The ubiquitin conjugation system is involved in the disassembly of cilia and flagella

Kaiyao Huang, Dennis R. Diener, and Joel L. Rosenbaum

Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520

The disassembly of cilia and flagella is linked to the cell cycle and environmental cues. We have found that ubiquitination of flagellar proteins is an integral part of flagellar disassembly. Free ubiquitin and the ubiquitin-conjugating enzyme CrUbc13 are detected in flagella, and several proteins are ubiquitinated in isolated flagella when exogenous ubiquitin and adenosine triphosphatase are added, suggesting that the ubiquitin conjugation system operates in flagella. Levels of ubiquitinated flagellar proteins increase during flagellar resorption, especially in intraflagellar transport (IFT) mutants, suggesting that disassembly products are labeled with ubiquitin and transported to the cell body by IFT. Substrates of the ubiquitin conjugation system include α-tubulin (but not β-tubulin), a dynein subunit (IC2), two signaling proteins involved in the mating process, cyclic guanosine monophosphate–dependent kinase, and the cation channel polycystic kidney disease 2. Ubiquitination of flagellar proteins is enhanced early in mating, suggesting that ubiquitination also plays an active role in regulating signaling pathways in flagella.

Introduction

Ubiquitination plays an essential regulatory role in many cellular processes in eukaryotes and is a multistep reaction catalyzed by at least three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). This set of enzymes can attach mono-, multi-, or polyubiquitin chains to substrate proteins (Hochstrasser, 1996; Hershko, 2005). Classically, polyubiquitination is a signal that directs substrates to the proteasome for degradation, and through this pathway, it is involved in cell cycle control (Hershko, 2005), apoptosis (Silva et al., 2007), major histocompatibility complex class I antigen presentation (Rock et al., 2002), intracellular signaling (Robinson and Ardley, 2004), and quality control of the ER (Hampton, 2002; Ye, 2005). Mono- and multiubiquitination have nondegradative cellular functions, including mediation of transcriptional activation (Dhananjayan et al., 2005), ubiquitination of histones and gene silencing (Weake and Workman, 2008), endocytosis and endosomal sorting (Mukhopadhyay and Riezman, 2007), and DNA repair (Weake and Workman, 2008). Given the diverse cellular roles of ubiquitination, we reasoned that it may also be active in the biology of cilia and flagella, particularly during their assembly and disassembly.

Cilia and flagella are hairlike organelles projecting from the surface of eukaryotic cells, where they have important motile and sensory functions. The core of these organelles, called the axoneme, is composed of a cylinder of nine outer doublet microtubules and appended proteins (Rosenbaum and Witman, 2002; Satir and Christensen, 2007). The axoneme is sheathed by the ciliary membrane, which is continuous with the plasma membrane, but is enriched with specific lipids and membrane proteins (Iomini et al., 2006), including receptors, ion channels, and pumps (Pazour et al., 2005). Because cilia project into the extracellular milieu, they are ideal for receiving signals and relaying them to the cell body. In keeping with this role, components of multiple signaling pathways reside on the ciliary membrane, e.g., platelet-derived growth factor receptor α, polycystin-1 and -2, members of the hedgehog and Wnt pathways in mammalian cells, and molecules active in the mating signaling pathway in *Chlamydomonas reinhardtii* (Pazour and Rosenbaum, 2002; Wang et al., 2006; Christensen et al., 2007; Huang et al., 2007; Yoder, 2007; Corbit et al., 2008; Spassky et al., 2008). The role of cilia in signaling and their widespread distribution in animals (see http://www.bowserlab.org/primarycilia/ciliumpage2.htm)

Correspondence to Joel L. Rosenbaum: joel.rosenbaum@yale.edu

Abbreviations used in this paper: IBMX, isobutylmethylxanthine; IFT, intraflagellar transport; NaPPi, sodium pyrophosphate.

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explains why defects in cilia result in such seemingly disparate human diseases as polycystic kidney and liver diseases, retinal degeneration, polydactyly, left–right patterning defects, airway diseases, hydrocephalus, and infertility (Pazour and Rosenbaum, 2002; Satir and Christensen, 2007; Yoder, 2007; Zariwala et al., 2007; Adams et al., 2008).

The length of flagella and cilia is tightly regulated, i.e., cilia of a given cell, be it the biflagellate alga *C. reinhardtii*, a ciliate like *Tetrahymena thermophila*, or a tracheal epithelial cell, tend to be of uniform length; yet, flagellar length is not static (Lefebvre, 2009). According to the balance point model, flagellar length results from the balance of the assembly and disassembly of axonemal microtubules. These microtubules reach a steady-state length in which precursors are continually added to the tip at the same rate that turnover products are removed (Marshall and Rosenbaum, 2001). We will refer to the disassembly that occurs during steady-state as "turnover" to differentiate it from the "breakdown" of the flagellar axoneme that occurs during disassembly/shortening/resorption as described in the following paragraph. A specialized motility system called intraflagellar transport (IFT) moves the precursors to the tip for assembly and removes the turnover products. The IFT machinery includes the anterograde motor (heterotrimeric and homodimeric kinesin-2), the retrograde motor (cytoplasmic dynein-2), and IFT particles (Kozminski et al., 1993; Pedersen et al., 2008; Scholey, 2008). IFT particles, first identified in *C. reinhardtii* (Kozminski et al., 1993), comprise complexes A and B (Cole et al., 1998). Complex B may be specialized in transporting axonemal precursors to the tip for assembly, whereas complex A may play a greater role in transporting turnover and breakdown products away from the tip to the cell body (Qin et al., 2004; Pedersen et al., 2006, 2008; Lee et al., 2008; Tsao and Gorovsky, 2008).

Flagellar length can change according to developmental and environmental cues. For example, *C. reinhardtii* flagella extend an additional 2 µm at the beginning of the mating process (Goodenough and Jurivich, 1978) and also lengthen beyond the normal wild-type length in response to lithium (Nakamura et al., 1987). Trypsin and theophylline cause the cilia of sea urchin blastula to grow to 1.5 times their normal length (Riederer-Henderson and Rosenbaum, 1979; Stephens, 1994). Likewise, in MDCK cells, cAMP causes primary cilia to elongate (Low et al., 1998). At the other end of the spectrum, flagellar resorption (also called shortening or disassembly) can be induced by several chemical agents in *C. reinhardtii* (Lefebvre and Rosenbaum, 1986; Wilson et al., 2008) and occurs normally in synchrony with the cell cycle; flagella resorb before the cell divides and regenerate after cell division is complete (Quarmby and Parker, 2005; Plotnikova et al., 2008). In mammalian cells, cilia must be removed before a cell can progress through mitosis perhaps because the ciliary basal body must be freed from the cilium to become the centriole that helps form the mitotic spindle (Quarmby and Parker, 2005; Plotnikova et al., 2008). Thus, ciliary length and even existence are sensitive to external conditions as well as to the cell cycle.

Compared with the recent bloom of research on flagellar assembly, the study of flagellar shortening has just begun, and players in this process have only recently been identified. CrKinesin-13, which belongs to a class of kinesins that depolymerize microtubules, enters the flagella during flagellar shortening in *C. reinhardtii* (Piao et al., 2009). During resorption, α-tubulin becomes deacetylated (L'Hernault and Rosenbaum, 1985a), and the amount of a methionine synthase increases in the flagella (Schneider et al., 2008). Furthermore, *C. reinhardtii* aurora-like kinase, a member of the aurora family of kinases involved in mitosis, is phosphorylated and activated during flagellar shortening and deflagellation, a severing of the flagella from the basal body distinct from resorption (Pan et al., 2004). In mammalian cells, aurora A kinase links cell cycle progression to ciliary disassembly, at least in part through stimulation of the tubulin deacetylase HDAC6, which accelerates the requisite loss of the cilium before cell division (Pugacheva et al., 2007). Ciliary resorption is required for cell cycle progression, and IFT plays a role in this process as more empty IFT particles enter the cilium to remove axonemal breakdown products (Pan and Snell, 2005); however, the mechanism of disassembly at the tip and how breakdown products are recognized by IFT are unclear.

While studying the assembly and disassembly of flagella in the green algae *C. reinhardtii*, we found that the ubiquitin conjugation system functions in flagella. Axonemal proteins, including α-tubulin, and a membrane protein, PKD2 (polycystic kidney disease 2), are polyubiquitinated during flagellar resorption. Ubiquitinated proteins accumulate dramatically in flagella of mutants defective in retrograde IFT. These results suggest that ubiquitination tags flagellar breakdown products as cargo for transport by retrograde IFT to the cell body. Because ubiquitination is involved in the continuous turnover of the flagellar tip, it is intimately related to control of ciliary and flagellar length; furthermore, the role of ubiquitination in flagellar shortening may provide another link between ubiquitination and cell cycle regulation.

