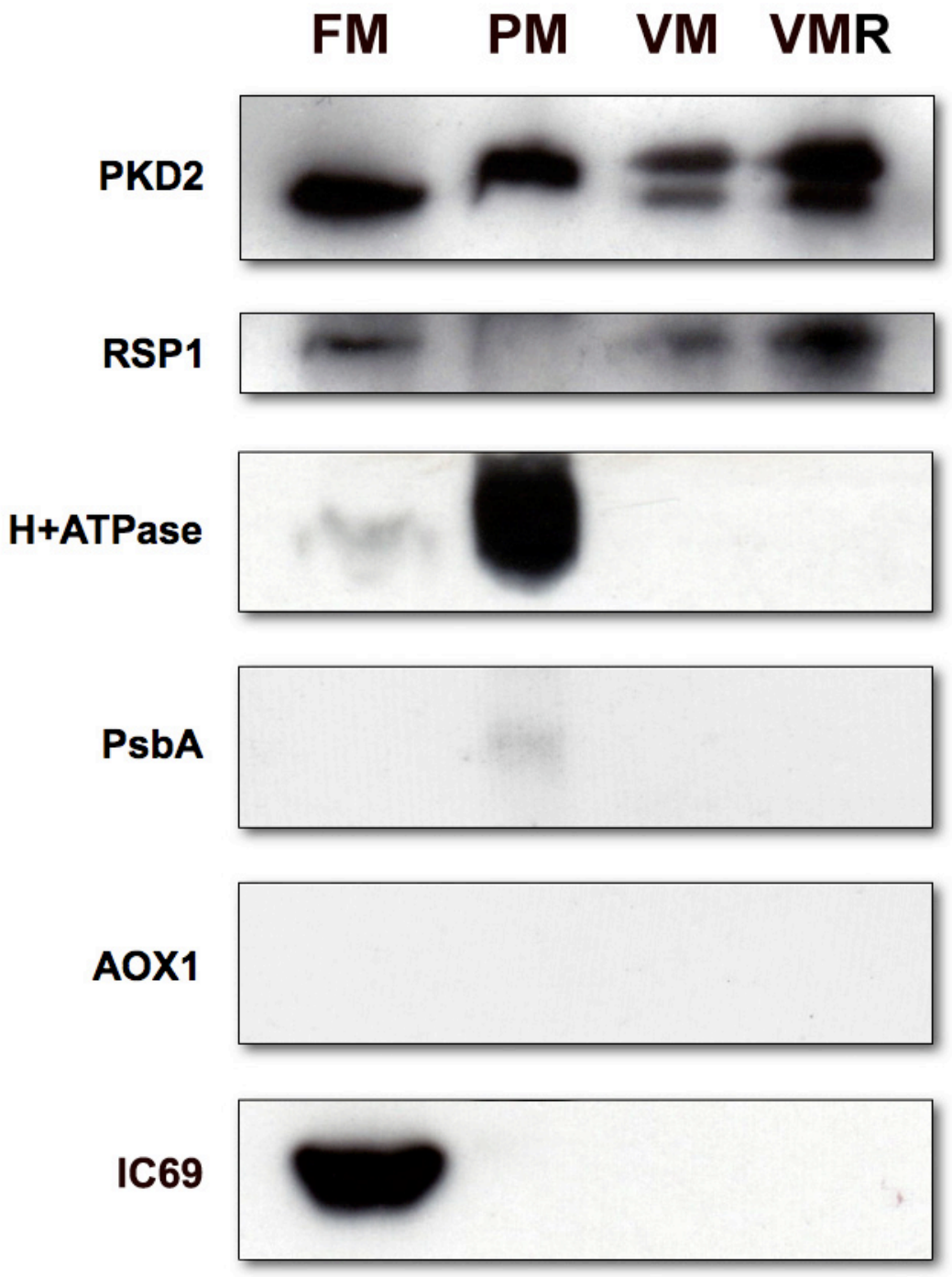

Figure S1. Assessment of membrane contamination of cytoplasmic vesicle preparations. Equal protein loads of flagellar membrane (FM) and plasma membrane (PM) were compared directly with cytoplasmic vesicles isolated from control cells (VM) and cells regenerating flagella (VMR) by western blotting. Vesicles isolated from cells during flagellar regeneration showed increased amounts of PKD2 and RSP1. Proteins of the plasma membrane (H+ATPase), chloroplast membrane (PsbA) and mitochondrial

