Quantitative analysis and modeling of katanin function in flagellar length control

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ABSTRACT Flagellar length control in Chlamydomonas reinhardtii provides a simple model system in which to investigate the general question of how cells regulate organelle size. Previous work demonstrated that Chlamydomonas cytoplasm contains a pool of flagellar precursor proteins sufficient to assemble a half-length flagellum and that assembly of full-length flagella requires synthesis of additional precursors to augment the preexisting pool. The regulatory systems that control the synthesis and regeneration of this pool are not known, although transcriptional regulation clearly plays a role. We used quantitative analysis of length distributions to identify candidate genes controlling pool regeneration and found that a mutation in the p80 regulatory subunit of katanin, encoded by the PF15 gene in Chlamydomonas, alters flagellar length by changing the kinetics of precursor pool utilization. This finding suggests a model in which flagella compete with cytoplasmic microtubules for a fixed pool of tubulin, with katanin-mediated severing allowing easier access to this pool during flagellar assembly. We tested this model using a stochastic simulation that confirms that cytoplasmic microtubules can compete with flagella for a limited tubulin pool, showing that alteration of cytoplasmic microtubule severing could be sufficient to explain the effect of the pf15 mutations on flagellar length.

INTRODUCTION Regulation of organelle size is a critical aspect of the determination of cell architecture, but the mechanisms that determine organelle size are largely unknown (Chan and Marshall, 2010; Levy and Heald, 2012). Control of organelle size involves regulation of two separate steps: the synthesis of organelle precursor molecules, and the assembly of these precursors into the final structure. Size control by limiting the quantity of organelle precursor has been clearly demonstrated for centrosomes (Decker et al., 2011) and has emerged as a potentially general scheme for organelle size determination (Goehring and Hyman, 2012). In cases in which organelle size is controlled by limiting quantities of precursors, the key question becomes what pathways exist to regulate the size of the precursor pool.

One tractable model system in which to study the interplay between precursor synthesis and assembly is the flagellum of the green alga Chlamydomonas reinhardtii. In this organism, flagella can be detached by pH shock and induced to rapidly regenerate, during which time genes encoding flagellar proteins are synchronously up-regulated (Keller et al., 1984; Lefebvre and Rosenbaum, 1986; Stolc et al., 2005). If new protein synthesis is blocked, flagella start regeneration with normal kinetics but cease growth prematurely and reach a shorter final length (Rosenbaum et al., 1969; Lefebvre et al., 1978), demonstrating that a preexisting pool exists that is sufficient to drive partial flagellar assembly but is insufficient to grow a full-length flagellum.

The existence of a limiting pool of precursor suggests that the size of this pool should play a role in determining the length of the flagella. In cells with increased numbers of flagella, the average...
flagellar length is decreased (Marshall et al., 2005), again suggesting that the flagella share a pool of precursor proteins whose size affects the steady-state length. In the most idealized limiting precursor model, in which 100% of the precursor is consumed in the assembly process, the average size should scale as 1/n, where n is the number of copies of the organelle. This is true for centrosomes (Decker et al., 2011) but not for flagella (Kuchka and Janvik, 1982; Marshall et al., 2005); however, such idealized scaling is predicted only if the pool is completely consumed, and if the pool is in a dynamic equilibrium with the organelles, the dependence on n is expected to be less steep than 1/n (Marshall et al., 2005). Overall we expect there to be a strong link between pool size and flagellar length, making it essential to understand the mechanisms that regulate pool size. One barrier to understanding pool size control is that we do not know which flagellar protein or proteins are limiting for flagellar growth. Because the structural core of the flagellum is composed of tubulin, we imagine that tubulin may be the limiting precursor, but this has not yet been demonstrated. If tubulin was the limiting precursor, then the protein machinery that regulates microtubule dynamics would be predicted to influence the effective pool size regulating flagellar length.

Better understanding of pool regeneration would provide an important missing link in our understanding of flagellar length control. One length control model that has been proposed is a balance-point mechanism in which length is set by the dynamic balance between turnover of axonemal microtubules and the inherently length-dependent transport of new tubulin to the tip by the intraflagellar transport system (Marshall and Rosenbaum, 2001; Marshall et al., 2005; Wemmer and Marshall, 2007; Engel et al., 2009). This model is supported by the fact that tubulin has been shown to be a cargo that is carried by the intraflagellar transport (IFT) system (Qin et al., 2004; Hao et al., 2011; Bhogaraju et al., 2013). An alternative model is that a length sensor is used to construct a feedback control system in which the flagellum grows until it reaches the correct length and then stops due to changes in activity of a length-responsive signaling pathway (Wilson et al., 2008). Such a feedback mechanism is supported by the fact that IFT particle injection into flagella is a function of length (Ludington et al., 2013) and that loading of some cargo proteins onto the IFT system may also be a function of length (Wren et al., 2013). Current models focus almost entirely on IFT itself and do not address the mechanisms that might regulate the size of the precursor pool.