Results

Free ubiquitin and ubiquitinated proteins are present in flagella of *C. reinhardtii*

Ubiquitin was identified in the flagellar proteome of *C. reinhardtii* (Ensembl accession no. C_330025; Pazour et al., 2005), although because of the way the proteome was generated, the ubiquitin peptides found could have been derived from free ubiquitin and/or ubiquitinated proteins in the flagella. To determine which form of ubiquitin is in flagella, we probed immunoblots of isolated flagella for ubiquitin (Fig. 1 A). Monomeric, 7-kD ubiquitin was found in the flagella along with two ubiquitinated proteins of ∼100 and 60 kD. When flagella were extracted with the nonionic detergent NP-40 to yield a soluble membrane/matrix fraction and an axonemal fraction, the free ubiquitin was soluble in the membrane/matrix fraction. However, more than half of the larger ubiquitinated proteins remained bound to the axoneme in the presence of detergent. To confirm these results, we expressed an HA::ubiquitin fusion protein in *C. reinhardtii* and repeated the aforementioned analysis probing immunoblots with an anti-HA antibody. Again, free ubiquitin was present in the membrane/matrix fraction (Fig. 1 B). The more sensitive anti-HA antibody detected additional ubiquitinated proteins that partitioned into both the soluble and insoluble fractions.

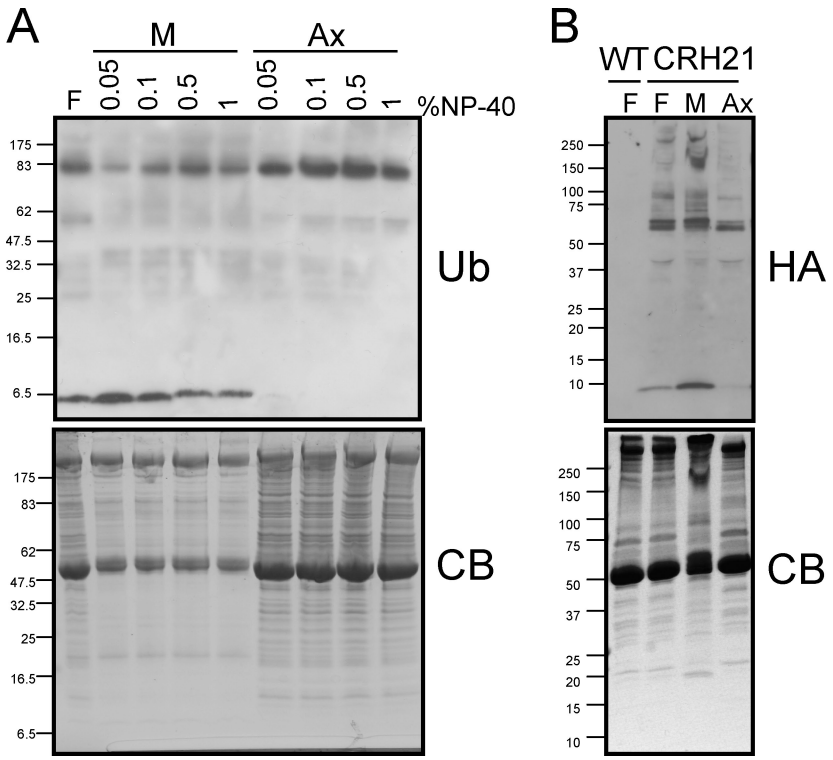

Figure 1. **Free ubiquitin and ubiquitinated proteins are present in the flagella of *C. reinhardtii*.** (A) Isolated flagella (F) were extracted with the indicated concentration of NP-40, and the soluble membrane/matrix (M) and insoluble axonemal (Ax) fractions were analyzed on immunoblots probed with an antibody to ubiquitin (Ub; P4G7; Covance). A Coomassie blue–stained gel (CB) of the loaded proteins is shown at the bottom. Monomeric ubiquitin is 7 kD. (B) Immunoblot analysis shows that HA::ubiquitin is present in the flagella and membrane/matrix of the strain CRH21, which harbors the HA::ubiquitin transgene. No signal of HA::ubiquitin was present in wild-type (WT) flagella. A Coomassie blue–stained gel of the loaded proteins is shown at the bottom. Mobilities of prestained molecular mass standards are indicated in kilodaltons.

These results demonstrate that free ubiquitin and ubiquitinated proteins are present in the flagella of *C. reinhardtii*.

A functional ubiquitin conjugation system is present in flagella

In addition to ubiquitin, the ubiquitin-activating enzyme (E1; Ensembl accession no. C_730071), three ubiquitin-conjugating enzymes (E2; Ensembl accession nos. C_610015, C_410080, and C_7006), and three ubiquitin ligases (E3; Ensembl accession nos. C_720049, C_70050, and C_610015) are also found in the flagellar proteome. Proteomic analysis identified 11 peptides of the E1 homologue, at least two peptides of each E2 homologue, and one peptide of each E3 ligase homologue in the membrane/matrix fraction. The alignments of ubiquitin, E1, and E2 from *C. reinhardtii*, yeast, and human are shown in Figs. S1 and S2. Both ubiquitin and E1 are conserved in these three organisms. E2 (Ensembl accession no. C_360048) and E2 (Ensembl accession no. C_410080) show high homology to yeast E2 Mms2 (UBEV1 in human) and Ubc13 (UBE2N in human), respectively. Mms2 and Ubc13 form a heterodimer that participates in the DNA damage tolerance pathway (Hofmann and Pickart, 1999). The other flagellar E2 (Ensembl accession no. C_70066) shows homology to Ubc7 in yeast (UBE2G1 in human), which is involved in ER-associated degradation (Biederer et al., 1997).

To verify the presence of ubiquitinating enzymes in the flagella, one of the E2-conjugating enzymes, CrUbc13, was fused at its C terminus to GFP and was expressed in wild-type *C. reinhardtii* cells. Flagella of the transgenic strain were isolated and separated into membrane/matrix and axonemal fractions. CrUbc13::GFP was present in flagella, and most of the fusion protein was in the membrane/matrix fraction (Fig. 2 A). Punctate CrUbc13::GFP fluorescence was visualized in the flagella (Fig. 2, B and C), confirming that E2 is present in flagella in vivo and is not a cytoplasmic contaminant released during flagellar isolation.

We performed an in vitro ubiquitination experiment to verify that the ubiquitin conjugation system was active in flagella. Isolated flagella were freeze thawed to open the flagellar membrane, and exogenous HA::ubiquitin and ATP were added. After a 5-min incubation at 30°C, at least six proteins were conjugated with HA::ubiquitin (Fig. 3 A, lane V). Many HA::ubiquitin–tagged proteins appeared after a 30-min incubation (Fig. 3 A, lane VIII). These proteins were tagged with HA::ubiquitin to a lesser extent even without adding ATP, indicating that there was enough ATP in the isolated flagella to drive the reaction (Fig. 3 A, lane VI). When ATP was depleted by adding hexokinase and D-glucose, the amount of ubiquitinated proteins decreased (Fig. 3 A, compare lane VII with lane VIII). Because ubiquitination can target proteins to the proteasome for degradation, we checked for evidence of the proteasome in the flagellar proteome; however, not a single peptide of any subunit of the proteasome was found in the flagellar proteome. This observation indicates that the proteins that are ubiquitinated in the flagella are not degraded in situ. They are most likely transported to the cell body either for degradation or reutilization.

