CDKL5 regulates flagellar length and localizes to the base of the flagella in Chlamydomonas

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ABSTRACT The length of Chlamydomonas flagella is tightly regulated. Mutations in four genes—LF1, LF2, LF3, and LF4—cause cells to assemble flagella up to three times wild-type length. LF2 and LF4 encode protein kinases. Here we describe a new gene, LF5, in which null mutations cause cells to assemble flagella of excess length. The LF5 gene encodes a protein kinase very similar in sequence to the protein kinase CDKL5. In humans, mutations in this kinase cause a severe form of juvenile epilepsy. The LF5 protein localizes to a unique location: the proximal 1 μm of the flagella. The proximal localization of the LF5 protein is lost when genes that make up the proteins in the cytoplasmic length regulatory complex (LRC)—LF1, LF2, and LF3—are mutated. In these mutants LF5p becomes localized either at the distal tip of the flagella or along the flagellar length, indicating that length regulation involves, at least in part, control of LF5p localization by the LRC.

INTRODUCTION Interest in the control of the assembly of cilia and flagella has greatly increased in recent years with the discovery that many human diseases are caused by defects in cilia (Badano et al., 2006; Berbari et al., 2009). A particularly intriguing aspect of flagellar assembly is the highly regulated control of flagellar length in the unicellular green alga Chlamydomonas (reviewed by Wemmer and Marshall, 2007; Wilson et al., 2008). The genetic regulation of flagellar length in Chlamydomonas has been uncovered by analyzing the phenotypes and underlying molecular lesions of mutants in which the flagella assemble to lengths either longer or shorter than the flagella of wild-type (WT) cells (Kuchka and Jarvik, 1987; Barsel et al., 1988; Asleson and Lefebvre, 1998). Mutants that cause flagella to assemble to excessive length, up to two to three times the 14-μm length of flagella of WT cells, have been shown to identify four unlinked genes that control the extent of flagellar assembly. Two of these genes, LF2 and LF4, encode kinases of the cyclin-dependent kinase (CDK) family (Tam et al., 2007) and the mitogen-activated protein kinase (MAPK) family, respectively (Berman et al., 2003).

Three of the four long-flagella (LF) genes encode proteins that interact with each other and are found exclusively in the cytoplasm and not the flagella. The proteins, LF1p, LF2p, and LF3p, are found together in complexes called length regulatory complexes (LRCs; Tam et al., 2003, 2007; Nguyen et al., 2005). These three proteins have been shown to interact by a variety of methods, including cofractionation and pairwise yeast two-hybrid experiments. All three proteins localize to punctate structures in the cytoplasm using immunofluorescence microscopy. In addition, any double-mutant combination of If1, If2, and If3 alleles produces cells with severe flagellar assembly defects (Barsel et al., 1988; Asleson and Lefebvre, 1998). Double-mutant cells assemble either a single flagellum or no flagella.

The fourth LF gene, LF4, encodes a MAPK localized in both the flagella and the cytoplasm (Berman et al., 2003). If4 mutants have been isolated repeatedly as insertionional mutants, presumably because the long-flagella phenotype is the null-mutant phenotype. By contrast, null mutants in If2 and If3 have been shown to have a more severe flagellar phenotype known as “ULF” for “unequal length flagella” (Tam et al., 2003, 2007). These mutants are either flagella-less or, if they have flagella, they often have two flagella of unequal length, each shorter than WT flagella.

We recently isolated two new insertionional mutant alleles that identify a fifth LF locus, LF5. If5 mutants have long flagella, but not
as long as lf4. LF5 encodes a protein kinase with a high degree of sequence homology in the kinase domain to human cyclin-dependent kinase–like (CDKL) kinase, CDKL5. A remarkable feature of LF5p is that it localizes to the proximal 1 μm of the flagella, a localization observed for very few flagellar proteins. This kinase is of particular interest in humans, as a number of different lesions in CDKL5 lead to severe juvenile epilepsy of unknown etiology (Kalscheuer et al., 2003; Rademacher et al., 2011; Kilstrup-Nielsen et al., 2012). The identification of LF5 in Chlamydomonas as a gene controlling flagellar length raises the possibility that ciliary length plays an important role in early brain development and that defects in ciliary length control might be involved in the development of juvenile epilepsy.

RESULTS
Phenotype of two new LF mutants
Exogenous DNA, when transformed into Chlamydomonas, can randomly integrate into the nuclear genome and cause mutations in different genes. Previously, all of the mutants with LF phenotype created by this method were alleles of a single gene, LF4 (Asleson and Lefebvre, 1998). We recently identified two insertional LF mutants, 3F12 and DKD6, that identify a previously unknown LF locus, LF5. When crossed to each other, 3F12 and DKD6 gave rise to progeny that only have the long-flagella phenotype (14 tetrads), indicating that these two mutants are either allelic or very closely linked.

In contrast to lf4 mutants, which have very long flagella at least twice the length of flagella of WT cells, 3F12 and DKD6 have moderately long flagella, with average lengths that are 1.3–1.5 times the length of flagella of WT cells (Figure 1A). All mutant cells move erratically and slowly. Among the previously identified if mutants, if4 mutants have flagella with tapered ends similar to those seen on the flagella of WT cells, whereas certain mutant alleles in if1, if2, or if3 have flagella with distal tips that appear to be swollen. The swollen flagellar tips in these mutants are accompanied by an accumulation of intraflagellar transport proteins at the tips (Tam et al., 2003). Mutants 3F12 and DKD6, however, have flagella that are more similar in morphology to those of if4 mutants, having no distal swelling (Figure 1B).

Many if1, if2, and if3 mutant alleles show severe impairment in their ability to regrow flagella after amputation (Barsel et al., 1988). To test whether DKD6 cells can regenerate their flagella, we measured the length of the flagella at different times after pH-induced deflagellation. In three separate experiments, we found that DKD6 cells were able to regrow their flagella, although less synchronously and more slowly than WT cells. A representative experiment is illustrated in Figure 1C, showing the lower average lengths and wider range of flagellar lengths of DKD6 compared with those of WT at time points between 15 and 60 min after pH shock.

