target sequence amplification and Opticon Monitor3 software (Bio-Rad) was used for data analysis. qPCR was carried out in triplicate and the average reading is represented as the final KAT1 transcript level.

Identification of the sites of insertion of the RNAi constructs

To determine the site of insertion of the RNAi constructs, we used the Vectorette PCR method (Riley *et al.*, 1990). Briefly, this method is based on annealed DNA adapters, that have large regions of mismatched nucleotides between the two DNA strands (the “bubble anchors”), to genomic DNA digested with blunt-cutting restriction endonucleases. Subsequent PCR, using one primer specific to one bubble anchor and the other primer

specific to the integrated construct, amplifies genomic DNA between the restriction enzyme cut site and the insertion. Primer sequences used are in Table 1.

Measurements of cell size

The length and width of at least 75 cells of each strain were measured by DIC microscopy and SoftWorx (v. 3.22) software package (Applied Precision, Issaquah, WA). Cell volumes were calculated using the formula $4/3\ \pi[L/2][W/2]^2$ (Umen and Goodenough, 2001), where L and W describe length and width of the cells, respectively.

Generation of Antibodies and Immunoblotting

KAT1 antibodies were raised against two synthetic peptides (Sigma-Aldrich), N-terminal (KGSAGEKAKKQY) and C-terminal (QVDGVHGSEKDK), conjugated to KLH and injected into four rabbits (Sigma-Aldrich). *KAT1* cDNA (KAT1 full length) and a derived construct lacking the sequence corresponding to the peptide epitopes (KAT1-Δ-epitope) were expressed using pET-DEST42 (Invitrogen) in BL21 *E.coli* (Novagen) and induced with 1mM IPTG (Invitrogen). Antisera from rabbit #8907 exhibited a reactive band of ~60 kDa, which is the predicted size of KAT1, from IPTG-induced cells expressing KAT1 full length but not cells expressing KAT1-Δ-epitope or uninduced cells (data not shown). Characterization of antisera recognizing PKG2 will be described in a future report. Protein levels in cell lysates were determined using QuickStart Bradford Reagent (Bio-Rad). Horseradish peroxidase-linked goat anti-rabbit IgG (1:10,000; Sigma-Aldrich) and ECL (GE Healthcare) were used for visualization of western blots. Densitometry was performed using Adobe Photoshop.

Immunofluorescence Microscropy

Indirect immunofluorescence was conducted as described (Mahjoub *et al.* 2004) using a DeltaVision imaging station (Applied Precision, Issaquah, WA). Primary antibodies used in this study were: mouse monoclonal anti-acetylated-tubulin Igγ2b (1:300; Sigma-Aldrich clone 6-11B-1), mouse monoclonal anti-α-tubulin (1:1000; Sigma-Aldrich clone DM1A) and anti-phosphohistone H3 (ser10, Cell Signalling). Secondary antibodies against mouse IgG2b, mouse IgG1, or rabbit were purchased conjugated to Alexa Fluor 488 or 594 (Invitrogen) and all were used at 1/500. Cells were also stained with DAPI.

For fixation of mitotic cells, a protocol graciously provided by Brian Piasecki and Carolyn Silflow (University of Minnesota, St. Paul, MN), and originally developed by M.A. Sanders, was modified. The fixative consists of 4% formaldehyde (added as formalin, Anachemia), 0.01% glutaraldehyde, 10 mM HEPES buffer pH 7.0, 1 mM EGTA, 1 mM $MgSO_4$ and 0.1% Igepal CA-630 (Sigma-Aldrich) and thus permeabilizes cells but also cross-links cyotological features. After incubation on ice, fixed cells were affixed to coverslips and methanol-extracted essentially as described previously (Mahjoub *et al.*, 2004), except coverslips were washed with ammonium chloride in addition to PBS.

For flagellar remnant determination, cells were counted as "mitotic" if either positive for phospho-histone H3, unhatched and in mother cell walls, or showing either clear reorganization of rootlet microtubules or separated centrosomes.

For the experiment shown in Figure 4E, cells were pre-stained with Lugol's iodine solution (Harris, 1989) and then fixed and permeabilized in MeOH (Cole *et al.*, 1998). Primary antibodies used were mouse anti-acetylated tubulin (Sigma), mouse anti-α tubulin (Sigma, clone DM 1A used at 1/300). Secondary antibodies (Jackson

ImmunoResearch, West Grove, PA) used were CY5-conjugated goat anti-mouse IgG, subclass I, FITC-conjugated goat anti-mouse IgG, subclass 2b (used at 1/100 dilution), and TRITC-conjugated goat anti-rabbit (1/300).

2.4 Results

Initial isolation of two KAT1 knock-down strains.

Currently, the most effective approach to RNA interference in *Chlamydomonas* involves the non-homologous insertion of a transgene which encodes the inhibitory RNA, into the nuclear genome (reviewed by Schroda, 2006). The insertion event is often associated with deletions of genomic sequence and part of the exogenous DNA (Gumpel *et al.*, 1994). Because our initial attempts using conventional methods did not yield katanin knock-down strains, we sought to increase the frequency of recovery of intact insertions. To do this we developed a vector wherein the inhibitory sequence (a hairpin loop corresponding to KAT1 sense and antisense sequence) is flanked by two different selectable markers (Figure 2-1A).

From fifteen independent transformation experiments, ~1500 colonies grew on plates containing both Zeocin and paromomycin. For initial characterization, we grew six of these isolates and assessed levels of KAT1 mRNA. Although all six had integrated the complete transgene, none of the colonies had reduced levels of KAT1 mRNA (data not shown). In *Chlamydomonas*, RNAi transgenes can be rapidly silenced or suppressed (especially those targeting genes involved in cell cycle progression; Schroda, 2006; Qin *et al.*, 2007) and we were concerned that in growing up sufficient quantities of cells for RNA analysis, suppressed strains would rapidly overtake the culture preventing us from observing authentic knockdowns. In order to narrow our search for KAT1 knock-down

isolates, we conducted a preliminary screen by phase-constrast microscopy. We examined live samples of the ~1500 isolates for gross defects in motility or for defects in deflagellation in response to weak acid (as described in Finst *et al.*, 1998). Fixed samples of each isolate were also examined by phase contrast microscopy for gross changes in ciliary length, number or position or for aberrant cell size (as a proxy for effects on cell cycle progression (Bradley and Quarmby, 2005).

Only two of the 1500 strains exhibited either flagellar or cell size defects, and both of these had the same phenotype: the cells were small relative to the cell wall-less parental strain (Figure 2-1B) and remained small after backcrossing into a wild-type (cell-walled) background (Figure 2-1C). In addition, both isolates also exhibited flagellar defects (Figure 2-1D). Katanin mRNA levels were dramatically reduced in both of these strains (Figure 2-1E). Although KAT1-RNAi#1 and #4 were clearly katanin knock-down strains, it was important to establish whether the phenotypes were due to the reduced levels of katanin. As described above, generation of transgenic strains in *Chlamydomonas* involves disruption of genomic DNA. We therefore set out to determine the sites of insertion of the RNAi transgenes in order to assess the possible role of these disruptions in affecting the observed phenotypes.

The two original KAT1 knock-down strains are also insertional mutants of flagellar assembly genes

We used a PCR-based approach to clone the DNA flanking the insertions (see Materials and Methods). This approach identified unique sites of insertion in each of the two back-crossed strains. We found that the KAT1-RNAi#4 transgene had inserted into the genomic sequence for the 3' UTR of the *IFT88* gene (C_500002 in version 3 of the *Chlamydomonas* genome), which encodes an intraflagellar transport protein that is

essential for ciliogenesis: *ift88* mutants are flagella-less (Pazour *et al.*, 2000). We confirmed that *IFT88* expression is lost in the KAT1-RNAi#4 strain by RT-PCR (Figure 2-1F). This result raised the important possibility that the flagella-less phenotype was not a bona fide KAT1-RNAi phenotype but rather was a consequence of the inadvertent knock-out of *IFT88*. It was thus imperative to determine the site of insertion of the transgene in the KAT1-RNAi#1 strain.

We determined that in the KAT1-RNAi#1 strain, the construct had inserted into the coding sequence of a predicted protein kinase G gene, which we will refer to as *PKG2* (C_740056). Primers specific to *PKG2* amplified cDNA from the *cw2* strain, but not from the KAT1-RNAi#1 strain (Figure 2-1F). We have subsequently determined that PKG2 plays an essential role in ciliogenesis, but does not affect cell size (Rasi *et al.*, unpublished observations.).

Is a defect in ciliogenesis permissive for knock-down of KAT1?

As described above, KAT1 knock-down strains were rare, and the only two that we identified were co-incident with mutations that disrupted ciliogenesis. These observations lead us to hypothesize that KAT1 might indeed be performing an essential function, but whatever this function might be, it is only essential in cells with normal flagella. In other words, we hypothesized that defects in flagellar assembly are permissive for knock-down of katanin. This hypothesis led us to predict that we would recover KAT1 knock-down strains at a much higher frequency if the recipient strain for the transformations was already defective in ciliogenesis.

In order to test this idea, we independently transformed two flagella-less strains, *ift88-1* and *bld1-1* (which is mutant for IFT52, Brazelton *et al.*, 2001; Deane *et al.*, 2001),

as well as wild-type cells, with the same KAT1-RNAi construct described in Figure 2-1A. In this experiment, we recovered only one isolate that was resistant to both selectable markers when the recipient strain was wild-type. In contrast, ten and eleven isolates were resistant to both Zeocin and paromomycin when the recipient strains were *ift88-1* and *bld1*, respectively. The single KAT1-RNAi isolate from a wild-type background that grew on double selection was not knocked down for *KAT1* mRNA (data not shown). In contrast, 9/10 and 8/11 double-selected isolates, from the transformations of *ift88-1* and *bld1* respectively, had significantly reduced levels of *KAT1* mRNA (Figure 2-2A and 2-2B; representative subset shown). Thus, seventeen new KAT1 knock-down strains were isolated. All of these strains lack flagella (as do the parental strains) and, distinct from the parental strains, all had substantially reduced cell size (see Figure 2-2C and D; representative subset shown).

In order to more directly assess levels of KAT1, we raised an antibody against two peptides of KAT1 (see Materials and Methods). Western blotting revealed ~60 kDa band from wild-type *Chlamydomonas* cell lysates, not observed using preimmune serum from the same rabbit (Figure 2-3A). With this antibody in hand, we repeated the transformation of WT and *bld1* cells with the KAT1-RNAi construct.

In this experiment, 52 isolates grew on double selection when the recipient strain was flagellated (137c). None of these 52 isolates had severely reduced levels of KAT1, but in a few lines KAT1 protein levels were moderately reduced (Figure 2-3B shows the data for eight of these 52 isolates, including two with moderately reduced levels of KAT1 protein). In this same experiment 14 isolates grew on double selection when the recipient strain was *bld1*. In contrast to the flagellated cells transformed with the same construct,

most of these flagella-less isolates showed reduced levels of KAT1 protein and in some the reduction in KAT1 levels was severe. Of the seven isolates shown in Figure 2-3C, three show substantial reduction in KAT1 levels, three are moderately reduced and in the seventh KAT1 levels are unaffected.

In this experiment the severely knocked down strains showed distorted cell size profiles (Figure 2-3E), but unlike the experiment in Figure 2-2, reduction in cell volume did not correlate with the extent of knock-down of KAT1. It is interesting to note that the knock-down of KAT1 is transient, even in the *bld1* cells. Figure 3D shows that after an additional two weeks, levels of KAT1 are no longer severely reduced. Concomitantly, the cell size profiles become closer to WT (Figure 2-3E).

What are the cellular consequences of KAT1 knock-down?

In addition to their ciliogenesis defects, the two original KAT1 knock-down isolates had small cells (Figure 2-1B,C). The other genes disrupted in these strains, *IFT88* and *PKG2*, are essential for ciliogenesis (Pazour *et al.*, 2000; Rasi *et al.*, unpublished observations), but do not affect cell size (data not shown). Furthermore, the seventeen additional KAT1 knock-down strains generated in *ift88-1* and *bld1* backgrounds also had small cells (Figure 2-2C,D). However, this pattern was not as clear in a subsequent experiment (Figure 3E). Figures 3C and 3D reveal that isolate #29 has the most severe knock-down of KAT1, yet this isolate has unusually large cells (Figure 2-3E). Nevertheless, the data in Figure 2-3E also show that the cell size profiles approach wild-type as the levels of KAT1 return to normal. Taking all of these data together, we conclude that reduced levels of KAT1 can influence cell size, but the cell size phenotype is not consistent and appears to depend on some yet-to-be-defined differences in culture conditions.

Aberrant cell size is often an indication of defects in cell cycle progression. Unfortunately, none of the katanin knock-down strains remained phenotypically stable long enough for us to assess their cell cycle characteristics as we have previously done for other *Chlamydomonas* flagellar mutants with cell size defects (Mahjoub *et al.*, 2002; Bradley and Quarmby, 2005). We have attempted several approaches to obtaining conditional KAT1 knock-down strains, but so far none of these strategies has succeeded. Nevertheless, an independent line of study has led us to a possible ciliary-cell cycle role for KAT1. The experiments in the next section may provide the explanation for the difference in susceptibility to KAT1 knock-down of WT compared to *bld* cells.

Basal bodies are released by severing at the proximal transition zone prior to complete resorption of flagella

Taken together, our data thus far indicate that KAT1 plays an important role in *Chlamydomonas* and that this role is obviated in cells that do not have flagella. This suggests that katanin may play a role in coupling the cycle of flagellar resorption and regeneration with cell cycle progression. Our earlier work predicted that the machinery of deflagellation plays a part in pre-mitotic resorption of flagella. Indeed, we predicted that disassembly at the base of flagella might contribute to resorption (Parker and Quarmby, 2003).

The generally accepted model for pre-mitotic resorption of flagella is that disassembly continues down from the tip, through the transition zone of the flagella, until all that is left is the basal body (Cavalier-Smith, 1974). Contrary to this view, the images in Figure 4 reveal that flagella or their derivatives (visualized with indirect immunofluorescence using an antibody against acetylated tubulin) remain with the mother cell wall when cells enter mitosis. In Figure 2-4, panels A-D reveal pairs of

acetylated tubulin spots associated with the mother cell walls of dividing wild-type cells. The spots, or flagellar remnants, were observed in the mother cell walls of 226 out of 291 (78%) dividing cells. Flagellar remnants are not observed if gluteraldehyde, a cross-linking reagent, is omitted from the fixative, or in a cell wall-less mutant background, suggesting that the remnants are loosely associated with the cell walls (data not shown). Mitotic cells with more substantial incompletely-resorbed flagellar remnants, in some cases almost full-length flagella, were uncommon, but could be found (Figure 2-4E; also see Piasecki *et al.*, 2007). Importantly, in all of these cells it is clear that the basal bodies have separated from the residual flagella (Figure 2-4).

These data indicate that a severing event occurs between the basal bodies and the axoneme, releasing the basal bodies for service at the spindle poles. Severing of the axoneme is a well-established event in deflagellation (reviewed in Quarmby, 2004) and we wondered whether the release of basal bodies might be using the same pathway. In order to test this idea, we examined the *fa2* deflagellation-defective mutant. The *fa2-3* strain is null for FA2; the cells are completely defective in the axonemal severing associated with deflagellation and exhibit a G2/M delay in cell cycle progression (Mahjoub *et al.*, 2004). Figure 2-4F-H show examples of *fa2* cells clearly in mitosis, while retaining flagellar remnants. We examined 157 dividing *fa2-3* cells and observed that 144 (92%) retained flagella or flagellar remnants associated with the mother cell wall. Thus the production of flagellar remnants is at least as common in *fa2* cells as it is in wild-type. Severing in deflagellation occurs at the SOFA, which is distal to the transition zone and it is likely that the separation of basal bodies observed here is a consequence of severing at the proximal end of the transition zone (Figure 2-6). The

proximal severing appears to be intact in *fa2* mutants, consistent with the observation that the null mutation is not lethal. Taken together with the data of Mahjoub *et al.* (2004), our data indicates that FA2 plays a role in distal but not proximal severing. Katanin may play a role at both sites. Indeed, immuno-gold studies with an anti-katanin antibody revealed katanin at both sites (Lohret *et al.*, 1999).

Knock-down of KAT1 prevents pre-mitotic release of flagella from basal bodies. We next asked whether the lethality of KAT1 knock-down in flagellated cells might be a consequence of failure to separate basal bodies from flagellar remnants. Our previous experiments indicated that flagellated recipient strains do not survive severe KAT1 knockdown, therefore we examined these colonies five days post-transformation. We used a *cw2* recipient strain in order to observe only flagella and flagellar remnants remaining attached to the basal bodies, without the complication of discarded flagellar remnants retained by association with the mother cell wall.