Ubiquitinated proteins increase in flagella during flagellar resorption

Reasoning that ubiquitination may be involved in flagellar growth or disassembly, we assayed the extent of flagellar protein ubiquitination in vivo during flagellar regeneration and resorption. When exposed to a brief pH shock, *C. reinhardtii* cells excise their flagella, and new flagella are regenerated in ~1 h once the normal pH is restored (Rosenbaum and Child, 1967). No change in the pattern of ubiquitinated proteins was detected in regenerating flagella,

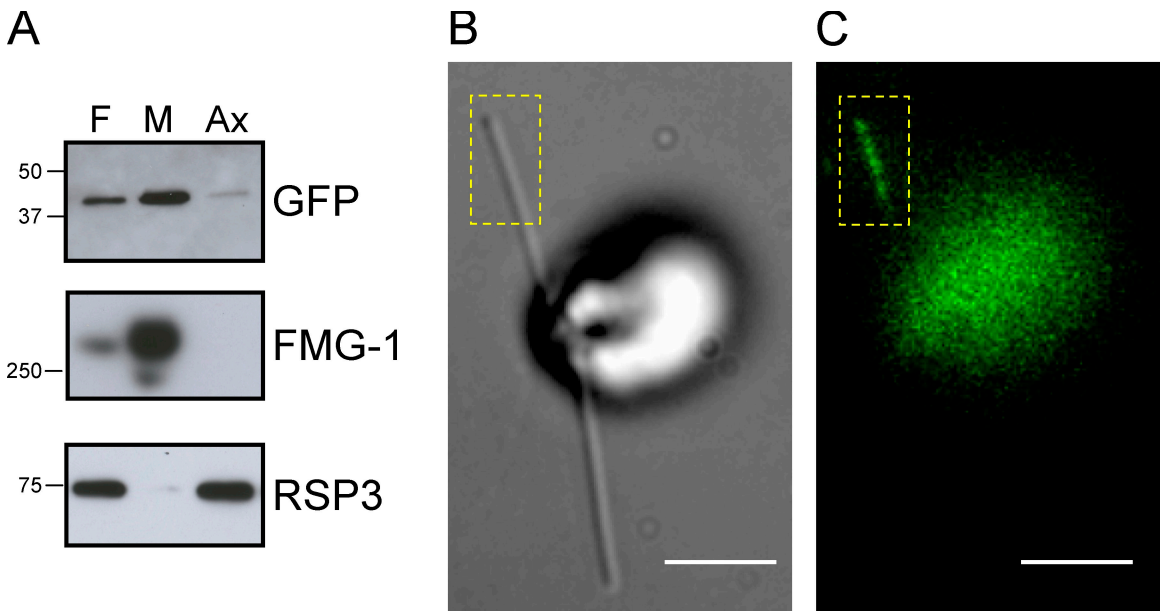

Figure 2. **CrUbc13 is present in the flagella of *C. reinhardtii*.** (A) Isolated flagella (F), membrane/matrix (M), and axonemal (Ax) fractions from cells expressing CrUbc13::GFP were analyzed on immunoblots probed with antibodies to GFP, FMG-1 (the major membrane glycoprotein in the flagella), and RSP3. FMG-1 and RSP3 were used to monitor the purity of the membrane/matrix and axonemal fractions, respectively. Molecular masses are given in kilodaltons. (B) The box in this differential interference contrast picture of a cell that expresses CrUbc13::GFP delineates the region illuminated with a 488-nm laser in C. (C) Fluorescence from the illuminated area (box) demonstrates that CrUbc13::GFP is present in the flagellum. Although only the distal two thirds of the flagella were irradiated, there is some spillover of the light, causing the cell body region to emit fluorescence. Bars, 5 µm.

although the amount of ubiquitination was reduced (Fig. 4 A). Conversely, in the presence of high Na^+/no-Ca^{2+} medium, sodium pyrophosphate (NaPPi), or isobutylmethylxanthine (IBMX), the flagella of *C. reinhardtii* can be induced to resorb over the course of 1–1.5 h (Lefebvre et al., 1978, 1980). As the flagella resorbed in high Na^+/no-Ca^{2+} medium, there was an increase in the amount of ubiquitinated proteins in the flagella (Fig. 4 B). The same was true when flagellar shortening was induced with NaPPi or IBMX, as shown more dramatically in the strain expressing HA::ubiquitin and probed with the HA antibody (Fig. S3). In addition to chemically induced flagellar shortening, we also monitored ubiquitination of flagellar proteins during naturally occurring flagellar resorption. During mating of *C. reinhardtii*, plus and minus gametes fuse to form quadriflagellate cells. Between 1 and 2 h after fusion, the four flagella of the quadriflagellates begin to resorb, becoming two-thirds of their original length after ∼3 h. During this time, high molecular mass–ubiquitinated proteins accumulated in the flagella (Fig. 4 C). In summary, these data show that ubiquitination is an integral part of flagellar shortening whether it is induced chemically or occurs naturally as part of mating.

We also measured the ubiquitin conjugation activity of flagella isolated during flagellar regeneration or shortening. As assayed in vitro, the ubiquitin conjugation activity increased during flagellar resorption (Fig. 3 B) and decreased during flagellar elongation (Fig. 3 C). Therefore, the changes in the level of ubiquitinated proteins seen in vivo during flagellar resorption and regeneration reflect parallel changes in the ubiquitination system in the flagella. In keeping with this observation, in cells expressing CrUbc13::GFP, the amount of CrUbc13::GFP increased during flagellar resorption; surprisingly, CrUbc13 also increased in regenerating flagella (Fig. S5 A).

Ubiquitinated proteins increase dramatically in flagella when IFT is blocked

Assembly and maintenance of almost all eukaryotic cilia and flagella are dependent on IFT. For example, when IFT is blocked in temperature-sensitive mutant strains of *C. reinhardtii* in which the anterograde IFT kinesin motor is affected, flagella cannot assemble, and existing flagella resorb. Fig. 5 A shows immunoblots of flagella isolated from such mutants as they were undergoing flagellar resorption at the nonpermissive temperature of 32°C. In two mutants, *fla10* and *fla8*, the resorbing flagella contained an accumulation of ubiquitinated proteins (Fig. 5 A), most of which formed a smear that extended from two distinct bands ∼40 kD to the top of the blot. Most of the ubiquitinated proteins were present in the membrane/matrix fraction (Fig. 5 C), with only a few high molecular mass proteins associated with the axoneme. We also expressed HA::ubiquitin in the *fla10* background to examine the effects of blocking IFT on ubiquitination of flagellar proteins. 1 h after moving this strain to the restrictive temperature, extensive ubiquitination of flagellar proteins was detected by both ubiquitin and HA antibodies (Fig. 5 B). In addition, a third anterograde motor mutant, *fla3*, was examined. Even at the permissive temperature, the flagella of *fla3* had only 10% as much IFT protein as those of wild type, *fla10*, or *fla8* (Fig. 5 A) and were replete with ubiquitinated proteins (Fig. 5 A). In spite of the increase of ubiquitinated proteins, there was no change in the amount of CrUbc13::GFP in the flagella of *fla10* cells at the restrictive temperature (Fig. S5 B). These results further implicate ubiquitination of flagellar proteins as having a role in flagellar shortening and suggest that IFT is required to remove the ubiquitinated proteins from the flagella.

To evaluate this model, we also examined ubiquitinated proteins in flagella of the IFT retrograde mutants *fla16*, *d1bic*,

