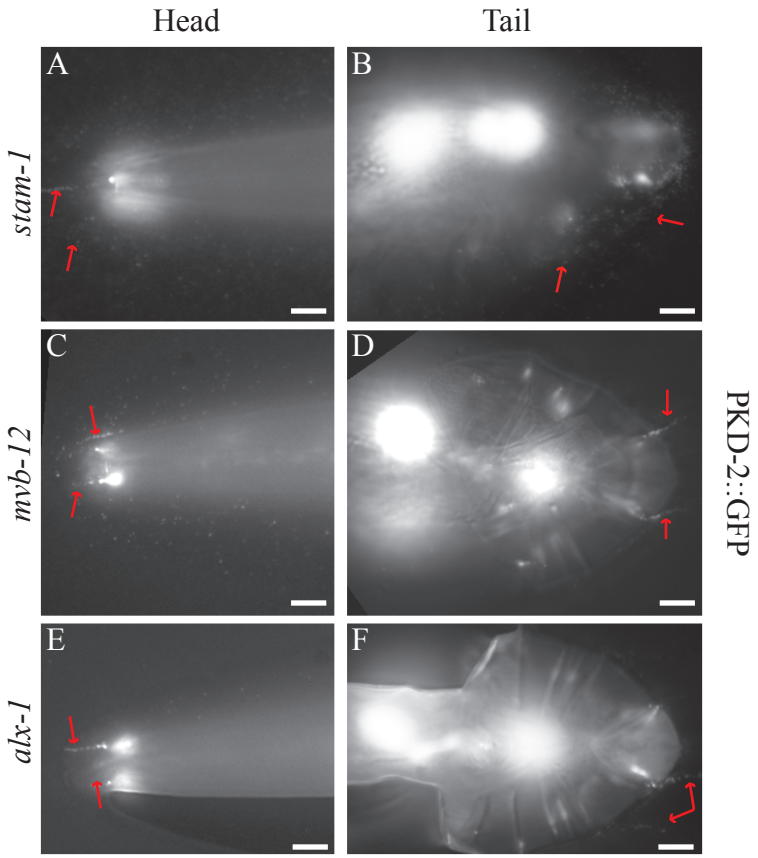
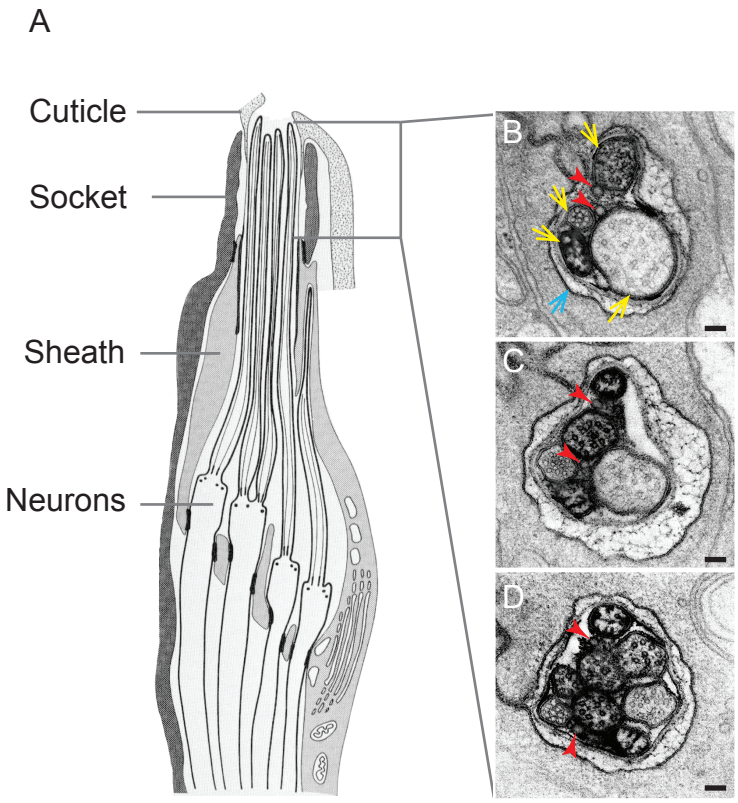
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 1, related to Figure 2B. MVB biogenesis pathway components STAM-1, MVB-12 and ALX-1 are not essential for ECV secretion. (A-B, C-D and E-F) Male head and tail images of PKD-2::GFP reporter in *stam-1, mvb-12* and *alx-1* mutants respectively. Red arrows point to ECVs surrounding the head and the tail. Scale bar is 10 μm.

