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Primary Cilia and the Cell Cycle

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Abstract

Cilia are microtubule-based structures that protrude from the cell surface, and function as sensors for mechanical and chemical environmental cues that regulate cellular differentiation or division. In metazoans, ciliary signaling is important both during organismal development and in the homeostasis controls of adult tissues, with receptors for the Hedgehog, PDGF, Wnt, and other signaling cascades arrayed and active along the ciliary membrane. In normal cells, cilia are dynamically regulated during cell cycle progression: present in G0 and G1 cells, and usually in S/G2 cells, but almost invariably resorbed before mitotic entry, to re-appear post-cytokinesis. This periodic resorption and reassembly of cilia, specified by interaction with the intrinsic cell cycle machinery, influences the susceptibility of cells to the influence of extrinsic signals with cilia-associated receptors. Pathogenic conditions of mammals associated with loss of or defects in ciliary integrity include a number of developmental disorders, cystic syndromes in adults, and some cancers. With the continuing expansion of the list of human diseases associated with ciliary abnormalities, the identification of the cellular mechanisms regulating ciliary growth and disassembly has become a topic of intense research interest. Although these mechanisms are far from being understood, a number of recent studies have begun to identify key regulatory factors that may begin to offer insight into disease pathogenesis and treatment. In this chapter we will discuss the current state of knowledge regarding cell cycle control of ciliary dynamics, and provide general methods that can be applied to investigate cell cycle-dependent ciliary growth and disassembly.

I. Introduction

A. Physical components of cilia

Cilia (also known as flagella) are found throughout the evolutionary tree, in organisms spanning the green algae *Chlamydomonas reinhardtii*, the protist *Tetrahymena thermophila*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and all vertebrates so far examined. Detailed studies of cilia date back over 50 years (Gilula and Satir, 1972; Lewin, 1952b; Wheatley, 1969; Wheatley, 1995; Wheatley et al., 1994). The structure of a cilium is highly conserved. The cytoskeletal scaffold of a cilium is composed of microtubule triplets termed the axoneme, arrayed in a 9+0 configuration in non-motile cilia (9 triplets in a hollow ring), or a 9+2 configuration (with 2 additional central triplets) in motile cilia. The ciliary axoneme is anchored at the cell body-proximal surface of the ciliary membrane by the basal body, which provides an anchor near the cell surface. As discussed in detail below, the basal body is derived from

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the mother centriole in a cell during specific phases of cell cycle, requiring its release from cilia and utilization as a microtubule-organizing center (MTOC) in a different capacity during mitosis. In vertebrates, this oscillation between basal body and MTOC identity is typically accompanied by extension of the cilium in post-mitotic (G1 or G0) cells, and resorption of the cilium later in cell cycle, prior to mitotic entry (Archer and Wheatley, 1971; Ho and Tucker, 1989; Quarmby, 2004; Sorokin, 1968a; Sorokin, 1968b; Tucker et al., 1983; Tucker et al., 1979a; Tucker et al., 1979b). The exact point of ciliary resorption during cell cycle depends on the cell type, with some cells resorbing cilia in S-phase, and others at the G2/M transition (Jensen et al., 1987; Rash et al., 1969; Rieder et al., 1979), with the latter pattern more typical (Figure 1).

The basic physical process of ciliary assembly and disassembly requires contributions of the cell secretory machinery, and of specialized motors that transport cargo towards and away from the cilia “tip”. The cilium typically emerges from the cell surface in close conjunction with the Golgi apparatus, with association of a vesicle with the distal surface of the basal body preceding extension (Sorokin, 1962). Recent studies have implicated the Arl and Rab GTPases of the Ras superfamily and their regulators in cilium formation and function (Grayson et al., 2002; Qin et al., 2007; Yoshimura et al., 2007). Rab family GTPases are known to promote interactions between membrane and the cytoskeleton, and to control specific interactions between membranes (Zerial and McBride, 2001). In the context of cilium formation, Rabs might therefore provide a means to control the interaction of the basal body with the cell surface, and integrate microtubule growth and membrane elongation. Intraflagellar transport (IFT), which delivers ciliary proteins to and from the ciliary tip, is mediated IFT complexes A and B: IFT complex B associates with the motor kinesin 2 to transport vesicles from the cell body towards the tip, while complex A associates with the motor dynein to transport packets from the tip towards the cell body. Cell cycle-regulated ciliary assembly and resorption relies in part on highly coordinated anterograde and retrograde cargo delivery system managed through the IFT proteins (Rosenbaum, 2002; Rosenbaum and Witman, 2002), and there are close connections between proteins involved in IFT and in core cell cycle controls. As we will discuss herein, a number of discrete signaling systems have now been identified as contributing to cell cycle coordination with ciliogenesis.

B. Normal and pathogenic cell cycle regulation of cilia

As cell cycle regulation of ciliary resorption and extension is strongly conserved through evolution, it is of interest to understand the underlying basis (if any) for selection of this coupling. It is possible to consider the needs of cell cycle, or the needs of ciliation, as primary in driving the connection. For example, the dual function of a centriole in the basal body and the MTOC/centrosome provides one obvious point of connection (Doxsey et al., 2005; Nigg, 2006). A cell cycle-centric perspective might ask if the selection is based on the need to “recover” the basal body for use as an MTOC in mitosis, or to “incapacitate” an MTOC in post-mitotic cells, by causing it to undergo differentiation to a basal body status. Further, centrosomes do not only act as MTOCs, but also serve as major signaling hubs for cell cycle regulators (Doxsey et al., 2005). The process of differentiation between centrosome and basal body may cause sequential displacement of factors that contribute to an earlier cell cycle stage, allowing them to be replaced by groups of proteins that control a later cell cycle stage. Ciliary resorption/shortening during the progression from G2 to M phase might be important for timing of cell cycle, due to altering accessibility of cells to growth factors that use cilia-localized receptors.