We examined sixty isolates that grew on paromomycin after transformation of cell wall-less (*cw2*) cells with the empty pNI-537 vector. We observed no flagella on cells with cytokinetic furrows (>20 presumptive dividing cells were examined in each colony; data not shown). These data support the idea that in cells with a normal allotment of katanin, release of the basal bodies from the flagella or flagellar remnants results in the shedding of the flagellar remnant from the cell. In the absence of cell walls, the flagellar remnants are washed away, whereas in walled cells remnants are observed because they are cross-linked to the mother cell wall (Figure 2-4). We next examined sixty isolates derived from pNI-537-KAT1-RNAi transformation of the same cell wall-less recipient strain. Dividing (furrowed) cells in 27/60 presumptive KAT1-RNAi

colonies had flagella attached to almost all of the furrowed cells observed. Because these cells are wall-less, flagella will only be retained if still anchored at the basal body. Figure 2-5 shows examples of *cw2* cells presumptively depleted for katanin. In addition, small malformed cells were common in these colonies (data not shown). These data suggest that depletion of KAT1 affects pre-mitotic loss of flagella and leads to gross cellular abnormalities. Most importantly, the presence of flagella on the KAT1-RNAi transformed wall-less cells with apparent cytokinetic furrows is consistent with a failure to separate basal bodies from flagella prior to mitosis.

2.5 Discussion

A phenomenological correlation between ciliary disassembly and entry into mitosis has been known for some time (Tucker *et al.*, 1979). More recently, several proteins have been implicated in coordinating ciliary resorption with cell cycle progression, including AurA, Hef1 and HDAC6 (Pugacheva *et al.*, 2007), and IFT27 (Qin *et al.*, 2007). The presumption has been that ciliary resorption is a consequence of down-regulation of anterograde intraflagellar transport (IFT) and possible upregulation of retrograde IFT, resulting in a net transport of disassembled ciliary components from the tip back to the cell body. While this is almost certainly the case based on the work of Qin *et al* (2004), our previous work with *Chlamydomonas* mutants revealed that events at the base of the cilium are also important (Parker and Quarmby, 2003). The most dramatic instance of ciliary deconstruction at the base is deflagellation (also known as deciliation; reviewed in Quarmby, 2004). Deflagellation, which usually occurs as a stress response, involves the activation of a signaling pathway that culminates in an increase in intracellular calcium concentration leading to activation of the severing machinery which breaks all nine outer

doublet microtubules at the SOFA. Although some elements of the signaling pathway have been established in *Chlamydomonas*, the actual machinery of severing has not been determined. Katanin has been implicated through biochemical and cell biological studies, but genetic evidence has been elusive. Because we were unable to sustain knock-down of katanin in flagellated cells, the current data do not allow us to test whether KAT1 or one of the other microtubule-severing proteins serves the stress-inducible deflagellation pathway.

Katanin is a conserved eukaryotic protein that has been implicated in the cell cycle since its original purification from M phase oocytes (McNally and Vale, 1993), but its specific role in cell cycle progression has been difficult to define. Evidence is accumulating that the microtubule-severing activity of katanin is required for rearrangement of the interphase microtubule cytoskeleton and concomitant spindle formation (Toyo-Oka et al, 2005), as well as spindle function and disassembly (Buster *et al.*, 2002; McNally *et al.*, 2006; Zhang *et al.*, 2007). Mitotic spindle formation involves multiple pathways, even in a single organism or cell type (Wadsworth and Khodjakov, 2004; O'Connell and Khodjakov, 2007). In flies, but not worms, multiple microtubule-severing enzymes, katanin, spastin, and fidgetin, all have different roles in contributing to mitotic spindle function (Zhang *et al.*, 2007; Srayko *et al.*, 2000). In mice, katanin p60 is recruited to splitting centrosomes by Ndel1 in an Aurora A-dependent manner (Toyo-Oka *et al.*, 2005; Mori *et al.*, 2007), but no obvious Ndel-like proteins are identifiable by tBLASTn in the *Chlamydomonas* genome (version 3.0, accessed July 2008). It is possible that the development of important roles for microtubule-severing in eukaryotic cell division preceded divergence of the microtubule severing proteins. This requires that, in

divergent lineages, different microtubule-severing proteins cooperate with diverse partner proteins to undertake the various specialized severing tasks.

Early evolutionary divergence could also serve to explain differences in subunit interactions. In animal cells katanin is usually isolated as a dimer, comprised of the p60 catalytic subunit and the p80 regulatory subunit (Hartman *et al.*, 1998). A human tumor suppressor, LAPSER, associates with the human p80 subunit and localizes preferentially to the mother centriole, which nucleates the primary cilium (Sudo and Maru, 2008). The *pf15* strain of *Chlamydomonas*, which carries a mutation in the sole katanin p80 subunit found in the genome, has defects in the formation and stability of the central pair of microtubules (Dymek *et al.*, 2004). Intriguingly, the central pair microtubules are nucleated close to the SOFA, where the p60 subunit is implicated in axonemal severing.

We observed that a substantial fraction of mitotic cells retain flagella or flagellar remnants, even though their basal bodies are no longer associated with the retained structures, indicating that the axonemes of these flagella have been successfully severed from their basal bodies. This is consistent with previous observations by electron microscopy of *Chlamydomonas* (Johnson and Porter, 1968) and *Chlorogonium* (Hoops and Witman, 1985) and immunofluorescence microscopy (Piasecki *et al.*, 2007). Based on these data, we propose that katanin-based severing of the axoneme proximal to the flagellar transition zone facilitates entry into mitosis (see Figure 2-6). We propose that severing proximal to the transition zone results in release of the basal bodies and abandonment of the transition zone at the mother cell wall. Consequently, *Chlamydomonas* cells without flagella do not require katanin.

To be more precise, our model predicts that transition zone-less cells will not depend upon katanin for the release of their basal bodies. In spite of this, we were able to knock-down KAT1 in *bld1* cells, which are capable of building transition zones (Brazelton *et al*., 2001; Deane *et al*., 2001). Our model suggests that the key event for mitosis is the release of basal bodies and that this it occurs by severing at the proximal end of the transition zone. In this case, why are flagella-less cells more tolerant of katanin knock-down than wild-type cells, even though both build transition zones? The higher frequency of recovery of knock-down strains might be indicative of a subpopulation of *bld1* cells that do not assemble transition zones, or transition zones that are less stable than wild-type. We think it more likely that there is an alternate mechanism to free basal bodies from transition zones, and in the absence of the need to disassemble flagella prior to mitosis, this alternate, albeit less efficient, pathway is taken.

It is interesting to note that in both *bld1* and wild-type cells, katanin may itself contribute to the process of RNAi-mediated knockdown. Recently, in the plant *Arabidopsis*, Argonaute-mediated translational suppression via the siRNA pathway was shown to be dependent on katanin (Broderson *et al.*, 2007) acting through an as yet unknown mechanism. If this is true in *Chlamydomonas,* then it is likely that katanin knockdown may be unsustainable, even in the absence of direct effects on cell cycle or growth.

We propose that severing at the proximal transition zone is an important activity of katanin in *Chlamydomonas* and possibly mammalian cells, but not necessarily all ciliated cells. For example, in *Tetrahymena*, another genetic model system for the study of cilia, misexpression of katanin affects ciliary length but does not result in a cell cycle

phenotype (Sharma *et al.*, 2007). However, in *Chlamydomonas* but not in *Tetrahymena,* ciliary basal bodies must be detached from their axonemes prior to mitosis, thus any proteins that affect this process in *Chlamydomonas* may cause a cell cycle phenotype, whereas such an effect would not be predicted in *Tetrahymena*.

Flagella-less cells can survive and divide without katanin, but in some of the isolates the cells are small (or large), and the KAT1 knock-down is quickly lost. To date, these cultures have not survived as KAT1 knock-down strains long enough for us to determine the reason for the cell size defects and our attempts to develop conditional strains have failed. It is possible that the cell size phenotype might not be related to severing of the proximal transition zone because katanin has been implicated in several other mitotic events (Buster *et al.*, 2002; Toyo-Oka *et al*., 2005; McNally *et al.*, 2006). In addition, microtubule severing by katanin regulates non-centrosomal microtubule arrays (Roll-Mecak and Vale, 2006) and in *Chlamydomonas*, a cortical array of non-centrosomal microtubles play important roles in cytokinesis (Ehler *et al.*, 1995). We speculate that katanin plays several fundamental roles essential to cell cycle progression in both *Chlamydomonas* and mammalian cells and that one of these roles is related to severing of the ciliary axoneme at the proximal transition zone prior to mitosis.

We have shown that *Chlamydomonas* cells free their basal bodies from flagella prior to mitosis. Katanin-mediated severing at the cell-proximal end of the transition zone is the likely mechanism for this disengagement, because of the relative ease with which katanin can be knocked down in flagella-less cells versus wild-type cells. This conclusion gains further support from our observation that presumptive KAT1 knockdown cells retain flagella during division. In retrospect, our initially perplexing observation that the

two original KAT1 knock-down isolates both coincidentally disrupted genes required for ciliogenesis is predictable.

2.6 Acknowledgements

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2.7 Figures and Tables

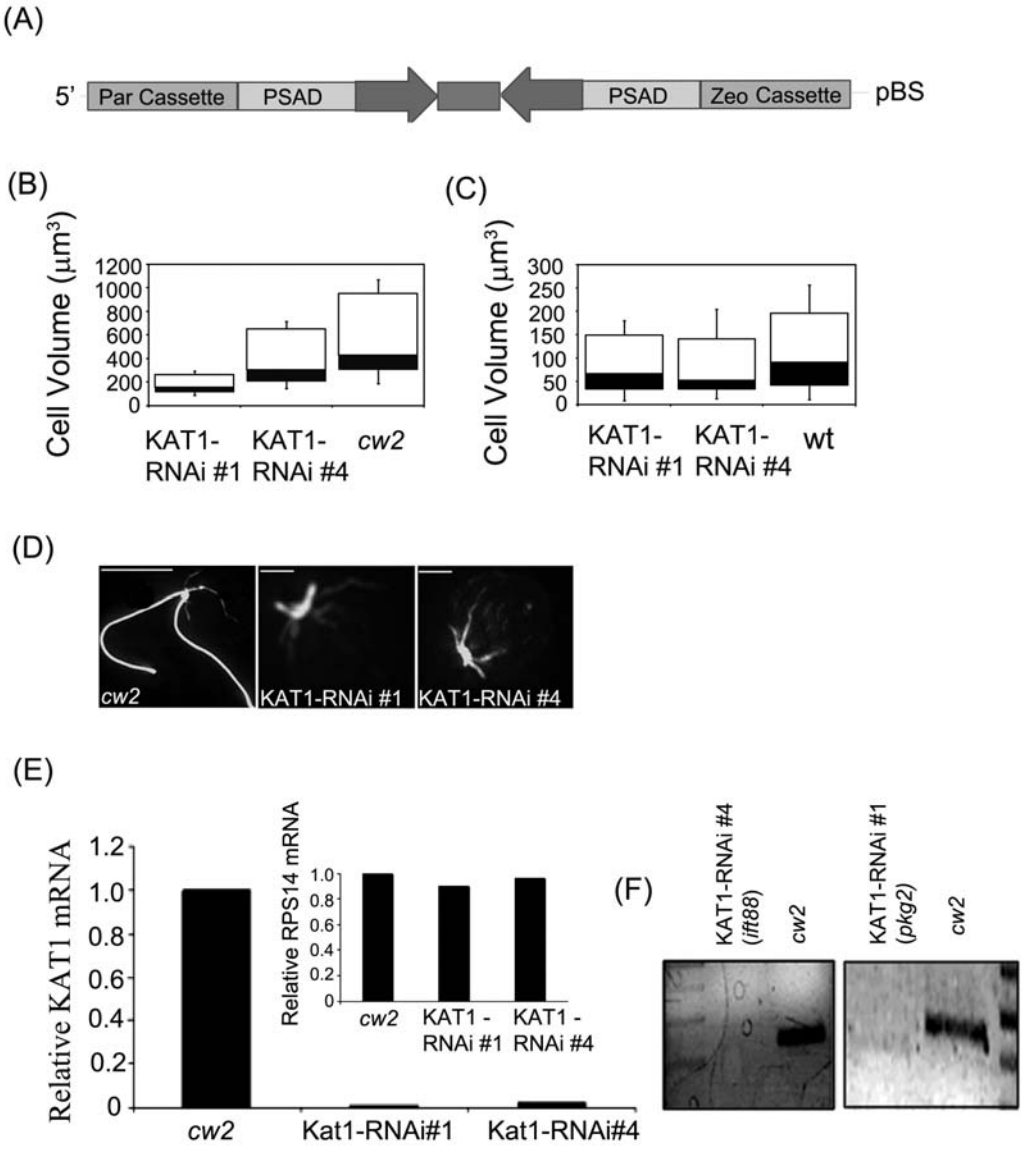

Figure 2-1. Isolation of two KAT1 knock-down strains.

(A). pEZ-KAT1-RNAi construct used to generate stable RNAi lines. Cells transformed with this construct were sequentially selected on plates containing paramomycin, then plates containing Zeocin, to confirm the presence of the PsaD-driven hairpin targeting KAT1 (large inward-pointing arrows).

Legend continued on next page.

Figure 2-1 Legend, continued.

(B). Box plots of cell volume distributions of two KAT1-RNAi isolates and the parental cw2 strain. Boxes encompass 50% of all data points (the interquartile range) and error bars encompass 90% of all data points. The boundary between the black and white boxes is the median cell volume.

(C). Box plots of cell volumes of both KAT1-RNAi isolates after backcross to a wild-type (cell walled) strain, as well as the wild-type cell volume. Boxes encompass the interquartile range and error bars encompass 90% of all data points.

(D). Anti-acetylated tubulin immunofluorescence visualization of typical cells from each isolate (right panels) shows lack of flagella in both KAT1-RNAi isolates. Scale bars, (cw2 panel) 7.5 μm, (KAT1-RNAi#1 and KAT1-RNAi#4, 2.5μm).

(E). Quantification of mRNA from *cw2* and the two KAT1-RNAi strains. Main graph shows relative KAT1 level (the amount of KAT1 mRNA in *cw2* cells is set at 1). The inset graph shows relative control transcript (RPS14) level.

(F). Agarose gel electrophoresis of reverse-transcriptase PCR products. Amplicons targeted putative genes disrupted by insertion of KAT1-RNAi constructs into strain KAT1-RNAi#4 (left panel) and KAT1-RNAi#1 (right panel). All cDNA pools showed accumulation of PCR products targeting a control gene (not shown).

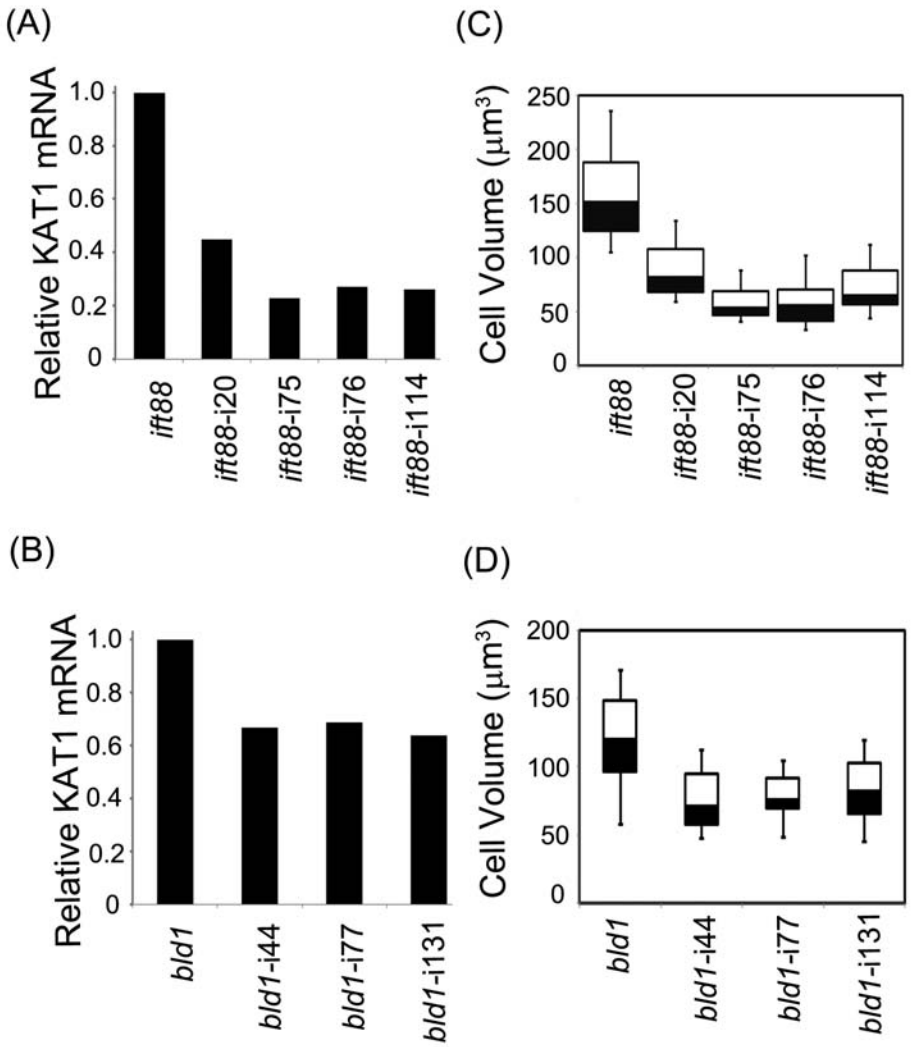

Figure 2-2. *KAT1* can be readily knocked down in flagella-less cells.

(A). Relative *KAT1* mRNA level, as determined by real-time PCR, from a representative subset of *ift88* strains transformed with, and selected for, pEZ-KAT1-RNAi.