In this study we used a set of short-flagella mutants in the unicellular green alga C. reinhardtii obtained in a previous screen for phototaxis-defective mutants (Feldman et al., 2007) as a starting point to search for mutations affecting precursor pool synthesis. This organism has long been a favored system for genetic studies of flagellar biology due to its ease of culture and tractable yeast-like haploid genetics. Chlamydomonas cells possess two flagella, each typically 10–12 μm in length. Chlamydomonas genetic analyses have yielded a handful of genes that alter flagellar length, including three SHF genes, whose mutants cause short flagella (Kuchka and Janvik, 1987), and four LF genes, whose mutants cause atypically long flagella but can also, paradoxically, cause cells to lack flagella, depending on the allele (McVittie, 1972; Barsel et al., 1988; Asleson and Lefebvre, 1998). Starting with a quantitative analysis of flagellar length distributions to identify a candidate mutant with defective pool regeneration, we identified a mutation in the p80 subunit of the microtubule-severing enzyme katanin. We found that katanin mutants impaired the mobilization of the effective cytoplasmic precursor pool during flagellar regeneration, suggesting that katanin-mediated severing of cytoplasmic microtubules might make tubulin available for flagellar assembly. A simple computational model of microtubule dynamics coupled with flagellar length control confirmed that reduced katanin activity could lead to shorter flagella due to impaired mobilization of the total tubulin pool.

RESULTS
Identifying short-flagella mutants with slowed regeneration kinetics
To identify genes regulating pool synthesis, we must first have an understanding of what phenotype we should expect to observe if such genes are mutated. When flagella are severed from wild-type Chlamydomonas cells after pH shock, they grow back to full length in <2 h. The regeneration kinetics is biphasic, with flagella growing rapidly to approximately half-length, followed by a period of gradually slowing growth during which flagella asymptotically approach the final steady-state length (Lefebvre et al., 1978). If new protein synthesis is inhibited before flagellar severing, the new flagella that regenerate can grow back only to roughly half-length, but their initial growth rate is very close to the initial rate of rapid growth in untreated cells (Lefebvre et al., 1978). Thus it is the second, slower phase of growth that is affected when protein synthesis is inhibited. We hypothesized, therefore, that mutants with defects in the induction of cytoplasmic precursor pool synthesis during flagellar regeneration would be recognizable by a much slower secondary growth phase compared with wild-type cells while retaining approximately normal rates of initial rapid growth.

Direct measurement of flagellar regeneration kinetics is time-consuming and difficult to perform in parallel for large numbers of mutants, so we first asked whether there is a simpler criterion that could be more rapidly assayed as a primary screen, to be then followed by direct measurement of regeneration kinetics. The relation of growth kinetics to the length distribution of linear structures has been formalized in the population-balance method, previously developed by Aizawa and colleagues for calculating growth kinetics of the bacterial flagellar hook from the distribution of hook lengths (Koroyasu et al., 1998). The principle of this method is that for a structure that undergoes monotonic growth, the fraction of such structures found within a given range of lengths in an asynchronous population is directly related to the growth rate over that size range. The faster the object is growing when at a particular size, the less frequent will be objects of that size when a population of objects is observed. For example, in a wild-type cell in which flagella grow rapidly to their final length and then remain there for a long time, few cells will have short flagella because any given cell will not spend a long period of time with short flagella, because they are rapidly growing. Therefore, in an asynchronous population, the distribution will be sharply peaked around the final steady-state length. For a hypothetical mutant defective in pool regeneration, it would not spend much time with very short lengths, since the rapid early growth phase does not involve pool regeneration. Thus the length distribution should not contain many individuals with less than half of the normal length. However, slow regeneration of the pool would mean that cells would spend more time with flagella at intermediate lengths between half-length and wild-type length, so we expect the overall distribution of lengths in a population of asynchronously dividing cells to be unusually broad and stretch between half-length and full length, with an average length that is shorter than wild type.

Although three short-flagella (shf) mutants have already been identified, they are difficult to clone because their mutations are not sequence tagged. We therefore screened a collection of 20 inser-
Roman numerals indicate the assigned length distribution class for insertional mutants.

*Significant deviation from a normal distribution, defined as a skewness statistic with absolute value greater than twice the SE of skew.

**Significant deviation from a normal distribution, defined as a kurtosis statistic with absolute value greater than twice the SE of kurtosis.

TABLE 1: Flagellar length distribution in short-flagella mutants.