Mapping and cloning of LF5
Unfortunately, the mutant lesions in the DKD6 and 3F12 strains were not caused by the insertion of the plasmids used in transformation, and so map-based cloning was used to obtain the LF5 gene. To map the mutation in 3F12, we performed a cross of 3F12 with the polymorphic C. reinhardtii strain S1 D2 and performed PCR to check the linkage of the lf mutation with molecular markers on each chromosome. This mapping placed the lf mutation on chromosome 12, linked to the markers TUB2 and RDB (Table 1). On the basis of the genomic sequences of the polymorphic C. reinhardtii strains, we designed additional mapping primers on chromosome 12 around the region of interest to further delineate the location of the mutation. The closest markers that recombined with this lf muta-
did not recombine with the mutation, probably due to suppression of recombination around the centromere. The mapped location of this new LF mutation is distinct from LF2, which is also on chromosome 12 but at the distal portion of one arm. Therefore 3F12 defines a new LF locus, designated as LF5.

Having obtained detailed mapping information for LF5, we next used a candidate gene approach to clone the gene. Because analysis of the four known LF genes strongly suggests the involvement of protein kinases in flagellar length control, we designed primers to test for genetic lesions in protein kinases among the candidate gene models. An insertion in the gene model FAP247—a protein kinase previously found in the flagellar proteome (Pazour et al., 2005)—was detected in 3F12 (Figure 2, A and B). Chlamydomonas DNA fragments covering this gene model cloned in lambda phage (λ5, λ6, λ12) or in pBluescript (pBS3830) were transformed into the mutant. Only clones containing the entire FAP247 gene were able to rescue 3F12 to the WT phenotype (Figure 2A). To determine the location of the insertion in 3F12, we made a bacteriophage lambda library using DNA from 3F12 and obtained and characterized clones containing the mutant gene. A piece of Chlamydomonas DNA from an unmapped scaffold was found in intron 8 of FAP247. Presumably, during the transformation experiment that produced this mutant, a

### Table 1: Mapping distance of molecular markers on chromosome 12 to 3F12 mutation.

| Marker | Location (base pairs) | Mapped distance (cM) |
|--------|-----------------------|----------------------|
| RDG    | 7,400,000             | 14.7 (34)            |
| TUB2   | 7,700,000             | 15.6 (45)            |
| 8820   | 8,820,000             | 6.8 (47)             |
| 14-3-3 | 9,000,000             | 4.3 (46)             |
| 9280   | 9,280,000             | 0 (79)               |
| 6330   | 6,330,000             | 0 (96)               |
| 6170   | 6,170,000             | 0 (124)              |
| ODA9   | 6,070,000             | 0 (43)               |
| 6000   | 6,000,000             | 0 (123)              |
| 5750   | 5,750,000             | 3.3 (92)             |

*Approximate position of markers is given according to the Joint Genome Institute (JGI) database for Chlre4 (http://genome.jgi-psf.org/Chlre4/Chlre4.info.html). The centromere is located between markers 9280 and 6330 and has apparently disrupted the linearity of sequence assembly within this region in the JGI database.

*The number of progeny used for mapping is given in parentheses.

![Figure 2](image-url)
CDKL5 regulates flagellar length

CDKLs, for example, have a TE/DY motif, characteristic of MAPK, in kinases that also share common characteristics with MAPK kinases. Different CDKL kinases in humans, which are distant relatives of CDK kinases, have FRAT, with the FRAT/DY motif. LF5p is a member of the CDKL kinase subfamily. There are five different CDKL kinases in humans, which are distant relatives of CDK kinases that also share common characteristics with MAPK kinases. CDKLs, for example, have a TE/DY motif, characteristic of MAPK, in the activation loop. Of interest, two other genes in which mutations produce a long-flagella phenotype, LF2 and LF4, encode a CDK-related kinase and a MAPK, respectively. Figure 3B shows the phylogenetic relationship of these three Chlamydomonas kinases with their closest human orthologues and the five human CDKL kinases. LF5p is most homologous to CDKL5 (Figure 3C; 80% similar/62% identical and 81% similar/65% identical to human and Tetrahymena CDKL5, respectively). The human CDKL5 protein exists in multiple isoforms, with different C-terminal regions produced by alternative splicing (Williamson et al., 2012). The longest isoform has a C-terminal tail of 750 residues. LF5p has a C-terminal domain of 331 residues (Figure 3A). This C-terminal domain does not share significant homology to other CDKL5 proteins, nor does it contain any obvious sequence motifs. Analysis of LF5p using NetPhos 2.0 (www.cbs.dtu.dk/services/NetPhos) identified 22 very likely sites of (scores >0.9) S/T/Y phosphorylation, of which 15 are in the C-terminal tail. Given the well-established roles of other kinases in regulating flagellar length, phosphorylation of LF5p might play important roles in LF5 function.

**LF5 is up-regulated by deflagellation and encodes a flagellar protein**

To identify the RNA transcript of LF5, we fractionated total RNAs from WT and lf5 mutants on RNA blots and hybridized them with a probe made from the entire coding region of LF5. In WT cells, a 4-kb transcript was detected (Figure 2C). This transcript was not detectable in either lf5-1 (3F12) or lf5-2 (DKD6); however, a small transcript of 1.5 kb hybridized to the LF5 probe when RNA from lf5-1 was examined (Figure 2C). Based on the location of the inserted DNA in lf5-1 and the size of this aberrant transcript, it appears that the insertion caused an early termination of transcription, producing a chimeric transcript containing only the first eight exons of LF5 (1 kb) and part of the inserted DNA. On the contrary, lf5-2, which appeared to have a deletion within the genomic region, was likely to be a null mutant not producing any stable RNA product. We detected no differences in flagellar length or function between the two lf5 mutant alleles, suggesting that they are both effectively null alleles of the LF5 locus.