In this context, it is striking that the majority of tumor cells arising from ciliated normal precursor cells do not possess cilia at any stage of the cell cycle (Seeley et al., 2009; Wheatley, 1995; Wheatley et al., 1996). The loss of ciliation in tumor cells may indicate either that the

deregulated cell cycle which is a hallmark of cancer cells requires loss of a cell cycle-restrictive signal mediated through cilia, or that the loss of tumor cells ability to readily enter quiescence causes defects in ciliogenesis. Besides tumor cells, it has also been found that the cystogenesis found in polycystic kidney disease (PKD) or due to other mutations is accompanied both by defects in cell cycle progression, and shortened or absent cilia (Deane and Ricardo, 2007; Veland et al., 2009). The increasingly close connections apparent between inappropriate ciliation and disease state make understanding of the mechanisms involved of potentially great therapeutic value.

Although it is not unreasonable to think of cell cycle requirements as a primary driver of the ciliation cycle, some studies argue against easy generalizations or development of absolute rules. For example, a number of vertebrate cell lineages, such as lymphocytes, lack cilia entirely, arguing that it is not necessary for a centriole to pass through a phase as a basal body for a cell to undergo a normal cycle. In exciting recent work, Wong and coworkers demonstrated that cilia could positively or negatively regulate tumorigenesis, based on the role of the Hedgehog pathway in distinct cell types (Wong et al., 2009). As noted above, different lineages of cells resorb cilia at different phases of cell cycle, again arguing against a strict requirement for a centrosome versus a basal body at a specific stage. In many cell types, cells with ablated centrosomes are able to undergo mitosis (Mahoney et al., 2006), while ablation of centrosomes is accompanied by severe defects in primary cilia assembly (Mikule et al., 2007). With few exceptions in highly specialized conditions, such as the acentriolar pathway used for the formation of hundreds of cilia from deuterosomes in multi-ciliated cells (Dirksen, 1991; Duensing et al., 2007; Habedanck et al., 2005; Hagiwara et al., 2004; Kleylein-Sohn et al., 2007; Rodrigues-Martins et al., 2007a; Sorokin, 1968b; Vladar and Stearns, 2007), a centriole is absolutely required for cilia formation. These latter observations suggests that centrioles may be more important for formation of cilia than cell cycle, an idea developed at length by Marshall (Marshall, 2008). Finally, in adult organisms, most cells exist in a quiescent state, with cell cycle considerations secondary to the need to respond to transient environmental signals sensed through the cilia. Hence, in a cilia-centric view, brief “borrowing” of the centriole to act as an MTOC during mitosis may be tolerated as an efficient way to optimize cell division.

III Signaling systems regulating ciliary protusion and resorption

A. Sources of mechanistic insight: the leading role of *Chlamydomonas*

Although insights into the regulation of cell cycle coordinated ciliary extension and disassembly have emerged from many model systems, most paradigms for these processes have emerged from analysis of the biflagellate, unicellular alga *Chlamydomonas reinhardtii* (Dutcher, 1995; Haimo and Rosenbaum, 1981; Mitchell et al., 2004; Mitchell and Rosenbaum, 1985; Rosenbaum and Carlson, 1969). Many structural characteristics of the cilia and basal body in *Chlamydomonas* are identical to those of higher eukaryotes. The short life cycle of *Chlamydomonas*, the genetic potential of the organism, and the ability to manipulate environmental stimuli combine to create a robust capacity to analyze signals controlling ciliary dynamics. Of the regulatory signals discussed below as relevant to ciliary control in vertebrates, a number first were identified based on experiments performed in *Chlamydomonas*, and it is likely that further “mining” of this source will be fruitful.

In comparing the regulatory controls of *Chlamydomonas* and vertebrates, it is important to remain aware that the stimuli leading to loss of cilia may not be identical, given the different life cycles of unicellular organisms and metazoan. *Chlamydomonas* can reproduce asexually (either by vegetative cell division, or more often by zoospore formation) or sexually. During the vegetative cell cycle, the flagella regress before cell division begins, gradually becoming shorter over a period of approximately 30 min (Lewin, 1952a). After the cell division occurs,

flagella grow again from the basal body. The related process of flagella resorption is directly coupled to the cell cycle, with shortening occurring in pre-prophase phase, and extension at the beginning of G1 phase. In the sexual cycle, flagellar resorption begins a few hours after biflagellate + and -gametes have fused to form a quadriflagellate zygote, and proceeds gradually (Cavalier-Smith, 1974). This disassembly and resorption occurs during pre-prophase, in parallel with basal body replication: the duplicated basal bodies remain associated with the plasma membrane, and serve as poles for the mitotic spindle (Quarmby, 2004). Genes involved in regulating this mating-associated resorption process contain many parallels with the cell cycle-regulated loss of cilia in mammals, as discussed below.

