

Supplementary Materials and Methods

Phylogenetic analyses

Sequences of TTLL6 protein homologs were obtained from NCBI databases. Conserved catalytic domains were aligned and neighbor-joining trees constructed as described in [1].

Germ line-based targeting

The gene targeting plasmids were made based on the macronuclear genome sequence of *Tetrahymena thermophila*, available at the *Tetrahymena* Genome Database [2]. Two fragments of macronuclear DNA were amplified for each locus. The fragments were designed to flank the genomic sequence that encodes the catalytic domain of TTLL6 (Table S1). Fragments were subcloned on either side of a selectable drug resistance cassette (*neo3* [3] or *mtt1-rpl29* (Bowen and Gorovsky, personal communication)). The cassette was embedded in a reverse transcriptional orientation. The targeting fragments were separated from the rest of the plasmid using restriction enzymes and transformed biolistically into mating CU428 and B2086 strains [4] at 3, 3.5 and 4 hr after mixing. After shooting, cells were incubated in SPP and 2.5 µg/ml CdCl₂ for 3 hr, and transformants were selected with appropriate drugs (*neo3*, paramomycin-100 µg/ml with CdCl₂ 2.5 µg/ml; *mtt1-rpl29*, cycloheximide 15 µg/ml with CdCl₂ 2.5 µg/ml). Putative germline transformants were identified by co-resistance to 15 µg/ml 6-methylpurine. Knockout heterokaryons were generated by allowing the disrupted alleles to assort from the macronucleus via phenotypic assortment and by making the micronucleus fully homozygous using a star cross [5]. Double knockout strains were made by multiple rounds of standard crosses. Total homozygotes were created by crossing appropriate heterokaryons and isolating progeny. The absence of the targeted genomic regions in the homozygote was confirmed by PCR of genomic

DNA with primers designed to amplify junctions between selectable cassettes and the non-targeted gene specific regions (Table S2).

To construct a triple mutant strain, 6AF-KO;*oadl-1*, a 6AF-KO heterokaryon was crossed to an *oadl-1* germline mutant strain [6], triple heterozygotes were matured and drug resistance was lost from the macronucleus by phenotypic assortment. Strain with homozygous micronuclei were made by a cross to A*III. Specific heterokaryons that were homozygous for both gene disruptions and the *oadl-1* allele were identified by an outcross to an *oadl-1* homozygote strain, and identification their progeny that was resistant to paromomycin, cycloheximide in the presence of cadmium and displayed ciliary paralysis after incubation at 38°C.

Immunofluorescence and western blotting.

The cells were stained by immunofluorescence as described in [7] with the following primary antibodies: 12G10 (1:50), GT335 (1:100), SG polyclonal anti-total tubulin (1:600) and polyE (1:100).

For western blotting, 5 µg of cilia protein per lane were separated on a 10% SDS-PAGE and western blots were done as described [8], with the primary antibodies at the following dilutions: GT335 anti-glutamylated tubulin mAb (1:1,000) [9], 12G10 anti- α -tubulin mAb (1:10,000) (Developmental Studies Hybridoma Bank), polyE anti-polyglutamic acid antibodies (1:1,000) [3], polyG, anti-polyglycine antibodies [10], 6-11 B-1 anti-acetyl-K40 on α -tubulin [11], and anti-SerH surface antigen antibodies [12] .

Electron microscopy

For standard transmission electron microscopy (TEM), cells were fixed as described [13]. For immunogold TEM, cells carrying an MTT1-GFP-TTLL6A transgene were grown in 5 ml of SPP medium (2×10^5 cells/ml) and induced with $2.5 \mu\text{g/ml}$ CdCl_2 for 3 hr, gently spun down briefly, fixed in an Eppendorf tube and stained as described above for immunofluorescence with anti-GFP antibodies (Abcam, 1:10,000) overnight followed by anti-rabbit IgG-10 nm gold (1:60) (Amersham Pharmacia) for 2 hr at room temperature. Cells were concentrated by centrifugation to $100 \mu\text{l}$ and post-fixed with 1 ml of 2% glutaraldehyde (in 0.1M sodium cacodylate buffer, pH7.2) on ice for 1h. Cells were washed 5 times in cold sodium cacodylate buffer (10 min each on ice) and fixed with 1 ml of 1% osmium tetroxide for 1 hour on ice. The pellet was washed 5 times in water, followed by dehydration in ethanol concentration/water series and embedding in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and analyzed on JEOL 1200 EX transmission electron microscope. For immunogold whole mount TEM, cilia were purified from 50 ml of cells (3×10^5 cells/ml) and demembrated in $500 \mu\text{l}$ of 1% NP40 in the axoneme buffer. After 5 min on ice, axonemes were collected at 23,000 g (15 min, 4°C), washed with 1 ml of axoneme buffer and, centrifuged and suspended in the axoneme buffer at 10 mg tight pellet/ml of. To initiate sliding of axonemal microtubules, $0.5 \mu\text{l}$ of 2 mM ATP was added to $50 \mu\text{l}$ of axonemal suspension (final concentration $40 \mu\text{M}$). After incubation for 3 min at room temperature, $10 \mu\text{l}$ of suspension was placed onto a formvar-coated grid for 1 min and the excess was absorbed by filter paper. Grids were processed for immunogold TEM [14] as described detail in the Supplemental Material and stained using uranyl acetate.