Previous genome-wide transcriptional analysis and real-time reverse transcription-PCR indicated that FAP247 (LF5) RNA transcript was induced upon deflagellation (Pazour et al., 2005; Stolc et al., 2005). We examined poly(A)+ RNA isolated from WT cells at different times after deflagellation to characterize in more detail the expression of LF5 transcript. We found that the level of LF5 transcript increased rapidly to greater than fourfold 15 min after deflagellation and reached a maximum level of greater than fivefold at 30 min (Figure 2D). This result shows that the increase of LF5 RNA accumulation occurs quickly as cells start to regenerate their flagella.

**LF5p can be dissociated from axonemes with high salt**

An affinity-purified antibody prepared using recombinant histidine-tagged LF5p as immunogen specifically recognized a major protein product that migrated at ~66 kDa and a minor product at ~45 kDa in WT flagella but not in lf5 mutant flagella (Figure 4A, left). The size of the major band is in agreement with the predicted size of LF5p from sequence analysis. The minor band may be a truncated or degraded fragment of LF5p. Some smaller proteins were also detected, but all of them were equally abundant in WT and both lf5 mutant flagella, indicating that they represent nonspecific interaction with the antibody. The 66- and 45-kDa protein bands were also detected in the flagella of lf1 or lf4 mutants.
suggesting that mutations in other LF genes do not affect the accumulation of LF5p in flagella.

To assess which flagellar compartment(s) contain LF5p, we extracted WT flagella with 0.5% NP-40 to obtain the soluble matrix/membrane fraction. Only a small fraction of LF5p was present in the NP-40–soluble fraction, with the majority of LF5p remaining in the demembranated axonemes (Figure 4A, right). The majority of LF5p could be solubilized from demembranated axonemes by treatment with 0.5 M NaCl or 0.6 M KI (Figure 4A), consistent with the earlier finding that the five unique tryptic peptides of FAP247 were identified, mostly from KCl extracts of axonemes (Pazour et al., 2005). The extractability of LF5p by 0.5 M NaCl indicates that ionic interactions are critical to axonemal binding of LF5p.

LF5p is localized to the very proximal region of flagella
We used the LF5 antibody to localize LF5p in flagella by indirect immunofluorescence, using an acetylated α-tubulin antibody for colocalization to identify the location of flagella, basal bodies, and microtubule rootlets. In WT cells, bright staining was observed in the very proximal region (∼0.5 μm) of the flagella (Figure 4B, a–c). A small amount of punctate staining was also observed on some of the flagella. Such flagellar staining was not observed in lf5-1 and lf5-2 cells, showing the specificity of the flagellar fluorescence signal (Figure 4B, d–f). Cytoplasmic spots of staining were observed with the antibody

and separated into soluble (S) and insoluble (P) fractions. The insoluble fraction was subsequently extracted with either 0.5 M NaCl or 0.6 M KI. The majority of the 66-kDa LF5p band was found in the NP-40–insoluble fraction and could be solubilized with 0.5 M NaCl or 0.6 M KI. A control antibody against RSP3 shows the solubility of that radial spoke component. (B) Indirect immunofluorescence microscopy of (a–c) WT cells, (d–f) lf5-1, (g–i) detached flagella, (j–l) deflagellated cells obtained by pH shock, and (m–o) uni2-3 mutant cells with a single flagellum, using an antibody to LF5p (green) and an antibody to acetylated α-tubulin (red). LF5p localized specifically to the proximal ends of WT flagella. The conspicuous tubulin staining on the distal ends of some of the flagella was caused by curling up of the distal ends of flagella, an occasional artifact produced during methanol fixation. In g–i, the bright LF5p staining was only found on the nonswollen proximal ends of detached flagella. Bar, 10 μm.

FIGURE 4: Western analysis and immunofluorescence localization of LF5p. (A) Western blot analysis of 37.5 μg of purified flagella identified a major protein band of 66 kDa and a minor band of 45 kDa in WT, If1, and If4 cells (left). Right, flagella were first extracted with 0.5% NP-40
in some experiments. However, these spots appeared to be present similarly in both WT and lfd5 mutant cell bodies, indicating that they do not represent LF5p localization in the cell cytoplasm.

In each Chlamydomonas cell there are two mature basal bodies that nucleate the formation of two flagella. When cells shed their flagella, the flagella break distal to a structure at the end of the body known as the transition zone (Craigie et al., 2010), so that the transition zone remains with the basal bodies in the cell (Sanders and Salisbury, 1989). Three observations indicate that the LF5p is localized to the flagella but not the transition zones or the basal bodies. First, bright proximal staining was observed on flagella that had been detached from the cells during the fixation process (Figure 4B, g–i). Second, when flagella were amputated by pH shock, all staining in the cell near the basal body region was lost (Figure 4B, j–l). Third, in the uni2-3 mutant, which often forms a single flagellum from one of the two basal bodies, only one bright spot was seen in the proximal region of the flagellum (Figure 4B, m–o) in cells that had only a single flagellum.

**Rapid sequestration of LF5p to the proximal region of growing flagella**

To determine how rapidly LF5p becomes proximally localized during flagellar assembly, we examined cells undergoing flagellar regrowth after deflagellation (Figure 5). At 15 min after pH shock, when the regenerating flagella were 3.4 μm long on average (~30% of predeflagellated length), we could detect proximal staining. However, 100% of the flagella also showed punctate staining along the flagella during this stage, probably reflecting the ongoing deposition of LF5p into the flagellum (Figure 5, A–C). As the flagella continued to grow, the proximal stain became brighter (Figure 5, D–F) and the percentage of flagella with punctate staining along the length in addition to proximal staining decreased to 82.1, 45.4, and 14.8% at 30, 60, and 120 min, respectively.