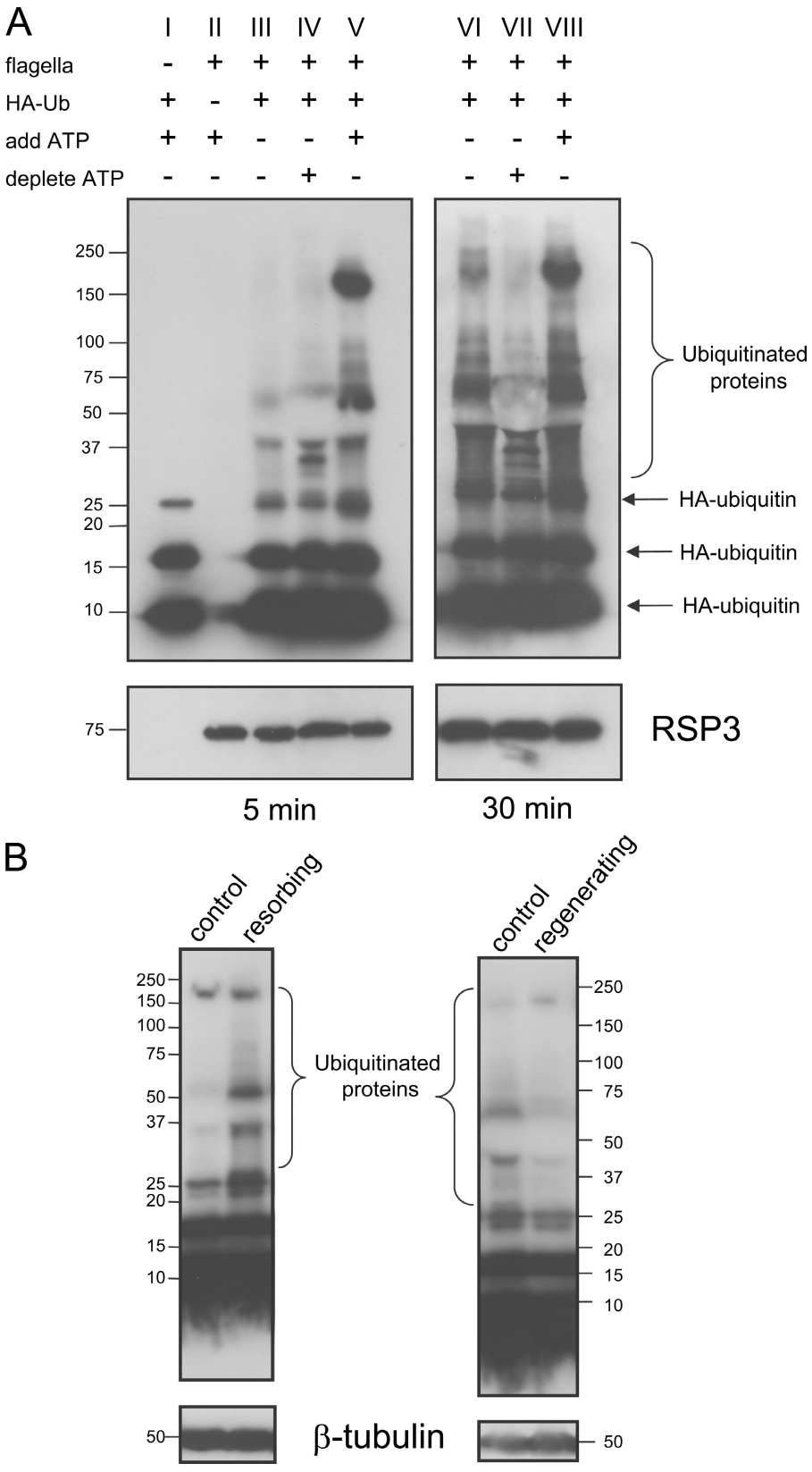

Figure 3. **The ubiquitin conjugation system is present in flagella, and its activity increases during flagellar shortening.** (A) 62.5 µg flagella isolated from wild-type cells was used for in vitro ubiquitination with or without added ATP or HA::ubiquitin (HA-Ub) and incubated for 5 or 30 min at 30°C. Flagellar proteins were analyzed on an immunoblot probed with an antibody to HA. Monomeric HA::ubiquitin is ~10 kD and formed dimers and trimers. The molecular mass of ubiquitinated proteins ranged from 27 to 200 kD. (B) The in vitro ubiquitination assay was performed on control, resorbing, and regenerating flagella and showed an increase in activity in resorbing flagella and a decrease in regenerating flagella. The reactions were incubated for 1 h at 22°C. The immunoblot was probed with HA antibody. Tubulin and RSP3 were used as loading controls. Molecular masses are given in kilodaltons.

and *dhc1b*^{ts}. *fla16* is a temperature-sensitive mutant affecting retrograde IFT (Iomini et al., 2001); *d1bic* encodes a mutant dynein light intermediate chain, D1bLIC, that is required for retrograde IFT (Hou et al., 2004); and *dhc1b* is a temperature-sensitive mutant of the heavy chain of cytoplasmic dynein, the retrograde motor of IFT (Pazour, G.J., B.L. Dickert, and G. Witman. 1999. American Society for Cell Biology meeting. Abstr. 369a). Consistent with the proposed model, even at the permissive temperature,

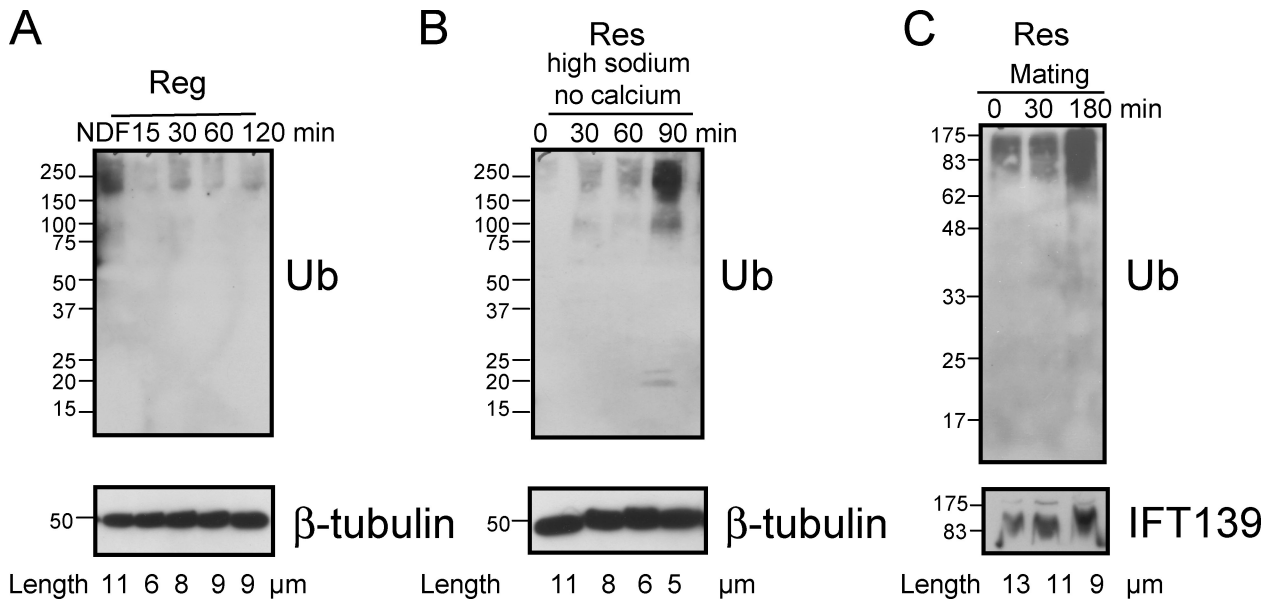

Figure 4. **Ubiquitinated proteins increase during flagellar resorption.** (A–C) Flagella were isolated at the indicated times (NDF, not deflagellated) during regeneration (Reg; A) or resorption (Res) induced by high Na^+/no-Ca^{2+} medium (B) or mating (C). Immunoblots were probed with ubiquitin (Ub) antibody. β-Tubulin or IFT139 was used as a loading control. The mean length of flagella is shown beneath each lane. Molecular masses are given in kilodaltons.

flagella of the three retrograde mutants contained more ubiquitinated proteins than wild type (Fig. 6 A). The amount of ubiquitinated proteins in these stable flagella, in which retrograde IFT was compromised, was comparable with the level in flagella induced to shorten by blocking IFT and was >10 times the amount in flagella induced to shorten while IFT functioned normally (Fig. 6 B). The molecular masses of the ubiquitinated proteins ranged from 30 to >250 kD, and although these flagella were not actively being resorbed, the pattern of ubiquitinated proteins was similar to that found during flagellar resorption of *fla10* and *fla8* at the restrictive temperature. These data corroborate the role of retrograde IFT in removing ubiquitinated proteins from flagella and indicate that ubiquitination is continuous even in flagella that are not resorbing.

Substrates of the ubiquitin conjugation system in flagella

To identify substrates of the ubiquitin conjugation system in the flagella, we probed samples of resorbing flagella of *fla10*, *fla8*, and *fla3* cells with various antibodies to look for the appearance of multiple bands, representing ubiquitinated forms of the corresponding protein. Resorbing flagella of IFT mutants were used because they have a greater accumulation of ubiquitinated proteins in the flagella (Fig. 5 A), which made the ubiquitinated forms of proteins easier to detect. Immunoblots probed with anti–α-tubulin antibody revealed a high molecular mass form of α-tubulin present after a 1-h incubation at the restrictive temperature (Fig. 7 A). In the flagella of *fla3*, a ladder of α-tubulin was seen (Fig. 7 A). The size of the α-tubulin band (>200 kD) suggested that >20 ubiquitin residues were added to the α-tubulin. Because α-tubulin only has 17 lysine residues, a small number of these residues must be polyubiquitinated.

α-Tubulin in the axonemal microtubules is acetylated at lysine 40 (L'Hernault and Rosenbaum, 1983, 1985b), and removal of this acetyl group may be involved in the disassembly of the primary cilium (Pugacheva et al., 2007). Because both acetylation and ubiquitination occur on lysine residues, it was of interest to see whether any of the ubiquitinated tubulin was also acetylated. Fig. 7 B shows that the pattern of ubiquitinated, acetylated α-tubulin is very similar to the pattern of total α-tubulin. This observation indicates that residues other than lysine 40 can be ubiquitinated. Tubulin ubiquitination was limited to α-tubulin; no evidence was found for ubiquitination of β-tubulin (Fig. 7 C). Using the same strategy, we found that IC2, an intermediate chain of outer arm dynein, was also ubiquitinated during resorption (Fig. 7 D); however, a radial spoke protein, RSP3, did not show any sign of a shift in molecular mass (Fig. 5 A).