For immunogold whole mount TEM, axonemes were purified and reactivated with ATP as described in the main text, and labeled with antibodies according to [14] with minor

modifications. Ten μl of the ATP-reactivated axonemes were placed on top of a formvar-coated copper EM grid, allowed to settle for 1 min, the excess of liquid was drained with a filter paper and the material was immediately fixed by floating the grid on top of a 50 μl drop of 2% paraformaldehyde in PHEM buffer. The grids were washed by dipping in 10 mM HEPES (pH 7.4) about 15 times and blocked by covering with 10 μl of 3% BSA, 0.01% Tween20 in PBS for 15 min. The grids were incubated in 50 μl drops of primary antibodies (1:100 polyE in the blocking buffer). The grids were washed by dipping in PBS about 15 times, incubated in 50 μl drops of the secondary antibody, anti-rabbit IgG-10 nm gold (GE Healthcare) (1:60 in the blocking buffer), washed 15 times in PBS, and negatively stained with 2% uranyl acetate.

Supplemental figures

Figure S1. Loss of Ttll6Ap and Ttll6Fp leads to a loss of elongated glutamyl side chains in axonemes. (A). A histogram that documents the average intensity of fluorescence signals detected for antibodies against tubulin or glutamylated tubulin using quantitative immunofluorescence. Wild type (labeled with India ink) and 6AF-KO cells were mixed and processed for double immunofluorescence for total tubulin and glutamylated tubulin (using either GT335 or polyE antibodies). Confocal images were obtained of pairs of wild type and mutant cells located near each other. Signal intensities were measured for individual axonemes using ImageJ. A total of 4 images (8 cells) were analyzed, Between 73 and 196 axonemes were measured for antibody. For any given pair of cells, the mutant signal average was calculated as a percentage of the adjacent wild type cell signal. The histogram contains an average of each image value. Error bars represent standard errors. (B) Images of portions of tubulin regains of 2D

gels of axonemal proteins. The arrowhead marks a region that contains a string of highly acidic protein forms migrating near the main spots of tubulin in the wild type that are missing in the 6AF-KO cilia. Note that on SDS-PAGE, ciliate tubulin migrates in an inverted order as compared to most other species (β -tubulin migrates more slowly).

Figure S2. AF-KO mutants respond to membrane-depolarizing treatments by turning.

Paths of live 6AF-KO cells in the control solution (A,B) and upon exposure to 20 μ g/ml SDBS. The slight turn in the path that occurs immediately after addition of SDBS is marked with an arrow. Bar - 200 μ m.

Figure S3. Ttl6Ap and Fp primarily affect the polyglutamylation levels on the B-tubule or outer doublets. Images of negatively stained immunogold-labeled (using polyE antibodies) wildtype and 6AF-KO axonemes. Bar = 100 nm.

FIG S4. *Oad1-1* allele decreases the frequency of outer dynein arms in 6AF-KO cells. TEM crosssections of wild type (A), 6AF-KO (B,C) and 6AF-KO;*oad1-1* cilia (38°C) (D-F).

Supplemental Movies

Movie S1A. High speed video recording of motile cilia in a wild type cell. Video was acquired at 500 frames per second and replayed here at 30 fps. The beating cilia produce metachronal waves.

Movie S1B. High speed video recording of motile cilia in a 6AF-KO cell. Video was acquired at 500 frames per second and replayed here at 30 fps. Many cilia appear stiff and appear to swivel at the base. The metachronal activity is not apparent.

Movie S2A. A high speed video used for measurement of the beat frequency of wild type cilia.

Movie S2B. A high speed video used for measurement of beat frequency of 6AF-KO^A (partially normalized after long growth) mutants.