Disassembly of flagella can also be induced by calcium chelators such as 20 mM sodium pyrophosphate (Lefebvre et al., 1978). Cells treated with pyrophosphate shorten their flagella, with ~50% of the flagellar length resorbed into the cell within 60 min of treatment. We examined the localization of LF5p in cells treated with pyrophosphate during a 2-h period in which the cells gradually resorbed their flagella. The proximal staining of LF5p remained prominent throughout resorption, independent of the length of the flagella (Figure 5, G–L). Unlike growing flagella, LF5p staining was infrequently observed along the length of resorbing flagella.

**Proximal LF5p localization is not affected by many flagellar mutations**

To determine whether LF5p might be associated with any of the known axonemal structures, we localized LF5p using indirect immunofluorescence in mutants missing or defective in radial spokes (pf14, pf27; Luck et al., 1977; Huang et al., 1981; Diener et al., 1993), the central pair (pf15, pf16, pf19, pf20; Witman et al., 1978; Dutcher et al., 1984; Smith and Lefebvre, 1996; 1997; Dynak and Smith, 2012), inner dynein arms (pf9, pf23, ida5; Piperno et al., 1990; Mastronarde et al., 1992; Kato-Minoura et al., 1997; King and Dutcher, 1997; Myster et al., 1997), and outer dynein arms (pf28; Kamiya, 1988), as well as the dynein regulatory complex (pf3; Piperno et al., 1992; Gardner et al., 1994). Bright proximal staining of LF5p was observed in all these cases, suggesting LF5p is not part of these structures (representative examples for each type of axonemal mutant are shown in Figure 6A). Proximal localization of LF5p also was observed in mbo2, which lacks doublet microtubule-specific beam projections residing in the proximal one-third of flagella (Segal et al., 1984). These results suggest that LF5p does not associate with axonemal structures we analyzed here, which all occur with
FIGURE 6: Immunofluorescence microscopy shows proximal localization of LF5p in various flagellar mutants. Green, LF5p; red, acetylated α-tubulin. (A) LF5p localization in various axonemal mutants defective in (a–c) radial spokes (pf14), (d–f) central pair microtubules (pf20), (g–i) inner-arm dyneins (pf23), (j–l) outer-arm dyneins (pf28), (m–o) dynein regulatory complex (pf3), and (p–r) beak projections (mbo2). (B) LF5p was localized to the proximal ends of flagella in (a–c) ida5 and (d–f) fa2 mutants, whose flagella lack DHC11 and FA2 proteins, respectively. Both DHC11 and FA2 have been shown to be localized to proximal ends of flagella, but their absence did not affect the localization of LF5p. LF5p localization also appeared normal in IFT mutants (g–i) fla11 and (j–l) fla14, which have been shown to overaccumulate IFT proteins in their flagella. Bar, 10 μm.
regular periodicity along the axonemes. It is possible that LF5p associates directly with the doublet microtubules of the axoneme.

Two Chlamydomonas proteins have been localized to the proximal regions of flagella. DHc11, an inner-arm heavy-chain dynein, was localized to the proximal 2 μm of flagella (Yagi et al., 2009). In the ida5 mutant, which does not assemble DHc11 in its flagella, localization of LF5p to the proximal ends of flagella was indistinguishable from the localization seen in WT flagella (Figure 6B, a–c; FA2, which encodes a NIMA kinase important for calcium-dependent delflagellation, localizes tightly to the proximal end of the flagella, as well as around the basal bodies (Mahjoub et al., 2004). We examined both fa1 and fa2 mutants, which are defective in the same pathway, but did not detect any change in LF5p localization (Figure 6B, d–f). At least two proximally localized axonemal proteins therefore play no role in proximal localization of LF5p.

Flagellar assembly and disassembly in Chlamydomonas requires intraflagellar transport (IFT), and in mutants with defects in IFT the localization of flagellar proteins can be altered (e.g., Pazour et al., 1998; Pedersen et al., 2005). We examined localization of LF5p on the flagella of two IFT mutants with defective IFT. The flα11 mutant has a genetic lesion in IFT172 and is believed to have defects in the remodeling of IFT particles at the flagellar tip to prepare for the change from anterograde to retrograde transport (Pedersen et al., 2005). The flα14 mutant is defective in the dynein light chain LC8, and as a result of defects in the cytoplasmic dynein complex flα14 lacks retrograde IFT (Pazour et al., 1998). Indirect immunofluorescence localization showed proximal localization of LF5p in both mutants, indistinguishable from the localization in the flagella of WT cells (Figure 6B, g–i). Although IFT might be involved in the transport of LF5p to the flagella, this result suggests that IFT, particularly retrograde IFT, probably has no direct regulatory role in the proximal localization of LF5p in flagella.

LF5p proximal localization is affected by mutations in LF1, LF2, and LF3

Previous studies showed that LF1p, LF2p and LF3p reside in a protein complex, the LRC, in the cell body (Tam et al., 2007), whereas LF4p is present in both the cell body and the flagella (Berman et al., 2003). To assess the effect of mutations of these four LF genes on the localization of LF5p, we performed immunofluorescence studies with the LF5 antibody on various If mutant alleles, including If1-1, If2-1, If2-2, If2-3, If3-2, and If4-9. It is striking that the proximal localization of LF5p was greatly diminished or absent in various mutant alleles of LF1, LF2, and LF3. Instead, in these mutants, bright staining of LF5p was often observed at the distal tips of flagella (representative examples are shown in Figure 7, A–P). Proximal and distal staining were not mutually exclusive—both could be seen on the same flagellum (Figure 7, E–H). In addition, punctate staining was observed along some flagella (Figure 7, M–P). In contrast, LF5p remained localized predominantly to the proximal ends of flagella in If4-9, although punctate staining also appeared to be present along the length (Figure 7, Q–T). We quantified the percentage of flagella showing different LF5p localization: proximal, distal, both proximal and distal, or only along the entire length of flagella (Table 2). Although the localization patterns were variable among different LRC mutants, all of them showed a striking shift of LF5p staining from proximal to distal ends of flagella. These results raise the interesting possibility that LF5p is a target of the LRC, perhaps regulating flagellar length, at least in part, by regulating the localization of LF5p in flagella.