To confirm the ubiquitination of α-tubulin during flagellar resorption, we used an anti–α-tubulin antibody to immunoprecipitate protein from an extract of flagella from *fla10* cells incubated at the restrictive temperature for 1 h. The immunoprecipitate was analyzed on immunoblots probed for α-tubulin and ubiquitin. The α-tubulin antibody pulled down nonubiquitinated α-tubulin as well as the higher molecular α-tubulin forms (Fig. 7 E). These high molecular proteins were also recognized by antiubiquitin antibody (Fig. 7 E), confirming that this high molecular mass protein form of α-tubulin is ubiquitinated.

Interestingly, the cation channel CrPKD2 and CrPKG (cyclic GMP–dependent protein kinase) were also ubiquitinated in the flagella (Fig. 8, A and B). Both of these proteins are involved in a cAMP-dependent signal transduction pathway initiated in flagella at the onset of mating (Wang et al., 2006; Huang et al., 2007). In addition to these signaling proteins, many flagellar proteins were ubiquitinated within 5 min of mixing plus and minus gametes (Fig. 8 C). Likewise, the amount of the ubiquitin-conjugating enzyme CrUbc13 also rapidly increased in the flagella after mating (Fig. 8 D). At this time, the flagella had not begun to shorten, but flagella of mating type + and − cells were interacting,

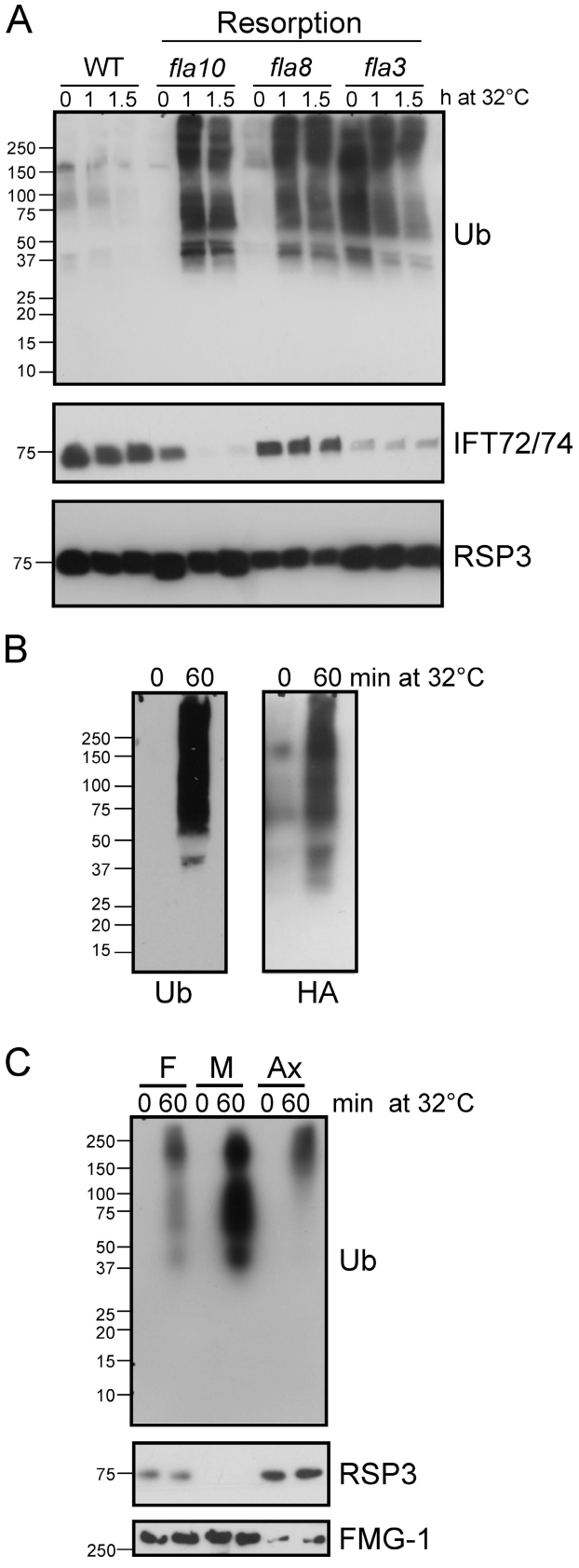

Figure 5. **Ubiquitinated flagellar proteins increase dramatically during flagellar resorption when IFT is blocked.** (A) IFT temperature-sensitive mutants *fla10*, *fla8*, and *fla3* were shifted to 32°C for 1 and 1.5 h, and immunoblots of the isolated, partially resorbed flagella were probed for ubiquitin (Ub) and IFT72/74. RSP3 was used as a loading control. WT, wild type. (B) HA::ubiquitin was expressed in *fla10* cells, and after the cells were incubated at 32°C for 60 min, resorbing flagella were isolated. Immunoblots of flagellar proteins were probed for ubiquitin and HA. (C) Isolated flagella (F), membrane/matrix (M), and axonemal fractions (Ax) of *fla10* cells after shifting to 32°C for 1 h were analyzed on immunoblots probed with antibodies to ubiquitin, RSP3, and FMG-1. Most of the ubiquitinated proteins were in the membrane/matrix fraction, although some were retained by the axoneme. Molecular masses are given in kilodaltons.

thereby initiating signals that lead to cell fusion and ultimately flagellar shortening. These data suggest that the ubiquitin conjugation system is not only involved in the dynamics of flagellar length but also plays a role in regulating signal pathways in flagella, in this case, the cAMP-dependent signaling pathway characteristic of mating (Snell and Goodenough, 2009).

Discussion

Ubiquitin and ubiquitin-conjugating system in flagella and cilia

In this study, we demonstrate that free ubiquitin and the ubiquitin-conjugating system, including E1, E2, and E3, are present in the flagella of *C. reinhardtii*. This system apparently exists in other motile and primary cilia because ubiquitin, E1, and E2 have also been identified in the ciliome of *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens* (Fig. S4; Inglis et al., 2006). In addition, the ubiquitin conjugation system and ubiquitin-dependent proteolysis have been shown to be involved in light signal transduction in the outer segment of the photoreceptor cell, which is a highly modified cilium (Obin et al., 2002). Furthermore, one E3 ligase, von Hippel-Lindau protein, controls ciliogenesis in kidney cells and is present in renal cilia (Lutz and Burk, 2006; Schermer et al., 2006). These observations suggest that the function of ubiquitination may be conserved in all cilia and flagella. Because there are no proteasome subunits in the flagella of *C. reinhardtii*, the function of the ubiquitin conjugation system may be different from the classical ubiquitin conjugation–proteasome pathway. More likely, ubiquitination functions in protein targeting and signal transduction in cilia and flagella.

Working model of the ubiquitin conjugation system in flagellar disassembly

The findings that the activity of the ubiquitin conjugation system and the level of ubiquitinated proteins increase in flagella that are shortening suggest a model for the function of protein ubiquitination in flagellar disassembly. Assembly and disassembly of the axoneme occur continuously at the tip of flagella (Marshall and Rosenbaum, 2001), so while IFT delivers axonemal precursors to the tip for assembly, other axonemal components disassemble. The transport system (IFT) must distinguish between the incoming cargo it releases and outgoing cargo being picked up. We propose that the ubiquitin conjugation system labels the turnover products with ubiquitin, thereby targeting them for transport to the cell body by retrograde IFT. Because flagellar proteins are constantly turning over, there will be some ubiquitinated proteins in flagella of stable length, but if retrograde IFT