Flagellar loss also occurs in response to a diverse set of environmental and stress-associated signals that are not always paralleled by stimuli occurring physiologically in a complex metazoan (Quarmby, 2004). For example, increasing intracellular concentration of Ca^{2+} (Quarmby and Hartzell, 1994) or addition 1 mM Ca^{2+} in the culture medium (Lohret et al., 1998) result in rapid deflagellation. Additional triggers of flagellar disassembly include exposure to low pH (Lewin et al., 1982), increase in temperature above 40°C (Lewin et al., 1982), or treatment with the local anesthetic dibucaine (Butterworth and Strichartz, 1990). For many of these stimuli, the mechanism of flagellar removal is not controlled resorption, but instead a rapid process termed variously deflagellation, excision, shedding, or autotomy (meaning “self-severing”) (Quarmby, 2004). This process involves direct severing of the axoneme and rapid changes in IFT (Parker and Quarmby, 2003) and vesicular transport (Overgaard et al., 2009). This process is much less well documented in vertebrates, although it clearly exists. The ciliated epithelia of the oviduct and lung transiently shed cilia in response to triggers including smoke and infection (Quarmby, 2004). A recent fascinating study documents chemical stress-induced ciliary shedding in cultured epithelial cells, and documents that the process of shedding is linked to enhanced tight junction association and transepithelial barrier function (Overgaard et al., 2009). The near-complete dearth of literature on this topic makes it a fertile ground for future studies: however, at present, it is not clear how directly genes involved in *Chlamydomonas* deflagellation, rather than flagellar resorption, will be relevant to cell cycle-regulation of the ciliary cycle in vertebrates.

B. IFT proteins

Intraflagellar transport (IFT) is the cellular process essential for the formation and maintenance of eukaryotic cilia and flagella (Figure 2). The IFT protein complexes are non-membrane-bound particles moving along the axonemal doublet microtubules from the base to the tip of the organelle, and subsequently returning. The relative rate of transport of building materials to and from the tip governs the length of the primary cilium. During resorption, disassembly of the axoneme counter-intuitively is associated with an increased rate of anterograde IFT particles entering the cilium, but with those particles having a higher rate of empty cargo sites (Pan and Snell, 2005; Rosenbaum and Witman, 2002). Defects in IFT proteins commonly results in failure to assemble cilia, with this the predominant activity of this protein class.

Interestingly, several studies have suggested that a limited number of specific IFT proteins may play dual roles in controlling ciliary protrusion and resorption, and in regulating cell cycle. Depletion of IFT27, a Rab-like small G-protein, results in the loss of flagella as well as the inhibition of cytokinesis (Qin et al., 2007). Rosenbaum and colleagues suggested that IFT27 may be involved in regulation of membrane dynamics during cytokinesis, limiting the vesicular trafficking necessary for abscission. Potentially, IFT27 might act as a cell cycle repressive checkpoint protein, limiting cell cycle progression until the cilia are resorbed and the centrioles can be used in cell division (Qin et al., 2007). As another example, mice with mutant IFT88/polaris/Tg737 (the Tg737/*orpk* mouse model) die soon after birth with cells containing abnormally short cilia, suggesting a defect in ciliogenesis or ciliary maintenance (Pazour et al.,

2000; Taulman et al., 2001), In mammalian cells, IFT88 is not only required for the formation of primary cilia, but also serves as a centrosomal protein that regulates G1-S transition in non-ciliated HeLa cells (Robert et al., 2007). The G1-S transition control may involve the interactions documented between IFT88 and Che-1, a regulator for S phase entry that binds and inactivates the tumor suppressor Rb: IFT88 overexpression causes failure to enter S, and stimulates apoptosis, while IFT88 depletion drives cell cycle progression through S and G2 (Robert et al., 2007). No cell cycle-specific functions have yet been described for other IFTs, nor have IFT mutations been specifically associated with resorption phenotypes.

C. NEK kinases

Although Rieder first predicted that ciliary resorption might be controlled by factors regulating mitotic progression in 1979 (Jensen et al., 1987; Rieder et al., 1979), more than two decades elapsed before this insight was validated (Figure 2). The “never-in-mitosis” or NIMA-related kinase (NRK) family of proteins are cell cycle kinases that are essential for mitosis regulation, G2-M transition and centrosome separation (Quarmby and Parker, 2005). In 2004, the Quarmby group first identified a NRK family member, Nek2/FA2, as important for both cell cycle progression and deflagellation in *Chlamydomonas* (Mahjoub et al., 2004; Quarmby and Parker, 2005). The Fa2p protein localizes to a specific region of the proximal cilium in interphase cells, the site of flagellar autotomy (SOFA). Fa2p is essential for Ca²⁺-mediated axonemal microtubule severing during deflagellation. Cells carrying a complete deletion of the Fa2 gene also have a significant delay at the G2/M transition, slowing their transit of the cell cycle (Mahjoub et al., 2004); whether the severing and cell cycle defects are directly related or partially independent needs further study. Nek2 kinases control centrosomal maturation in non-ciliated cycling cells (Fry et al., 2000), suggesting a possible independent function; further, subsequent studies by the Quarmby group indicated the ciliary function is kinase-dependent, whereas the cell cycle function is kinase-independent (Mahjoub et al., 2004). During resorption, Fa2p relocates from the SOFA to the proximal end of the basal bodies; during mitosis, it is associated with the polar region of the mitotic spindle. As the cells exit mitosis, Fa2p accumulates at the proximal end of the basal bodies and moves out to the SOFA as soon as ciliogenesis is initiated. These localizations position Fa2p appropriately to directly phosphorylate proteins that regulate severing and resorption, although at present, critical substrates have not been identified.