The possibility that the proximal localization of LF5p is critically important for its function in length control is supported by experiments to localize the protein during mating. When Chlamydomonas cells mate, the flagella adhere to each other, leading within ∼10 min to cell fusion to form a single cell with four flagella and two nuclei, known as a dikaryon. When If mutants are mated to WT cells, the long flagella are resorbed to WT length by 90 min, indicating that flagellar length control is a dynamic and regulated process and that the If mutations are recessive (Barsel et al., 1988). We localized LF5p in flagella before and at different time points after mating If1 mutants to WT cells. In flagella of If1 gametes before mating, most labeling using the LF5 antibody was not at the proximal region but at the distal tip or along the length of the flagella (92% of cells). At 15 min after mating, 32% of mating dikaryons still showed two flagella with proximal labeling (the WT flagella) and two flagella with label along the length or at the distal tip (the If1 flagella; Figure 8A, a–c). However proximal labeling was clearly observable on all four flagella in 40% of dikaryons (Figure 8A, g–i). In addition, 28% of dikaryons also showed partial relocalization of LF5p to one of the two long flagella that originate from the If1 parent (Figure 8A, d–f). The percentage of dikaryons showing four proximal spots increased to 60 and 73% at 30 and 45 min, respectively. Of importance, the movement of LF5p to the proximal end of the long flagella preceded the complete resorption of the flagella to WT length. Although striking relocalization of LF5p was observed as early as 15 min after mating, the length of the longer pair of flagella in these dikaryons was largely indistinguishable from the length of the flagella of the If1 parent (Figure 8B). The kinetics of movement of the LF5 protein to the proximal end before flagellar resorption indicates that the proximal labeling is not a consequence of restoration of length control and might, in fact, be causal for resorption.

DISCUSSION

What does the proximal localization of LF5p tell us about flagellar length control?

LF5p is unique among the proteins known to be involved in flagellar length control in its localization to the very proximal end of the flagella. Of interest, the function of the other LF gene products identified as components of the LRC might be involved in LF5p localization. Proximal localization of LF5p is either reduced or eliminated by the defects in If1, If2, or If3 mutants. The fact that If1, If2, and If3 mutants cause LF5p to be mislocalized along the length and even at the distal end of flagella suggests that one role of the LRC might be to restrict LF5p to the proximal end of the axoneme. It is unlikely that the sole role for the LRC proteins is to control LF5p localization, however, because If1, If2, and If3 mutants are all signifi cantly longer than If5 mutants (Barsel et al., 1988). One possible role for a proximally located kinase might be to regulate entry of proteins into the flagella, including length control proteins such as LF4p. A kinase at the proximal end of the flagella could also be part of a length-sensing mechanism. If, for example, phosphorylation of some unidentified sensor protein occurs as it enters the flagella and dephosphorylation of such a protein by phosphatases occurs at a constant rate as it is transported to the distal end, then the phosphorylation state of this protein could be used to inform an assembly-regulating mechanism at the tip of the length of the flagella. A potential candidate protein whose phosphorylation state is regulated by flagellar length has been identified (Luo et al., 2011). The Chlamydomonas flagellar Aurora-like kinase CALK exists in either a phosphorylated or a nonphosphorylated form, and the phosphorylation state appears to be directly related to flagellar length. When flagella are ≤6 μm in length, CALK is phosphorylated, but longer flagella...
contain only nonphosphorylated CALK. If phosphorylated CALK is itself involved in regulating a protein essential for flagellar growth, presumably by phosphorylation, then CALK and LF5p, along with LF4p, might work together to tightly regulate flagellar length.

Although the placement of LF5p at the proximal end of the flagella suggests that regulation of flagellar length occurs at that end, there are a number of reasons to predict regulation at the distal tip as well. Assembly of both tubulins, the major structural proteins of the axoneme (Witman, 1975), as well as of other flagellar proteins (Johnson and Rosenbaum, 1992), occurs at the tip. Anterograde IFT has been shown to be required for transport of flagellar proteins to the flagellar tip for assembly (reviewed by Scholey, 2003; Qin et al., 2004). Proteins at the flagellar tip have been implicated in remodeling the IFT protein machinery for the transition from anterograde to retrograde transport (Pedersen et al., 2005), and this remodeling and its implications for control of assembly have been used as the basis for a model for control of flagellar length (Engel et al., 2009). The movement of LF5p from the proximal to the distal end of the flagella in \( \text{lf1} \), \( \text{lf2} \), and \( \text{lf3} \) mutants might play a role in the assembly of flagella of excessive length, or it could be a consequence of loss of flagellar length control. Movement of LF5p to the distal end is not a requirement for assembling aberrantly long flagella, however, because LF5p remains at the proximal end of long flagella in \( \text{lf4} \) mutants.

We do not yet know how LF5p localization is restricted to the proximal end of the flagella of WT cells. The protein could be actively transported (from the tip?) to the proximal end and retained there by binding to a proximally localized binding target or it could simply bind to the proximal end soon after the protein enters the flagella. The former possibility is consistent with the observation (Figure 5) that during flagellar regeneration LF5p is initially localized along the length of the flagella and then later becomes exclusively localized to the proximal end. Also supporting this active movement is the observation that when mutants with long flagella, in which LF5p is localized at the distal tip or along the flagellar length, are mated to WT cells, LF5p rapidly moves to the proximal end of the flagella, even before the long flagella are restored to WT length (Figure 8A). It is surprising, however, that retrograde IFT is not an absolute requirement for localization of LF5p to the proximal end of the flagella.