(B). Relative *KAT1* mRNA level, as determined by real-time PCR, from a representative subset of *bld1* strains transformed with, and selected for, pEZ-KAT1-RNAi.

(C). Box plots of cell volume distribution of the same *ift88*-KAT1-RNAi isolates examined in (A). Error bars capture 90% of cells.

(D). Box plots of cell volume distribution of the same *bld1*-KAT1-RNAi isolates examined in (A). Error bars capture 90% of cells.

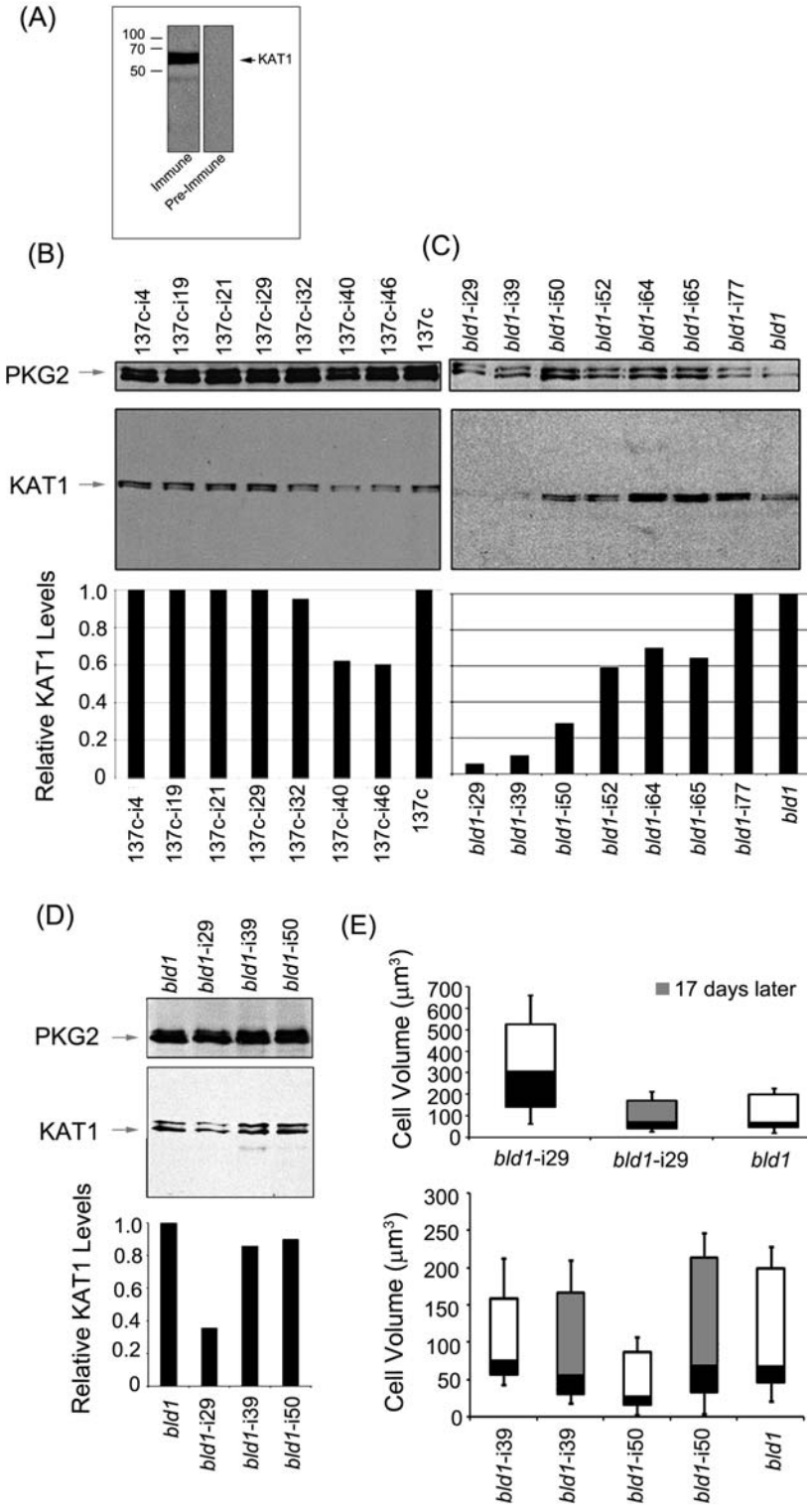

Figure 2-3. KAT1 protein can be readily knocked down in *bld1* mutants**.**

Full Legend on next page.

Figure 2-3. KAT1 protein can be readily knocked down in *bld1* mutants.

(A). Characterization of anti-KAT1 antiserum. Wild-type cell lysate was blotted in duplicate and probed with either immune antiserum (left panel) or pre-immune serum from the same rabbit (right panel). The expected molecular weight of KAT1 is 60kDa.

(B). Quantification of relative knock-down of KAT1 protein levels by anti-KAT1 western blot (middle panel) in seven random isolates recovered from transformation of wild-type cells with pEZ-KAT1-RNAi. Anti-PKG2 was used as a loading control by re-probing the same blot (top panel). Gel films were scanned and densitometric analysis (bottom panel) performed as described in Methods.

(C). Quantification of relative knock-down of KAT1 protein levels by anti-KAT1 western blot (middle panel) in seven random isolates recovered from transformation of *bld1* cells with pEZ-KAT1-RNAi. Anti-PKG2 was used as a loading control by re-probing the same blot (top panel). Gel films were scanned and densitometric analysis (bottom panel) performed as described in Methods.

(D). Quantification of relative knock-down of KAT1 protein levels in a subset of the same isolates examined in (C) after an additional two weeks of growth. Analysis as described for (C).

(E). Box plots showing cell volumes of the same *bld1*:KAT1-RNAi isolates examined in (B) and (C). White boxes show cell volume distribution data from the time of the experiment shown in (B) and grey boxes are data from the time of the experiment shown in (C). Boxes encompass the interquartile range and error bars encompass 90% of the total data points.

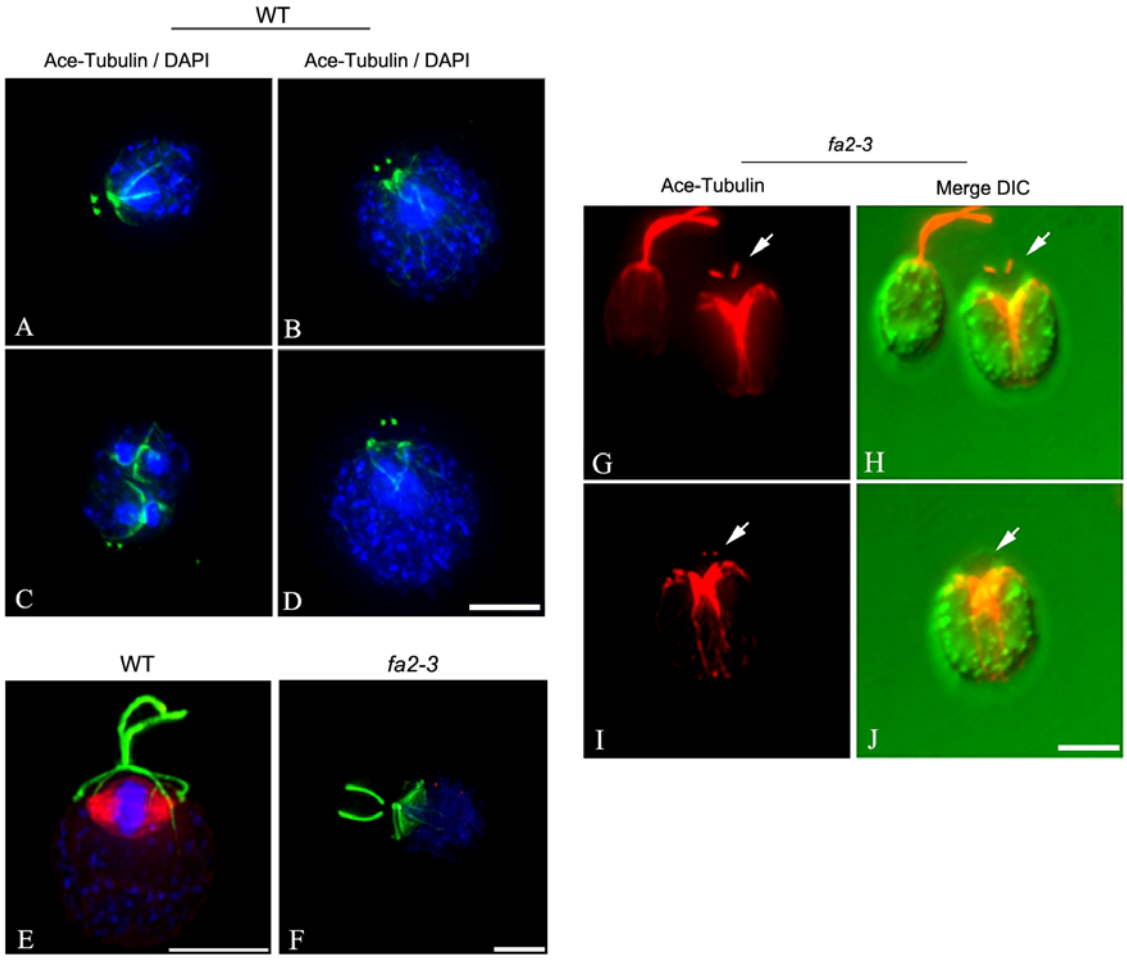

Figure 2-4. Basal bodies are freed from flagella by severing prior to mitosis.

(A-D). Representative images of flagellar remnants associated with representative wild-type mitotic cells. Remnants are visible as dots staining for acetylated-α-tubulin (green) and are found at the anterior end of the cell (A, B, D) or in a position consistent with the prior anterior of the mother cell, in cells that have completed at least one round of division (C). Blue is DAPI, which stains both nuclear and plastid DNA.

(E). Longer flagella (stained with anti-acetylated tubulin; green) are observed more rarely in wild-type mitotic cells. The spindle of this cell visualized with anti-α-tubulin (red).

(F-J). Remnants are found in mitotic *fa2* mutant cells. (F) a *fa2* cell early in cell division has relatively long flagella as stained by acetylated tubulin (green), counterstained with DAPI (blue). (G, I), *fa2* cells later on in cell division, as indicated by the presence of cleavage furrows, have flagellar remnants as visualized by acetylated-tubulin staining (red; the primary antibody for these cells was a mixture of anti-acetylated- and anti-α-tubulin) (H,J), overlay with corresponding DIC images.

Scale bars, 5 μm.

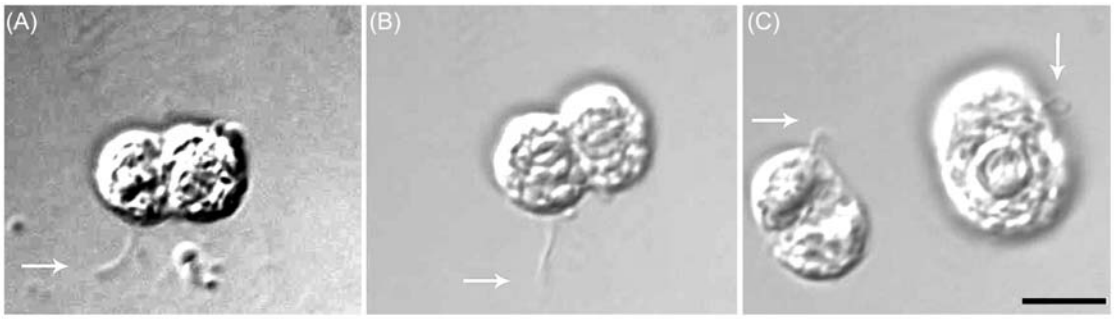

Figure 2-5. DIC images of presumptive *cw2*:KAT1-RNAi cells.

(A-C). Examples of presumptive KAT1 knock-down cells in a *cw2* (cell wall-less mutant) background. Note the presence of flagella on dividing cells. Similar cells were not seen in any control colonies. Scale bar, 5 μm.

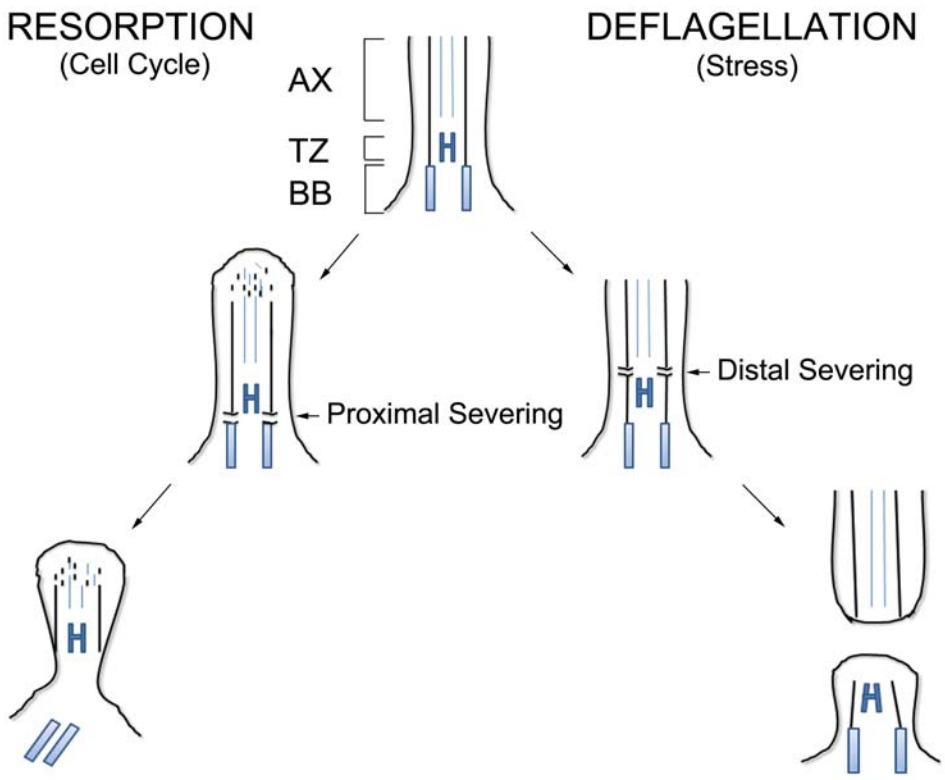

Figure 2-6. Severing may occur at either end of the flagellar transition zone.

Deflagellation in response to stress involves severing at the distal end of the transition zone (the SOFA), after which the flagellum is cast away from the cell while the transition zone remains associated with the basal body and can nucleate the assembly of a new flagellum.

Resorption before mitosis involves both shortening of the flagellum from its distal end and severing at the proximal transition zone. This proximal severing frees the basal body, leaving the transition zone. Any residual flagella, or flagellar remnants, remain associated with the cell wall. (Abbreviations: AX, axoneme; TZ, transition zone; BB, basal body)

Table 2-1. Sequences of the oligonucleotides used in this work.

All sequences are listed in the 5'-3' direction. Plus and minus signs denote forward and reverse primer orientation respectively.

Primer Name	Sequence
Spe-par(+)	5' ATAACTAGTGAGCTCGCTGAGGCTTGA 3'
Spe-par(-)	5' ATAACTAGTGGTACCCGCTTCAAATAC 3'
Kat-EcoRI(+)	5'TAAGAATTCATCACTCGG TACGTCCGC 3'
Kat-Spacer(-)	5' TAAGGATCCCGCCGAGCCGGCGCGCCC 3'
Kat-IR(+)	5' ATATCTAGAGAATTCATCACTCGGTACGTCCGC 3'
Kat-IR(-)	5' ATTGGATCCCAGACCCGCAATGTCGTC 3'
K311A-1 (+)	5' CGGGACCGGCGCGACCATGCTCGCC 3'
KAT 1440-64 (-)	5' GGACCCGGCTCCTTGTCCTTTTCGC 3'
PKG-RT(+)	5' AACCAAGCTTTTTGTTGTGAGTGG 3'
PKG-RT(-)	5' CGGAAGACGTCGCGCTCCAGCA 3'
IFT88-RT(+)	5' ATAGTGATATCATTCAAGCCCTGTTCCTAC 3'
IFT88-RT(-)	5' GTGTGTGAATGTATGTGTGTGCTAGGTAAG 3'
Qcp60(+)	5' AACAGCTACCCCGAGATGAA 3'
Qcp60(-)	5' TTGTTGTTGTTAGCCCAGGA 3'
Bubble1	5'GAGAGGGAAGAGAGCAGGCAAGGAATGGAA-GCTGTCTGTCGCA GGAGGAAG 3'
Bubble2	5'GACTCTCCCTTCTCGAATCGTAACCGTTCGTAC-GAGAA TCGCTGTCCTCTCCTTC 3'
B-specific	5'CGAATCGTAACCGTTCGTACGAGAATCGCT 3'
Par-specific	5' TGGCGTTTTACCGGCTGTTGGACGAGTTC 3'

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CHAPTER 3
CHLAMYDOMONAS CYCLIC GMP-DEPENDENT PROTEIN KINASE 2 HAS A ROLE IN CILIOGENESIS

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M. Qasim Rasi, Jeremy D.K. Parker, Gabriel Alfaro, Christopher T. Beh and Lynne M. Quarmby

Department of Molecular Biology and Biochemistry, Simon Fraser University, Canada

Author Contributions:

MQR identified the genomic mutation in *pkg2*, performed immunofluorescence microscopy, RNA interference of PKG2, biochemical fractionating of PKG2, PKG2 localization and flagellar length measurements. JDKP helped with the phylogenetic tree and manuscript editing. GA performed the yeast two-hybrid screen. LMQ contributed intellectually to all experiments.