membrane (AOX1) did not contaminate the cytoplasmic vesicle preparations. Dynein arm intermediate chain, IC69, a potent marker of the flagellar membrane, does not react with the cytoplasmic vesicle fractions.

A

	nucleus	chloroplast	cytoplasm
RSP3-HA cells	0.55 (17/30.82) [1]	1.33 (208/156.14)	**2.38 (179/75.35)**
control cells	0.43 (10/23.09)	1.28 (357/278.19)	0.32 (37/113.05)

[1] gold particles or clusters/um^2

B

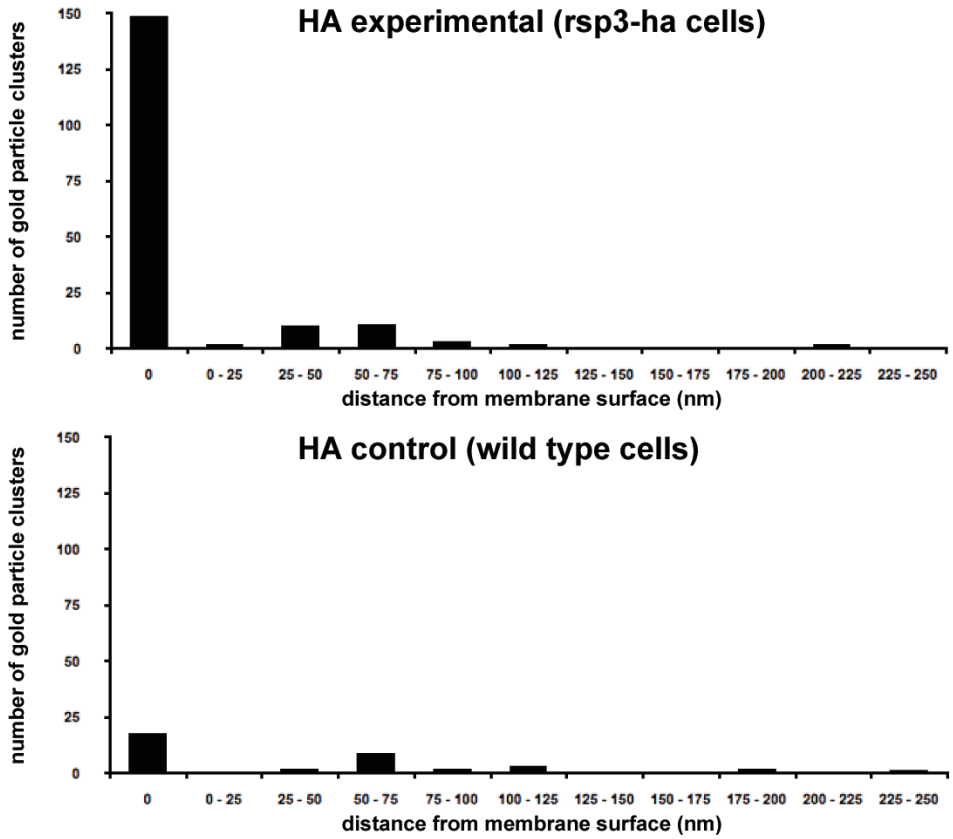

Figure S2. Quantitation of RSP3-HA gold labeling. A. Sections of RSP3-HA tagged and wildtype untagged cells were subjected to immunogold labeling with HA antibodies. The amounts of HA gold clusters per unit area of nucleus, chloroplast, and cytoplasm were quantified in RSP3-HA cell sections and in wildtype control cell sections lacking the HA epitope. **B.** The distance between each RSP3-HA-specific gold particle cluster found in the cytoplasm and the nearest membrane surface was measured and displayed as a distribution. A distance measurement of zero indicates that the gold particle was directly adjacent to a membrane surface.