Supplemental Tables

Table S1. Primers used for construction of targeting fragments.

Gene	Primer sequence (5'-3')	Restriction sites used to release the targeted fragment	Size of targeting fragment	Selectable cassette	TTL catalytic domain region replaced
TTLL6A	Forward: ATATTGGGCCCGAGGAAGATGATGATGAGA Reverse: ATAAACCCGGGGCTAAAGAAAACATACCAG	ApaI / SacI	1.5 Kb	Neo3	926 bp
	Forward: AATTTACTAGTAGCCATGGGTTT TAGAAGT Reverse: ATTATGAGCTCCTTTTGGGAAGTA ATGTCAG		1.45 Kb		
TTLL6F	Forward: ATATTGGGCCCGAGCTAATCAAA CATA CGA Reverse: ATTATATCGATTTCCTAGCTATTC TGGTTA	ApaI / SacII	1.47 Kb	mtt1-rpl29	1.6 Kb
	Forward: ATATTCCCGGGAAAAAGCCTGAT GTTGAAG Reverse: ATATTCCGCGGGGCTACAAATAA AGTCCAT		1.44 Kb		

Table S2. Diagnostic primers used for verification of gene disruptions (amplify deleted regions).

Type of Gene	
TTLL6A	Forward 5'-TATCTTTTGGACTGATAATGCT-3' Reverse 5'-CTCTTAATATCTTTCCACAG-3'
TTLL6F	Forward 5'-AGATCTCTAAAGGAAAATGC-3' Reverse 5'-TTCATGTAGTTATCTGGTTG-3'

Table S3. Phagocytic activity is restored by re-introduction of GFP-Ttll6Ap transgene into 6AF-KO cells.

	Wild Type	6AF-KO	GFP-TTLL6A ^R
Average number of labeled food vacuoles/cell	42.8	0	36.3
Standard deviation	3.7	-	3.5
N=	25	25	25

Food vacuoles were labeled with India ink for 30 min, cells were fixed and the average number of food vacuoles per cell was determined.