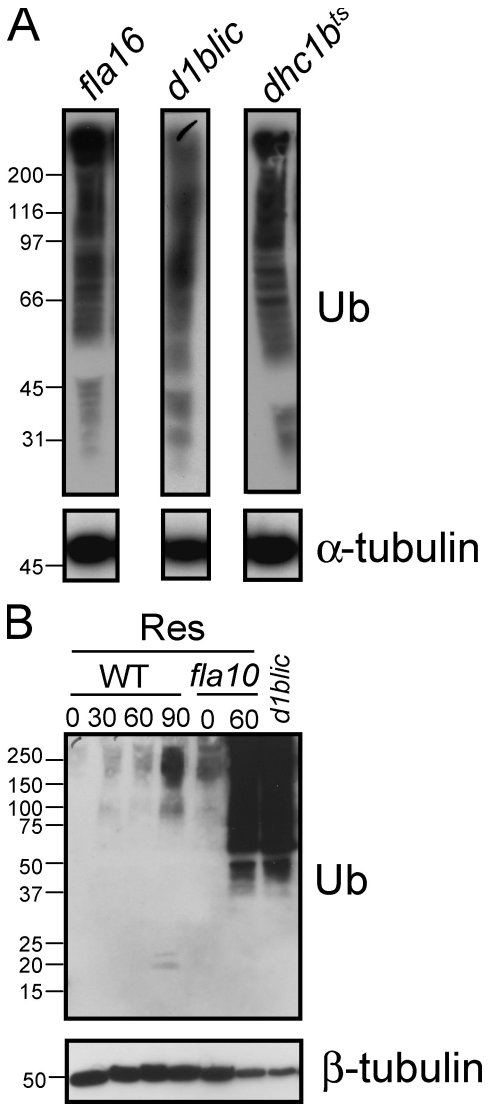

Figure 6. **Ubiquitinated proteins increase dramatically in the flagella of retrograde IFT mutants.** (A) Flagella of three retrograde IFT mutants, *fla16*, *d1blic*, and *dhc1b*^{ts}, were isolated at the permissive temperature and analyzed on immunoblots probed for ubiquitin (Ub). α-Tubulin was used as a loading control. (B) The amounts of ubiquitinated proteins in flagella were compared directly on a single immunoblot. The samples were flagella induced to resorb (Res) in wild-type (WT) cells with high Na^+/no-Ca^{2+} medium for the indicated times (minutes), flagella induced to resorb in *fla10* by incubation at 32°C for the indicated times, and flagella of the retrograde IFT mutant *d1blic* isolated at the permissive temperature. Molecular masses are given in kilodaltons.

is defective, ubiquitinated proteins will accumulate in the flagella as was observed in *fla16*, *dhc1b*^{ts}, and *d1blic* (Fig. 6 A). When flagella undergo shortening, breakdown products are produced as the axoneme disassembles; these are ubiquitinated, so more ubiquitinated proteins are found in resorbing flagella. Because retrograde IFT efficiently transports these ubiquitinated proteins out of the flagella, only a modest build up of ubiquitinated proteins occurs during shortening; however, when IFT is blocked during flagellar shortening, the ubiquitinated proteins cannot be transported to the cell body and they accumulate dramatically in flagella, as was seen in the anterograde IFT motor mutants (Fig. 5 A). In these anterograde motor mutants, both anterograde and retrograde IFT are slowed at the restrictive temperature because anterograde IFT is needed to bring IFT particles and the retrograde motors to the flagellar tip.

In addition to the known role of ubiquitination as a director of protein trafficking, ubiquitination might also play a more active, specialized role in the disassembly of flagella. Resorption of flagella can be brought about by a shift in the balance of microtubule assembly and disassembly (Marshall and Rosenbaum, 2001). Disassembly could be driven by ubiquitination of flagellar components in the polymerized state, thereby altering their conformation so as to favor depolymerization and inhibiting the reassembly of disassociated subunits. Supporting this mechanism, a fraction of the ubiquitinated flagellar proteins remained associated with the axoneme (Fig. 5 C), indicating that at least some proteins are ubiquitinated while still assembled in the axoneme. Furthermore, acetylated α-tubulin was a substrate of ubiquitination, and this modified tubulin is generally a component of microtubules rather than soluble tubulin dimers (L'Hernault and Rosenbaum, 1983).

Substrates of ubiquitination in flagella

Perhaps the most obvious way for ubiquitination to affect assembly/disassembly kinetics of flagellar microtubules would be to ubiquitinate tubulin and thereby influence its ability to polymerize. Indeed, α-tubulin was found to be polyubiquitinated in shortening flagella. Interestingly, β-tubulin was not ubiquitinated, demonstrating that ubiquitination, like acetylation and detyrosination, is specific to the α-tubulin subunit. Ubiquitination of only the α-tubulin subunit would be sufficient to target the tubulin dimer to retrograde IFT or to alter the ability of the tubulin dimer to assemble. Although the specific lysine residue that is ubiquitinated in α-tubulin was not determined, the fact that acetylated α-tubulin was ubiquitinated demonstrates that lysine residues other than lysine 40 can be ubiquitinated.

Two other axonemal proteins, IC2 and RSP3, were tested for ubiquitination. Both proteins are subunits of larger protein complexes in flagella (Kamiya, 2002; Yang et al., 2006). The IC2 subunit of the outer dynein arm was polyubiquitinated, whereas RSP3, a subunit of the radial spoke, was not. One might anticipate that ubiquitination of only one subunit of a complex would be sufficient to target the entire complex to retrograde IFT or to inhibit its assembly onto microtubules. Thus, ubiquitination of IC2 could target the dynein arm for removal from the flagellum. Radial spokes are a complex of 23 different proteins, and perhaps a subunit other than RSP3 is ubiquitinated.

In addition to these axonemal proteins, one membrane protein, PKD2, and one soluble matrix protein, CrPKG, were tested and found to be ubiquitinated. Ubiquitination is known to be a part of internalization and recycling of membrane proteins and likely plays a similar role with proteins in the flagellar membrane. In *C. elegans*, a ubiquitin-PKD2 fusion protein is largely absent from cilia and the ciliary base where wild-type PKD2 is concentrated (Hu et al., 2007), suggesting that ubiquitin may be a tag for directing PKD2 out of cilia. This observation supports our hypothesis that in *C. reinhardtii*, ubiquitination of flagellar proteins, including the integral membrane protein PKD2 (Huang et al., 2007), targets them for transport to the cell

