Table S1. Reproducibility and Significance of Flagellar Length Differences at 90 minutes Post pH Shock Between Wild-Type and ida5 Mutant Cells

Experiment #	T-test p-value
1	1.9175E-08
2	5.2717E-11
3	5.8328E-07
4	1.1081E-04
5	3.3682E-10
6	3.2542E-10
7	9.3871E-04
8	3.5931E-05
9	7.0468E-04

Supplemental Experimental Procedures

Compounds

LatrunculinB, Arp2/3 inhibitor CK-666 and inactive analog CK-689 were purchased from Sigma and used at indicated concentrations. Formin inhibitor SMIFH2 and inactive analog KVSM18 were a generous gift from David Kovar at the University of Chicago. Myosin II inhibitor blebbistatin and inactive enantiomer were purchased from Tocris Bioscience. Myosin V inhibitor, myoVin-1, was used at 200μM and was purchased from Millipore. Unless otherwise indicated, compounds were incubated with cells for two hours.

Strains

ida5 mutants and strains expressing KAP-GFP on a KAP (*fla-3*) mutant background were previously described [S9-S11] and were obtained from the *Chlamydomonas* stock center as CC-3420 and CC-4296 respectively. The *lf4v86* mutant was obtained from George Witman and Greg Pazour and reported previously [S12]. KAP-GFP/*fla-3/ida5* and *lf4-ida5* triple and double mutants were generated by mating. Progeny were screened by PCR and/or sequencing to determine genotype.

Inhibitor Treatment and Flagellar Length Measurements

Wild-type CC125 *Chlamydomonas* cells were grown in liquid TAP medium for 24 hours prior to 2 hour incubation with various inhibitor concentrations. For pretreatment experiments, cells were pre-treated for 30 minutes prior to incubation in 25mM LiCl for 1.5 hours. Cells were fixed in 1% glutaraldehyde and imaged by DIC microscopy at 40x magnification. Flagellar lengths were measured following line segment tracing and spline fitting in ImageJ.

Flagellar Regeneration

Flagellar regeneration was induced by deflagellating cells via pH shock. 60μl 0.5N acetic acid followed by 60μl 0.5N KOH were added to 1ml cells in liquid TAP (45 second incubation at low PH). Cells were fixed with 1% glutaraldehyde at 0,15, 30, 45, 60, 90, 120, and 240 minutes or every 30 minutes post-pH shock. Flagella were measured as described above. Double deflagellation experiment for evaluation of flagellar precursor pool was performed as described previously [S9]. Briefly, cells were pH shocked once to remove flagella, and then pH

shocked a second time at varying time points but regrown in cycloheximide to determine the amount of protein was available for flagellar incorporation at various time points. Unlike previously published protocol, cells were allowed to regrow flagella for three hours rather than two hours in cycloheximide following second pH shock because of slow *ida5* regeneration.

IFT Injection Analysis

KAP-GFP expressing cells were imaged live on a Nikon Ti-E microscope using a 100x 1.49 NA TIRF oil objective. Images were taken with an Andor DU897 EMCCD camera at 27.22fps with 0.16μm per pixel. Kymographs were generated using NIS-Elements software (v4.1) and analyzed using custom software in Matlab as described previously [S2]. Briefly, to measure injection sizes relative to flagellar length from TIRF data, lengths of flagella were first measured from single frames from movies. Kymographs generated from frames captured of IFT trains were used to generate smoothed time series using custom MATLAB software (background subtraction included a photobleaching component). The injection sizes were found by determining the area under the time series peaks and normalizing by minimum peaks to eliminate noise.

KAP-GFP Quantification

Cells expressing KAP-GFP or KAP-GFP on an *ida5* mutant background were pH shocked and fixed in 100% methanol 2x 5 minutes immediately and at 30 minutes, 60 minutes, 3 hours, 8 hours, and 24 hours following pH shock. Samples were imaged at 100x on a Deltavision microscope with a 0.2μm z-step. Raw TIFF images were analyzed using custom MATLAB software as described previously [S2] to determine the fluorescence area at the flagellar base.

Fluorescence microscopy of Lifeact-Venus and Myo2-Venus

The Venus-tagging plasmids were constructed based on pLM005, which contains the paromomycin-resistance gene AphVIII (expressed from the HSP70A/RBCS2 promoter) and Venus-FLAG that has been codon-optimized for *Chlamydomonas* (expressed from the PsaD promoter). Details of construction of this vector will be described elsewhere (Mackinder and Jonikas, unpublished data). To fuse Lifeact at the N-terminus of Venus, a coding sequence of Lifeact (codon-optimized for *Chlamydomonas*) was generated by dimerizing the