Supplemental References

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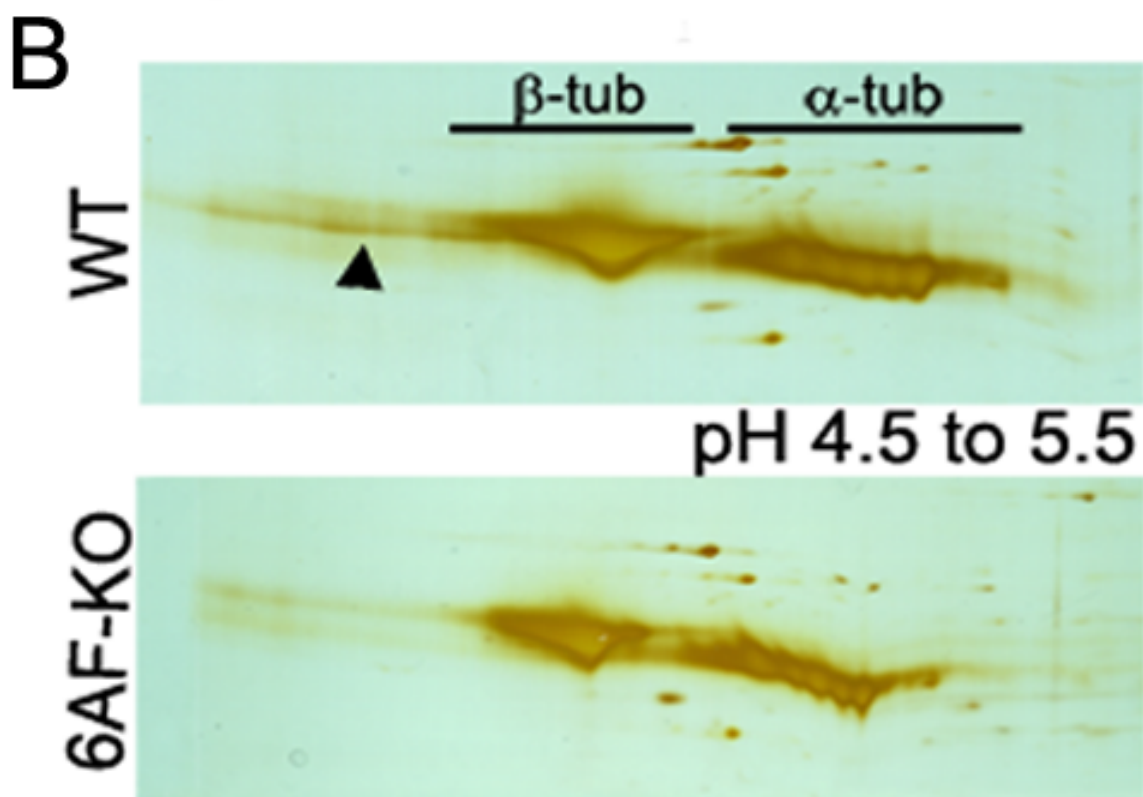
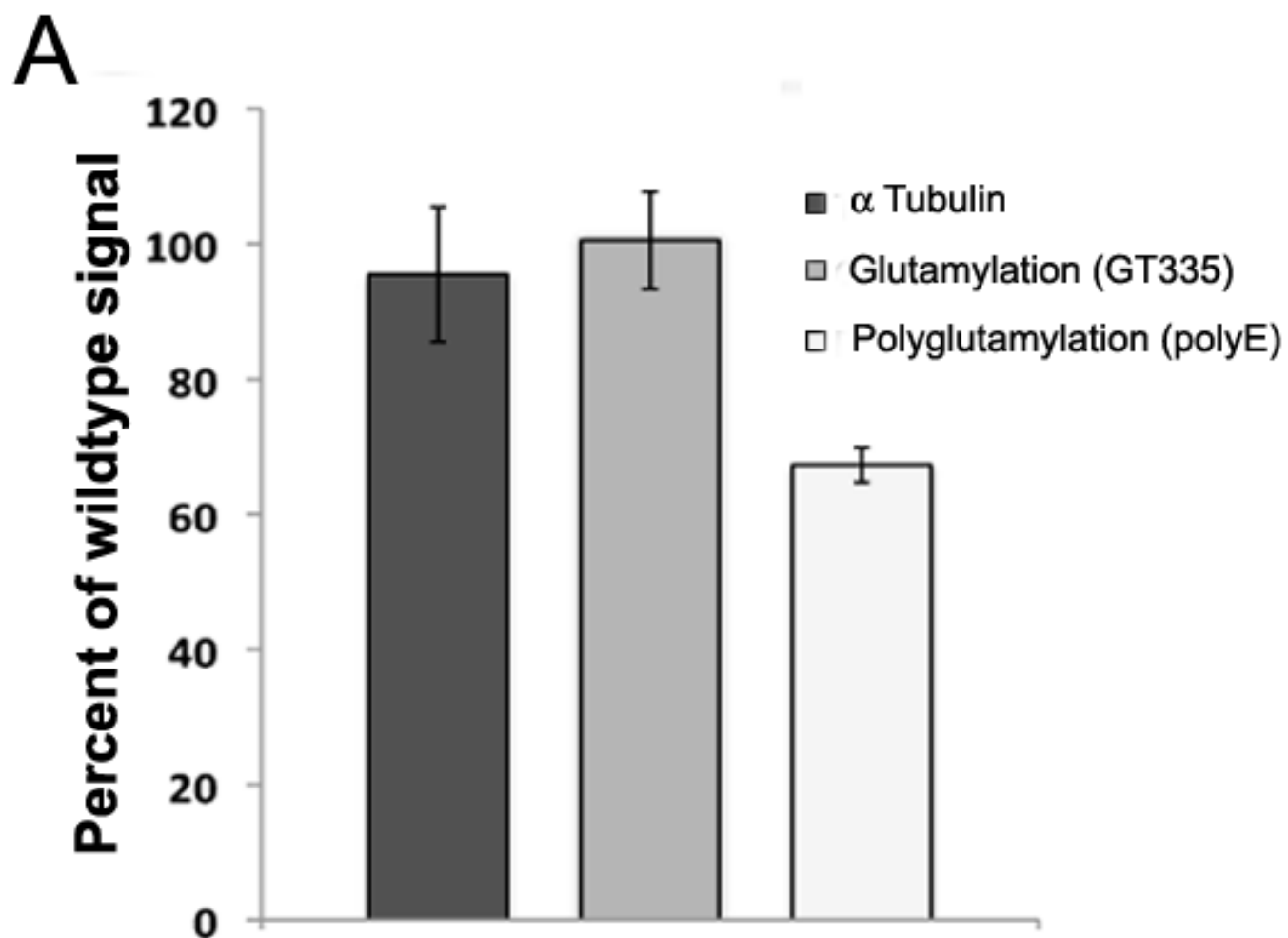


Fig. S1.