| Strain  | <L> (μm) | σ (μm) | Skew | Kurtosis | n  |
|---------|----------|--------|------|----------|----|
| cc-125  | 11.0     | 1.1    | 0.06 | 0.43     | 166 |
| shf1-253| 5.2      | 0.3    | −1.38| 0.42     | 60  |
| shf3-1851| 10.4    | 0.2    | −0.35| −0.26    | 60  |
| pf14    | 2.8      | 1.7    | 1.02 | 1.23     | 21  |
| pf15a   | 6.1      | 3.5    | 0.61 | −0.66    | 153 |
| pf18    | 4.2      | 3.0    | 1.53 | 1.37     | 62  |
| pf19    | 1.9      | 1.0    | 1.07 | 0.14     | 90  |
| pf20    | 6.1      | 4.3    | 0.34 | −1.48    | 54  |
| pf26    | 11.7     | 3.4    | 1.71 | 2.21     | 20  |
| 802 I   | 3.1      | 2.0    | 2.15 | 5.28     | **114** |
| 267 I   | 2.7      | 2.0    | 1.70 | 2.74     | **110** |
| 6648 I  | 2.5      | 2.0    | 2.01 | 4.97     | **49** |
| 6773 I  | 2.2      | 1.1    | 1.25 | 1.82     | 50  |
| 5834 I  | 1.9      | 1.5    | 2.01 | 5.16     | **110** |
| 5840 I  | 1.8      | 2.2    | 2.29 | 4.98     | **39** |
| 5836 I  | 0.7      | 0.3    | 1.74 | 3.67     | 60  |
| 9111 II | 10.5     | 1.6    | −0.88| 0.49     | 109 |
| 7107 II | 10.2     | 3.2    | −1.23| 2.68     | 130 |
| 9484 II | 10.0     | 1.4    | −0.12| 0.19     | 108 |
| 6894 II | 9.8      | 1.6    | −0.94| 1.72     | **110** |
| 8488 II | 9.4      | 2.7    | −0.81| 0.71     | 110 |
| 8333 II | 9.4      | 2.1    | −0.94| 0.74     | 114 |
| 6444 II | 9.2      | 3.5    | −1.26| 0.80     | 112 |
| 8796 II | 9.1      | 3.5    | −1.48| 1.22     | **83** |
| 784 III | 6.8      | 4.0    | −0.09| −1.42    | **168** |
| 4580 III| 5.9      | 3.0    | −0.28| −1.36    | **170** |
| 1463 III| 5.1      | 3.3    | 0.74 | −0.81    | **199** |
| 5899 III| 5.0      | 3.7    | 0.49 | −1.05    | **112** |
| 3584 III| 4.7      | 3.7    | 0.43 | −1.46    | **170** |

By plotting length versus time normalized to predeflagellation length, we found that four of the five mutants regenerated to half of their steady-state length within ~40 min, whereas mutant 784 had reached only one-fourth of its predeflagellation length by 100 min (Figure 2B). This suggested a severe defect in flagellar regeneration rates even at short lengths for that mutant, which does not appear to be consistent with a primary defect in pool synthesis or length regulation per se but instead may represent a more fundamental failure in one of the many necessary steps of flagellar assembly. Therefore this mutant was excluded from further analysis.

Impaired recovery of flagellar precursor pool in shf mutants

Slower regeneration kinetics could indicate a reduced precursor pool size but could also be caused by many other types of defects, such as reduced intraflagellar transport. Thus we next sought to measure pool regeneration in the four remaining candidate mutants. The length-limiting protein component of the flagellum is not known, and therefore it is not possible to measure the regeneration of the relevant precursor protein by direct protein analysis. However, the regeneration kinetics of the functional precursor pool can be indirectly assessed using an elegant method developed by Lefebvre and Rosenbaum (Lefebvre et al., 1978), which entails performing two sequential deflagellations. In this procedure (Figure 2C), flagella are detached by pH shock and allowed to regenerate. Aliquots of cells with actively regenerating flagella are removed from the culture and subjected to a second pH shock, this time in the presence of cycloheximide, at a series of time intervals. These samples are then allowed to fully regenerate after the second shock. Because regeneration during the second shock depends on whatever precursor pool was present before the second shock was initiated (since further protein synthesis was inhibited at the same time that the second shock was performed), a plot of the final length versus the time at which the second shock was performed yields a plot of the effective pool size as a function of time. Analysis of pool dynamics using this dual-shock assay in wild-type cells showed that the pool of flagellar precursors is depleted during the first 40 min of regeneration and then begins to accumulate again (Lefebvre et al., 1978).

When we applied this dual-shock measurement method to our mutants, we found that all four of the class III mutants analyzed showed pool depletion by t = 40 min, just as was seen in wild type, but then failed to recover the pool size back to the initial level (Figure 2D). Thus all four class III mutants tested showed an apparent effect on the regeneration of the effective precursor pool in comparison to wild-type cells. We emphasize that this measurement is not a direct...
measurement of any particular protein component of the flagellum but instead is a functional measurement of the effective pool size in terms of the flagellar length that can be grown with a particular quantity of precursor.

**Gene induction during flagellar assembly**

Given the foregoing results, it appears that the class III mutants have a defect in regeneration of the flagellar precursor pool. The precursor pool is known to be under transcriptional control, such that during the process of regeneration, expression of flagellar genes is dramatically up-regulated (Lefebvre and Rosenbaum, 1986; Stolc et al., 2005). Because a failure to induce flagellar gene expression would prevent regeneration of a precursor pool, one obvious reason for the failure to regenerate the pool in the class III mutants would be that perhaps the genes defective in these mutants are part of the pathway controlling transcription of flagellar protein–specific genes. None of the components of this pathway was known, so we were extremely interested in determining whether the class III mutants might reveal genes that regulate flagellar precursor protein–encoding genes. To do this, we tested whether the shf mutants in our collection were capable of proper gene induction, using quantitative reverse transcription PCR using the radial spoke protein 3 (RSP3) gene as a representative flagellar gene and the RBCS2B gene, which encodes a subunit of the abundant photosynthesis enzyme ribulose-1,5-bisphosphate carboxylase oxygenase, commonly known as RuBisCo, as a control “housekeeping” gene for normalizing expression levels.