3.1 Abstract

We have recently reported the serendipitous isolation of a new *Chlamydomonas* insertional mutant with ciliary defects. We identified an insertion in a gene encoding PKG2, a member of the type II family of cyclic GMP-dependent serine/threonine protein kinases. PKG2 is one of the three PKGs identified in *Chlamydomonas* ciliary proteome. Localization and fractionation studies show that PKG2 is a cytosolic protein, with a small fraction appearing in the flagella. We have identified SAS1 (C_750005) as a PKG2 interacting protein using yeast two hybrid analysis. SAS1 (Adenosyl methionine synthase) is an evolutionarily protein required for catalytic formation of adenosyl methionine from ATP and methionine. In mammals, the PKGs are implicated in a variety of cellular functions including intestinal and renal fluid secretion, bone

growth and regulation of circadian rhythm. Our results suggest that these phenotypes might be derived from ciliary defects.

3.2 Introduction

Cilia are microtubule based structural extensions that project from cell surfaces and play roles in motility and sensing the environment (Christensen *et al*. 2008). Cilia are anchored at the cell surface by other microtubule-based structures called basal bodies or centrioles (Dutcher, 2008). During early stages of ciliary assembly, basal bodies dock at the plasma membrane and by a process generally referred to as IFT (Intra Flagellar Transport) cilia are assembled (Sorokin 1968; Kozminski *et al*. 1993). IFT is defined as a bidirectional movement of ciliary cargo within the spaces between the membrane and the axoneme of the cilia (Kozminski *et al.,* 1993; Kozminski *et al.,* 1995). The cargo, consisting of IFT complexes and ciliary precursors, are mobilized to the tip of the cilia (anterograde direction) by a hetrotrimeric kinesin-2 and the turn-over products are shipped back to the cytoplasm (retrograde transport) by a cytoplasmic dynein 1b (reviewed by Cole, 2008).

Most of our current understanding of ciliary assembly comes from *Chlamydomonas reinhardtii* – a haploid, bi-flagellated alga in which IFT was first discovered (Kozminski *et al.* 1993). Mutations affecting components of the IFT complex (for example, IFT88, IFT146, IFT140, IFT27, kinesin-2 subunits, cytoplasmic dynein subunits) or basal body proteins (for example, Bld10, Bld2, Bld1) yield ciliary assembly defects such as complete loss of cilia or presence of short cilia-like structures (Cole, 2008; Dutcher 2008).

Cellular mechanisms that regulate ciliary assembly also play important roles in regulating ciliary length. Cilia length mutants such as those causing long flagella (*lf*), unequal length flagella (*ulf*) or short flagella (*shf*) have been isolated (Marshall *et al*., 2005). The *shf* genes have not yet been cloned, but four *lf* genes have been identified and characterized, and *ulf*s have been found to be allelic with some *lf*s (Wilson *et al*. 2008). LF2 is a cyclin-dependent protein kinase-like (CDK-

like) protein, and may be activated by a LF1/LF3 heterodimer rather than a cyclin (Tam *et al*. 2003). LF4 is a member of the mitogen-activated protein (MAP) kinase family (Berman *et al*. 2003; Burghoorn *et al*. 2007). Two other kinases have been shown to be involved in regulating ciliary length control – GSK3β (Glycogen Synthase Kinase β ,Wilson and Lefebvre, 2004) and CNK2, a *Chlamydomonas* NIMA-related Kinase (Bradley and Quarmby, 2005).

We have previously described that a mutation in a gene encoding a cyclic GMP-dependent protein kinase II (PKG2) results in ciliary assembly defects (Rasi *et al*. 2009). In this report we present the results of our characterization of this mutant. We find that PKG2 is one of the three *Chlamydomonas* PKGs localizing to the flagella, and cells lacking PKG2 show a spectrum of ciliary assembly defects. Using yeast two hybrid we find that PKG2 interacts with a component of flagellar methylation pathway, and we discuss the relevance of this interaction in the context of ciliary assembly.

3.3 Materials and Methods

Strains, culture conditions and nuclear transformations:

Chlamydomonas wild type strain 137c and B214 were obtained from the *Chlamydomonas* Genetics Center (Durham, NC) and G. Pazour (University of Massachusetts, Amherst, MA) respectively. Cell cultures were maintained on TAP (Tris-acetate-Phosphate; Harris, 1989) supplemented with 1.5% agar. Nuclear transformation with glass beads (Kindle et al. 1989) were performed in the presence of 2μg of plasmid DNA or H_2O as control.

Molecular Constructs:

To make pAc-PKG2RNAi, we used pAc (generously supplied to us by Don Weeks, University of Nebraska); this vector contains a fragment of the *Chlamydomonas* acetamidase gene flanked by inverted rubisco promoters. A hairpin of 426 base pairs representing the 5' end of the PKG2 3'UTR was PCR-amplified using primers PKG-RNAi(+) and PKG-RNAi(-) (see Table X) and cloned into the X site adjacent to the acetamidase fragment. PKG2 cDNA was

obtained from Kazusa Database (Accession# AV 395805; Kazusa DNA Research Institute, Chiba, Japan).

For PKG2 rescue experiments. A 14kb genomic DNA spanning PKG2 along with 3kb 5' upstream seqeuences and 2kb 3' downstream sequences, were cloned in SpeI and ScaI sites of pBluescript. Since *pkg2* mutants were generated by an insertion of a plasmid containing genes conferring resistance to both parmomycin and zeocin, *pkg2* mutants were therefore backcrossed into *Chlamydomonas arg-7* mutants (argininosuccinate lyase gene mutants unable to synthesize arginine, Debuchy *et al*. 1989) to generate *pkg2* x *arg-7* double mutants. *pkg2* x *arg-7* were co-transformed with pBluescript-PKG2 and pARG (pBluescript containing argininosuccinate lyase gene).

Vectorette PCR:

To determine the site of insertion of the RNAi constructs, we used the Vectorette PCR method (Zhong S. and Dean AM., 2004).

Antibodies, Western Blotting and Immunofluorescence:

The PKG2p antibodies were raised against two synthetic peptides (AgriSera, Vannas, Sweden), N-terminal (YADGEGGQVAYIAQRC) and C-terminal (CSPQEAAEWDRVFKDF), conjugated to KLH and co-injected into two rabbits. Antisera from both rabbits exhibited a reactive band of ~81kDa, which is the predicted size of PKG2p, by Western blot. Primary antibodies were used at 1:10,000 dilution (in 5% skim milk) and incubated, overnight, with the nitrocellulose blots at $4^{o}C$. Horseradish Peroxidase-conjugated anti-rabbit secondary antibody was used at 2:10,000 (Sigma Aldrich). Rabbit IC140 antibodies were generously provided by Dr.Winfield S. Sale (Emory University School of Medicine) and it was used at 1:5000 dilution.

Indirect immunofluorescence was conducted as described (Mahjoub *et al.* 2004) using a DeltaVision imaging station (Applied Precision). Monoclonal mouse primary antibodies raised against acetylated and alpha-tubulin were used at 1:500 (Sigma Aldrich). Secondary antibodies used were goat anti-mouse Alexa 488 Igγ2b (1:200; Molecular Probes, Eugene, OR).

Subcellular Fractionation:

Subcellular fraction was carried out as previously described (Mahjoub *et al.* 2004). Cell equivalents of each fraction were used for immunoblot analysis.

Flagellar Length Measurements:

Flagellar length of 137c, short-flagella mutants (*shf*1), *pkg2* and PKG2-RNAi were measured by DIC microscopy and Softworx (v. 3.22) software package (Applied Precision, Issaquah, WA).

PKG2 Evolutionary Tree Analysis:

Protein sequences for Protein kinase A (PKA), Protein kinase C (PKC), Cyclic GMP-dependent protein kinase type I (PKG-I) and type II (PKG-II) were obtained from a broad range of eukaryotic lineages for our phylogenetic analysis. Using computational methods outlined in Parker *et al.* 2007, PKG2 tree was assembled.

Yeast Two Hybrid Analysis:

Volvox c. cDNA two hybrid library was generously provided by Dr. Stephen Miller (University of Maryland). In brief, plasmid pGBKT7-PKG2, pACTII and pAS2 (control plasmids) were transformed into yeast AH109 strains containing the cDNA library in pGADT7-Rec (Clonetech Matchmaker, CA). Transformation was performed as described in Schiestl and Gietz 1989. *Saccharomyces cerevisiae* were cultured as described by Kozminski *et al*. (2003). Interactions were identified by growth on synthetic media lacking adenine, histidine, and supplemented with 3mM 3-AT (A8056 Sigma-Aldrich, St. Louis, MO). LacZ reporter assayed as described in Breeden and Nasmyth 1985. Plamids were isolated as described in Amberg *et al.* (2006). cDNA clones were sequenced (Macrogen Inc. Seoul, Korea) and identified by Blast (NCBI).

3.4 Results and Discussions

***Chlamydomonas* has three flagellar type II PKGs**

The *pkg*2 mutant has an insertion of an exogenous plasmid DNA at exon#2 of gene C_740056 (V3: 153242), which encodes a type II cyclic GMP-dependent protein kinase (Figure 3-1A; Rasi *et al*., 2009). PKG2 is one of the three protein kinase Gs found in the *Chlamydomonas* genome. One of the others, CrPKG (V2: C_50062, v3:181974), has been characterized and localizes to the ciliary membrane matrix (Wang *et al*. 2006). CrPKG does not appear to affect flagellar assembly, but is required for flagellar adhesion during early stages of *Chlamydomonas* mating, and was the first signalling molecule identified to be a cargo for the IFT machinery (Wang *et al*. 2006). The third PKG (V2: C_60149, V3: 131695) has not been studied yet, but is one of the most abundant proteins found in the flagellar proteome (Pazour *et al*. 2005).
To determine the relatedness of *Chlamydomonas* PKGs to one another and to the human PKGs, we used Bayesian methods (Huelsenbeck *et al*. 2001; Ronquist *et al*. 2003) to construct a phylogenetic tree of PKG proteins from *Chlamydomonas* and several animal species (Figure 3-1B). All three *Chlamydomonas* PKGs are members of the same clade as animal PKG2s, while PKA and PKC sequences form a separate clade, indicating that the *Chlamydomonas* PKGs are bona fide PKG2s. Our phylogenetic tree also indicates that the PKG1 family is animal-specific, and is derived from a PKG2-like ancestor. *Chlamydomonas* has retained and expanded the ancestral, type-II PKGs.

_pkg_2 mutants have a flagellar defect:

To better define the phenotype associated with flagellar assembly defects of *pkg*2, we visualized the cilia of *pkg*2 cells by indirect immunofluorescence. Consistent with our earlier observation by DIC (Rasi *et al*. 2009), the *pkg*2 mutants either lacked cilia completely or assembled short and unequal length flagella (Figure 3-2A and 3-2B).

PKG2-RNAi phenocopies _pkg2_ mutant flagellar defects:

As described previously, our *pkg*2 mutant strain was generated by insertion of an exogenous DNA plasmid. Although the mutant phenotype co-segregated with the insertion when backcrossed to wild-type cells, it was also important to confirm that the flagellar assembly

phenotypes were specific to loss of PKG2 function, and not a consequence of rearrangement of neighbouring DNA sequences (Gumpel *et al*. 1994). The original plasmid insert contained two selectable markers and because there is a paucity of selectable markers in *Chlamydomonas*, we did not have the tools for a direct rescue experiment. However, we reasoned that if the flagellar phenotype is a direct consequence of loss of function of PKG2, then it should be phenocopied by an RNAi-induced knock-down of PKG2.

We designed and constructed an RNAi construct (see methods and materials) and transformed wild type cells in order to knock down PKG2. We obtained four independent transformants, only one of which showed substantial knock down of PKG2 when assessed by western blot analysis (Figure 3-3A).

The lengths of flagella from wild type cells, *pkg*2 mutants, a short flagella (*shf*1) mutant and *PKG2*-RNAi are shown in Figure 3B-E. *shf*1 mutant cells have flagella that are short but of equal length, where as *pkg*2 and *PKG2*-RNAi populations have cells with no flagella, short flagella and unequal length flagella. The mechanism for maintaining equal length flagella is not well understood, but is thought to be a consequence of a common pool of precursors, constitutive flagellar turnover, and a length-dependent rate of flagellar assembly and length-independent rate disassembly (Marshall *et al*. 2005). Thus, if one flagellum is longer than the other, its rate of assembly drops while the assembly rate of the shorter flagellum increases, until the two flagellar lengths are again equal. Our data indicate that PKG2 may play a role in regulating the processes that maintain flagella of equal length.

PKG2 partially localizes to the flagella:

Of the kinases known to affect flagellar assembly or length control, two (Cnk2 and LF4) predominantly localize to the cilia, while the other two (LF2 and GSK3β) are predominantly cytosolic. To address the localization of PKG2, we fractionated *Chlamydomonas* cells into cell bodies and flagella. Using anti-PKG2 and anti-IC140 (Flagellar dynein Intermediate Chain 140kDa), western blots containing cell equivalent fractions were probed. The data in Figure 3-4A

reveal that most of the cellular PKG2 is in the cell body and a small fraction appears in the flagella. Furthermore, PKG2 appears as a doublet in the whole cell fractions and fractionation reveals that the lower molecular weight PKG2 is associated with the cell body whereas the higher molecular weight PKG2 sorts mostly to the flagellar fraction (Figure 3-4A and 4B). Whether these different forms of PKG2 represent different splice variants or post-translational modifications PKG2 is currently under investigation.

We next fractionated flagella into membrane-matrix and axonemal fractions. Under low ionic conditions, flagellar PKG2 appeared to be primarily in the membrane-matrix fraction, although some remained associated with the axoneme (Figure 3-4C). These data are consistent with data available from the *Chlamydomonas* flagellar proteome, which shows the presence of 3 peptides of PKG2 in the membrane-matrix fraction (Pazour *et al*., 2005).

As predicted by our biochemical analysis, indirect immunofluorescence reveals PKG2 appears to be mostly in the cell body and localizes to distinct puncta (Figure 3-4D and E). We detect only faint staining of flagella (not shown). This is not inconsistent with our biochemical data because relative to the cytosol only a small fraction of PKG2 localizes to the cilia.

***pkg2* mutant phenotype rescue:**

Mutant *pkg2* cells were transformed with a genomic DNA containing PKG2. In total 276 transformants were assayed phenotypically and biochemically for rescue of flagellar defects and expression of PKG2, respectively. Isolate 3R6 had a wild type flagellar phenotype and this was associated with a concomitant expression of PKG2 (Figure 3-4F).

Studies from mammalian PKG2 have shown that in oppossum proximal tubule cell lines, PKG2 inhibits Na^+/H^+ exchanger NHE3 (Cha *et al*. 2005) and in mouse intestinal cells it is involved with trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) (Goline-Bisello *et al*. 2005). Mice mutant in PKG2 exhibit dwarfism and both sox9 and GSK3β have been shown as downstream substrates of PKG2 in chondrocytes (Kawasaki *et al*. 2008). Given the punctuate localization of PKG2 that we observed (Figure 3-4), and flagellar assembly

defects in *pkg2* mutants, we were particularly interested to learn the identity of proteins that associate with PKG2 in *Chlamydomonas*. We therefore performed a yeast two-hybrid screen to search for interactors of PKG2.

PKG2 interacts with SAS1:

Volvox carteri is closely related to *Chlamydomonas reinhardtii*, and shares high synteny and homology at the chromosomal and protein levels, respectively. A volvox yeast two hybrid library was generously provided by Dr. Stephen Miller (University of Maryland). We screened 65,000 clones of the library with full length PKG2 as bait and identified two independent isolates that showed a strong interaction (Figure 3-5). Sequencing revealed that both of the PKG2-interacting clones encode volvox SAS1 (Adenosyl methionine Synthase : C_750005 and V3: 182408). SAS1 (also called Methionine adenosyltransferase) is evolutionarily conserved across all phyla and is 92% identical to *Chlamydomonas* SAS1. SAS1 is responsible for the catalytic formation of Adomet (Adenosyl methionine) from ATP and methionine and is the principle methyl group donor in cellular reactions such as compartmentalization of proteins (Boisvert *et al*. 2005), regulation of transcription (Prokhortchouk and Defessoz 2008), post-translational prenylation of proteins destined for membrane targeting (Perez-Sala *et al*. 1992) and regulation of protein turnover (Chuikov *et al*. 2007).

Next, we tested various truncation mutants of PKG2 to determine the domain(s) required for PKG2 and SAS1 interaction (Figure 3-5). Our data indicate that only full-length PKG2 interacts with SAS1. Since, most of our current data is based on PKG2 and volvox SAS1 interaction, we are currently testing PKG2 and *Chlamydomonas* SAS1 interaction in a new yeast two-hybrid assay, where *Chlamydomonas* SAS1 is the bait and PKG2 is the prey.