A growing number of NRK proteins have been implicated in linked to control of ciliary resorption and/or deflagellation coordinated by cell cycle. The Quarmby group has more recently shown the NRK Cnk2p is found along entire length of the axoneme of cilia/flagella, and regulates flagellar length by promoting flagellar disassembly as well as regulates cell size, by affecting the assessment of cell size prior to mitosis (Bradley and Quarmby, 2005). An extensive phylogenetic analysis of NRKs in the highly ciliated organism *Tetrahymena* has nominated multiple NRKs from three different sub-families as regulating ciliary resorption (Wloga et al., 2006): in this case, specific NRKs induced the resorption of specific sets of cilia on the organismal surface, suggesting specialized control mechanisms. Although analysis of the NRKs in vertebrates is not as advanced as in lower eukaryotes, Fa2p localization and likely activity are equivalent in murine kidney cells and *Chlamydomonas* (Mahjoub et al., 2004). In both mice and humans, mutations in the genes Nek1 and Nek8 cause polycystic kidney disease, (Liu et al., 2002; Upadhyay et al., 2000), and these proteins have been shown to localize to cilia and regulate both centrosomal and ciliary integrity (Otto et al., 2008; Trapp et al., 2008; White and Quarmby, 2008). More work to establish the downstream targets of the NRKs is clearly required.

D. Aurora-A and HEF1/NEDD9

Another source of insight into ciliary resorption in humans came from studies of the *Chlamydomonas* kinase CALK, which was identified by the Snell group as an important trigger for flagellar resorption (Pan and Snell, 2003; Pan and Snell, 2005; Pan et al., 2004; Pan et al., 2005). Kinesin II translocates CALK into the flagella in response to fertilization, where its action promotes ciliary shortening. Beyond fertilization, the CALK kinase becomes activated by a number of different stimuli inducing loss or shortening of flagella, and depletion of CALK by RNAi inhibits both resorption and deflagellation.

Although CALK is as much an environmental sensor as a cell cycle kinase in *Chlamydomonas*, it is evolutionarily related to the cell cycle regulatory Aurora-A kinase in vertebrates (Figure 2). Aurora-A is a centrosomally associated kinase that becomes activated at mitotic entry to phosphorylate substrates including such as Cdc25B, TPX2, Eg5, histone H3 and others that promote progression through the stages of mitosis (Vader and Lens, 2008). Aurora-A activity is reduced and much of the kinase pool is degraded as cells re-enter G1. Given the known tendency of ciliary resorption to coincide with G2/M transition, the suggestive timing of this activity profile, and the centrosomal localization of Aurora-A, together led our group to investigate Aurora-A as a regulator of ciliary resorption (Pugacheva et al., 2007). We found that microinjection of Aurora-A into ciliated cells caused rapid (<2 minutes) ciliary disassembly, while treatment with a specific small molecule inhibitor of Aurora-A (PHA-680632) or siRNA depleting Aurora-A prevented serum-induced disassembly of primary cilia, indicating activated Aurora-A is necessary and sufficient for ciliary shortening. In addition, stimulation of cells with serum to initiate a disassembly program induced Aurora-A activation and auto-phosphorylation at the basal body of the cilium immediately preceding ciliary resorption. Unexpectedly, this study demonstrated that cilia were resorbed in two waves: a first at 1–2 hours post-serum stimulation, when cells were still in G0/G1 phase, and a second at 18–24 hours post-serum stimulation, when cells were entering mitosis. Aurora-A became active before disassembly even in the earlier, non-mitotic wave: providing the first evidence for a non-mitotic activation of this kinase in vertebrates, but paralleling the environmental activation of CALK seen in *Chlamydomonas*.

The mechanism of Aurora-A action in promoting disassembly is likely to involve Aurora-A dependent activation of the tubulin deacetylase HDAC6, which deacetylates α -tubulin in vitro and in vivo leading to destabilization of microtubules. Supporting this idea, pre-treatment of cells with the HDAC6 inhibitor tubacin (Haggarty et al., 2003) limited Aurora-A induced ciliary resorption, while Aurora-A phosphorylation of HDAC6 stimulated its activity in vitro (Pugacheva et al., 2007). However, it is uncertain whether HDAC6 is the only substrate of AurA involved in ciliary resorption, while studies of *Chlamydomonas* with non-acetylatable mutants of α -tubulin do not have notable defects in ciliary dynamics (Kozminski et al., 1993); this topic requires more investigation.

The activation of Aurora-A for ciliary disassembly following serum stimulation is mediated in part by interaction with the adaptor protein HEF1/NEDD9 (Singh et al., 2007), which was previously shown to directly bind and activate Aurora-A during G2/M transition (Pugacheva and Golemis, 2005). Serum stimulates HEF1 expression and hyper-phosphorylation preceding each wave of Aurora-A activation, while siRNA depletion of HEF1 reduced ciliary resorption (Pugacheva et al., 2007). Upstream of HEF1, constituents of serum previously associated with HEF1 activation that might be relevant to ciliary disassembly include ligands of integrin receptors (Law et al., 1996), GPCR (Zhang et al., 1999), Ca^{2+} (Zhang et al., 2002), and growth factors such as TGF β (Liu et al., 2000). We have assessed a number of these and other components individually for ability to induce ciliary disassembly in starved ciliated cells ((Pugacheva et al., 2007), and unpublished results). In tests of PDGF, TGF β , EGF, estrogen, hydrocortisone, progesterone and ionic calcium, zinc, and magnesium, only PDGF elicited a

partial response. The role of PDGF α at cilia has been discussed in detail in (Christensen et al., 2008).