We then examined transcriptional induction of RSP3 in our collection of mutants, as well as in several known short-flagella mutants (shf) and paralyzed-flagella mutants (pf) for comparison. We expected to find a defect in class III mutants, which showed a defect in pool regeneration as assessed in Figure 2D. Because peak induction of most flagellar genes occurs 30 min after deflagellation (Stolc et al., 2005), we selected this time point for comparison of induction across strains. Figure 2, E and F, depicts the results of this analysis, which revealed extensive variation between mutant lines. The shf3 strain and mutants 802 and 7107 had significantly higher RSP3 expression than the wild-type strain, and the strains pf18 and shf1 and mutants 784, 5836, and 5840 had significantly lower expression than the wild-type strain. Of interest, shf2 mutants showed virtually no induction of flagellar genes, raising the interesting possibility that the SHF2 gene (Kuchka and Jarvik, 1987), whose product is unknown, might be involved in the transcriptional control circuit regulating flagellar gene expression. Of the low inducers, all but strain 784 were found to have significant numbers of flagella-less (bld) cells.

Surprisingly, of the class III mutants, only mutant 784 showed decreased induction of RSP3. This mutant had already been
FIGURE 2: Identifying a short-flagella mutant with impaired precursor pool regeneration kinetics but normal transcriptional induction. (A) Regeneration kinetics in class III short-flagella mutants. Graph shows flagellar length vs. time after pH shock. Time 0 indicates length before pH shock. Error bars indicate SEM. Error bars smaller than the radius of the data-point marker are not visible. All data points are based on measurement of flagella from 60 cells. (B) Length vs. time after deflagellation normalized to predeflagellation length; red circles, wild type; orange squares, mutant 5899; blue squares, mutant 1464; green circles, mutant 4580; gray circles, mutant 3584; white circles, mutant 784. (C) Assay for flagellar pool regeneration as described in Lefebvre et al. (1978). Initial culture is subjected to pH shock (red arrow) and allowed to recover. At 10-min intervals, aliquots are removed, cycloheximide is added to the aliquot (vertical blue arrows), and then it is subjected to a second pH shock. Aliquots after the second aliquot are incubated for 2 h to reach steady-state length. (D) Result of pool regeneration assay. Time indicates the time at which the second pH shock was performed relative to when the initial pH shock was performed, that is, the time during which the cells were regenerating their pool before inhibition of protein synthesis. Length indicates the final steady-state length reached after regenerating from the second pH shock and is an indicator of the size of the protein pool at the time of cycloheximide addition; red circles, wild type; orange squares, mutant 5899; blue squares, mutant 1464; green circles, mutant 4580; gray circles, mutant 3584. (E) Induction of flagella-specific gene expression during regeneration in...
Mapping and identification of katanin p80 mutation in shf strain 1464

To determine the regions flanking the inserted sequence in our collection of insertional mutants, we used restriction enzyme site-directed amplification PCR (RESDA-PCR; Gonzalez-Ballester et al., 2005). Our initial RESDA-PCR results identified flanking sequence in four mutants: 1464, 3584, 4580, and 9111. However, only in line 1464 did the insertion occur in an annotated gene (Figure 3A), namely PF15, which encodes the p80 subunit of katanin (Hartman et al., 1998; McNally et al., 2000; Dymek et al., 2004), and we therefore chose this insertional line for further analysis. Genomic PCR revealed that the insertional mutagenic event deleted all but the first three predicted exons of the PF15 gene (Figure 3B). Mutations in the PF15 gene were previously identified in screens for paralyzed mutants. The central-pair defect seen in mutant 1464 is thus comparable to that seen in pf15a mutants.

We outcrossed mutant line 1464 containing the insertion in PF15 with wild-type cells and analyzed the genotype and phenotype of random progeny from 135 independent tetrads using a quadruple-redundant PCR strategy to identify the presence of the 1464 mutant–specific insertion in the PF15 gene and a visual screen for flagellar length and motility to identify the flagellar phenotype. No recombinants were identified that separated the insertion from the shf or paralyzed phenotypes, giving high confidence that the insertional event that we tracked was linked to the mutation causing the shf phenotype in line 1464.

However, the insertion in mutant 1464 was accompanied by deletion of several genes (Figure 3A), as judged by genomic PCR using primer pairs spanning the region. To determine whether mutation in the PF15 gene itself is sufficient to cause the length and pool regeneration defects seen in mutant 1464, we examined another independent allele of the PF15 gene, pf15a (Dymek et al., 2004), to ask whether it shows the same length and pool regeneration phenotype. The pf15a strain contains a point mutation in the second exon of the PF15 gene, giving rise to a single amino acid substitution, V43D (Dymek et al., 2004). We measured the flagellar length distribution in pf15a mutants and found that the pf15a strain exhibits heterogeneous flagellar lengths with a negative kurtosis, virtually identical to mutant 1464 (Figure 3D). We also found that pf15 mutant cells share the same slow regeneration kinetics (Figure 3E) and the same defect in regenerating the functional pool of flagellar precursors (Figure 3F) as in mutant 1464, and both mutant strains induce similar RSP3 expression (Figure 3G). The identical phenotypes of our insertional mutant and the pf15 point mutant indicate that PF15 mutation is indeed the cause of the shf phenotype. We designated our insertional allele of PF15 as pf15-2. The fact that the shf and pool regeneration phenotypes are virtually identical in two completely independent PF15 mutations indicates that katanin somehow plays a role in flagellar length and precursor pool mobilization.