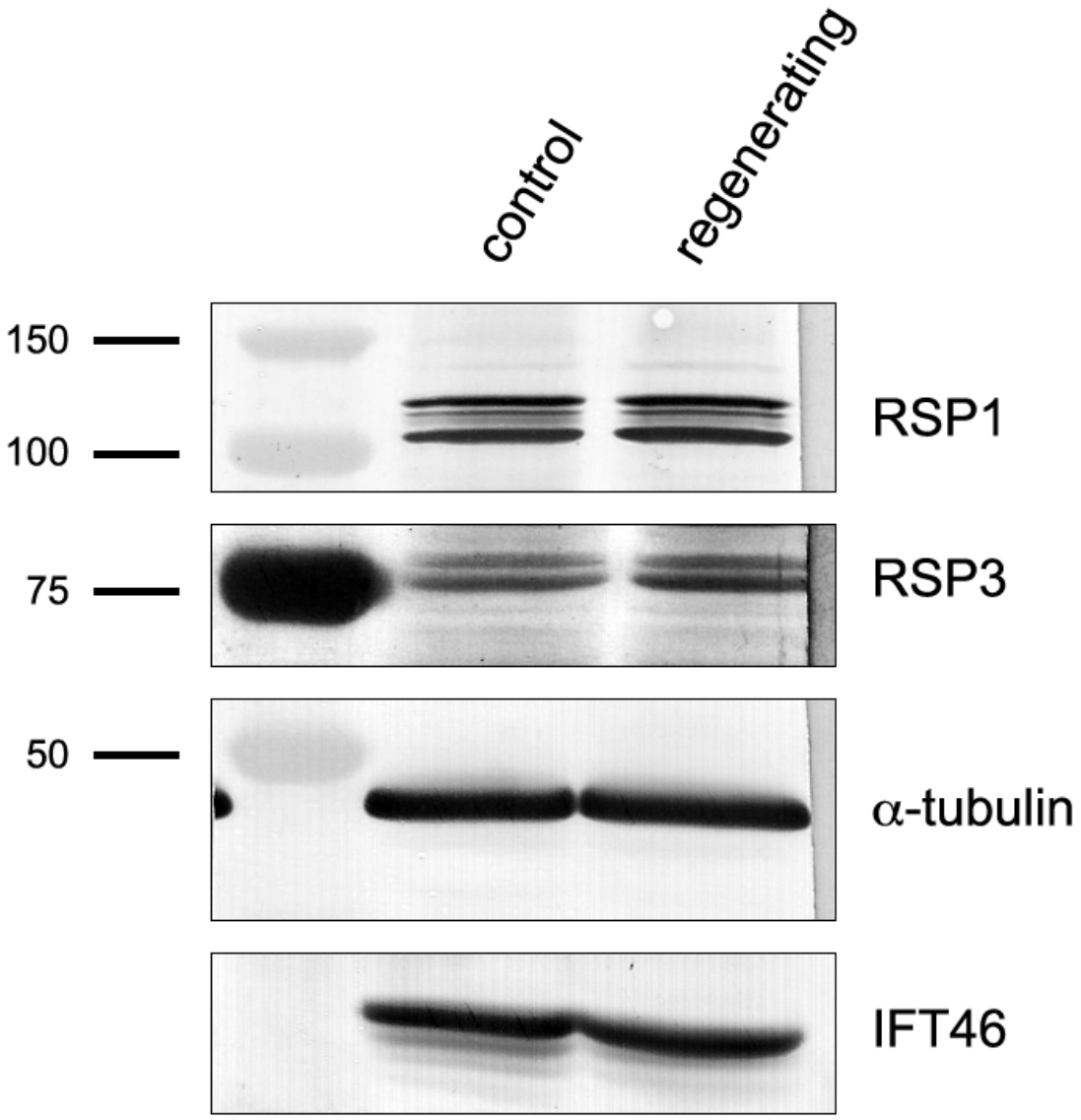

Figure S3. Ciliary proteins found in the bottom (30%) layer of the OptiPrep density gradient do not change in amount during flagellar regeneration. The 30% OptiPrep gradient fraction was obtained from control (full length flagella) and cells undergoing flagellar growth (regenerating) and compared by SDSPAGE and immunoblotting. Shown are immunoblots resulting from equal protein loads of control and regenerating 30% OptiPrep layer fractions probed with antibodies specific for the indicated ciliary proteins. Pre-stained protein marker bands that transferred during blotting are seen to the left and labeled with their molecular weight in kDa where they appear.

Supplemental Experimental Procedures

Immunolectron Microscopy

For *in situ* immunogold labeling, 10 ml of cells in the process of regenerating flagella were harvested by centrifugation at 500 g. Cell pellets were resuspended in M1 medium and fixed by addition of 4% formaldehyde and 0.5% glutaraldehyde for 30 min at room temperature. Following three washes in 50 mM Hepes, pH 7.4, cells were incubated in 0.05% osmium tetroxide on ice for 30 minutes. Cells were pelleted at 500 g and pellets were washed two times with distilled water then dehydrated to 100% ethanol (15 minute incubations each of 30% and 50% ethanol on ice; then 70%, 95%, and 100% ethanol at -20°C). Infiltration of the sample with LR Gold resin (Plano, Marburg, Germany) was performed at −20°C according to the following scheme: 0.4% Benzil activated LR Gold/ethanol 1:1 for 16 hours followed by 3:1 for 24 hours, then 24 hours in 100% resin. Polymerization was performed under fluorescent light for 24 h at 4°C. Ultrathin sections (50 to 80 nm) were cut and collected on formvar coated, nickel grids. The sections were subjected to a saturated solution of sodium metaperiodate for 4 minutes, washed four times for 5 minutes each with distilled water, then washed two times for 5 minutes each in phosphate-buffered saline (PBS). Blocking