E. GSK3 and VHL

In contrast to the preceding examples, in which the identified proteins have clear roles in ciliary resorption in cell cycle, GSK3 and VHL have been described as associated with the process of ciliary maintenance (Figure 2), and their cell cycle function is less clear (Thoma et al., 2007). von Hippel-Lindau disease is marked by renal cysts and high incidence of renal cell carcinomas: the VHL gene mutated in this syndrome encodes a protein that regulates oxygen sensing, but also microtubule dynamics. Although the VHL protein localizes to the ciliary axoneme, loss of VHL as a single event does not affect formation of cilium in serum starved cells, but does increase the rate of ciliary loss in cells stimulated with serum (Thoma et al., 2007). VHL is a substrate of the kinase GSK3 β , which regulates its functions at microtubules (Hergovich et al., 2006). Moreover, while *Chlamydomonas* has no ortholog for VHL, the GSK3 β ortholog in this organism regulates flagellar assembly and length control (Zhou and Hung, 2005). Thoma and colleagues determined that simultaneous loss of GSK3 β and VHL intensified serum-induced ciliary loss, and in some contexts (GSK3 β -deficient MEFs) reduced the overall formation of ciliation following serum starvation. These activities were specifically linked to the microtubule-stabilizing rather than oxygen-sensing activities of VHL. Finally, renal cells from the cysts of patients with VHL disease tended to become deciliated more readily when treated with agents that inhibited GSK3 β activity.

It is interesting to note that Aurora-A and GSK3 β have been shown to directly interact in a number of studies (Dar et al., 2009; Fumoto et al., 2008), with phosphorylation by Aurora-A altering some functional activities of GSK3 β (for example, towards β -catenin). At present, potential interactions between these proteins, or between NEK kinases, GSK3 β , and VHL have not yet been examined in regard to cilium or cell cycle: such studies would be of considerable interest, particularly given the observation that inactivation of VHL contributes to ciliary loss. In future experiments, it may also be interesting to consider that GSK3 β is regulated by Lkb1, a tumor suppressor that influences cell cycle and cell polarity (Green, 2004), and regulates expression of HEF1/NEDD9 (Ji et al., 2007), the Aurora-A activator (Pugacheva et al., 2007).

F. CEP97, CPP110, CEP290, CEP164

In the past two years, a series of studies have identified an interacting cluster of proteins that specifically suppress ciliary protrusion (Figure 3) (Graser et al., 2007; Spektor et al., 2007; Tsang et al., 2008). CP110, a centrosomal protein required for centrosomal duplication and cytokinesis, was identified as binding the additional centrosomal proteins Cep97 (Spektor et al., 2007), and subsequently CEP290 (Tsang et al., 2008): mutations in CEP290 have been implicated in a set of “ciliary diseases”, including Bardet-Biedl Syndrome (BBS), nephronophthisis, and others. Importantly, depletion of Cep97, or CP110 caused protrusion of cilia in quiescent cells, while overexpression of CP110 repressed ciliary protrusion in quiescent cells without affecting their cell cycle (Spektor et al., 2007). Mutants of CP110 unable to bind CEP290 were unable to repress ciliary protrusion (Tsang et al., 2008). CEP290 binds to the Rab8a GTPase, which controls vesicular trafficking into the centrosome; Tsang and co-workers showed that Rab8a binding to CEP290 was necessary for ciliogenesis. Taken together, these results supported a model in which ciliogenesis is actively inhibited in cycling cells, based on the action of CP110+CEP97 and CP110+CEP290+Rab8a complexes acting at the centrosome. Serum starvation led to the removal of CP110 from these complexes, allowing a CEP290+Rab8a complex to contribute to ciliary protrusion (Tsang et al., 2008). Importantly, these results indicated that it is possible to at least partially decouple ciliogenesis from cell cycle in

vertebrate systems, although some abnormalities associated with the cilia-like structures emerging from CP110-depleted cells have been noted (Keller and Marshall, 2008).

An independent study based on the siRNA depletion of proteins associated with the basal body (Graser et al., 2007) also identified CEP290 as required for ciliogenesis. This thorough study showed that a considerable number of basal body and centrosomal function influenced ciliogenesis, with depletion of CEP290, pericentrin, and CEP164 yielding particularly strong phenotypes. Interestingly, CEP164 localizes specifically to mature centrioles, and associates with distal appendages (Graser et al., 2007). In the normal centrosome/ciliary cycle, as cells emerge from quiescence and resorb cilia, the CDK2-cyclinE complex progressively associates with the centrosome and triggers the G1/S transition. Centrosome duplication and DNA synthesis are tightly connected through cyclin E/Cdk2 activation (Hinchcliffe and Sluder, 2001; Schnackenberg et al., 2008). Both centrioles disengage at this point as a new “daughter” is formed from the “mother”, or older, centriole. Cilia invariably protrude from mothers rather than daughters; significantly, in normally cycling cells, no centriole is capable of forming a cilium until it has gone through one phase of mitosis (Rodrigues-Martins et al., 2007b; Rodrigues-Martins et al., 2008). Of relevance to CEP164, between S phase and mitosis, the new mother centriole undergoes a maturation process during which it gains a set of mother centriole-specific proteins and appendages (Pearson et al., 2009; Pearson and Winey, 2009). The appendages, which include sub-distal proteins such as ninein and CEP170, and distal proteins including ODF2, are necessary for anchoring the cilium to the apical side of the cell, and nucleation of the axoneme (Bornens, 2002; Bornens and Piel, 2002; Palazzo et al., 2000; Pedersen and Rosenbaum, 2008; Piehl et al., 2004). The identification of CEP164 as a novel distal appendage protein suggests it may serve as a mark allowing protrusion of the ciliary axoneme in post-mitotic cells, connecting the basal body and plasma membrane.