Supplemental Figure 2, related to Figure 1. Examples of the ECVs in the amphid channel lumen. (A) A schematic model of amphid cilia and the extracellular lumen formed by their surrounding glial sheath, socket cell and the cuticle (revised from [S1], Figure 18). (B-D) are taken from electron tomograms (ETs) of amphid region, showing the examples of the ECVs. Red arrowheads point to the ECVs, yellow arrows point to cilia, blue arrow points to the plasma membrane of the socket cell enclosing the lumen. Scale bar = 100 nm.

Supplemental Table 1, related to Figure 1 and 2. **Size comparison of ECVs from different preparations.**

ECV type	TEM method	Sizes (mean ± stdev, nm)	n
Preparation from culture by ultracentrifugation	negative staining TEM	91.7 ± 92.5	329
Preparation from culture by ultracentrifugation	Ultra-small gold labeled LOV-1 antibody immunostaining	111.2± 31.1	210
Cephalic lumen (fixed animals)	Electron tomography	104.7±46.7	647

Supplemental Experimental Methods

Nematode strains.

The following transgenic reporters were used: *myIs4 [PKD-2::GFP+Punc-122::GFP]V* [S2], *syEx301[lov-1::GFP1+pBX1]* [S3], *Ex[CWP-1::GFP + pBX1]* [S4], *nIs133[Ppkd-2::GFP]I; him-5(e1490)V* [S4], *myEx647 [Pklp-6::KLP-6::GFP + pBX1]* [S5], *myEx825[Posm-*

6::GFP::LMP-1 +pBX1], Ex [OSM-3::GFP + pRF4] [S6]*, Ex [KAP-1::GFP + pRF4],* [S6]*; myEx10[CHE-11::GFP + pRF4]* [S7], *mnIs17[OSM-6::GFP]* [S7], *fsEx166 [TBB-4::YFP + pBX1], kyIs53[ODR-10::GFP]* [S8]. The following alleles were used: LGI: *stam-1/pqn-19(ok406), che-3(e1124);* LGIII: *bbs-7(n1606), klp-6(my8), alx-1(gk275);* LGIV: *mvb-12(ok3482), daf-10(p821), osm-3(mn391), klp-11(tm324);* LGX: *osm-5(m184) X.*

Imaging

C. elegans and ECVs were imaged using a Zeiss Axioplan 2 microscope with a100x 1.4NA oil Zeiss Plan-APOCHROMA objective and Photometrics Cascade 512B EMCCD (Roper Scientific) camera. All images, stacks and streams were acquired using Metamorph (Version 7.6.1.0, Molecular Devices) software.

Young adult males or hermaphrodites were synchronized by picking L4 larvae one day before imaging. Late L4 males with developed fan and rays just before molting were picked from a population of L4 males and imaged within 1-5 hours. Worms were anesthetized with 10mM levamisole solution, transferred to 2% agarose mounting slides and imaged within 30 min of mounting. The ECV preparation was imaged by putting two pieces of double adhesive tape on a glass slide, adding 1 µl of ECV suspension on the glass slide between the two pieces of tape, and then placing a cover slip on top.

Transmission Electron Microscopy (TEM), electron tomography (ET), and serial thin sections

Young adult animals were subjected to high-pressure freeze fixation and freeze substitution in 2% osmium tetroxide + 2% water in acetone as the primary fixative [S9]. Samples were slowly treated to freeze substitution in an RMC freeze sub device, before infiltration with Embed812 plastic resin. For TEM, serial sections (80 nm thickness) of fixed animals were collected on copper mesh grids and stained with 4% uranyl acetate in 70% methanol, followed by washing and incubating with aqueous lead citrate. Images were captured on a Philips CM10 transmission electron microscope at 80kV with a Morada 11 megapixel TEM CCD camera driven by iTEM

software (Olympus Soft Imaging Solutions). For tomography, data were acquired from thicker sections (250nm) using an FEI Technai20 TEM, using SerialEM software, collecting 100 images per tilt axis. These data was Fourier processed using Protomo software, and the markerless backprojection method to generate dual tilt tomograms [S10]. Up to 20 serial section tomograms were stitched to generate a volume spanning 5 microns and annotated using iMod software to generate models [S11].