**FIGURE 7:** Immunofluorescence microscopy shows altered LF5p localization in \( \text{lf1} \), \( \text{lf2} \), and \( \text{lf3} \) mutants. Green, LF5p; red, acetylated \( \alpha \)-tubulin. (A–H) LF5p staining was often present at the distal tips of flagella in \( \text{lf1-1} \) mutants. Sometimes both distal and proximal LF5p staining could be seen on the same flagellum. Arrowheads in E–G point to the dual fluorescent spots of LF5p on a single flagellum. (I–L) \( \text{lf2-1} \) is a severe allele that is not expected to produce any functional LF2p and often has a pair of flagella that greatly differ in length. LF5p staining was present on the distal tips of the two unequal-length flagella on one cell but on the proximal ends of another pair of flagella on another cell. In both \( \text{lf1-1} \) and \( \text{lf2-1} \) mutant flagella, the distal ends are often swollen, as indicated by the arrowhead in H. (M–P) In the \( \text{lf3-2} \) mutant, LF5p also localized abnormally to the distal ends of flagella (arrowheads). Punctate staining of LF5p could be seen on these flagella. (Q–T) LF5p localization remained predominantly proximal in \( \text{lf4-9} \) mutants. More punctate staining was observed along the length of flagella in \( \text{lf4-9} \) cells compared with WT cells (see Figure 4B). Bar, 10 μm.
The role of protein kinases in flagellar length control

The characterization of a new protein kinase associated with flagellar length control adds to a growing realization that this control is a highly regulated and complex process involving numerous cell-signaling inputs. Not only have three kinases (LF2p, LF4p, and LF5p) been shown directly by mutation to be involved in length control, but also several other kinases have been shown directly by mutation to be involved in length control, which results in increased cell volume and increased flagellar length, up to 40%, accompanied by increased cell volume (Bradley and Quarby, 2005).

A unique feature of LF5p relative to the other length control genes is that flagellar length appears to still be regulated in LF5 mutants but to a longer length than WT. Whereas mutants in $lf1$, $lf2$, $lf3$, and $lf4$ have a broad distribution of flagellar lengths, from shorter than WT to two to three times WT, a population of $lf5$ cells has a tighter distribution of flagellar lengths centered around a point ~50% longer than the flagella length of WT cells (Figure 1). From the length distribution curves it appears that mutants in the other LF genes lose flagellar length control but $lf5$ mutants retain it, although the target length is increased relative to WT. The intriguing possibility raised by this observation is that LF5p might be involved in setting the appropriate flagellar length as opposed to enforcing length control.

The majority of LF5p is tightly associated with doublet microtubules in an NP-40-insoluble fraction. Given that we cannot find evidence for association of the protein with any known axonemal structures, such as dynein arms or radial spokes, the interesting possibility exists that LF5p binds directly to the doublet microtubules of the axoneme, localizing to only the proximal 1 μm by an as-yet-unknown mechanism. Clearly the proteins of the LRC in some way help localize LF5p to the proximal end of the axoneme, as $lf1$, $lf2$, and $lf3$ mutations cause LF5p to localize along the length of the axoneme and at the distal tip. Perhaps these proteins modify LF5p, most likely through the kinase activity of LF2p, to make it bind to its proximal location. Alternatively, they could modify the proximal end of the axoneme to provide a binding site for LF5p.

The role of protein kinases in flagellar length control

Because in the flagella of $fla11$ and $fla14$, which have defective retrograde IFT, LF5p is still localized to the proximal end.

Another protein kinase in Chlamydomonas, the NIMA-related kinase CRK2p, was implicated in flagellar length control by experiments in which levels of the kinase were reduced by RNA interference. Reduced levels of CRK2p resulted in increased flagellar length, up to 40%, accompanied by increased cell volume (Bradley and Quarby, 2005).

### TABLE 2: Percentage of cells with different localization pattern of LF5p.

|       | Proximal only | Distal only | Proximal and distal | Along flagella only |
|-------|---------------|-------------|---------------------|---------------------|
| WT    | 100/98.1      | 0/0         | 0/1.9               | 0/0                 |
| $lf1$-1 | 1.8/0        | 65.5/77.8   | 32.7/20.4           | 0/1.9               |
| $lf2$-1 | 3.4/0        | 44.3/56.3   | 49.2/40.0           | 3.5/3.6             |
| $lf2$-5 | 10.4/6.8     | 37.9/44.4   | 46.5/42.6           | 5.2/5.6             |
| $lf3$-2 | 0/0          | 90.2/88.0   | 7.8/2.0             | 2.0/10.0            |
| $lf4$-9 | 96.4/83.6    | 0/0         | 3.6/16.4            | 0/0                 |

Numbers denote percentage of flagella with one of the four localization patterns from two different experiments. Punctate staining might also be present with the first three localization patterns. Between 50 and 61 flagella were scored.

Control experiments in which levels of the kinase were reduced by RNA interference. Reduced levels of CRK2p resulted in increased flagellar length, up to 40%, accompanied by increased cell volume (Bradley and Quarby, 2005).