G. New candidates: katanin, kinesin, and screens

In addition to the regulatory pathways discussed above, a number of other proteins have been implicated in the gain and loss of cilia in *Chlamydomonas* and/or in metazoans. Some interesting recent findings are summarized here.

As noted above, resorption likely involves activity at the base of cilia, in addition to the well-established disassembly that occurs at the ciliary tip (Marshall and Rosenbaum, 2001; Parker and Quarmby, 2003), while deflagellation is caused by precise severing of the axoneme at a specific site between the axoneme and the flagellar transition zone, known as the site of flagellar autotomy (SOFA) (Mahjoub et al., 2004). The microtubule-severing ATPase katanin induces breakage of the outer doublet microtubules during deflagellation in *Chlamydomonas* (Lohret et al., 1998). In vertebrates, katanin is best known for a role in cell cycle progression (Buster et al., 2002; Toyo-Oka et al., 2005; Zhang et al., 2007). Now, using RNA interference (RNAi) approach to reduce levels of katanin expression in *Chlamydomonas*, Rasi et al have recently demonstrated that there is a second site of axonemal severing proximal to the transition zone, and that severing occurs at this site before complete pre-mitotic resorption of flagella (Rasi et al., 2009) (Figure 3). Cells deficient in katanin fail to release resorbing flagella from the basal bodies. These findings suggest that there are at least two parallel programs active during resorption: 1) reducing the axoneme from the tip modulation of IFT, resulting in a net transport of disassembled ciliary components from the tip back to the cell body; and 2) severing of the basal body from ciliary remnants before functional reassignment of the basal bodies to the spindle poles/centrosome. It will be interesting to establish if katanin-dependent loss of cilia occurs in mammalian cells.

Kinesins are important regulators of microtubule dynamics in mitosis (Figure 3), and one member of the family, kinesin-2, is well-established for a role of anterograde IFT in the flagella of *Chlamydomonas*. In a recent study by Piao and colleagues (Piao et al., 2009), a new

Chlamydomonas kinesin, Crkinesin-13, was identified as phosphorylated upon the initiation of flagellar protrusion, and dephosphorylated when flagella reached maximal length. Depletion of Crkinesin-13 delayed flagellar shortening after physiological stimuli such as recovery from pH shock. CrKinesin-13 physically is moved into the flagella by an IFT process during flagellar shortening, while depletion of Crkinesin-13 also inhibited the shortening process. Taken together, these data indicate a central role for this protein in regulating microtubule dynamics during all phases of flagellar remodeling. No studies of Crkinesin orthologs have been undertaken in regulation of metazoan cilia.

Finally, data from a number of high throughput profiling and screening screens have begun to identify a diverse set of proteins that influence ciliary resorption, maintenance, and protrusion. Examples of these approaches include a difference gel electrophoretic (DIGE) analysis of the flagellar tip complex of *Chlamydomonas* with long versus short flagella, which identified a novel protein methylation pathway specifically activated during flagellar resorption (Schneider et al., 2008). Proteins in the MetE complex are associated with the axoneme, and appear to be cell cycle regulated; a role for methylation in regulation of metazoan cilia remains to be investigated. Stolc and co-workers performed a genome-wide transcriptional analysis of *Chlamydomonas* cells undergoing flagellar regeneration (Stolc et al., 2005), following an earlier more limited profiling screen (Li et al., 2004). This screen identified 220 strongly induced genes, additional repressed genes, and a number of orthologs of genes previously identified in zebrafish studies as associated with polycystic kidney disease, almost none of which have been studied in detail.

An siRNA-based kinome screen to identify modulators of Hedgehog (Hh) signaling (Evangelista et al., 2008) predictably identified a number of known and new regulators of ciliogenesis, given the known dependence of the Hh signaling cascade on cilia-localized receptors. Implicated kinases included the cell cycle regulator Nek1, but also Prkra: the latter kinase is best known as a regulator of Pkr, which regulates interferon response, and also controls microRNA processing. As Evangelista and colleagues note, some microRNAs have been localized to the basal body of cilia (Deo et al., 2006); what functional role they might play in resorption or ciliogenesis remains obscure. Finally, repeated analyses of the *Chlamydomonas* centriolar proteome (Keller et al., 2009; Keller et al., 2005) have led to identification of many proteins orthologous with ciliary disease genes. The rich results of these various studies in *Chlamydomonas* suggests the next several years will be extremely rich in disease-relevant discoveries, as findings are used to inform studies in humans.

III. Methods

A number of immortalized cell lines can readily be made to form cilia. Features that make cell lines particularly useful for studying ciliary function and structure include a homogeneous growth profile, a high frequency of ciliation under induced conditions, and availability of reagents (e.g., antibodies) suitable for use in the species of derivation. Cell lines we have evaluated and which are suitable for studies include MDCK (canine polarized epithelial kidney cells) (ATCC, CCL-34) (Praetorius and Spring, 2003), IMCD3 (mouse kidney collecting duct cells) (ATCC CRL 212) (Mai et al., 2005) Caki-1 (human renal cells) (ATCC, HTB-46) (Glube and Langguth, 2008), LLCPK1 (porcine kidney cells) (ATCC, CL-101) (Bendayan et al., 1994), hTERT-RPE1 (human retinal pigmented epithelial cells) (ATCC, CRL-4000) (Rambhatla et al., 2002), MEFs (primary mouse embryo fibroblasts) (Wheatley, 1972) and HK-2 (normal human proximal tubule cells) (ATCC, CRL-2190) (van Rooijen et al., 2008). Typically, only 10–30% of the overall population of these cells is ciliated while they are exponentially growing in medium with 10% fetal bovine serum, while >90% of the population can become ciliated under starvation conditions.