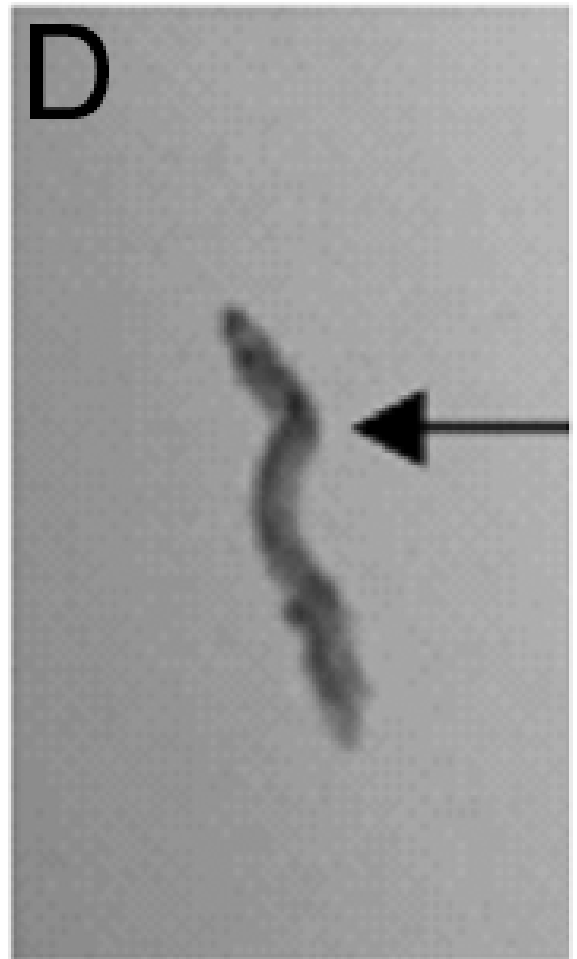
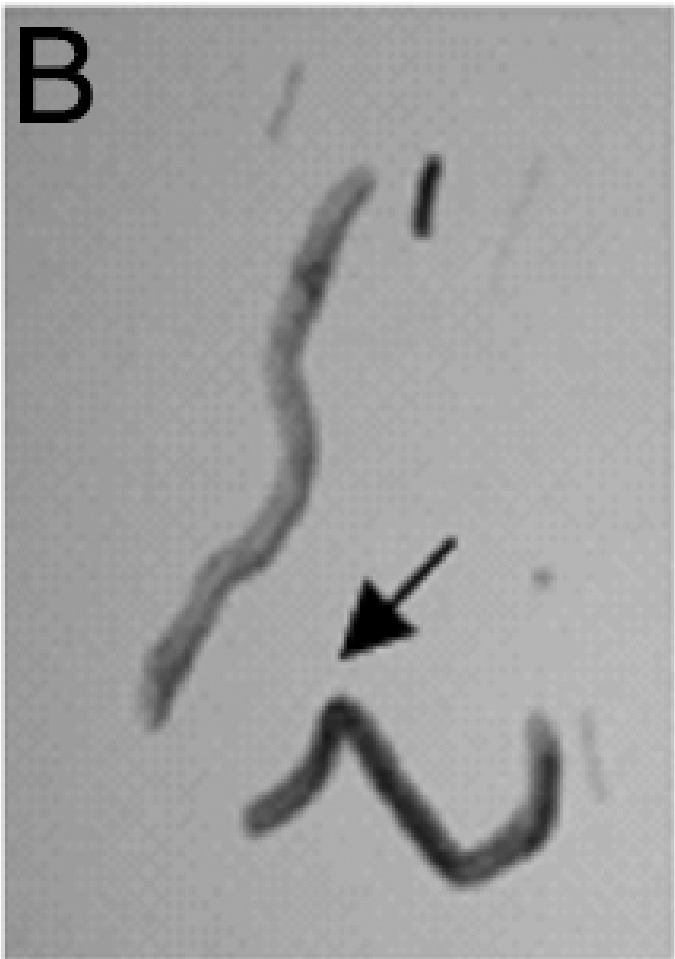