Lumen size quantification of *klp-6* mutants and wild type

22 cross sections around transition zones of CEM neuronal cilia were obtained and quantified for area measurement in *klp-6* mutants. From four wild-type CEM neuron tomographs, four cross sections around CEM transition zones in wild type (total=16) were selected for measuring area.

Quantification of PKD-2::GFP distribution

L4 males were isolated the day prior to recording. From adult males, we made a Z-stack of each male head to encompass cilia and cell bodies of all four CEM neurons. We then flattened the Z-stacks into max stack projections and thresholded the areas of CEM cell bodies and cilia bases separately for each projection to ensure the selected area corresponded to the fluorescence boundaries. Mean fluorescence intensity of cilia base and cell bodies (including nuclei) were quantified using Metamorph software and then averaged to achieve a single mean value for each male. Cilia base/cell body fluorescence intensity ratio was calculated for each male using the following formula: average cilia base fluorescence intensity/average cell body fluorescence intensity. 12 animals were analyzed for wild type and *klp-6* mutant respectively.

ECV preparation

Mixed adult (male and hermaphrodite) populations from *him-5* (high incidence of males) strains were washed off plates with M9 buffer and spun at 3000 g in a centifuge to pellet worms and bacteria. Supernatants were passed through 0.45 µm filters and centrifuged at 25,000 g for 30 minutes to pellet debris from the culture. The supernatant was centrifuged at 200,000 g for 70 minutes, and the pellet was washed with M9 buffer once and centrifuged again at 200,000 g for 70 minutes [S12]. The pellet was resuspended in M9 buffer for use in behavior assays, negative staining, immunostaining and fluorescent microscopy. The CBQCA protein quantification kit (C-6667) from Molecular Probes was used to quantify protein content in the ECV preparation. ECV preparation concentration was normalized to 100 ng/ml.

Gold labeling anti-LOV-1 antibody immunostaining and negative staining

10 µl of the above vesicles were put on glow discharged Formvar-carbon-coated nickel grids (EMS FCF200-Ni) for 10 minutes. Methylamine Tungstate was used for negative staining (Nano_W Nano-Probes). For immunostaining, Ultra-Small Starter kit (EMS, CAT# 25550-10) was used. The grids with ECVs were blocked with Block Buffer (EMS, CAT# 25596) according to the procedure instruction. The monoclonal anti-LOV-1 antibody was generated by Abmart using peptides corresponding to the first 900 amino acids of the LOV-1 extracellular domain. Silver enhancement was done according to the above kit instruction. In whole worm mount immunostaining, the anti-LOV-1 antibody labels the 21 B-type male-specific ciliated sensory neurons in wild-type and *lov-1(sy582)* mutant males (not shown). The *lov-1(sy582)* deletion is

predicted to produce a 2530 aa prematurely truncated protein (as opposed to a 3284 aa full length protein) that is be recognized by the anti-LOV-1 N-terminal antibody.

Behavioral assays

The DAGE DC-220 digital camera was mounted on the Zeiss Stemi SV11 dissecting microscope to record behavior. Pinnacle Studio HD V15 software was used to process the video file. ECV preparation from wild type and *klp-6* mutants and M9 buffer as control were added to OP50 *E. coli*-seeded agar plates. For the choice assay, three spots of 1 ul ECV suspension from wild type and *klp-6* mutants, and M9 buffer control were added to the center of the plate one centimeter apart, forming an equilateral triangle with one of the three spots at each vertex. A single male was put in the center of the triangle. For the tail chasing assay, one big spot of ECV suspensions or M9 buffer control was made by putting four 1 ul dots side by side in the center of the plate (supplemental movie 3). A single male was placed on the center of the spot. A video of male locomotion was recorded for five minutes. Male reversal movements and tail chasing behaviors (male moving backward in a circle with tail touching male's own head) were scored for each video recording.

Supplemental References

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