The *Chlamydomonas* flagellar proteome indicates the presence of three peptides of SAS1 in the membrane matrix fraction of the flagella (Pazour *et al*., 2005). Interestingly, other proteins that are involved in the synthesis of adomet and transfer of methyl group from adomet are also present in the flagella (Pazour *et al*., 2005). For example, there are eight peptides of adenosyl

homocystein hydrolase, four peptides of cobalamin adenosyltransferase and 23 peptides of MetE (methionine transferase) in the flagella (Pazour *et al*., 2005). The MetE transcript was initially found to be up regulated during flagellar adhesion of the gametes and recently it was discovered that MetE is also upregulated during flagellar resorption (Kurvari *et al*. 1995; Schneider *et al*. 2008). Furthermore, it was also shown that during flagellar resorption several other proteins are arginine-methylated (Schneider *et al*. 2008).

Our finding that PKG2 interacts with SAS1 offers further support that a protein methylation pathway could play an active role in ciliary assembly and/or disassembly. This activity could be mediated by affecting protein stability, sorting or perhaps by mediating a cross talk between cilia and the nucleus. Although Schneider *et al*. 2008 showed that certain proteins are arginine methylated, it remains to be tested whether the other forms of methylation such as lysine methylation could also occur in the cilia. Lysine methylation plays an important role in stabilizing proteins by competing with lysines which may otherwise be ubiquinated (Chuikov *et al*. 2007). *Chlamydomonas* cilia contain ubiquitin, ubiquitin activating (Fab124) and ubiquitin conjugating (E2 ligase) enzymes (Pazour *et al*. 2005). In *pkg*2 mutants, failure to assemble a cilium could be due to a rapid turn over of proteins by the ubiquitination machinery which may otherwise be circumvented in wild type cells by the protein methylation pathway. Alternatively, because protein methylation is an important event during farnesylation and gernaylgernaylation of proteins destined to the plasma membrane, it is possible that failure to assemble a flagella in *pkg*2 mutants could be due to lack of proper membrane targeting and sorting of proteins important for ciliary assembly.

Closing Remarks:

In this report we extend the role of yet another signalling molecular, a cGMP-dependent protein kinase II, and show that it is required for proper ciliary assembly in *Chlamydomonas*. Although cGMP-dependent protein kinases have been studied in mammalian systems, the possible role of PKG2 in cilia biogenesis has not been considered. For instance, mice (and rats)

mutant in *pkg2*, are dwarf as a result of impaired chondrocyte hypertrophic differentiation during skeletal growth (Chikuda *et al*. 2004; Kawasaki *et al*. 2008). This defect seems to arise as a result of failed phosphorylation-dependent inhibition of GSK3β in *pkg*2 mutants, which leads to chondrocyte specific inactivation of β-catenin (Kawasaki *et al*. 2008). Interestingly, β-catenin /wnt signalling pathway has already been established as a determinant of cell proliferation (Clevers, 2006) and the cellular levels of β-catenin are shown to be regulated by the presence of a cilium (Reviewed by Xie, 2008). Taken together, we propose that, as we have discovered for *Chlamydomonas* PKG, cGMP-dependent protein kinase IIs may have roles in signalling or biogenesis of cilia in mammals as well.

3.5 Acknowledgements

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3.6 Figures

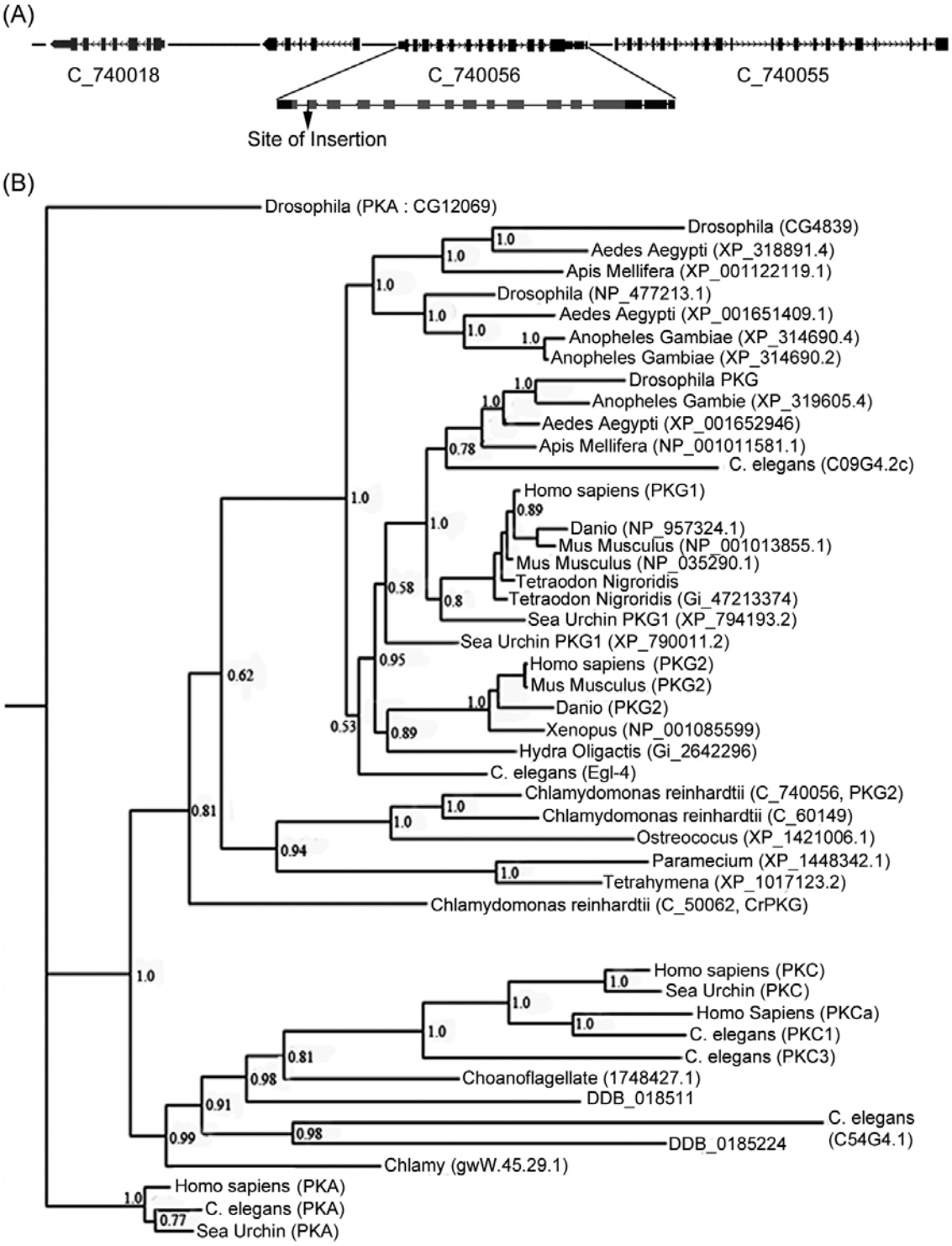
(A)
C_740018
C_740056
C_740055
Site of Insertion
(B)
Drosophila (PKA : CG12069)
Drosophila (CG4839)
Aedes Aegypti (XP_318891.4)
Apis Mellifera (XP_001122119.1)
Drosophila (NP_477213.1)
Aedes Aegypti (XP_001651409.1)
Anopheles Gambiae (XP_314690.4)
Anopheles Gambiae (XP_314690.2)
Drosophila PKG
Anopheles Gambie (XP_319605.4)
Aedes Aegypti (XP_001652946)
Apis Mellifera (NP_001011581.1)
C. elegans (C09G4.2c)
Homo sapiens (PKG1)
Danio (NP_957324.1)
Mus Musculus (NP_001013855.1)
Mus Musculus (NP_035290.1)
Tetraodon Nigroridis
Tetraodon Nigroridis (Gi_47213374)
Sea Urchin PKG1 (XP_794193.2)
Sea Urchin PKG1 (XP_790011.2)
Homo sapiens (PKG2)
Mus Musculus (PKG2)
Danio (PKG2)
Xenopus (NP_001085599)
Hydra Oligactis (Gi_2642296)
C. elegans (Egl-4)
Chlamydomonas reinhardtii (C_740056, PKG2)
Chlamydomonas reinhardtii (C_60149)
Ostreococus (XP_1421006.1)
Paramecium (XP_1448342.1)
Tetrahymena (XP_1017123.2)
Chlamydomonas reinhardtii (C_50062, CrPKG)
Homo sapiens (PKC)
Sea Urchin (PKC)
Homo Sapiens (PKCa)
C. elegans (PKC1)
C. elegans (PKC3)
Choanoflagellate (1748427.1)
DDB_018511
C. elegans (C54G4.1)
DDB_0185224
Chlamy (gwW.45.29.1)
Homo sapiens (PKA)
C. elegans (PKA)
Sea Urchin (PKA)

Figure 3-1. Identification of a *Chlamydomonas* insertional mutant:

(A) Schematic representation of a region within *Chlamydomonas* scaffold #74 (Version2) / #56 (version3) showing the site of a non-homologous insertion of a plasmid DNA and disruption of a *Chlamydomonas* Cyclic GMP-dependent Protein Kinase II (PKG2) at exon#2.

(B) Various cyclic GMP-dependent protein kinase sequences, as well as some Protein Kinase A and Protein Kinase C outgroups, were aligned using Clustal and phylogram produced using Bayesian inference (MrBayes). Type-I PKGs are restricted to Deuterostoma.

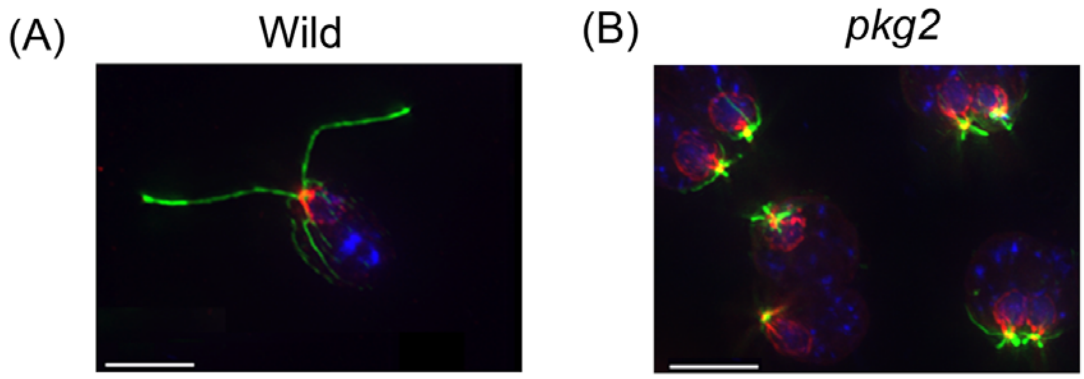

Figure 3-2. *pkg2* mutants have flagellar defects:

(A) Wild type (137c strains)

(B) *pkg*2 mutants were stained for centrin (red), acetylated Tubulin (green) and DNA (blue). Note that compared to wild-type cells, *pkg*2 exhibit short and stumpy flagellar defects (see Figure 3-3 for flagellar length quantification). Scale bar = 5 μm.

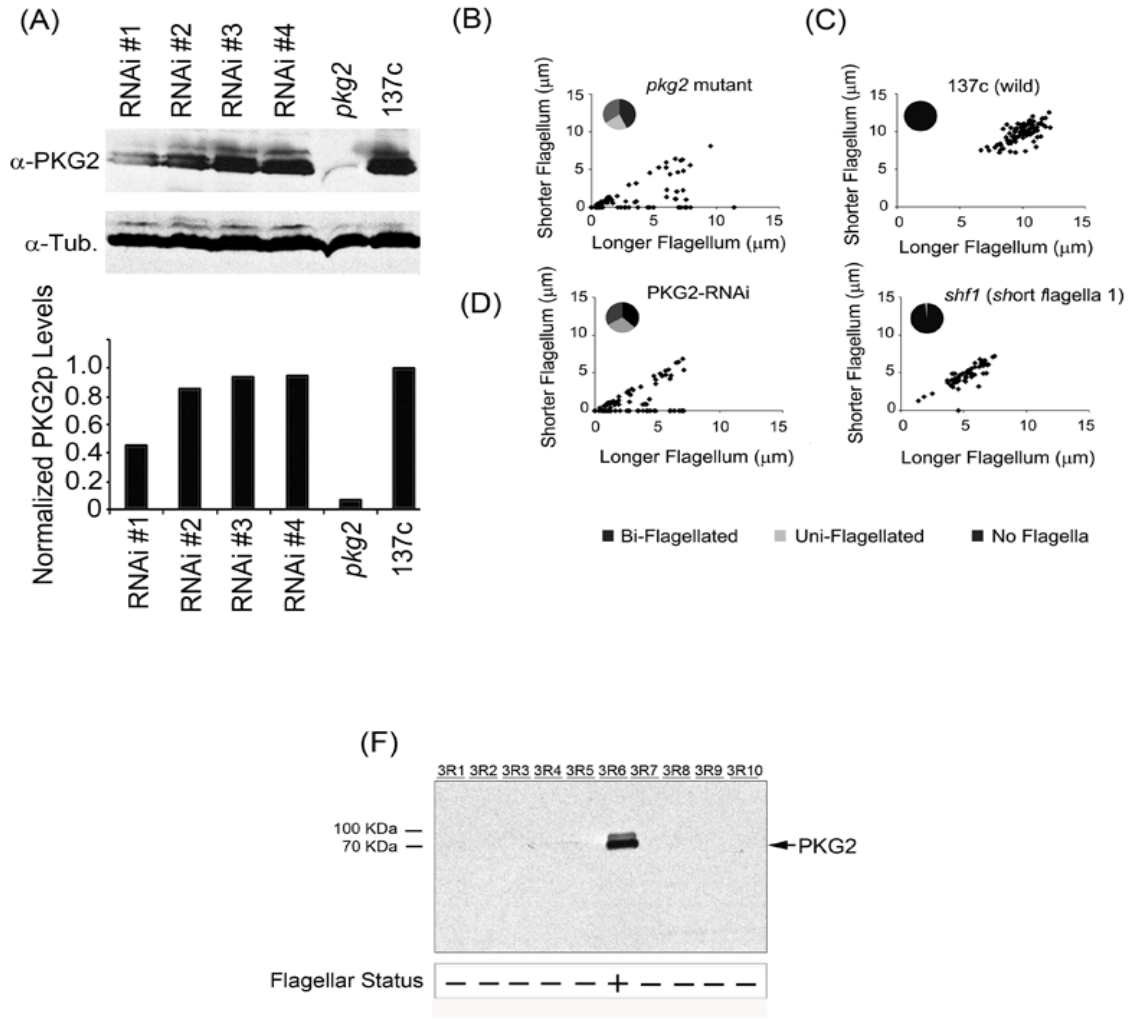

Figure 3-3. RNA interference of PKG2 , Quantification of flagellar defects and PKG2 Rescue:

(A) A short-hairpin construct was transformed into wild type *Chlamydomonas* cells. Knock-down of PKG2 was assessed by western blotting using anti-PKG2 antibodies.

(B - E) Flagellar length was measured in *pkg*2 mutants (B), wild type (C), *PKG2*-RNAi (D) and *shf*1 (E). Pie charts represent population of cells with one flagellum only (uni-flagellated), two flagella (either stumpy or wild type length) and cells with no flagella at all [*pkg2* mutants: n = 122, 42% bi-flagellated, 22.9% uni-flagellated, 34.4% no flagella. PKG2-RNAi: n = 118, 36% bi-flagellated, 30.5% uni-flagellated, 33% no flagella. *shf1* mutants: n = 57, 98.2% bi-flagellated, 1.7% uni-flagellated. 137c (wild): n = 96, 100% bi-flagellated].

(F) PKG2 mutant phenotype was rescued in isolate # 3R6, by transforming mutant cells with genomic DNA (see methods) [(-) indicates mutant flagellar photype, (+) indicates wild type flagella, transformants screened = 276].

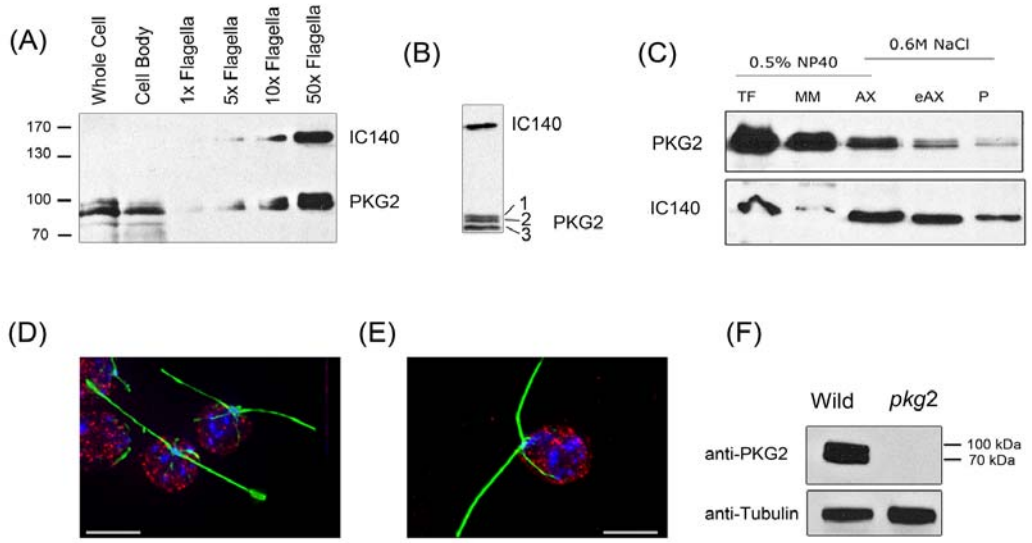

Figure 3-4. Localization and Fractionation of PKG2

(A) Whole cell *Chlamydomonas* was fractionated into cell body and flagella. Although most of the PKG2 appears cytosolic, a small fraction of PKG2 of higher molecular weight appeared to be flagellar.