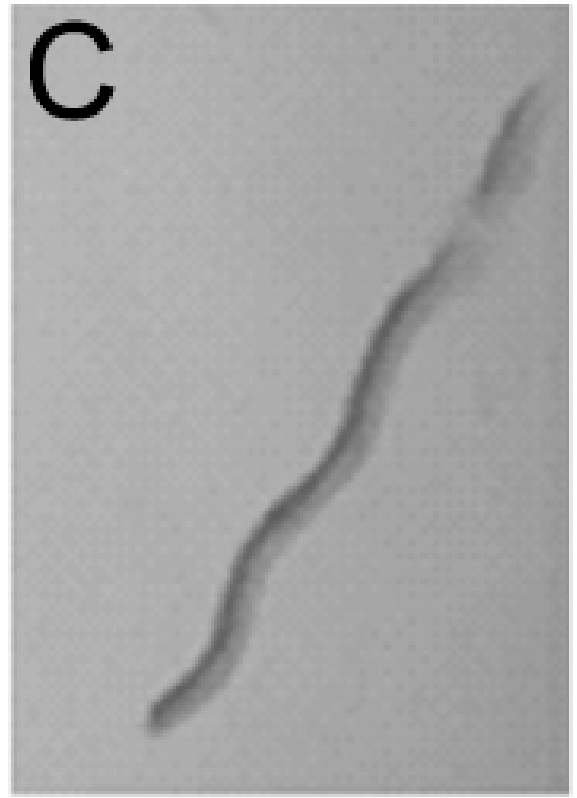
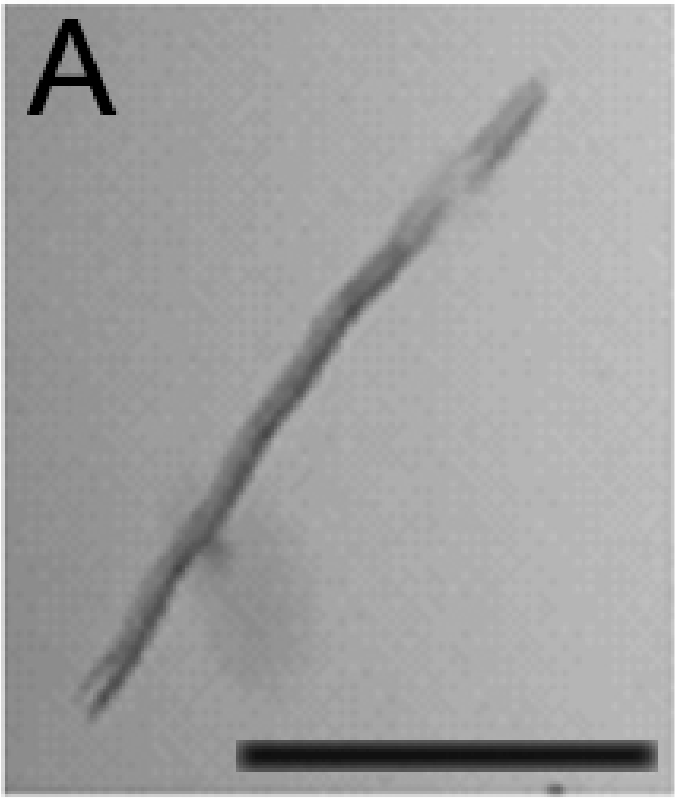