### FIGURE 8: Relocalization of LF5p to the basal region of $lf1$ flagella in dikaryons precedes rescue of flagellar length. (A) Immunofluorescence images showing different patterns of LF5p staining in dikaryons with a pair of shorter and a pair of longer flagella during a cross of $lf1$ to WT gametes. Green, LF5p; red, α-tubulin. Arrowheads point to the position of LF5p determined by immunofluorescence. (a–c) No relocalization, with only two proximal stained spots on the shorter pair of flagella from the WT parent; (d–f) incomplete relocalization, with proximal staining on the shorter pair of flagella and one of the pair of long flagella, with distal staining on the other long flagellum; (g–i) complete relocalization, with four proximal stained spots on all four flagella. One pair of flagella is longer than the other pair. Bar, 10 μm. (B) Flagellar length distribution of the parental WT and $lf1$ cells and dikaryons at different times after mating. Because two measurements were made for each dikaryon and only one per single cell, the data for the WT and $lf1$ cells were halved so that the heights of the graphs are comparable. In dikaryons, the flagella from WT and $lf1$ cells are clearly distinguishable, as indicated by the bimodal distribution pattern. At 15 min after mating, the longer flagella in dikaryons are similar in length to those of the $lf1$ parent. The mutant pair of flagella gradually shortened in dikaryons over time but had not completely resorbed to WT length even at 45 min.
Possible role of CDKL5 in juvenile epilepsy
A role for CDKL5 in human brain development is suggested by the association of epileptic seizures in early childhood with defects in this protein. It is not known how defects in CDKL5 lead to juvenile epilepsy. CDKL5 in human cells localizes to both the nucleus and the cytoplasm (Rusconi et al., 2008), with nuclear localization being associated with speckles and therefore perhaps with the splicing machinery (Ricciardi et al., 2009). Human CDKL5 has been shown to interact with a number of other proteins, including DNA methyltransferase I protein (Dnmt1), which in turn has been shown to interact with the methyl-cytosine-binding protein MeCP2 (Kameshita et al., 2008). The gene for MeCP2 is the gene affected in the majority of cases of Rett syndrome, a form of juvenile epilepsy with similar features to CDKL5 disease (reviewed by Grillo et al., 2012). Another protein interacting with CDKL5 is Rac1, an important protein in actin remodeling and neuronal patterning (Chen et al., 2010). Inhibition of Rac1 in tissue culture cells has been shown to alter the localization of the basal bodies that subtend the cilia (Hashimoto et al., 2010), suggesting a possible link between human CDKL5 and cilia. Although there is as yet no evidence that CDKL5 is a ciliary protein in any system besides Chlamydomonas, a ciliary localization could help clarify the unexplained role of CDKL5 mutations in causing juvenile epilepsy. A suggestion that a different form of epilepsy, juvenile myoclonic epilepsy, might be associated with defects in the ciliary axoneme also came from studies in Chlamydomonas. Genetic studies indicated that juvenile myoclonic epilepsy is associated with the EFHC1 gene, which structural analysis suggested was a closed structural homologue of the Chlamydomonas Rlb272 gene, which encodes a protein required for the assembly of the flagellar axoneme (King, 2006). The possibility that CDKL5 could be a ciliary protein in humans would add another set of diseases—juvenile epilepsies—to the growing list of diseases whose etiology appears to be associated with defective cilia and flagella, diseases that have been termed “ciliospathies” (Badano et al., 2006; Berbari et al., 2009).

MATERIALS AND METHODS
Strains, culture conditions, and genetic analysis
Strains obtained from the Chlamydomonas Resource Center (University of Minnesota, St. Paul, MN) are as follows: CC-1690 (21gr), CC-125 and CC-621 (WT), CC-1952 (polymorphic strain S1 D2), CC-1678 (lf1-1), CC-803 (lf2-1), CC-2287 (lf2-5), CC-2289 (lf3-2), CC-1026 (pf3), CC-1032 (pf14), CC-1033 (pf15), CC-1038 (pf20), CC-1877 (pf28), CC-1383 (pf23), CC-3421 (ida5), CC-3898 (pf9), CC-1370 (fa1), CC-3751 (fa12), CC-1920 (fla11), CC-3937 (fla14), CC-2377 (mbo2), and CC-4162 (uni2-3). Cells were grown in liquid M or TAP medium under continuous light. 3F12 and D12 (lf4-9) were obtained by DNA insertion into the genome of strain 21gr. D KD6 was a gift from Karl Johnson (Haverford University, Haverford, PA). Genetic crosses to determine allelism were performed using standard methods to produce stable diploids (Levine and Ebersold, 1960), and progeny were scored for motility and flagellar length phenotype using a Zeiss DR-C stereomicroscope (400x magnification; Carl Zeiss, Jena, Germany) and a Zeiss Standard phase contrast microscope (400x magnification), respectively. For physical mapping, strain 3F12 was crossed to polymorphic strain S1 D2 (Gross et al., 1988). Only one progeny from each tetrad was picked for PCR analysis, so that each represented independent recombination events. Primers used for mapping (Supplemental Table S1) were from Kathir et al. (2003) and Rymarquis et al. (2005) or were developed in this project.

DNA and RNA analysis
Chlamydomonas DNA and Southern blot analysis were performed as described (Tam and Lefebvre, 1993). Chlamydomonas genomic libraries in bacteriophage lambda vectors were constructed using λFixII/Xhol kit (Agilent Technologies, Santa Clara, CA), using MboI partially digested DNA from 21gr or 3F12. For physical mapping of LF5, PCR was performed using Failsafe enzyme with premix K (Epizentrum Biotechnologies, Madison, WI) on DNA prepared with DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Primer sequences for mapping and PCR amplification of LF5 are given in Supplemental Table S1.

Total RNA and poly(A+) RNA were prepared as described (Nguyen et al., 2005; Tam et al., 2007). Then 25 μg of total RNA or 5 μg of poly(A+) RNA was size fractionated on 1% formaldehyde gels and transferred to Brightstar Plus membrane (Ambion, Austin, TX) and hybridized successively with 32P-labeled DNA from the entire LF5 coding region (amplified from cDNA using 3830-5’ and 3830-3’ primers) or from CRY1. Radioactivity on membranes was quantified by exposing them to a phospho screen, which was scanned with a STORM 840 Imager and analyzed with ImageQuant v1.2 (Molecular Dynamics, Sunnyvale, CA).