(B) Western Blot showing the presence of 3 distinct bands of PKG2. Band #1 and #2 are enriched in the flagella as seen in Figure 4A

(C) Flagella (TF) were further fractionated into Axoneme (AX) and Membrane Matrix (MM) using a low ionic detergent NP-40. Axonemal pellet was further treated with 0.6M NaCl and the supernatant was regarded as extracted Axoneme (eAX) and the pellet (P).

(D and E) Anti-PKG2 antibody (red) reveals localization of PKG2 to puncta in the cell body of the wild type cells. Cells are also stained for acetylated Tubulin (green) and DNA (blue). **(F)** A Western Blot showing specificity of PKG2 antibodies and absence of PKG2 in *pkg*2 mutants [n = 100, Average number of spots/cell = 41, scale bar = 5 μm].

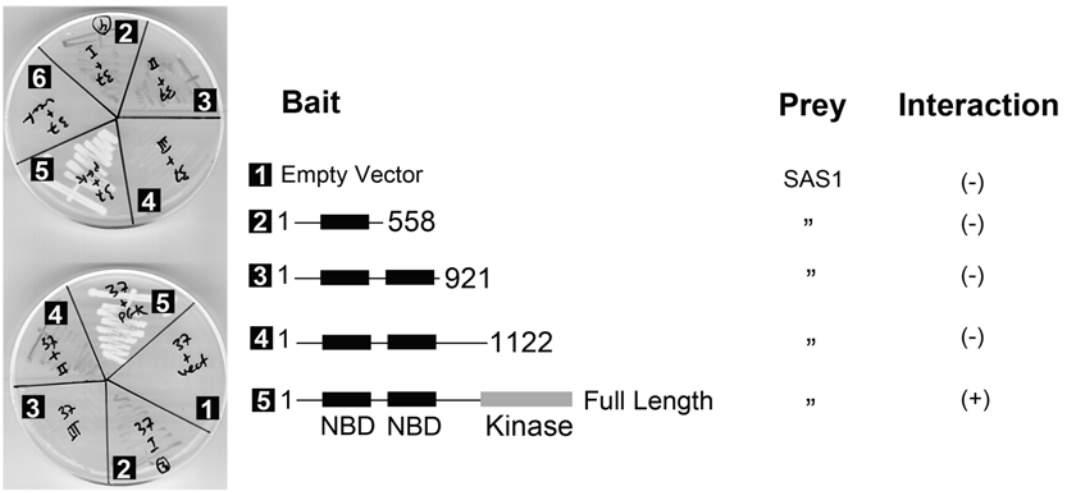

Figure 3-5. PKG2 interacts with SAS1

A Yeast Two hybrid screen identified SAS1 as PKG2 interacting partner. Two different isolates were obtained in our screen and sequencing data confirmed that they both code for SAS1. Truncations of PKG2 along with empty vector were used as 'Bait' and only full-length PKG2 tests 'positive' for interaction with SAS1 on $His^-/Ade^-/Leu^-/Tryp^-$ plates supplemented with 3-AT (see methods) [Yeast Plates show positive interaction between full-length PKG2 and two isolates of SAS1 as assessed by growth on selective media. NBD: Nucleotide Binding Domain].

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CHAPTER 4
CONCLUSIONS AND FUTURE WORK.

4.1 IFT, Resorption, and Deflagellation

The intimate relationship between cilia and cell cycle is dependent on the dual role of centrioles (or basal bodies) which act as a template for building a cilium during interphase and spindle microtubule organizing centres during mitosis (Tucket *et al*., 1979; Quarmby and Parker, 2005). Cilia are assembled and maintained at G0/G1 and disassembled at the onset of G2 (Sorokin, 1968; Tucker *et al*., 1979; Rieder *et al*., 1979) by a process referred to as IFT (Intra Flagellar Transport) (Kozminski *et al*., 1993, 1995; Pazour *et al*., 2000). Flagellar assembly requires flagellar precursors to be shipped to the tip of the growing cilia and flagellar disassembly may involve either inhibition of anterograde IFT, an increase in the rate of retrograde IFT, or perhaps both (Cole, 2008).

Much of our current understanding of IFT comes from studies of flagellar assembly mutants (*fla*) such as *fla10*, where a lesion in the motor subunit of kinesin-II, leads to flagellar assembly defects at the restrictive temperature of 33^{o}C (Huang *et al*., 1977; Lux and Dutcher, 1991; Walther *et al*., 1994; Kozminski *et al.,* 1995). The majority of *fla* mutants undergo flagellar disassembly (or resorption) at 33^{o}C (restrictive temperature) and are unable to regenerate their flagella at 33^{o}C with the exception of *fla2* (Huang *et al*., 1977; Adams *et al*., 1982). At the restrictive temperature, *fla*2 cells undergo deflagellation and can regenerate their flagella (Adams *et al*., 1982; Parker and Quarmby, 2003). As described in the introductory chapter of this thesis, deflagellation is

the severing of the flagella at a site distal to the transition zone, also called the SOFA (Blum, 1971; Lewin and Lee, 1985; Mahjoub *et al*., 2004). Deflagellation is a Ca^{+2} dependent process and so far multiple alleles of only three mutants have been identified, namely *fa1*, *fa2* and *adf1* (Lewin and Burrascano, 1983; Finst *et al.*, 1998; reviewed by Quarmby, 2008) which are unable to undergo deflagellation.

Parker *et al*., (2003) made the unexpected observation that although *fla2* was the only known *fla* mutant to undergo deflagellation at the restrictive temperature, other *fla* mutants actually behaved the same. For example, in the presence of Ca^{+2}, *fla10* mutants undergo deflagellation in addition to resorption at the restrictive temperature (Parker *et al*., 2003). To confirm that *fla10* mutants were indeed undergoing deflagellation Parker *et al*., crossed *fla10* mutants with that of *fa* mutants (deflagellation mutants or flagellar autotomy mutants). As predicted, in the presence or absence of Ca^{+2}, *fla10*/*fa1* and *fla10*/*fa2*, were unable to undergo deflagellation at the restrictive temperature and instead the cells underwent resorption albeit at a much slower rate, indicating that deflagellation was no longer contributing to flagellar disassembly (Parker and Quarmby, 2003). Furthermore, when *fa* mutants (*fa*1 and *fa*2) were subjected to stimuli that normally induce deflagellation in wild type cells, they instead underwent resorption. Flagellar regeneration experiments in *fa* mutants also indicate they are slow to assemble their cilia (Mahjoub *et al*., 2002; Quarmby, 2008).

These observations supported the idea that perhaps there is a link between IFT-mediated ciliary disassembly and deflagellation. The model for ciliary disassembly prior to entry into mitosis has been ciliary shortening by resorption, but electron microscopy of *Chlamydomonas* cells undergoing division has already revealed that quite often mitotic

cells display flagella while the basal bodies are in mitosis. In these EMs, the flagella and the basal bodies are separated as if by deflagellation and the flagella remain attached to the cell wall (Johnson and Porter,1968).

Thus, I wanted to ask the question whether cells undergo ciliary loss via deflagellation or IFT-mediated resorption when they are planning to go through mitosis, or do the two pathways progress in a complementary fashion to facilitate pre-mitotic ciliary shortening? Secondly, does katanin, a protein predicted to be involved in deflagellation and cell cycle, play any role(s) in pre-mitotic ciliary disassembly?

As we were pursuing the above questions, we noticed pre-mitotic cells containing two distinct 'bulges' in their cell wall. Further investigation by means of immunofluorescence revealed that these 'bulges' could be stained by antibodies raised against acetylated tubulin (Figure 4-1 and 4-2A). Finally, by electron microscopy we confirmed that the 'bulges' were in fact vesicular pockets containing transition zones (Figure 4-2C to I). The flagellar transition zone (TZ) is a unique region between the basal bodies and the flagella proper that docks the basal bodies to the plasma membrane via the TZ fibers (Dutcher, 2008). Also, distal to the transition zone is the region our lab has previously identified as SOFA (Mahjoub *et al*., 2004).

Live imagining microscopy of cells undergoing pre-mitotic resorption revealed that vesicle-bound transition zones are indeed 'separated' from the basal bodies (Figure 4-1). We hypothesized that this is a novel and previously uncharacterized mechanism, which allows the basal bodies to be disengaged from their site of anchorage and thus allowing the basal bodies to perform their cell cycle duties. Furthermore, this also

predicts that deflagellation may indeed have a role in pre-mitotic resorption and that the microtubule severing protein, katanin, could involved.

Immunofluorescence of katanin-deficient cells, expressing a Kat-RNAi, displayed spindles organized with their poles at the base of the flagella as well as cells with aberrant spindles (Figure 4-3). Furthermore, we found that katanin could efficiently be knocked-down in cells with a second site mutation that would affect ciliogenesis. For instance, RNAi could efficiently reduce katanin levels in mutants such as *ift*88, *pkg*2 and *bld*1 in contrast to wild type cells where the knock down of katanin was either lethal or rapidly suppressed. Our results suggested that the permissive nature of cilia-less mutants for efficient knock down of katanin is that in the absence of a cilium, the basal bodies are no longer sequestered at their anchoring sites.

A consequence of basal bodies being sequestered during cell divison or confined as a result of their attachment to the transition zone could lead to defects in spindle microtubule organization and cytokinesis defects. In *Chlamydomonas*, aside from being important for nucleating the flagella, the basal bodies are also important for the organization of rootlet microtubules, which in-turn are important for positioning of the cleavage furrow (reviewed by Dutcher, 2003). Although, *Chlamydomonas* cells lacking basal bodies or displaying too many or misplaced basal bodies are viable (Dutcher *et al*., 2002; Adams *et al*., 1985; Feldman *et al*., 2007), it appears as though, the presence of basal bodies and their confinement may exert a 'dominant-negative effect' on spindle assembly. It is important to note that spindle assembly can occur in the absence of basal bodies (for example in plants: reviewed by Marshall, 2008) or in the presence of too many basal bodies (Sluder and Nordber, 2004). However, when the right number of basal

bodies are present and if they are held confined at a place in the memberane where they should not be during cell division it could lead to aberrant spindle assembly.

As described in Chapter 2, we also determined that pre-mitotic deflagellation does not occur at the SOFA, as *fa2* mutant cells were able to disengage their basal bodies similar to wild type cells. Instead, pre-mitotic severing occurs at a site proximal to the transition zone. Interestingly, *fa2* mutants display a G2/M delay (Mahjoub *et al*., 2002) and although immunofluorescence images of *fa*2 mutants undergoing pre-mitotic severing appeared normal it is quite possible that the G2/M delay is a consequence of delayed basal body disengagement. One way to determine if there is indeed a delay would be to look at live resorption movies of *fa*2 mutant cells. Measuring various periods such as the time for a cilium to resorb, the time between disengagement of basal bodies from transition zones and the basal body mobilization, could help us determine the temporal and spatial co-ordinates of G2/M delay in *fa*2 mutants. An alternative experiment that Jeremy Parker attempted while he was a graduate student in our laboratory was to use a long flagella mutant such as *lf4* (Aleson and Lefebvre, 1998; Berman *et al*., 2003) crossed with *fa*2 mutants to decipher the G2/M delay. The premise behind this experiment was that the presence of longer flagella in *fa*-2 mutant background, would lead to cells taking longer to resorb their flagella before entry into the cell cycle. This would either manifest itself as a longer pause period in G2/M or that *lf*4/*fa*2 double mutants would simply sever off their slow resorbing flagella (proximal to the TZ severing site) and enter the cell cycle. Jeremy observed that fixed culture samples of *lf*4/*fa*2 double mutants, synchronized to undergo division, contained a large fraction of dead cells. Although this experiment needs to be repeated since such observation could

have been caused by culture conditions, culture handling and processing, nevertheless, it would offer valuable piece of data into understanding the possible role of *fa*2 in pre-mitotic resorption. The other possibility is that there are two distinct deflagellation complexes (deflagesomes) which may be localizing at the distal and proximal ends of the transition zone respectively. For instance, we can predict the presence of a Fa2-like kinase, which may be required for proximal severing of the TZ. A phylogenetic analysis of the *Chlamydomonas* Neks reveals that Cnk4 is a NIMA kinase closely related to Fa2 (Parker *et al.,* 2007). Thus, it would be interesting to test whether Cnk4 has any roles in pre-mitotic release of the basal bodies or ciliary disassembly prior to entry into cell cycle.

Recently I discovered that katanin may also have a role in gametogenesis (Figure 4-4A). Western blots of vegetative cells show that katanin usually runs as a doublet where as in gametic cells katanin only appears as a `modified` singlet (Figure 4-4A). In Hela cells, mammalian katanin has been shown to undergo phosphorylation (Maddika and Chen, 2009) by DYRK2 kinase and this is required for proper mitotic progression. Assuming the `modified`form of katanin, represents a phosphorylated form of katanin, I hypothesize that phosphorylation of katanin during gametogenesis may be required for its activation. Gametogenesis is an event that primes *Chlamydomonas* cells for mating, usually triggered by adverse envinronmental conditions or in a laboratory setting by growing cells in a nitrogen-free media (reviewed by Harris, 2008). Aside from important changes within the cytosol such as degradation of chloroplast and cytoplasmic ribosomes and dimished photosynthetic activity (reviewed by Harris, 2008), certain important structural changes occur to the flagella during gametogenesis and mating. One such change is the flagellar tip elongation and activation (Mesland *et al*., 1980). During tip

activation, the central pair and the A-tubule of outer-doublet microtubules elongate and form singlet-microtubules at distal end of the flagella (Figure 4-4B). Elongation of the central pair is as intriguing as its initial nucleation during flagellar assembly. It is believed that the central pair microtubules are nucleated by gamma-tubulin within the transition zone (Silflow *et al*., 1999; McKean *et al*., 2003). Central pair mutants such as *pf15* are unable to assemble their central pair (Starling and Randall, 1971). *PF15* codes for the katanin regulatory subunit p80 (Dymek *et al*., 2004). Studies have shown that katanin p80, is required for targetting of katanin (also called katanin p60) to the centrosomes and this is important for gamma-tubulin dependent nucleation of microtubules and regulation of spindle length during mitosis (McNally *et al*., 2006; Roll-Mecak and Vale, 2006). Therefore, it is possible that in *pf15* mutants, katanin p80 dependent localization of katanin p60 at the site of nucleation of central pair microtubules is affected. Furthermore, in relation to elongation of central pair microtubules during mating, I hypothesize that activation of katanin (p60) may be required. Alternatively, katanin activation may be required for microtubule re-organization during mating, or disassembly of the flagella post mating (Note: After two *Chlamydomonas* cells mate, they will fuse and form a cell with four flagella. Soon after, the four flagella will resorb (Snell, 2008)).To decipher the role of katanin in gametogenesis, I have currently set up experiments comparing katanin protein levels and modification state in vegetative as well as gametic cells of central pair mutants *pf15*, *pf18*, basal body mutants *bld1* and *bld10*, as well as deflagellation mutants *fa1* and *fa2*.

Another experiment that I designed to study katanin was in *fla10*^{ts} conditional mutants. The premise behind this experiment was that we could further confirm that

knock down of katanin in cells with normal flagella is lethal, is by expressing katanin RNAi in *fla10*^{ts} cells kept at the restrictive temperature (33^{o}C). At this temperature, *fla10*^{ts} cells would be unable to assemble their cilia and katanin knock down could be efficiently achieved. Furthermore, transferring *fla10*^{ts} cells to a permissive temperature, would lead to cells growing their flagella and based on our model (Chapter 2) could cause lethality of *fla10*^{ts} cells expressing katanin RNAi. Unfortunately, this experiment failed. Keeping newly transformed cells for extended period of time at the restrictive temperature caused cells to get sick and die. It is possible that cells could not tolerate the absence of FLA10 for extended periods. This is supported by data that mutations in FLA10 lead to chromosomal loss (Miller *et al.*, 2005).

Other proteins with potential roles in ciliary disassembly are the predicted KAT2 and Spastin in the *Chlamydomonas* genome. Kat2 is a member of the AAA family of ATPases (Lupas and DeLaBarre, 2003) and shares homology to *Chlamydomonas* and mammalian katanin. Spastin is also a microtubule severing protein, a member of the AAA family of ATPases, and is implicated in an axonal degenerative syndrome known as spastic paraplegia (Salinas *et al*., 2007). Although both genes are predicted in the genome, we determined that both are expressed in *Chlamydomonas*. Since I had difficulties expressing Kat2 cDNA in *Chlamydomonas*, I tried expression of Kat2 in ciliated mammalian IMCD3 (Intermedullary Collecting Duct) cells, and localization data revealed that Kat2 localizes at both mother and daughter centrioles (Figure 4-5). At this stage, whether Kat2 (or spastin) have any roles in deflagellation, resorption or basal body disengagement is still a pending question.