Fig. S2.

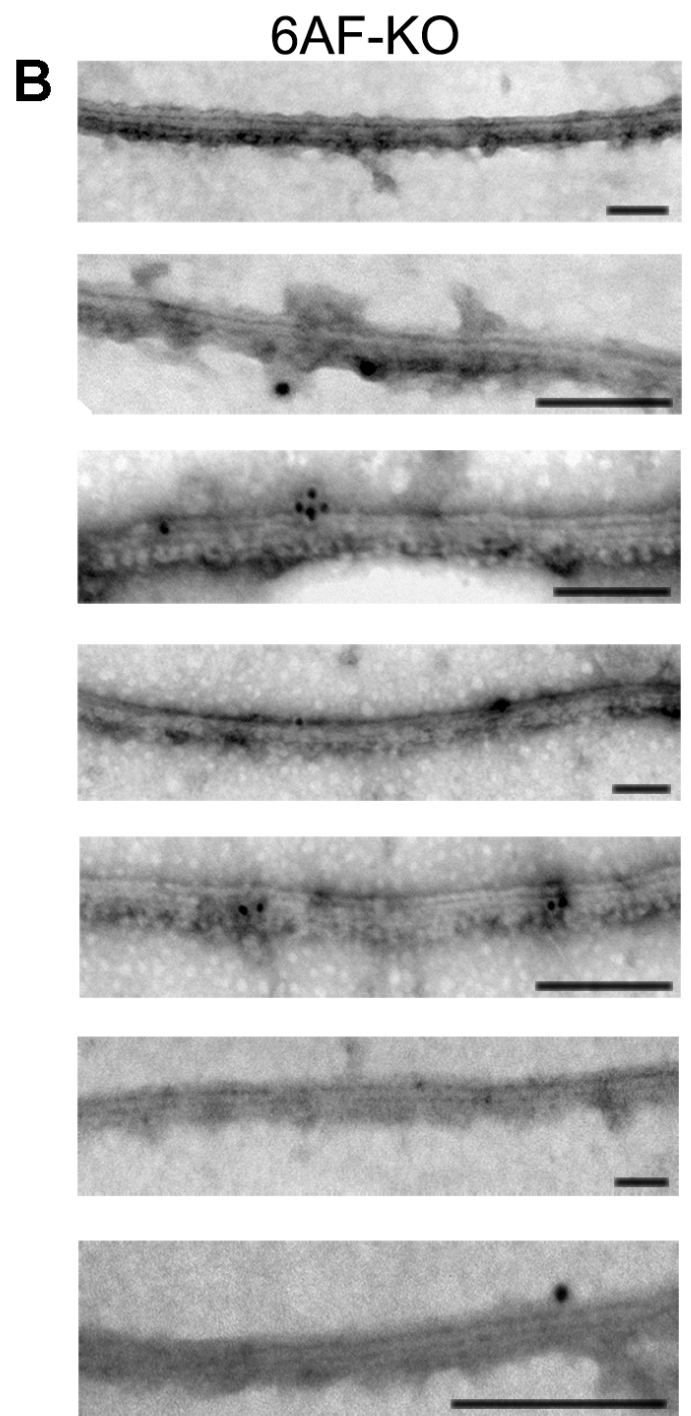
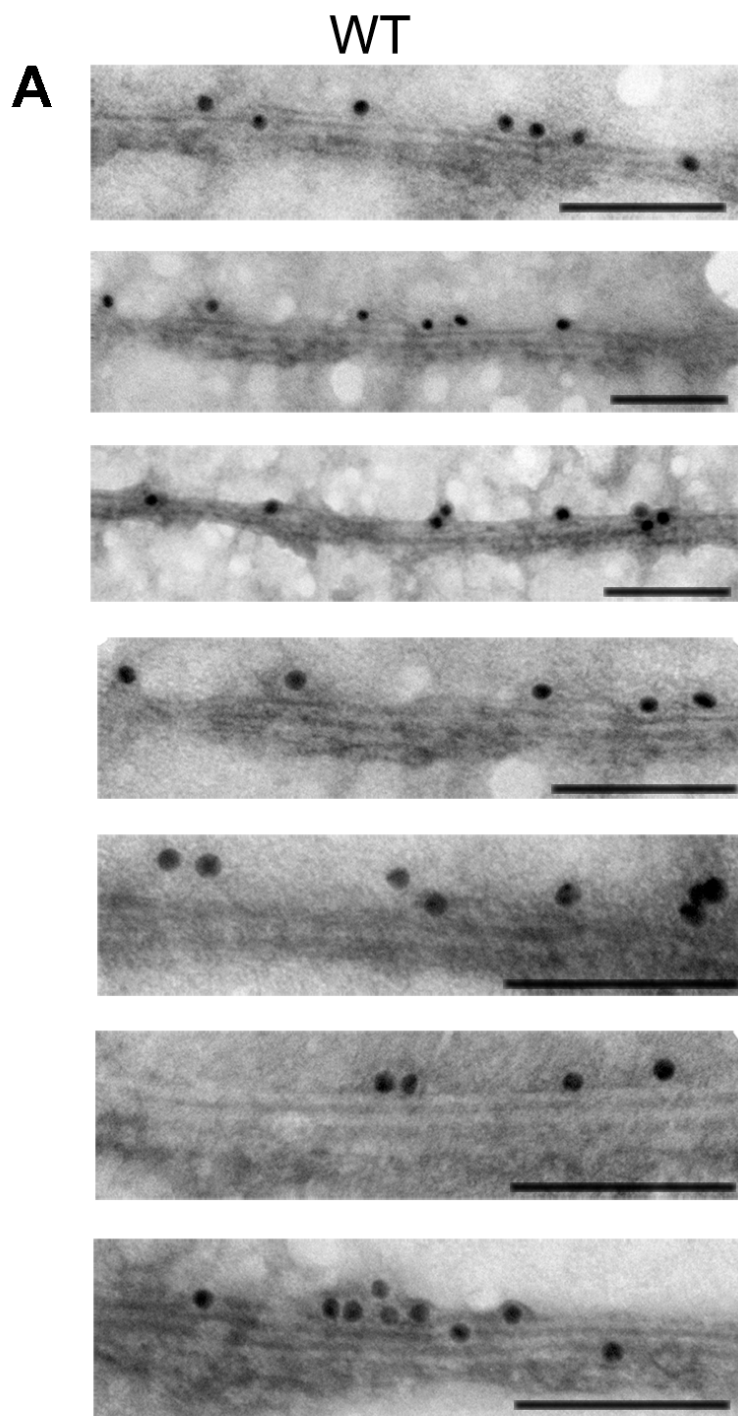


Fig. S3.