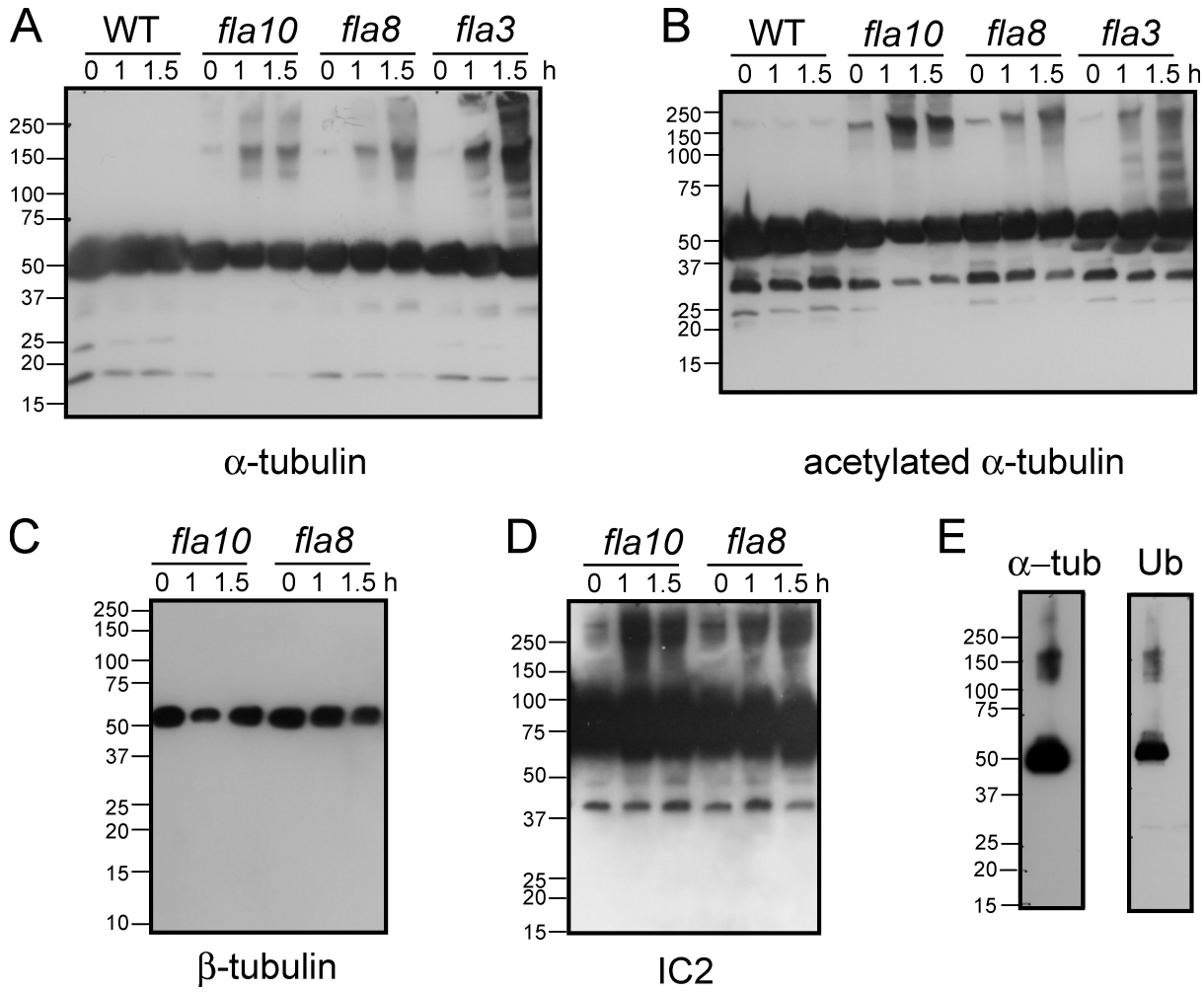

Figure 7. **α-Tubulin is a substrate of the ubiquitin conjugation system in flagella.** (A–D) Wild-type (WT), *fla10*, *fla8*, and *fla3* cells were shifted to 32°C for 1 and 1.5 h (as indicated on top of the blots), and immunoblots of flagella from these cells were probed with antibodies to α-tubulin (A), acetylated α-tubulin (B), β-tubulin (C), and IC2 (D). (E) Anti–α-tubulin antibody was used to immunoprecipitate tubulin from an extract of flagella isolated from *fla10* after shifting to 32°C for 1 h. Immunoblots of the precipitate were probed for α-tubulin (α-tub) and ubiquitin (Ub). The 50-kD bands include the heavy chain of the precipitating IgG. Molecular masses are given in kilodaltons.

body, possibly by IFT. CrPKG, a protein kinase, is involved in signaling during mating (Wang et al., 2006), and ubiquitination could possibly inactivate this kinase as well as direct it back to the cell body.

The fate of ubiquitinated proteins

Once they reach the cell body, the fate of polyubiquitinated flagellar proteins, such as α-tubulin, poses an interesting question. Misfolded and depolymerized cytoplasmic α-tubulin can be ubiquitinated by parkin (an E3 ligase), and polyubiquitinated α-tubulin goes to the proteasome for degradation (Feng, 2006). Does the polyubiquitinated flagellar α-tubulin share this fate? In the early seventies, Coyne and Rosenbaum (1970) showed that when one of the two flagella was amputated from *C. reinhardtii*, the remaining flagellum first shortened, then regenerated in consort with the newly forming flagellum. When new protein synthesis was inhibited by cycloheximide, the final length of the two regenerated flagella was dependent on the initial length of the unamputated flagellum (Coyne and Rosenbaum, 1970). Similarly, in the related biflagellate *Polytomella agilis*, the length of the flagella regenerated in the absence of protein synthesis is proportional to the length of flagella resorbed before regeneration (Brown and Rogers, 1978). These two experiments suggest that products of flagellar disassembly can be reutilized in making new flagella. In support of this conclusion, two of the flagellar E2s (Ensembl accession nos. C_360068 and C_410080) are orthologues of Mms2 and Ubc13 in yeast. Mms2 and Ubc13 comprise a heterodimeric E2 enzyme that exclusively forms K63-linked chains, which are primarily associated with trafficking and substrate regulation rather than degradation (Hofmann and Pickart, 1999; Sun and Chen, 2004). Clearly, more experiments are needed to clarify the fate of ubiquitinated flagellar tubulin once it reaches the cytoplasm.

Other functions of ubiquitin conjugation system in cilia and flagella

Cilia and flagella can serve as sensors and also as information-processing centers. PKD1 is cleaved in the primary cilium, and the C-terminal fragment goes into the nucleus to regulate gene expression (Chauvet et al., 2004; Low et al., 2006). Modification

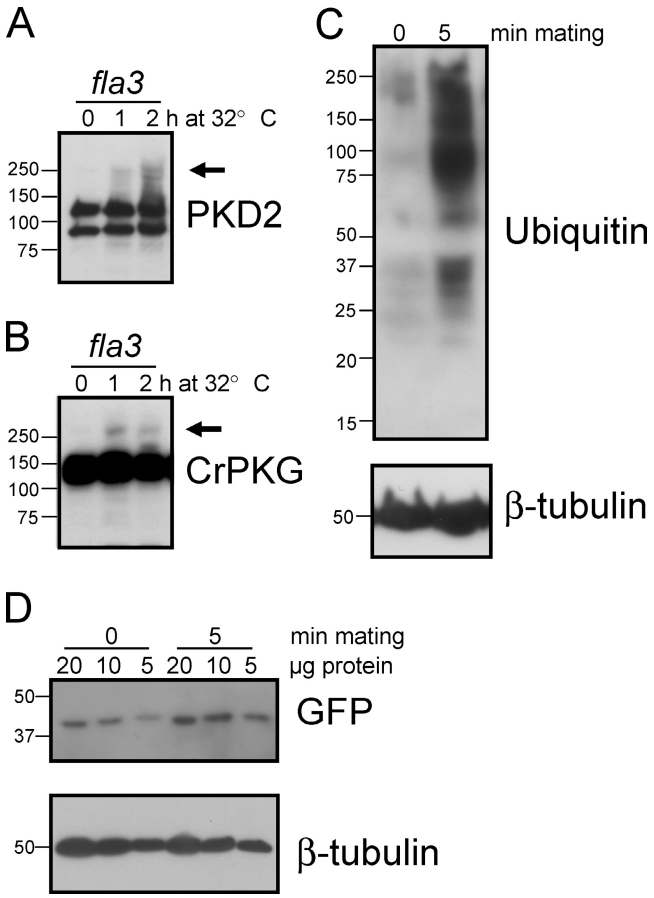

Figure 8. **The ubiquitin conjugation system is involved in a signal transduction pathway in flagella.** (A and B) *fla3* cells were shifted to 32°C for 1 and 2 h, and immunoblots of flagellar proteins were probed with antibodies against PKD2 (A) and CrPKG (B). Arrows point to putative ubiquitinated forms of PKD2 and CrPKG. (C) The flagella of plus and minus gametes before (0 min) and after mating for 5 min were isolated and probed with ubiquitin antibody. The amount of ubiquitinated proteins increased after 5 min of mating. β-Tubulin was used as a loading control. (D) Flagella were isolated from mating-type minus and plus gametes expressing CrUbc13::GFP before (0 min) and after mating for 5 min. Flagellar proteins (20, 10, and 5 μg) were probed for GFP. The amount of the CrUbc13::GFP increased quickly during mating. β-Tubulin was used as a loading control. Molecular masses are given in kilodaltons.

of the transcriptional factor Gli also occurs in cilia as part of the hedgehog signaling pathway in mammals (Haycraft et al., 2005). Ubiquitination may also regulate signaling pathways in cilia and flagella. For example, the ubiquitination of the βγ subunit of transducin is important in visual signaling in the outer segment of photoreceptor cell (Obin et al., 2002). In biflagellate *C. reinhardtii*, mating begins when the flagella of gametes adhere to one another through mating type–specific agglutinins. This initiates a signaling cascade that ultimately leads to cell fusion. Ubiquitinated flagellar proteins increase within 5 min of flagellar adhesion of *C. reinhardtii* gametes (Fig. 8 C), which is long before the flagella begin to shorten after cell fusion, suggesting that ubiquitination plays an important role in signal initiation and amplification in the flagella during the mating process. In keeping with this hypothesis, two components of the mating signaling pathway, PKD2 and CrPKG1 (Wang et al., 2006; Huang et al., 2007), are substrates of the ubiquitin conjugation system in the flagella.

We have demonstrated that the ubiquitin conjugation system is present in flagella and adds a ubiquitin tag to axonemal turnover and breakdown products, which are then removed from the flagella by IFT. Such a quality control mechanism is not only important for the disassembly of flagella, but may also be a critical component of the sensory and signal transduction functions of cilia and flagella.

Materials and methods

Cell and culture media

The *C. reinhardtii* wild-type strains CC-125 (mt+) and CC-124 (mt−) and the temperature-sensitive flagellar assembly mutants *fla10* (*fla10-1* allele, CC-1919, mt−) and *fla8* (CC-1396) were obtained from the *Chlamydomonas* Genetics Center. *fla3-1B* was provided by M.E. Porter (University of Minnesota, Twin Cities, MN), *d1blic* (dynein1b light intermediate chain mutant) and *dhc1b*^{ts} (dynein heavy chain mutant) were provided by G.B. Witman (University of Massachusetts Medical School, Worcester, MA), and *fla16* was supplied by G. Piperno (Mount Sinai School of Medicine, New York, NY). Cells were grown on solid media supplemented with 1.5% agar or in a liquid minimal medium, MI (Harris, 1989), MI-N (MI medium without nitrogen to induce gametogenesis), or Tris-acetate-phosphate media (Harris, 1989) at 18°C with a 12/12 h light/dark cycle and constant aeration.