Cloning of LF5
A bacteriophage lambda library prepared with DNA from WT cells was screened with a LF5 PCR probe amplified from a cDNA library using primers 3830-F1 and 3830-R2. The resulting clones were tested for their ability to rescue lf5 mutants by cotransformation with pSI103 (Sizova et al. 2001). A 16-kb BamHI-XbaI fragment from a lambda clone was subcloned into pBluescript II KS+ to produce plasmid pBS3830. To determine the insertion in 3F12, we screened a lambda library of 3F12 with the same probe. To verify the mRNA sequence, we screened a Chlamydomonas cDNA library (G2, from Chlamydomonas Resource Center) with a PCR probe made with 3830-F3 and R3. Six overlapping clones covering the entire coding region were recovered and sequenced.

Preparation of antibodies to LF5p
The entire coding region of LF5 was amplified by PCR from a cDNA clone using 3830-5’ and 3830-3’ primers and Pufo Ultra DNA polymerase (Agilent Technologies) and cloned into pCR8/GW/TOPO (Invitrogen, Carlsbad, CA). After the sequence was verified, the cDNA was recombinated using LR clonase (Invitrogen) into expression vector pMCSCG19C (Donnelly et al., 2006) or pMAL, each of which was made Gateway-cloning compatible by the insertion of the conversion cassette (a generous gift from Neil Olszewski, University of Minnesota, St. Paul, MN). Affinity-purified maltose-binding protein/LF5p or insoluble histidine-tagged LF5 fusion protein run and excised on acrylamide gel was used to immunize rabbits, UMN182 and UMN183, respectively. Both antibodies recognized the same protein bands on Western blots, but only UMN183 was used for subsequent studies, because its titer was higher. For affinity purification, maltose-binding protein/LF5 fusion protein was covalently linked to amylose resin in a column to purify UMN183. For Western analysis, flagella were isolated as described in Tam et al. (2007). For fractionation studies, purified flagella were first extracted with 0.5% NP-40 in HMEDNa (10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid [HEPES], pH 7.0, 5 mM MgSO4, 0.5 mM EDTA, 1 mM dithiothreitol, 30 mM NaCl) supplemented with 1/100 volume of protease inhibitor cocktail (p-8340; Sigma-Aldrich, St. Louis, MO), separated into soluble and insoluble fractions. The insoluble fraction was extracted with either 0.5 M NaCl or 0.6 M KI in HMEDNa before
separating into soluble and insoluble fractions. Equivalent amounts of each fraction corresponding to 37.5 μg of flagella were electrophoresed on 8% acrylamide gels and transferred to polyvinylidene fluoride membrane. Affinity-purified UMN183 and goat anti-rabbit horseradish peroxidase–conjugated secondary antibody (Sigma-Aldrich) were used at 1:250 and 1:22,000, respectively. Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ) were used as the chemiluminescent reagents.

**Light microscopy and flagellar length measurements**

Cells were fixed in 1% glutaraldehyde and visualized with differential interference microscopy (DIC; Diaplan; Leica, Wetzlar, Germany) with a 100×/numerical aperture 1.25 objective (Leica; Leica) using a digital camera (ProRes MAC, Jenoptik, Jena, Germany) and ProRes MAC CapturePro software. Images were opened with Image J, version 1.45s (National Institutes of Health, Bethesda, MD), and flagellar length was measured using the segmented line tool. For deflagellation experiments, cells were transferred to M medium and induced to shed their flagella by adding 0.005 volume of 1 M acetic acid to cells. As soon as deflagellation was complete, as verified by microscopy, 0.005 volume of 1 M potassium hydroxide was added to neutralize the medium. Cells were harvested by centrifugation and resuspended in fresh M medium to allow for flagellar regrowth. Regenerating cultures were sampled at different times and fixed for flagellar length measurement.

**Immunofluorescence microscopy**

For immunofluorescence, cells were treated with autolysin for 1 h to remove cell walls, transferred to a solution of 10 mM HEPES, 5 mM MgSO$_4$, 0.5 mM ethylene glycol tetraacetic acid (EGTA), and 25 mM KCl, and allowed to settle on polyethyleneimine-coated printed slides. The slides were dipped in methanol twice for 10 min at −20°C. Air-dried cell samples were blocked in blocking buffer (5% glycerol, 10% normal goat serum, 1% bovine serum albumin, 1% cold water fish gelatin, 5% dimethyl sulfoxide in phosphate-buffered saline) for 30 min at 37°C. Primary antibodies were affinity-purified UMN183 at 1:100 dilution and 6-11-B1 (monoclonal acetylated α-tubulin; a gift from G. Piperno [Mt. Sinai Medical School, New York, NY]) at 1:50 dilution. Secondary antibodies were goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR) and goat anti-mouse Texas red (ICN-Cappel) or goat anti-mouse Alexa Fluor 568 (Molecular Probes), all at 1:400 dilution in blocking buffer. Samples were mounted in SlowFade (Invitrogen). DIC and fluorescence images were captured with the same microscope and software setup described for light microscopy and assembled and adjusted with Photoshop CS5 (Adobe, San Jose, CA).

**Dikaryon analysis**

To make gametes, we resuspended ift1 (CC-1678) and WT (CC-125) cells in 1–2 ml of autolysin made in minimal medium without nitrogen and incubated them under lights for 4 h. To start the mating reactions, we mixed together 200-μl samples of each gamete culture in a microfuge tube. At the indicated times, the reactions were mixed together 200-μl samples of each gamete culture in a microfuge tube. At the indicated times, the mating cultures and resuspended in 10 mM HEPES, 5 mM MgSO$_4$, 0.5 mM EGTA, and 25 mM KCl, mounted on polyethyleneimine-coated coverslips, and processed for immunofluorescence as described. Samples of the mating cultures and parent strains were fixed in glutaraldehyde for flagellar length measurement. For each parent cell and each dikaryon, the length of one flagellum of each pair of flagella was measured. If the two flagella of a cell were different in length, as in some cases with ift1, the longer flagellum was measured.

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