To test for an effect of katanin p80 mutation on microtubule stability, we cultured cells on solid media containing either the microtubule-depolymerizing herbicide oryzalin (James et al., 1993) or the microtubule-stabilizing compound Taxol. Neither the insertional mutant pf15-2 nor the pf15a point mutant showed enhanced resistance or susceptibility to the antimicrotubule agents as compared with a wild-type strain, suggesting that the effect of the mutations on microtubule dynamics, if any, may be fairly subtle. It should be noted, however, that Taxol is less effective in most algae, including Chlamydomonas, than it is in animal cells, so a negative result for Taxol sensitivity is not informative.

Computational modeling of katanin activity in flagellar length control

It was previously shown that when flagella regenerate, cytoplasmic microtubules shorten transiently (Wang et al., 2013), suggesting that cytoplasmic microtubules and flagella are competing for a common pool of tubulin. Because the pf15 mutants in katanin show reduced mobilization of the precursor pool, we hypothesized that this slow mobilization might reflect the competition for a limited quantity of total tubulin between flagellar microtubules and cytoplasmic microtubules described by Wang et al. (2013) with katanin-mediated cleavage, making tubulin more available for flagellar growth. The Chlamydomonas cell contains two types of microtubules in the cytoplasm: four highly stable microtubule bundles known as rootlets (Ringo, 1967; Holmes and Dutcher, 1989) and ~10–20 nonroot microtubules (Schibler and Huang, 1991; Horst et al., 1999). Because the rootlet microtubules are highly stable, we postulate that the nonroot microtubules contain a dynamic pool of tubulin and that growth of flagella competes with...
plasmic microtubules (Figure 4A). We implemented a stochastic simulation of flagellar dynamics coupled to cytoplasmic microtubule dynamics. For this simulation, we implemented the model of microtubule dynamics described by Goodson and coworkers (Gregoretti et al., 2006), in which each microtubule is represented as a linear polymer of subunits that can be either GTP or GDP bound. We recognize that such single-protofilament-type models do not capture the full richness of microtubule dynamics and that in the future, more realistic models should be applied (Bowen-Anderson et al., 2013). Here we use this model simply to determine whether modulation of microtubules is at least in principle capable of affecting the apparent rate of flagellar precursor pool regeneration. At each time step, a decision is made to either release the final subunit, based on the GTP state of the penultimate subunit, or bind a new subunit based on the free tubulin concentration and the GTP state of the terminal subunit. The simulation of microtubules and the parameters of the model were based on those previously reported (Gregoretti et al., 2006) and are given here as probability per unit time of a given event: probability of GTP hydrolysis, \( K_{\text{hydrolysis}} = 0.1 \text{s}^{-1} \); probability to add a subunit when the GTP cap is present, \( K_{\text{grow, GTP}} = 2.0 \text{s}^{-1} \); probability to add a subunit when there is no GTP cap, \( K_{\text{grow, GDP}} = 0.10 \text{s}^{-1} \); and probability to remove a subunit when there is no GTP cap, \( K_{\text{shrink, GDP}} = 48 \text{s}^{-1} \). Following the approach of Gregoretti et al. (2006), we assume that shrinkage never occurs when the GTP cap is present. We note that for Chlamydomonas, none of the relevant kinetic parameters of microtubule dynamics has been reported; hence we use these parameters based on approximate values for mammalian cells discussed in Gregoretti et al. (2006). As was done by Gregoretti et al. (2006), we assume that regeneration of soluble GTP tubulin from disassembled GDP tubulin is instantaneous.

In our model, we simulated 10 cytoplasmic microtubules at once. The number of microtubules chosen for the simulation was based on three-dimensional microscopy images of Chlamydomonas cytoplasmic microtubules (Horst et al., 1999). We did not implement a constraint on microtubule length because even though the cell has a finite diameter, its round shape allows microtubules to bend and wrap back around it when they become too long, as directly seen in certain Chlamydomonas mutants having unusually long microtubules (Horst et al., 1999).

We augmented the existing microtubule dynamics model with a random cutting process to simulate katanin-mediated severing. At each time step, there was a constant probability of cutting a microtubule at any subunit. If a cut occurred, the site of the cut became random cutting process to simulate katanin-mediated severing. At each time step, we implemented a stochastic model for flagellar length (Marshall and Rosenbaum, 2001) with a stochastic model for cytoplasmic microtubule dynamics (Gregoretti et al., 2006). In this model, a single shared pool of tubulin is used for assembly of flagella and cytoplasmic microtubules (Horst et al., 1999). We did not implement a constraint on microtubule length because even though the cell has a finite diameter, its round shape allows microtubules to bend and wrap back around it when they become too long, as directly seen in certain Chlamydomonas mutants having unusually long microtubules (Horst et al., 1999).

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Katanin regulates flagellar length

\[
\frac{dL}{dt} = \frac{AF}{L} - D
\]

where \(L\) is the length of the \(i\)th flagellum (normal *Chlamydomonas* cells have just two flagella, but we preserve the option to simulate mutants with altered flagellar number) and \(F\) is the quantity of free tubulin in the cytoplasm. Here \(D\) is the rate of flagellar microtubule disassembly, and \(A\) is a constant that describes the efficacy of intraflagellar transport.