To further our understanding of basal body disengagement prior to entry into cell cycle, it would be very interesting to identify other proteins, which may be involved in this pathway. Because the release of basal bodies marks an important and early determinant of successful entry into the cell cycle in *Chlamydomonas*, mutations in the genes that regulate the release of basal bodies may be lethal. Therefore, using a temperature sensitive mutagenesis screen in *Chlamydomonas* we could potentially uncover genes encoding essential proteins, mutations in which could block basal body release. Furthermore, the identity of such proteins can help us elucidate the enigmatic cilia-cell cycle connection.

4.2 The role of PKG2 in Ciliogenesis

During the course of my thesis work on katanin-mediated ciliary disassembly I serendipitously discovered that *pkg*2 mutants fail to assemble cilia. As discussed in Chapter 3 of my thesis, PKG2 is a cyclic GMP-dependent protein kinase and is one of the three PKGs found in *Chlamydomonas*. I discovered that there are two distinct pools of PKG2 – one found in the cytosol and the other, in the flagella (Chapter 3; See also Figure 4-6). The flagellar pool of PKG2 appears to have a higher molecular weight than the cytosolic fraction. The higher molecular weight form of PKG2 is either a post-translationally modified form of PKG2 or a splice variant of PKG2. Using a cDNA pool obtained from wild type flagellated *Chlamydomonas* culture, we tested for the presence of spliced forms of PKG2 by using various primer pairs targeting various regions of the PKG2 mRNA. Although we did not detect any other forms of PKG2, it is quite possible that one form of mRNA is predominant over the other one, which may have lead to only one form of PKG2 being amplified.

PKG2 contains a predicted myristoylation site as well as putative phosphorylation sites. Myristoylation is a lipid moiety addition, important for protein targetting to the plasma membrane. Pazour *et al.* (2005) has found that out of 19,832 *Chlamydomonas* proteins, only 103 proteins are predicted to be myristoylated and 18 of these 103 proteins are found in the flagellar proteome (Pazour *et al*., 2005). The 18 predicted proteins constitute 2.7% of all flagellar proteins and 8% of all membrane matrix proteins (Pazour *et al*., 2005). Therefore, it is likely that instead of a spliced form of PKG2, a myristoylated form of PKG2 is targeted to the flagellar membrane. Using biochemical fractionation assays of the *Chlamydomonas* flagella, we find that flagellar PKG2 is predominantly in the flagellar membrane-matrix and our data is supported by the *Chlamydomonas* Flagellar Proteome dataset (Pazour *et al*., 2005): 3 peptides of PKG2 were recovered only in the flagellar membrane fraction. Confirmation of Myristoyl group addition to PKG2 and flagellar localization of PKG2 being dependent on myristoylation is currently under investigation.

Chlamydomonas PKG2 and mammalian PKG2 are 30% identical and 50% similar. I therefore hypothesized that *Chlamydomonas* PKG2's localization in a mammalin cell line such as IMCD3 (Intemedullary Collecting Duct) cells could reflect on mammalian PKG2's localization. Expression of a GFP-tagged *Chlamydomonas* PKG2 as well as truncation studies of it revealed that PKG2 localizes to the centrioles (Figure 4-7)**.** Truncation mutants, which lacked the sequence (AELRDAERKLV) within the protein could not localize to the centrioles and their localization was classified as non-centrosomal. Interestingly, the sequence that we identified by deletion analysis, corresponds to a nuclear export sequence (As predicted by NESbase, Cour *et al*. 2003).

Therefore, it is likely that PKG2 moves between the nucleus and the centrioles and constructs that do not have the nuclear export sequence could get trapped in the nucleus. The alternative hypothesis is that targeting of proteins to the centrioles (or cilia) shares a similar mechanism as that of nuclear export and import. In fact such models have been proposed for IFT-mediated transport of proteins across the ciliary necklace (Chapter 1) (Christensen *et al*., 2007; Satir *et al*., 2008).

The next important question that we asked was how does a cyclic GMP-dependent kinase regulate ciliary assembly? We took the approach of yeast two-hybrid to find putative interactors of PKG2. We screened ~65000 clones and two independent isolates both coding for SAS1 (S-adenosyl methionine synthase) were recovered as strong PKG2 interactors (Chapter 3).

SAS1 (or MAT – Methionine Adenoysl-Transferase) catalyzes the formation of AdoMet (Adenosyl Methionine) from ATP and Methionine (reviewed by Walsh, 2006). AdoMet is the primary methyl group donor to biological reactions (Schubert *et al.,* 2003). Methylation is a post translational modification important for compartmentalization of proteins (Bosivert *et al.,* 2005), modulation of protein-protein interaction (Walsh, 2006), regulation of gene expression (Prokhortchouk and Defessov, 2008) and modifying the 'age' of a protein (Chuikov *et al*., 2007).

Chlamydomonas SAS1 is a ~42 kDa protein and 3 peptides of SAS1 appear in the membrane-matrix fraction of the *Chlamydomonas* flagellar proteome (Pazour *et al*., 2005). Within the flagella, other peptides that correspond to proteins in the methylation pathway are 8 peptides of adenosyl-homocysteine hydrolase, 4 peptides of cobalamin adenosyltransferase and 23 peptides of MetE (Pazour *et al*., 2005). MetE is a

Chlamydomonas methionine transferase identified by Kurvari *et al*. 1995 to be upregulated in gametes. Recently, Schneider *et al*. 2008 showed that MetE levels dramatically increase during ciliary resorption and western blots revealed the presence of four distinct proteins that undergo argenine-methylation during resorption (Schneider *et al.,* 2008).

The interaction of SAS1 with PKG2, the flagellar localization of enzymes in the protein methylation pathway and the implication of metE in ciliary resorption raises important questions into the role of protein methylation in ciliogenesis. Therefore, to understand the ciliary roles of methylation and the context in which this pathway may be affected in *pkg2* mutants, I suggest a few testable models, which could have a potential role in regulation of ciliary events such as assembly or disassembly.

First, adomet-dependent farnesylation and geranylgeranylation of proteins, such as Rab GTPases containing a C-terminal CAAX motif, could play an important role in ciliogenesis (Pfeffer and Aivazian, 2004). For example, *Chlamydomonas* IFT27 is a small Rab protein with a predicted C-terminal CAAX motif (Qin *et al*., 2007). RNAi-mediated knock down of IFT27 leads to ciliary defects similar to *pkg*2 mutants. Expression of a C-terminal GFP tagged IFT27 also leads to defects in ciliary assembly. This intriguing observation could be due to dosage sensitivity of IFT27 or perhaps the presence of the GFP tag leads to improper folding of IFT27, which could act as a dominant negative construct by titrating out the endogenously expressed IFT27. Alternatively, the C-terminal GFP tag could interfere with the IFT27 CAAX motif from being prenylated and this may lead to improper membrane sorting. The ciliary assembly defects of *pkg*2 looks similar to that of IFT27-RNAi cells, therefore, it would be interesting to look at IFT27

localization in *pkg*2 mutants. Perhaps a PKG2 mediated, SAS1 dependent methylation of prenylated proteins required for membrane sorting is missing in *pkg*2 mutants. Furthermore, this would suggest that an important role of protein methylation pathway in *Chlamydomonas* is for proper membrane sorting.

Secondly, it is possible that PKG2 mediated SAS1-dependent protein methylation pathway regulates ciliary assembly and disassembly by mediating a cross-talk between cilia and the nucleus. In other words, the methylation of the genome (or the histones) could act as a software code for the hardware of ciliary assembly and disassembly pathway. Therefore, another experiment would be to generate a transcript micro-array profile of *pkg*2 mutants, PKG2-RNAi and compare to that of a wild type micro-array profile. Such profiling can be accomplished with much ease since micro-array chips consisting of 10,000 unique *Chlamydomonas* oligonucleotides are publicly available (Arthur Grossman, Carnegie Institution of Washington).

Thirdly, protein stability, longevity and sorting could be an important aspect of ciliary assembly regulated via a PKG2 dependent SAS1-mediated protein methylation pathway. In this scenario, lysine methylation events could regulate protein stability via ubiquination. An example of this sort of regulation comes from studies of p53 where a c - terminal p53 lysine methylation sequesters p53 to the nucleus and increases its stability by interfering with ubiquination (Chuikov *et al*., 2007). The *Chlamydomonas* flagellar proteome contains ubiqutin, ubiquitin activating (11 peptides of FAP124) and conjugating (3 peptides of E2 ligase) enzymes (Pazour *et al.*, 2005). To test this model, western blots of Flagellar Basal Body Complexes (FBBC) of wild, *pkg*2 cells, resorbing and regenerating *fla*10^{ts}flagella would be probed with an anti-ubiqutin antibody as well as

commercially available antibodies raised against methylated lysines. My model predicts that ubiqutination and methylation are important for flagellar assembly and disassembly. During resorption, proteins are ubiqutinated for being sorted out of the flagella (disassembly) and during assembly, proteins are methylated to first prevent their ubiquitination and second increase their life span. Furthermore, the mechanism of flagellar length regulation (Marshall and Rosenbaum, 2001) could be a consequence of competing methylation and ubiqutination events.

It is important to point out that we can generate many models and hypotheses to understand how PKG2 regulates ciliogenesis. The interaction of PKG2 with SAS1, the presence of methylation pathway proteins in cilia, and the regulatory role of methylation in other model systems led me to propose certain models. It is also possible that PKG2-SAS1 interaction may actually have nothing to do with ciliogenesis. Both proteins are present abundantly in the cytosol and their interaction may be irrelevant to our topic of research.

Immunofluorescence microscopy of *pkg2*, stained with anti-acetylated tubulin, revealed that *pkg2* mutants may have defects in their microtubule organization, since the rootlet microtubules quite often appeared short and disorganized. To determine if *pkg2* mutants does indeed have a problem with their microtubule organization, I plated *pkg2* mutant cells as well as wild type cells on media plates containing various concentrations of a microtubule destablizing agent – oryzalin. The premise behind this experiment was that if cells have defects in their microtubule organization or assembly they would not be able to tolerate drug-induced microtubule instability. Figure 4-8 shows the result of our experiment whereby, *pkg2* mutants were highly sensitive to oryzalin in comparision to

wild type cells. This supports our immunofluorescence microscopy observations and perhaps leads to a new hypothesis, whereby ciliary assembly defects in *pkg2* mutants could be a consequence of defects in microtubule stability.

Understanding the role of PKG2 in ciliary assembly could offer valuable insights into the role of cGMP-dependent pathways in relation to an important organelle, which has seen a sudden emergence of interest in the last decade. Although, it has yet to be established , the evolutionary emergence of the requirement for a second cylic nucleotide, the presence of large numbers of guanylyl cyclases in ciliated organisms such as *Chlamydomonas*, *Paramecium*, *Tetrahymena*, *C. elegans* and absence from non-ciliated living forms such as yeast and plants (Roelofs *et al*., 2002) could implicate cGMP-mediated signalling in relation to the emergence of cilia.

4.3 Figures

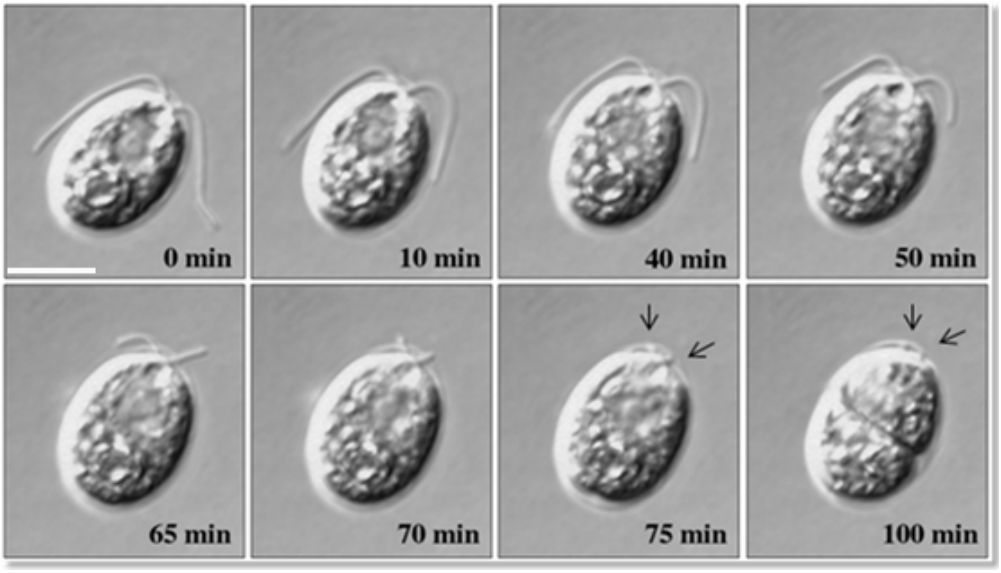


Figure 4-1. Pre-mitotic flagellar resorption

Time 0 min to 70 min shows resorption of flagella in anticipation of cell division. At 75 min. the flagellar resorption is complete and immediately the protoplasm rotates and the cell subsequently undergoes cytokinesis at 100 min. Notice the membrane 'bulges' that are still present, even after cell division is complete. Images courtesy of Moe Mahjoub (Quarmby, 2008) [Scale bar = 5μm].

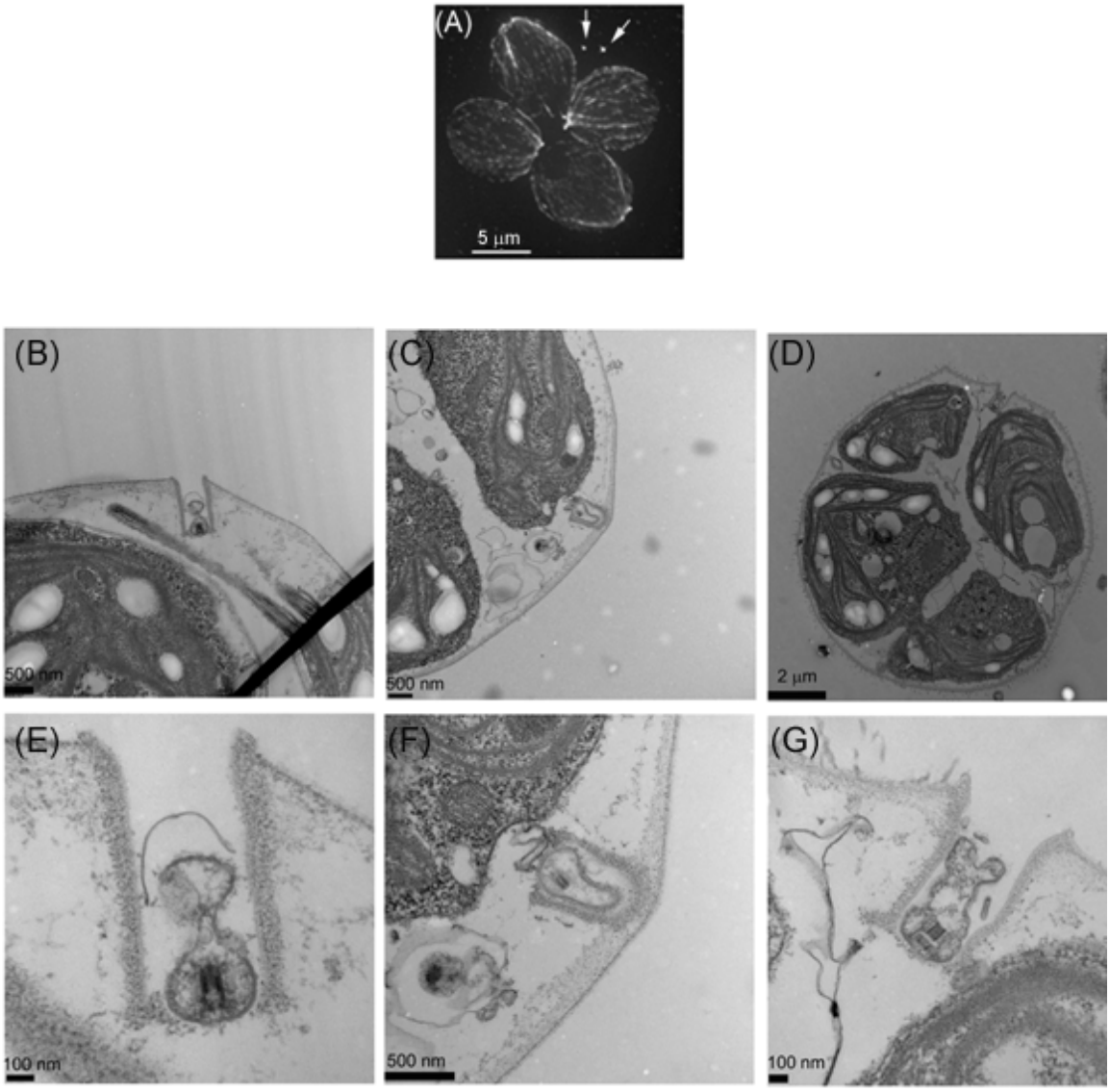


Figure 4-2. Flagellar remnants

Panel A : Immunoflourescence of dividing cells stained with antibodies against acetylated tubulin.

Panel B-D, C-F, D-G are illustrations of flagellar remnants present inside the vesicular pockets at the flagellar tunnel. These remnants are the spots or dots that appear in the IF images of Panel A. Notice that in D-G, within the flagellar tunnel the vesicles appears as though the flagella + transition zone were severed from the basal bodies before the flagella could undergo complete resorption pre-mitosis. TEM were done in collaboration with Drs. Dennis Diener and J. Rosenbaum (Yale University).