Flagellar length measurements

fla10, *fla8*, and *fla3-1B* cells were maintained at 22 (permissive) or 32°C (nonpermissive) to induce flagellar resorption. Wild-type cells were induced to resorb their flagella using 20 mM NaPPi, 0.5 mM IBMX, or by incubating in high Na^+/no-Ca^{2+} medium (Lefebvre et al., 1978, 1980). Aliquots of cells were fixed with 1% glutaraldehyde and were attached to microscope coverslips with 0.1% poly-L-lysine (Sigma-Aldrich). Images were recorded with a microscope (Eclipse TE2000; Nikon) equipped with a Plan Apo 40× objective lens and a camera (Cascade 512B; Photometrics). The lengths of at least 50 flagella were measured for each time point using the MetaMorph software package (MDS Analytical Technologies).

Flagellar isolation and fractionation

Flagella were isolated from *C. reinhardtii* by the pH shock method as described previously (Huang et al., 2007). The flagella from 16 to 48 liters of wild-type or mutant cells were resuspended in HMDEK buffer (10 mM Hepes, pH 7.2, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EDTA, and 25 mM KCl, including the protease inhibitors 1.0 mM PMSF, 50 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, and 1 μg/ml leupeptin) for a protein concentration of 2–4 μg/μl, and NP-40 (EMD) was added to a final concentration of 0.05 to 1% vol/vol. The mixture was incubated at 4°C for 30 min with shaking. The membrane/matrix fraction was separated from the axonemes by centrifugation at 16,000 *g* for 10 min at 4°C. Protein concentration determination and immunoblot analysis were performed as described previously (Huang et al., 2004). Immunoblots were scanned, and the relative protein concentrations were determined using Image J (National Institutes of Health).

In vitro ubiquitination

The in vitro ubiquitination assay was adapted from a published protocol (Xie et al., 2007). 10× ubiquitination buffer (500 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, and 5 mM DTT) was freshly prepared. The reaction components, i.e., the same amount of flagella (resuspended in 10 mM Tris-HCl, pH 7.5), ATP (final concentration of 1 mM), and HA::ubiquitin (final concentration of 0.15 μg/μl), were added to the 10× ubiquitination buffer and diluted with water to yield a 1× concentration. A typical 100-μl reaction was incubated at 30°C for 5 or 30 min, and 5× SDS loading buffer was added to terminate the reaction. The samples were denatured at 100°C for 3 min and loaded on 5–20% gradient SDS-PAGE gels. To deplete the ATP in the flagella, 50 U hexokinase (Sigma-Aldrich) and 50 mM glucose (Sigma-Aldrich) were added to the reaction.

GFP construct and transformation with CrE2::GFP

To make the CrE2::GFP fusion, a universal vector (pHK85) was generated to fuse GFP to either the N or C terminus of a protein. pHK85 includes the PsaD promoter (Fischer and Rochaix, 2001), two tandem GFP-coding sequences separated by a flexible protein linker (GASGQGASGADIGASGQGASGA), and the selectable marker gene HSP70A-RSC2::APHVIII

(Sizova et al., 2001). There is an NdeI site between the PsaD promoter and the first GFP, a unique EcoRV site in the middle of the linker, and an EcoRI site between the second GFP and the 3′ untranslated region of the PsaD gene. For expression of an N-terminal GFP fusion protein, the second GFP can be replaced with a cDNA using the EcoRV and EcoRI sites; for expression of a C-terminal GFP fusion protein, the first GFP can be replaced with a cDNA using the NdeI and EcoRV sites.

To amplify the C_410080 cDNA, NdeI and EcoRV sites were added to the primers 5′-**CATATG**AACCCGGATCAGGCAGCG-3′ and 5′-**GATATC**-GCCCTGAGCGTGCATGGCA-3′ (added sites are in bold), respectively, AV386983 was used as the template for PCR, and the PCR product was cloned into the TOPOII vector (Invitrogen), generating a new plasmid, pHK97. pHK97 was cut with NdeI and EcoRV, and a fragment that contained the cDNA of C_410080 (~400 bp) was exchanged with the first GFP in pHK85, generating pHK99. pHK99 was linearized with KpnI and used for transformation. The methods for transformation, screening the transformants with GFP antibody, and recording GFP fluorescence in the flagella were performed as described previously by Huang et al. (2007). The cells were immobilized with 20 mM NaPPi and 0.75% low melt agarose in MI medium at 20° for imaging. Images were recorded with a microscope (Eclipse TE2000) equipped with a Plan Apo 100× 1.4 NA objective lens and a camera (Cascade 512B). An argon ion 488 laser controlled by a Mosaic System (Photonic Instruments, Inc.) was used for illumination. Data were acquired using MetaMorph software, and Photoshop (Adobe) was used to adjust image brightness and contrast and to crop images.

Nucleic acid manipulations and transformation of HA-tagged ubiquitin

The HA-tagged ubiquitin was amplified by PCR using the polyubiquitin cDNA (AV643935) as a template with the forward primer 5′-GC**CATATG**-*TACCCCTACGACGTGCCCGACTACGCCTACCCCTACGACGTGCCC-GACTACGCC*ATGCAGATTTTCGTGAAG-3′ (NdeI is in bold, and the sequence in italics encodes the double HA tag, YPYDVPDYAYPYDVPDYA) and the reverse primer 5′-GC**GAATTC**TCAGAACATGCCACCGCGCAG-GCGCAG-3′ (EcoRI is in bold). The PCR product was cloned into the TOPO II vector, generating pHK62. pHK62 was cut with NdeI and EcoRI, and the HA::ubiquitin fragment was harvested and exchanged for the IFT52::GFP fragment in pHK24 using the NdeI and EcoRI sites, generating pHK64. pHK24 had been generated previously to express a IFT52::GFP fusion protein and includes the PsaD promoter, IFT52::GFP, the PsaD 3′ untranslated region, and a selectable marker gene, HSP70-ARSC2::APHVIII. The IFT52::GFP coding sequence was flanked by NdeI and EcoRI.

The plasmid pHK64 was linearized with Kpn1 and transformed into wild-type cells (CC-125). Because ubiquitin is ~7 kD, the chlorophyll light chain interfered with the migration of the ubiquitin during SDS-PAGE, so the chlorophyll was removed from cytoplasmic extracts by TCA precipitation (Shimogawara and Muto, 1989).

To construct the *fla10* (mt−) strain expressing HA::ubiquitin, the original HA::ubiquitin transformant (mt+) was crossed with *fla10*, and the progeny were analyzed to obtain the desired phenotype.

Immunoprecipitation

The immunoprecipitation protocol was modified from Spector et al. (1997). Isolated flagella were resuspended in RIPA buffer (1% NP-40, 1% DOC, 0.1% SDS, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.4) with protease inhibitors and incubated at RT for 20 min. The supernatant (protein concentration of 2–4 mg/ml) was clarified for 10 min at 16,000 *g* twice. Typically, 1 ml supernatant was incubated with 50 µl antibodies at 4°C for 1–2 h, and 5 mg protein A beads was added. After overnight incubation with shaking, the supernatant was removed, and the beads were washed with 1 ml RIPA buffer for 30 min at 4°C. After three washes, the beads were resuspended in 50 µl of 2× reducing SDS-PAGE buffer. After incubating at RT for 10 min with shaking, the beads were boiled for 3 min to release all of the bound proteins. The elution step was repeated twice, and the combined eluates were used for SDS-PAGE. Mouse TrueBlot ULTRA (eBioscience) was used as the secondary antibody to reduce the signal of the precipitating antibodies on the immunoblots.

Online supplemental material

Fig. S1 shows the alignment of ubiquitin and three E2s from *C. reinhardtii*, yeast, and human. Fig. S2 shows the alignment of E1 from *C. reinhardtii*, yeast, and human. Fig. S3 shows that ubiquitinated proteins increased during flagellar shortening induced by 20 mM NaPPi and 0.5 mM IBMX. Fig. S4 shows that ubiquitin and the ubiquitin-conjugating enzymes (E2) are present in the proteomic database of cilia. Fig. S5 shows that the amount of CrUbc13::GFP increased during flagellar shortening and regeneration and that the amount of flagellar CrUbc13::GFP did not change when IFT was blocked in *fla10* cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200903066/DC1.

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