In our simulations, we modeled two flagella but assume them to always have equal length; hence we have a single equation for flagellar length. We modified this equation to incorporate a first-order dependence of flagellar growth rate on free tubulin concentration. We augmented this equation with a single equation for the quantity of free tubulin dimer:

\[
\frac{dL}{dt} = \frac{A v K [T_{\text{free}}]}{L} - D
\]

\[
T_{\text{free}} = T_{\text{total}} - T_{\text{polymer}} - \frac{bnL}{s}
\]

where \(L\) is the flagellar length, \(D\) is the rate of disassembly from the tip (0.011 \(\mu\text{m/s}\)), \(n\) is the number of flagella (two for a typical cell), \(K\) is an affinity constant describing first-order binding of free tubulin to IFT particles, \(v\) is the velocity of IFT particles (strictly speaking, the harmonic mean of the anterograde and retrograde speeds), and \(A\) is a constant that combines the number of IFT particles and their effective cargo-carrying capacity (for our simulations, we used \(A = 0.022\) and \(K = 0.001\)). The constant \(b\) indicates the ratio of the number of tubulin subunits per unit length of axoneme versus per unit length of cytoplasmic microtubule (~18), and \(s\) is the size in micrometers of a tubulin dimer within the microtubule lattice. We assume that disassembly occurs at a constant rate \(D\), independent of flagellar length. This is a simplifying assumption based on the fact that flagellar shortening is length independent when IFT is inactivated (Marshall and Rosenbaum, 2001); however, recent studies demonstrated that the disassembly rate can be up-regulated when flagella become too long via a pathway regulated by the cnk2 kinase (Hilton et al., 2013). Because our model addresses situations in which flagella are shorter than wild type, we assume that this cnk2-mediated pathway will not be activated, and therefore it is not included in the model. We note that this model does not take into account the nucleotide state of the tubulin dimers, a simplifying assumption that we make based on the fact that axonemal microtubules do not undergo spontaneous dynamics when isolated and instead seem to rely on enzymatic catalysis for turnover.

At all times, the total quantity \(T\) of tubulin in the cell, which is the sum of the flagellar tubulin content, the cytoplasmic microtubule content, and the soluble tubulin pool, is kept constant.
It has been reported that depletion of a depolymerizing kinesin, kinesin-13, from Chlamydomonas leads to cells with regeneration defects and short flagella (Piao et al., 2009; Wang et al., 2013). Wang et al. (2013) proposed that these phenotypes could arise if kinesin-13 acts in the cytoplasm to help release tubulin from cytoplasmic microtubules for use in flagellar growth, a claim supported by the observation that cytoplasmic microtubules shorten less during flagellar regeneration when kinesin-13 is depleted by RNA interference (RNAi; Wang et al., 2013). Because the proposed role for kinesin-13 in flagellar growth is similar to our model for katanin function, we modified the stochastic simulation of Figure 4 to simulate the effects of kinesin-13. This was done by adding a variable $k_{\text{cut}}$, which determines the probability per unit time of kinesin-13 removing the final tubulin from a microtubule (regardless of GTP state). We assume that the probability of terminal tubulin removal by kinesin-13 is independent of microtubule length, in contrast to the length-dependent activity of kinesin-8 (Varga et al., 2006). With this additional term added to the model, we find that as the rate of kinesin-13 activity is increased, microtubules undergo higher frequency of catastrophe and gradually shorten during simulated flagellar regeneration (Figure 6A). When we plot steady-state flagellar length versus kinesin-13 activity (Figure 6B), we find that just as with katanin, increased activity leads to increased flagellar length. Decreased activity leads to short flagella. Our computational model thus recapitulates the key result of Wang et al. (2013) and further confirms that modulation of enzymes affecting cytoplasmic microtubule dynamics can alter flagellar length via a competition mechanism.

**DISCUSSION**

**Relation to previous studies**

Katanin has been shown to affect flagellar length in other organisms. Mutations in the $p60$ subunit of katanin cause short cilia length in *Tetrahymena* (Sharma et al., 2007). The *Chlamydomonas* PF19 gene encodes one of the $p60$ catalytic subunits of katanin contained in the *Chlamydomonas* genome (Dymek and Smith, 2012). Our measurements of length distributions (Table 1) showed that the $p$19 mutant has a short average length and a broad length distribution comparable to $p$15 mutants. This result further supports the idea that katanin activity plays a role in determining flagellar length. We also note that prior studies showed that reduction of katanin function can cause increased microtubule polymer mass (Sayko et al., 2006) and increased length of mitotic spindle microtubules (Goshima and Scholey, 2010; Loughlin et al., 2011).

Analysis of the microtubule-depolymerizing kinesin, CrKinesin-13, of *Chlamydomonas* supports the idea that turnover of cytoplasmic microtubules facilitates availability of tubulin for flagellar growth. RNAi-mediated knockdown of the CrKinesin-13 gene caused a short-flagella phenotype similar to what we see in $p$15, and these cells exhibit abnormally slow regeneration kinetics (Piao et al., 2009). After deflagellation, CrKinesin-13 knockdown cells undergo a lag phase of $\sim$1 h before beginning to regenerate their flagella, after which the flagella grow very slowly, reaching preshock length only after 4 h, suggesting that mobilization of the flagellar precursor pool is impaired. More recently, Pan and coworkers found that cytoplasmic...
microtubules depolymerize during flagellar regeneration and that this depolymerization is mediated in part by kinesin-13 (Wang et al., 2013). They are thus able to explain the previous results of kinesin-13 RNAi by arguing that in the knockdown cells, cytoplasmic microtubules become more stable and thus compete more effectively for tubulin against flagellar microtubules. Our experimental and computational results suggest that a similar mechanism is likely to explain the short-flagella phenotype in pf15 mutants.