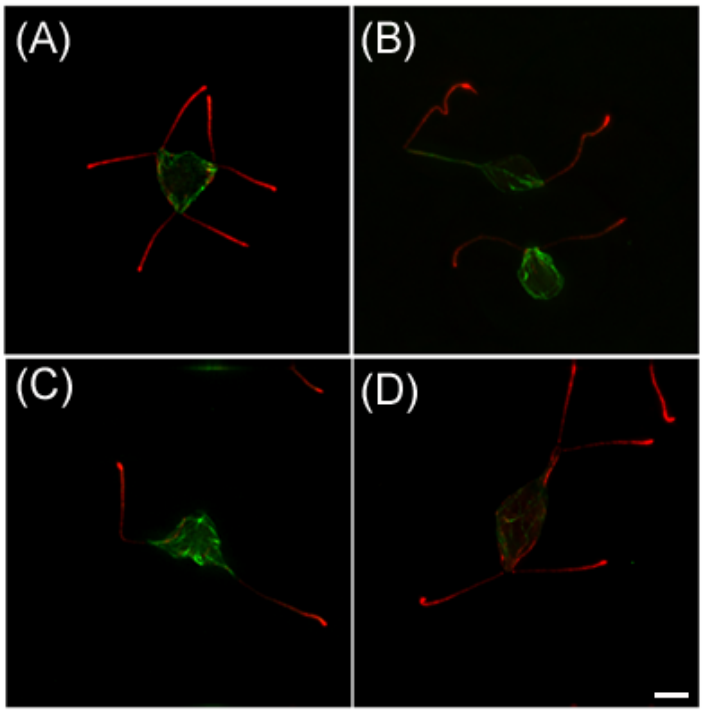


Figure 4-3. Katanin-RNAi in cell wall-less mutants

Above is an example of cells in which Kat-RNAi was expressed. Green = alpha tubulin and Red = acetylated tubulin. In Kat-RNAi, 16% of the cells were mitotic (n=200) and 50% of the mitotic cells still had flagella– indicative of absence of Katanin mediated release of basal bodies from the transition zone. (Immunoflourescence courtesy of Laura Hilton (Quarmby Lab). Scale bar = 5 μm.

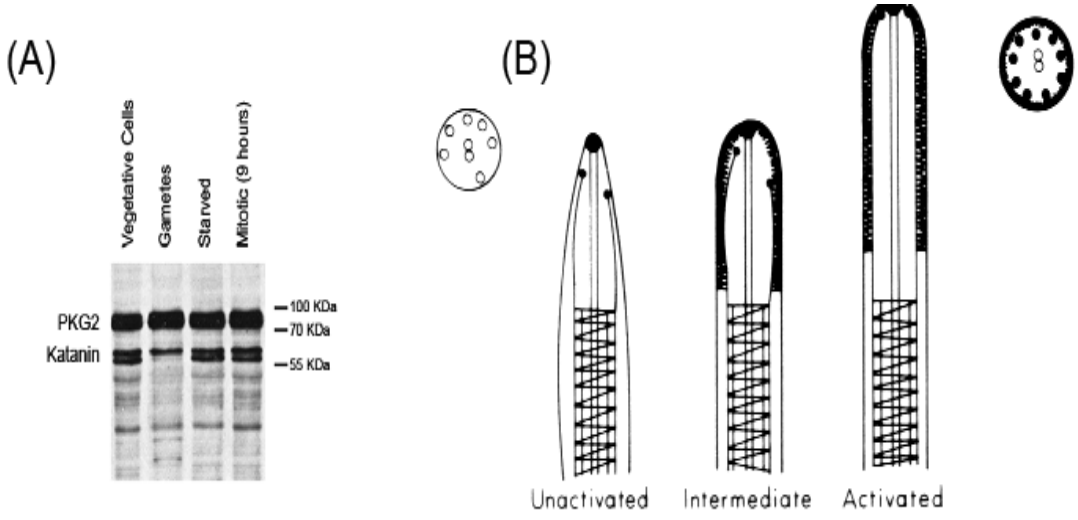


Figure 4-4. Katanin modification in gametes

(A) Katanin levels in vegetative cells, gametes, starved and synchronized mitotic cells, 9 hour after return to light (time when majority of the cells were undergoing division). In vegetative, starved and mitotic cells, Katanin appears as a doublet. Intriguingly, in gametes Katanin appears only in one form, which could be a phosphorylated form of Katanin.

(B) Figure obtained from Mesland *et al*., (1980) showing flagellar tip activation. A-tubules and the central pair extend and form a newly activated flagellar tip, which mediates mating in *Chlamydomonas*.

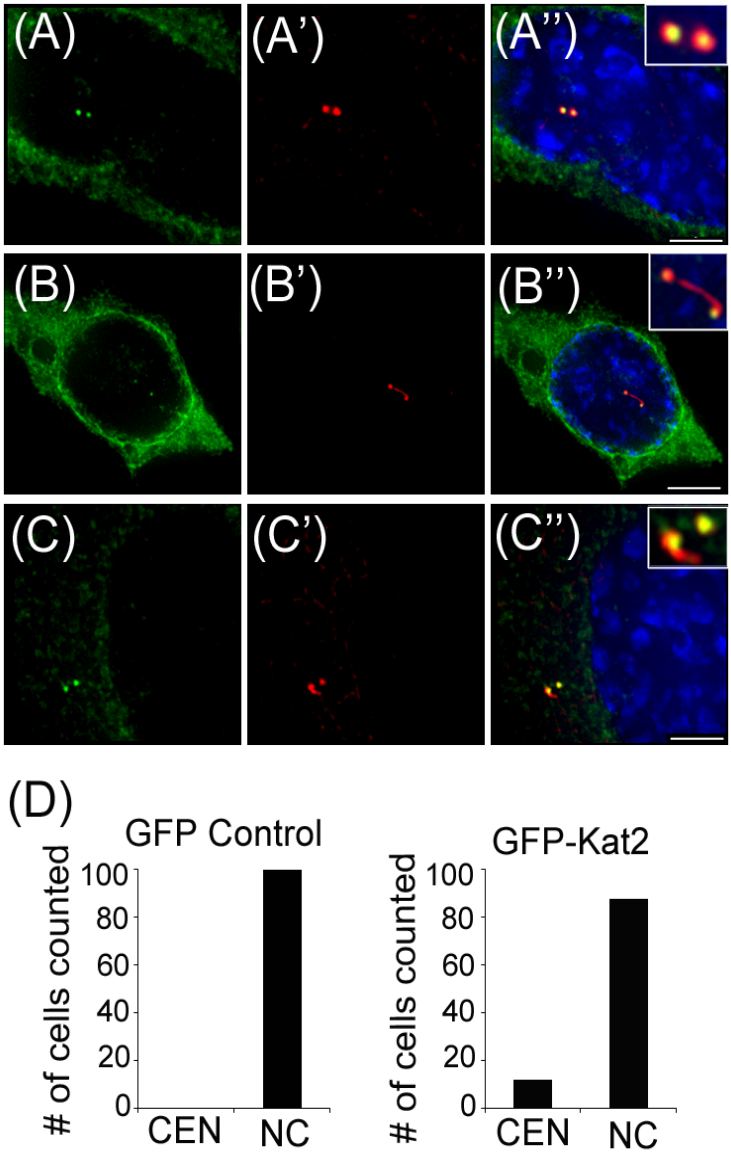


Figure 4-5. GFP-Kat2 localization in IMCD3 cells.

Panel (A to C''): Immunofluorescence of GFP-tagged *Chlamydomonas* Katanin2 in mouse IMCD3 cells. Green=GFP-Kat2, Red=Acetylated Tubulin, Blue=DAPI. GFP-Kat2 localizes to the centrioles. **Panel (D):** In 12% of the cells, GFP-Kat2 localizes to the centrioles (CEN) and expression of GFP alone was non-centrosomal (NC) in 100% of the cells. Scale bar = 5 μm, n = 100, Inset magnification = 3X.

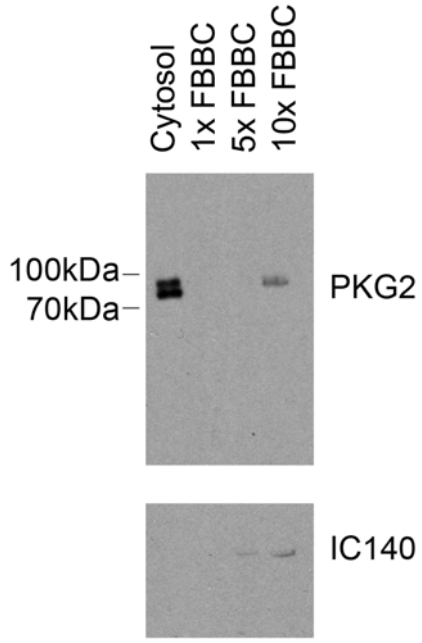
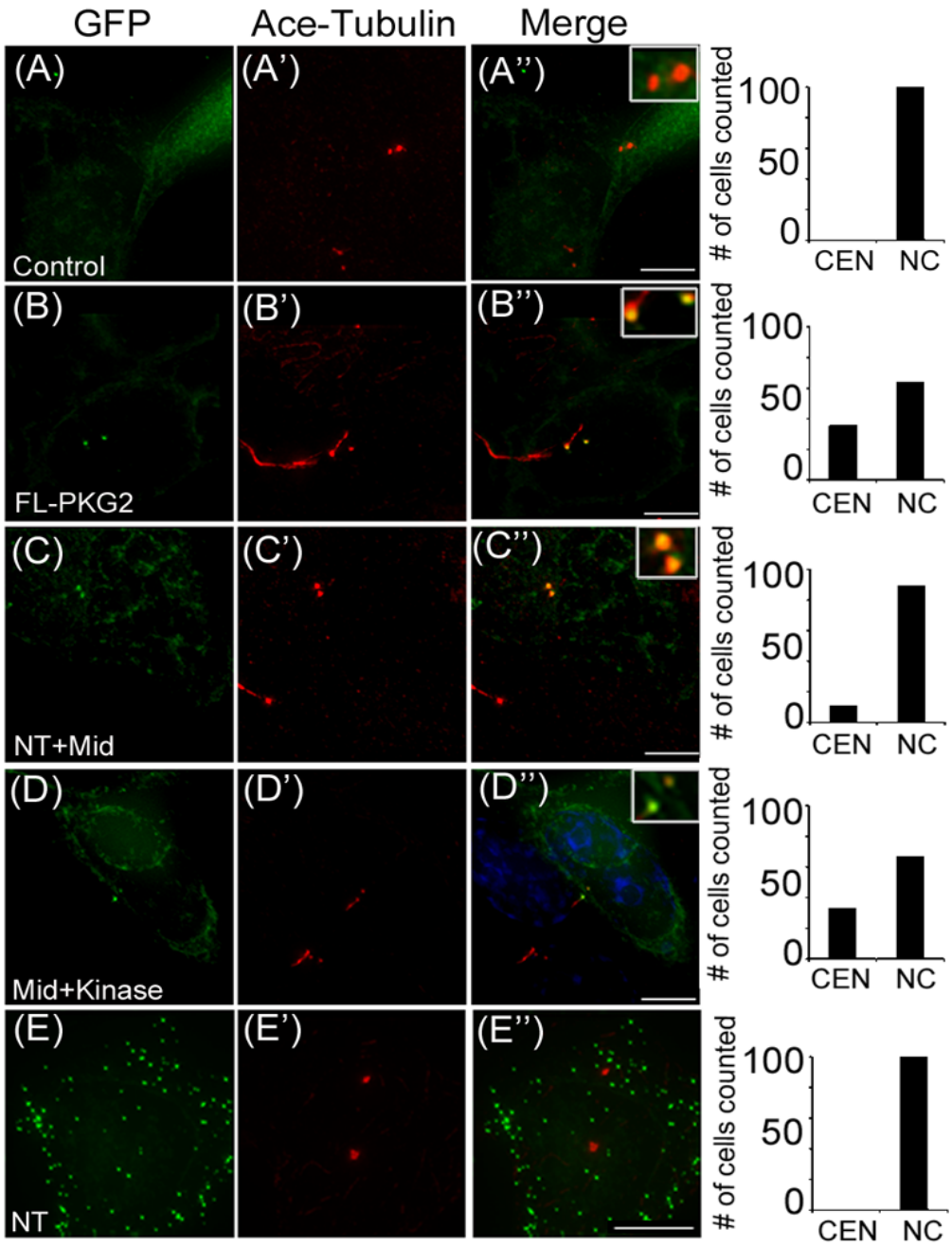


Figure 4-6. Comparison of PKG2p in the Cytosol vs. FBBC

In the cytosol, PKG2 appears as a doublet. In the FBBC (Flagellar Basal Body Complex) preparations, only the upper band is visible. This further supports our previous fractionating studies (Chapter 3) that a modified form of PKG2 associates with the flagella. Although the western blot shown above have been exposed for 1 min., longer exposures reveal that the upper band PKG2 in the FBBC fraction is a doublet in its own (not shown). Antibodies used: Rabbit anti-PKG2 and Rabbit anti-IC140 (Control). Western Blot courtesy of Bryan Tennant (Quarmby Lab).


GFP
Ace-Tubulin
Merge
(A)
(A')
(A'')
Control
(B)
(B')
(B'')
FL-PKG2
(C)
(C')
(C'')
NT+Mid
(D)
(D')
(D'')
Mid+Kinase
(E)
(E')
(E'')
NT
# of cells counted
100
50
0
CEN
NC

(F)

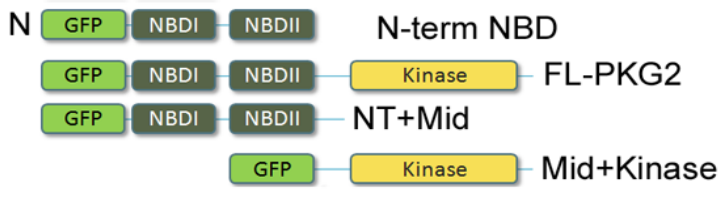

N
GFP
NBDI
NBDII
N-term NBD
GFP
NBDI
NBDII
Kinase
FL-PKG2
GFP
NBDI
NBDII
NT+Mid
GFP
Kinase
Mid+Kinase

Figure 4-7. GFP-PKG2 localization in IMCD3 cells

Immunofluorescence of GFP-tagged PKG2 in IMCD3 cells. Green=GFP-PKG2, Red=Acetylated Tubulin and Blue = DAPI. Panel **(A-A'')** is control eGFP expression in IMCD3 cells. Panel **(B-E'')** shows different trunctions of PKG2 expressed in IMCD3 cells and localization was scored as centrosomal (CEN) and non-centrosomal (NC). Full-length (FL), N-terminal+middle (NT+Mid), as well as C-term kinase (Mid + kinase) localizes to the centrioles (See panel **(F)** for constructs). The N-terminal construct containing only the NBDs (nucleotide binding domains) do not go to the centrioles. Scale bar = 5 μm. Inset Magnification = 3X. n=100 for each experiment.

Truncation series identified AELRDAERKLV along with the kinase domain to be important for proper targetting of PKG2 to the centrioles. Nuclear Export Prediction program identified AELRDAERKLV as a Nuclear export sequence.

(Prediction based on NESbase : http://www.cbs.dtu.dk/databases/NESbase-1.0/) ,Cour *et al*., 2003)

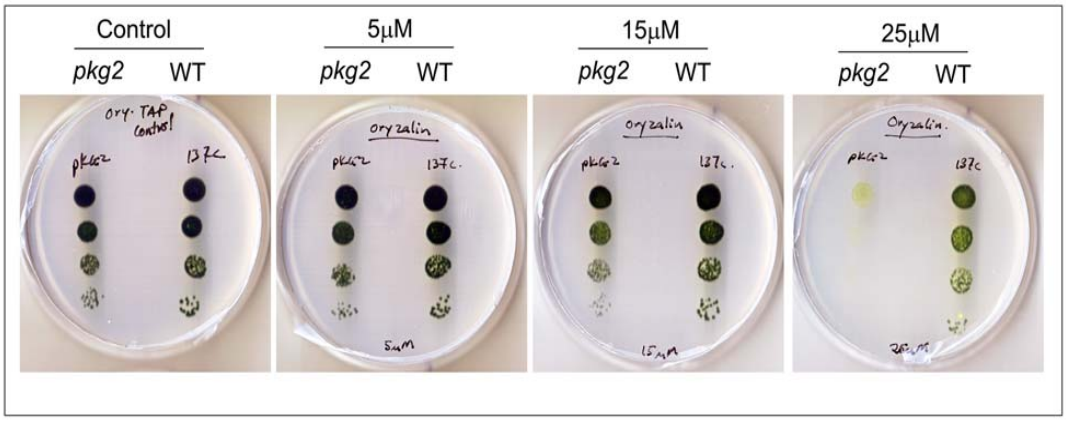


Figure 4-8. *pkg2* mutant cells are sensitive to Oryzalin

pkg2 mutant cells as well as wild type cells were plated on different concentrations of microtubule de-stablizing agent oryzalin. On control plates containing only regular media, both *pkg2* and wild type cells grow equally well. At increasing concentrations of oryzalin, *pkg2* mutants show sensitivity.

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