In 1979 Tucker showed that 80–90% of 3T3 fibroblasts form cilia after growing for 48 hours in low serum medium, at a confluence of 80–100% (Tucker et al., 1979a). For the cell lines discussed here, the time of incubation in serum free medium required for induction of cilia varies, and should be adjusted for each cell type individually. For example, for hTERT-RPE1 cells full ciliation is typically achieved in 48h, while for renal cell carcinoma cell lines containing re-expressed VHL, primary cilium formation required maintenance of confluent cultures for 7 days in serum-free medium (Lutz and Burk, 2006). Some cell lines, such as LLCPK1 cells, need a prolonged starvation period of up to 15–20 days to form cilia.

For most of the cell lines we have examined, there is a separate contribution of high cell density to the ciliation process: many cell lines require maintenance at >70% confluence for efficient ciliation. However, particularly when studying disassembly of cilia, it is useful to spend some time investigating minimum and maximum densities contributing to efficient deciliation, as plating at too high a density may in some cases limit re-entry into cell cycle and ultimate degree of ciliary resorption.

Working with fibroblasts, Tucker described three phases following stimulation with serum: “first, an initial but transient deciliation within 1–2 hr; second, a return of the cilium by 6–8 hr; and third, a subsequent final deciliation of the centriole coincident with the initiation of DNA synthesis at 12–24 hr”. In our work with other cell lines, we have more typically observed two phases: an initial loss at 1–2 hours, and a more complete loss at 18–24 hours, which in a number of cases we have determined coincides with mitotic entry rather than initiation of DNA synthesis. We strongly suggest parallel measurement of cell cycle status when using live cell imaging or fluorescence microscopy of fixed cells to measure ciliary resorption in cell lines that have not previously been assessed. The protocol presented below was initially optimized in the hTERT-RPE1 cell line, but also works well for IMCD3, Caki-1, MDCK, HK-2 cell lines and MEFs.

Compared to the well-studied list of chemical and physical stimuli that induce flagella resorption in *Chlamydomonas*, relatively little is known about the specific chemical factors that may play a role in induction of disassembly of primary cilium in mammals. As alternative to full serum, PDGF causes partial ciliary disassembly for at least some cell types (Christensen et al., 2008; Pugacheva et al., 2007). Some cell lines, such as human umbilical vein endothelial cells (HUVEC) disassemble under such mechanical stimulation as laminar shear stress (Iomini et al., 2004). Flow chambers suitable for generating defined amounts of flow forces can be purchased from Biopetechs, Butler, PA (the FC2 system).

Cilium Disassembly Protocol

1. Plate cells on cover slips in medium containing full serum (typically 10% fetal bovine serum), and grow for 24–48 hours, or until they have achieved 50–70% confluency. For cell lines that adhere poorly or unevenly, coating slides with collagen, fibronectin or poly-L-lysine before plating can assist in the subsequent visualization of cilia using immunofluorescence.
2. To induce formation of cilia, replace the medium to serum free (Opti-MEM medium, Invitrogen) and culture for 48 hrs. With hTERT-RPE1 cells, >85% of cells normally have clearly visible cilia, and little further increase is seen with additional days of culture. For a new cell line, it would be prudent to initially compare cultures maintained for 48 and 96 hours in Opti-MEM. If a low degree of ciliation is observed (<50%), or more cilia are seen at 96 than 48 hours, it would be useful to also explore longer incubation periods. Fresh Opti-mem should be added to cells every 2 days for longer culture periods.

3. To initiate ciliary disassembly, replace Optimem with medium containing 10% fetal bovine serum. Ciliary resorption should commence within 1–2 hours, and extend over the following 24 hrs (see Figure 4). Usually addition of serum leads to resorption of the primary cilium in hTERT-RPE1, Caki, MDCK or IMCD3 cells in two waves: at 2 hr (G0/G1 phase) and at 18–24 hr (most cells entering mitosis). Standard techniques for FACS analysis, BrDU staining, and direct observation of condensed DNA and mitotic figures (following staining with DAPI and antibodies to α -tubulin) are useful approaches to establish cell cycle phase during the resorption process.
4. Cilia can be visualized by immunofluorescence microscopy, using primary antibodies to mark the axoneme and adjacent basal body. For example, acetylation of tubulin results in stabilization of tubulin polymers during mitosis and in cilia (Kannarkat et al., 2006; Matsuyama et al., 2002), and in quiescent interphase cells, acetylated tubulin is strongly enriched in microtubules within cilia. Tubulin within the ciliary axoneme also accumulates additional post-translational modifications, such as glutamylation (Wheatley et al., 1994; Wloga et al., 2008). Hence, anti-acetylated α -tubulin (clone 6-11B-1, Sigma, or clone K(Ac)40, Biomol) or alternatively, anti-glutamylated α -tubulin (T9822, Sigma) (Million et al., 1999) are useful to mark axoneme. Antibodies against the ciliary proteins katanin, IFT and tektin can also be used to visualize the axoneme. Antibodies to γ -tubulin, centrin and other proteins associated with the centriole and/or pericentriolar mass (PCM) are useful to visualize the basal body and (in cells with disassembled cilia) the centrosome.
5. Antibodies to T²⁸⁸-phosphorylated Aurora-A (Cell Signaling Technology, Beverly, MA) allow measurement of Aurora-A activation during the ciliary resorption process (Pugacheva et al., 2007). These antibodies can be used as a control for use with immunofluorescence to mark the timing of peak ciliary disassembly. The antibodies currently available work well in human cell lines, but are less effective in the mouse cell lines we have tested. Unfortunately, there are currently no commercially available antibodies to endogenous phosphorylated Aurora-A that work well on western blots.