A similar role has been discussed for proteins involved in actin filament turnover; for example, Aip1 promotes generation of actin monomers from partially disassembled actin filaments, thus providing an assembly-competent precursor pool to support assembly of other actin filaments (Okreglak and Drubin, 2010).

Studies in other protists indicate an opposite role of microtubule-severing or microtubule-depolymerizing proteins in flagellar length control. Mutations affecting microtubule-depolymerizing kinesins affect flagellar length in Leishmania (Blaineau et al., 2007), but in that case, the effect is the opposite of what is seen in Chlamydomonas: depletion of kinesin-13 in Leishmania results in flagella that are longer, not shorter. Similarly, dominant negative alleles of kinesin-13 in Giardia cause flagella to become abnormally long (Dawson et al., 2007). For katanin itself, overexpression in trypanosomes and Leishmania caused flagella to become shorter, not longer, again a result that seems to be the opposite of what we observe in Chlamydomonas (Cassanova et al., 2009). The authors who reported these findings favor a type of model in which katanin or kinesin-13 functions primarily within the flagellum, and this could indicate a fundamental difference in flagellar length regulation between these organisms and Chlamydomonas.

Finally, we note that katanin has been shown to play a role in mitotic spindle length regulation (Loughlin et al., 2011), but in that case it is believed that katanin is acting directly on the spindle microtubules themselves, such that a decrease in severing activity makes the spindle as a whole longer. Thus, although the spindle represents a clear case of katanin regulating the size of a microtubule-based structure, it is by an entirely different mechanism than the tubulin competition model we hypothesize for flagellar length regulation.

Role of precursor competition in organelle size determination

Our results with katanin, as well as published results with kinesin-13 in Chlamydomonas (Wang et al., 2013), are consistent with the idea that within a single cell, there are differentially regulated sets of microtubules with differing functions, such as the stabilized microtubules of the flagellar axoneme and the more dynamic population in the cell body. In the biflagellate golden-brown alga Ochromonas, distinct sets of microtubules give rise to different aspects of the cell’s characteristic pyriform shape (Brown and Bouck, 1973), and in this system it was clearly shown that as flagella grow, specific sets of cytoplasmic microtubules transiently shorten. Our model of competition between flagellar and cytoplasmic microtubules in Chlamydomonas thus has a precedent in Ochromonas. Consistent with the results in Chlamydomonas and Ochromonas, Sharma et al. (2011) showed that increasing the pool of soluble tubulin in mammalian cells with a carefully titrated dose of nocodazole correlates with longer cilia. Competition for precursor between different cytoskeletal structures has recently been reported for the actin cytoskeleton (Burke et al., 2014). Taken together, these results argue that the size and assembly of individual cellular components cannot be treated in isolation and that the size of a particular cellular structure will depend at least in part on the competition between that structure and any other structures sharing common limiting pools of key components, much as the population sizes of distinct predators depend in part on the dynamics of shared prey populations.

MATERIALS AND METHODS

Strains and culture

C. reinhardtii cells were maintained on Tris-acetate-phosphate (TAP) plates (Harris, 1989), and experimental cultures were grown in M1
mineral medium (Harris, 1989). Insertional mutants were obtained in our lab in a previously reported screen (Feldman et al., 2007), and additional strains were obtained from the Chlamydomonas Genetics Center (Duke University, Durham, NC). Cells were cultured at 21°C with constant light and in a rotating roller drum to provide aeration. Short-flagella (shf) reference alleles used in this study were shf1-253, shf2-1249, and shf3-1851.

Length measurements
Cells were fixed in 1% glutaraldehyde in phosphate-buffered saline (PBS) and imaged using differential interference contrast (DIC) optics with a 60x objective on a DeltaVision microscope. Measurements were obtained by hand tracing flagella using Softworx software. Only cells with two visible, measurable flagella were included in the analysis.

Analysis of length distributions
Length measurement data were analyzed using Excel (Microsoft) to calculate skew and excess kurtosis values (Taylor, 1996). Skew was determined to be significant if the skew value was greater than twice the SE of skew, defined as $\mu_s$. Kurtosis was determined to be significant if the excess kurtosis value was greater than twice the SE of kurtosis, defined as $\mu_k$.

Deflagellation by pH shock
Cells were spun down at 1500 x g for 2 min in a bench-top centrifuge and resuspended in M1 medium acidified to pH 4.5 with acetic acid. After a 1-min incubation in acidic M1, medium was returned to neutral pH by addition of KOH, cells were centrifuged again, supernatant was decanted, and cells were resuspended in fresh neutral M1 medium.

Flagellar regeneration assay
Cells were subjected to acidic pH shock as described. Resuspended cells in neutral M1 medium were incubated at 21°C with constant light in a rotating roller drum. Aliquots of cells were withdrawn at 10-min intervals and mixed with an equal volume of 2% glutaraldehyde in 1x PBS to fix. Length measurements were performed as described.

Quantitative reverse transcription PCR
To measure gene expression during flagellar assembly, total RNA was isolated from Chlamydomonas cells before deflagellation and at 30, 45, and 60 min after deflagellation as previously described (Stolc et al., 2005), with an additional purification step using a PureLink RNA minikit (Life Technologies, Grand Island, NY). RNA was reverse transcribed using the SuperScript VILO kit (Life Technologies), with 1 μg of total RNA per reaction. cDNA was diluted 1:20 and used for quantitative PCR with Taq and SYBR Green and analyzed with a DNA Engine Opticon system (MJ Research, St. Bruno, Quebec, Canada). Ct values were obtained using Opticon software (MJ Research).