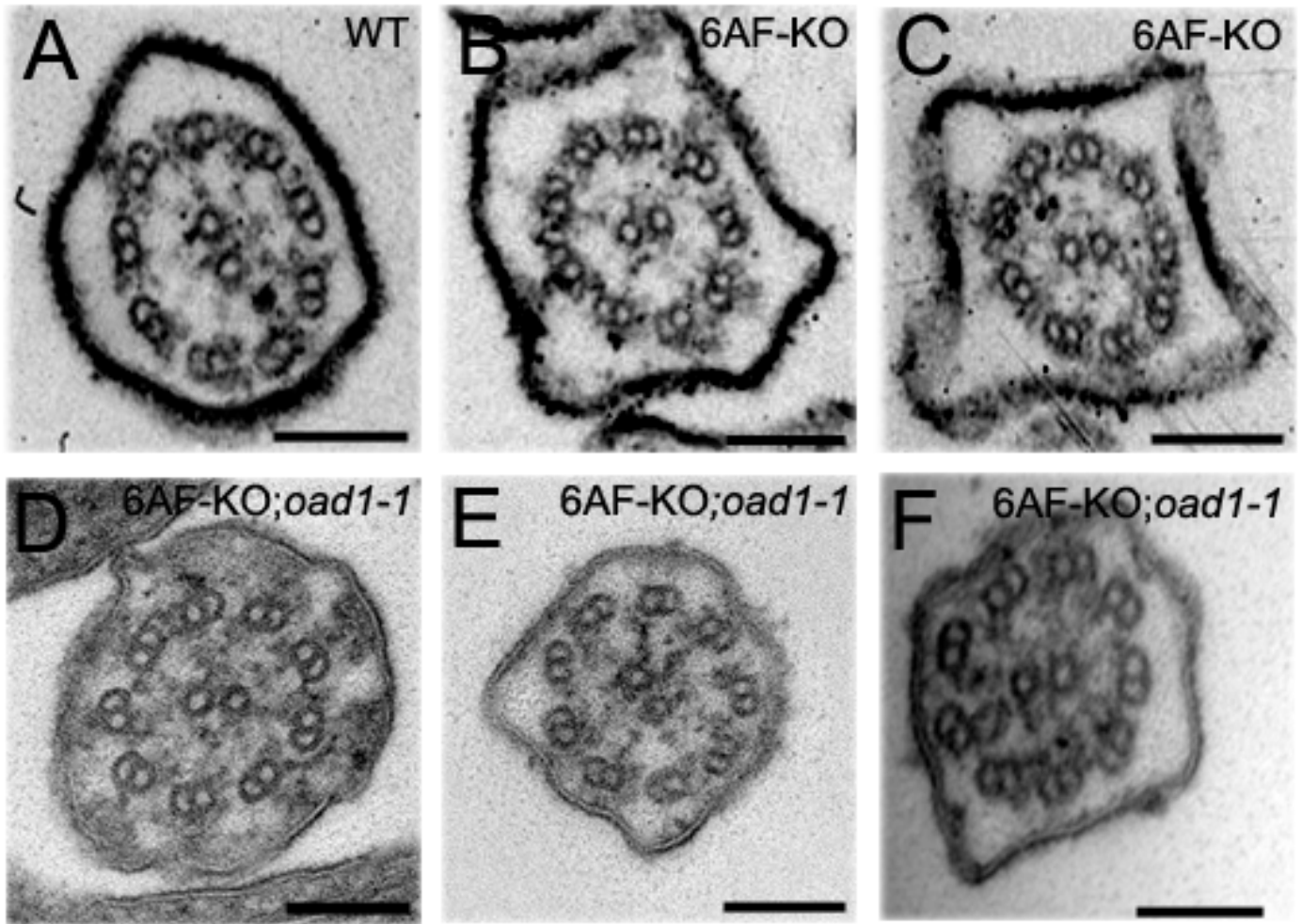


Fig. S4.