oligonucleotides 5'-GCTACTCACAACAAGCCCAGTTATGGGCGTGGCTGACCTGATCAAGAAGTTCGAGTCGAT-3' and 5'-GAGCCACCCAGATCTCCGTTCTCCTCCTTGCTGATCGACTCGAACTTCTTGATCAGGTCA-3' and then integrated into HpaI-digested pLM005 using Gibson Assembly Master Mix (New England Biolabs), yielding pMO424-Lifeact-Venus. Similarly, the genomic sequence encoding MYO2 (Cre09.g416250) was PCR-amplified from purified genomic DNA of strain CC-4533/CMJ30 using oligonucleotides 5'-GCTACTCACAACAAGCCCAGTTATGACCGAAGGCCTAGCGGGCCTTGGGCGT-3' and 5'-GAGCCACCCAGATCTCCGTTCTTGCCCCCGCTAAACAGTTTGCCCAACAG-3' and then integrated into pLM005, yielding pMO431-Myo2g-Venus (Venus at C-terminus). The plasmids were digested with EcoRV (pMO424) or ScaI (pMO431) and transformed into CC-124 by electroporation. Venus-positive cells were cultured in TAP + 1 µg/ml paromomycin, mounted between TAP + 1% low-melting-agar (SeaPlaque; FMC Corporation) pad and a coverslip, then observed using the Nikon Eclipse 600-FN microscope equipped with an Apochromat 100x/1.40 NA oil immersion objective lens, an ORCA-2 cooled CCD camera (Hamamatsu Photonics), and Metamorph version 7.0 software (Molecular Devices). Images were post-processes using the Metamorph, Photoshop (Adobe), and/or ImageJ (National Institutes of Health) software.

Homology Modeling and Docking Protein sequences for the three putative *Chlamydomonas* myosins were taken from the Phytozome database (http://www.phytozome.net): Myo1/Cre16.g658650.t1, Myo3/Cre13.g563800.t1, and Myo2/Cre09.g416250.t1 [S13]. The alignments for the construction of the *Chlamydomonas* myosins models were generated using PROMALS3D [S5], and homology models were built with MODELLER 9v8 [S14], using the crystal structure of *Dictyostelium* myosin II in complex with (-)-blebbistatin (PDB ID: 1YV3) as the template (see Figure S4 for full alignment including Uniprot [S15] identifiers for other myosins referenced in [6, 7]). Modeled sequences were truncated to only the relevant portion of myosin involved in (-)-blebbistatin binding. We used DOCK 3.7 [S16] to dock (+)- and (-)-blebbistatin into the binding site of each *Chlamydomonas* myosin, as in previously published protocols [S17]. The flexible-ligand sampling algorithm in DOCK 3.7 uses a graph-matching technique to superimpose atoms of the docked molecule onto binding site matching spheres, which represent favorable positions for individual ligand atoms. Complementarity of each ligand pose is scored as the sum of the receptor-ligand electrostatic and van der

Waals interaction energy and corrected for context-dependent ligand desolvation. Individual ligands were sampled until 20,000 favorable conformations were found and scored.

Precursor Pool Quantification

In order to determine if the basal body accumulation impairment of IFT machinery in the *ida5* mutant is due to a defect in generation of flagellar precursor protein, we performed a double deflagellation experiment to evaluate the amount of flagellar precursor present in wild-type and *ida5* cells at various points during regeneration [S18]. Quantification in this experiment, an initial deflagellation removes all material incorporated in flagella. At various points during flagellar regeneration, cells are deflagellated again and allowed to grow in cycloheximide to inhibit protein synthesis. The length of flagella prior to the second deflagellation represents the amount of protein incorporated into flagella. The length of flagella following the second deflagellation reflects how much flagellar precursor protein existed within cells that was not incorporated into flagella. Therefore the total flagellar protein in the cell is the amount incorporated in the flagella plus the amount expressed but not incorporated into flagella. Unlike previously published protocols, we allowed flagella to grow for an additional hour in cycloheximide (3 hours total) because *ida5* initial assembly rate is slower than wild-type cells. The double deflagellation experiment can also be used to extrapolate the amount of new flagellar protein synthesis during regeneration. This value is calculated by subtracting the amount of protein in the unassembled precursor pool at post deflagellation time=0 (when no new synthesis has taken place) from the total amount of flagellar protein (incorporated plus unassembled). It is important to note that flagellar lengths are only a readout for the amount of protein that is limiting. That is, an excess of flagellar protein may exist beyond what is assembled into flagella after each deflagellation but the length of flagella is restricted by the amount of the least abundant protein required for flagellar assembly.

Vector design and construction and transformation of *ida5*

A plasmid “pKF18-CrAct”, which contains the whole conventional *Chlamydomonas* actin gene in the pKF18k-2 vector (TaKaRa), was kindly provided from Dr. Haru-aki Yanagisawa (University of Tokyo). A *Chlamydomonas* GFP tag was introduced to the ScaI site in the last exon of the actin gene in “pKF18-CrAct”. In addition, for selection, a paromomycin resistance cassette, AphVIII, was introduced to the EcoRI site and the vector

referred to as “pKF18-CrAct-GFP-AphIII”. This vector was introduced to the *ida5* strain using the electroporation method to obtain the rescued strain *ida5*-GFP (F1), expressing GFP tagged conventional actin.

Supplemental References

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