Flagellar precursor pool estimation
Cells were subjected to deflagellation by pH shock as described. At 10-min intervals after the initial shock, cells were subjected to a second shock with cycloheximide at 10 μg/ml final concentration in both the acidic shock medium and neutral recovery medium. Cells were permitted to recover from the second shock for 2 h before fixation, and flagellar length measurements were performed as described.

Isolation of genomic DNA
Two procedures were used for isolation of genomic DNA: a dual extraction process for harvesting clean genomic DNA for sensitive downstream applications such as RESDA-PCR, and a rapid, crude preparation for genotyping PCR. Clean genomic DNA was obtained using a CTAB/chloroform extraction protocol, followed by a second extraction with phenol chloroform. Cells were grown in TAP medium with shaking under continuous light and harvested by centrifugation. Cells were then frozen at −80°C and lyophilized overnight. Lysis was achieved by vortexing with glass beads in CTAB extraction buffer (100 mM Tris-HCl, pH 7.5, 0.7 M NaCl, 10 mM EDTA, 1% cetyltrimethyl ammonium bromide [CTAB], 1% β-mercaptoethanol), followed by incubation at 65°C for 2 h. Subsequent chloroform extraction, isopropanol precipitation, phenol chloroform extraction, and ethanol precipitation were performed using standard methods. The resulting DNA was treated with 50 μg/ml RNase A at 37°C for 1 h and 0.5 mg of proteinase K at 55°C for 1 h. Crude DNA was prepared using a modified smash-and-grab protocol for Saccharomyces cerevisiae.

Identification of flanking sequence
RESDA-PCR was performed as described in Gonzalez-Ballester et al. (2005) using the following primers specific for the mutagenizing plasmid:

- HYG 5′ R7 TTGCCGAGGAGCTAGATGAAAG
- HYG 5′ R9 GGCATCCGTAAGATGCTTTTC
- HYG 5′ R6 ATACGGGAGGCTTACCATCT
- HYG 3′ F6 CAGAGGTTTTTCACCGTACTA
- HYG 3′ F8 CGAGTGGGTATACATCGAACTG
- HYG 3′ F5 GCTGCATGTTGCAAGGTTTT
- HYG 3′ F7 TTTTGGCTCTGTTTGGTG

Nested PCR products were subjected to gel electrophoresis, followed by excision of discrete bands. PCR products were isolated using a gel purification kit (Qiagen, Valencia, CA), cloned using a TOPO-TA cloning system (Life Technologies), and sequenced. Identified sequence was compared with the Chlamydomonas genome, version 3.0.

Genotyping PCR
Primer pairs were designed such that the insertion present in mutant 1464, located in genomic scaffold 34, would be amplified:

- 1464 insert+ F1 GACACCAGTCGTCGGTGAGAGTT
- 1464 insert+ R1 TCCGGTGTGGCCTATTCCTTTT
- 1464 insert– F1 AGCTTTGTAAGTGGAGGGCAACC
- 1464 insert– R1 GAGGTTATGGGGGTTGGCGAGT

Motility phenotype assessment
After 2 d in liquid culture, aliquots of live cells were subjected to DIC microscopy using a 20× objective, and swimming behavior was assessed visually.

Electron microscopy
Exponentially growing wild-type and PF15 mutant Chlamydomonas were collected by filtration and processed as described (Schuck et al., 2009). In brief, cells were frozen using a Leica (Wetzlar, Germany) EM PACT high-pressure freezer, fixed with 1% osmium tetroxide, 0.1% uranyl acetate, and 3% water in acetone using a Leica EM AFS2 freeze substitution machine, and embedded in Epon resin. Sections 40 nm in thickness were cut, stained with 2% aqueous uranyl acetate and Reynolds’ lead citrate, and viewed under a
FEI (Hillsboro, OR) Tecnai T12 electron microscope. To quantify the fraction of flagella with a central pair, micrographs were acquired of flagella cross-sectioned such that all nine outer doublet microtubules were visible, and the presence of a discernible central pair was scored.

Microtubule drug resistance assay

TAP agar was prepared and autoclaved as described previously (Harris, 1989). Before pouring plates, the desired drug was added to the medium (oryzalin at final concentrations of 2, 20, and 40 μM). Cells were streaked onto cooled plates and incubated in constant light for 1 wk before growth was assessed visually.

Stochastic simulation of competition for tubulin

The equations were solved using the Euler method with a time step of 0.02 s, and at each time step, the free tubulin pool size $T_{\text{free}}$ was updated based on the stochastic microtubule dynamics simulation running in parallel. We deliberately chose a time step small enough that the probability of more than one microtubule dynamics event (catastrophe, rescue, or cutting) was negligible.

We simulate stochastic cutting by katanin by generating a uniformly distributed random number and comparing it to the probability of cutting a given microtubule during any single time step. Once the decision is made to cut a microtubule, the position of the cut is chosen at random from a uniform distribution of positions spanning the full length of the microtubule. All tubulin subunits contained in the cut portion are returned to the free pool and set to the GTP-bound state. The fact that this occurs instantaneously is a simplification of the model.

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