Blocking ciliary disassembly

Small molecule inhibitors targeting specific molecules such as U0126 (a MEK1/2 inhibitor, EMD Chemicals Inc., Cat. No. 662005), roscovitine (a Cdk1/2 inhibitor, EMD Chemicals Inc Cat. No. 557360), TSA (a broad spectrum histone deacetylase (HDAC) inhibitor, Sigma, T1952), PHA-680632 (an Aurora-A inhibitor, (Soncini et al., 2006)) and tubacin (an HDAC6 inhibitor, (Haggarty et al., 2003)) have been used for study of the contribution of growth factors or specific cell cycle regulators to ciliary resorption. It needs to be taken into account that some of these inhibitors will block cell cycle progression at certain stages. Depending upon length pre-incubation with the drug, and at which stage characteristic blockage occurs, it is important to minimize these potentially complicating effects for the interpretation of studies of resorption (i.e. in establishing whether resorption effects are secondary to, or independent of, effects on cell cycle). Controls for cell cycle stage are particularly important when using inhibitors.

For studies we have performed in hTERT-RPE1 cells, we first established IC₅₀ curves for inhibitors of Aurora-A and HDAC6 inhibitors (0.3 μ M for PHA-680632, and 2.5 μ M for tubacin, respectively), then selected doses at approximately IC₇₅ values, followed by a relatively short incubation period. To achieve effective inhibition but minimize both drug toxicity and secondary effects, drugs are added in Optimem typically 1–3 hours before addition of serum to starved cells ensure inactivation of their target of interest prior to initiation of ciliary disassembly. Depending upon the stability of a particular inhibitor, it is sometimes necessary to repeat treatment during the course of study, since a full cell cycle in some cells might take

30–40 hours. Effective inhibition should be confirmed using an assay for a well-defined substrate for the targeted protein, at the beginning and end of the time of ciliary assay.

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Figure 1. Ciliary and the cell cycle in metazoans

In most cells, primary cilium formation first occurs post-cytokinesis, during G1. Upon entry into S phase, the centrioles and the DNA initiate replication. Ciliary disassembly occurs at the G2/M transition. Once cell division is complete, the cilia reassemble in G1. Typically, the length of cilia and the proportion of a cell population with cilia are increased in quiescent (G0) rather than cycling cells.

Figure 2. Cell cycle and signaling proteins associated with control of ciliogenesis and disassembly

See text for details: PDGF-AA binding to the PDGFR α receptor located at the ciliary membrane induces phosphorylation and activation of the number of different signaling pathways that have a role in ciliary resorption and cell cycle regulation. Among these, HEF1 binds to Aurora A kinase, promoting its activation. Aurora A in turn phosphorylates and activates cilia-associated HDAC6, resulting in HDAC6 mediated deacetylation of substrates in the ciliary axoneme, causing ciliary resorption. IFT proteins (IFT27 and IFT88) directly coordinate cell cycle machinery and trafficking of particles within the axoneme, contributing to timed disassembly. Nek1 and Nek8 act at the basal body and axoneme during cell cycle regulated disassembly. pVHL and GSK-3 β localize to primary cilia, and act together to maintain the primary cilium, and may be targeted during the disassembly process.

Figure 3. The centrosome-basal body transition in Cell Cycle

Following mitotic exit, primary cilium formation occurs as cells move into G0/G1 following centrosomal docking to the membrane. Distal appendages that includes ODF2 and CEP164 help to anchor of mother centrosome (basal body) to the cell membrane. Sub-distal appendages serve as a major site for microtubule (MT) anchoring, an activity requiring component proteins ninein and CEP170. CEP290 cooperates with the vesicular transport regulatory GTPase Rab8a to promote cilium formation: in phases of the cell cycle at which active cilium assembly is not occurring, CEP290, CP110 and Rab8a form an inactive centrosomal complex. Ciliary assembly is accomplished by action of the intraflagellar transport (IFT) proteins, which relies on the microtubule motor proteins kinesin 2 for anterograde transport of vesicles from the trans-Golgi

network (TGN), and dynein for retrograde transport from the ciliary tip. Anterograde transport builds the ciliary axoneme, which extends triplet microtubules directly from the mother centriole. Ciliary resorption (which invariably occurs at G2/M, and in some cases occurs in cells in G1/S) involves decreased IFT-dependent transport of cargo into the cilia, activation of retrograde transport of IFT complexes, and katanin-dependent separation of the basal body via severing of axonemal microtubules. With the maturation and separation of the two centrosomes at the G2/M transition, the cilium is disassembled and the centrioles function in mitotic spindle formation.

Figure 4. Ciliary disassembly curve

Ciliated cells were induced by serum stimulation after 48 h of serum starvation in hTERT-RPE1 and HK2 cell lines. An average of 150 cells were counted in each of three experiments, error bars show the standard deviation.