was performed for 1 hour at room temperature with blocking buffer (5% BSA, 1% cold water fish gelatin, and 10% goat serum in 1x PBS; pH 7.4). Incubation of grids in primary antibody mixtures was performed overnight at 4°C alongside control experiments in which blocking buffer alone was added with no primary antibody. Grids were washed five times with PBS for 10 min each and processed using Aurion Ultra Small Immunogold goat-anti-rabbit, or goat-anti-mouse IgG (H+L) with silver enhancement according to the manufacturer's instructions (Aurion, Wageningen, The Netherlands). Specimens were stained with lead citrate for 2 minutes and photographed with a JEOL JEM-1230 electron microscope (JEOL USA, Inc. Peabody, MA). For quantitation of gold particles in situ, digital TEM micrographs were measured using ImageJ software (http://rsbweb.nih.gov/). For immunogold labeling of isolated cytoplasmic vesicle whole-mounts, vesicles were resuspended in PBS buffer and transferred directly to glow discharged, formvar-coated, nickel grids. Grids were then floated on droplets of blocking buffer for 30 min at room temperature and transferred to droplets of primary antibody in Aurion incubation buffer (0.2% Aurion BSA-c, 15 mM NaH3, in PBS of pH 7.4), for 1 hour at room temperature. Grids were then washed, transferred to Aurion Ultra Small Gold secondary antibody, and processed as above according to manufacturer's instructions. Following silver enhancement, specimens were negative stained with 2% aqueous uranyl acetate and photographed in the electron microscope as above. Double labeling experiments were performed using two ultra small gold conjugates and differential silver enhancement according to Aurion's R-Gent SE-EM instructions (Aurion Newsletter 5). A duration of 40 minutes was employed for the first silver enhancement in the sequence, and a duration of 15 minutes was employed for the second silver enhancement; producing two different sized particles specific for each secondary antibody conjugate.