Sensory-evoked extracellular vesicle release and targeting

Juan Wang[1], Inna A. Nikonorova[1], Amanda Gu[1], Paul W. Sternberg[2], Maureen M. Barr[1]

1. Department of Genetics and Human Genetics Institute of New Jersey, Rutgers University, Piscataway, NJ 08854, USA
2. The Division of Biology and Biological Engineering, California Institute of Technology, Pasadena CA 91125, USA
Correspondence to Maureen M. Barr (barr@dls.rutgers.edu)

Abstract

Extracellular vesicles (EVs) are emerging as a universal means of cell-to-cell communication and hold great potential in diagnostics and regenerative therapies [1]. The urgent need of the field is precise understanding of physiological mechanisms driving EV generation and function. Ciliary EVs act as signaling devices in *Chlamydomonas* and *C. elegans* [2–4]. Mammalian cilia shed EVs to eliminate unwanted receptors [5] or in the process of cilia retraction when cultured cells enter the cell cycle [6]. Here we used our established *C. elegans* model to study sensory-evoked ciliary EV release and targeting using a fluorescently labeled EV cargo polycystin-2 (PKD-2). In *C. elegans* and mammals, the Autosomal Dominant Polycystic Kidney Disease (ADPKD) gene products polycystin-1 and polycystin-2 localize to both cilia and EVs, act in the same genetic pathway, and function in a sensory capacity, suggesting ancient conservation. We find that males deposit PKD-2::GFP-carrying EVs onto the vulva of the hermaphrodite during mating. We also show that mechanical stimulation triggers release of PKD-2::GFP-carrying EVs from cilia. To our knowledge this is the first report of mechanoresponsive nature of the ciliary EV release and of ciliary EV directional transfer from one animal to another animal. Since the polycystins are evolutionary conserved ciliary EV cargoes, our findings suggest that similar mechanisms for EV release and targeting may occur in other animals.

Results

C. elegans male mating involves stereotyped behavior steps including response to hermaphrodite contact, location of the hermaphrodite's vulva, spicule insertion, and sperm transfer to the hermaphrodite's uterus [7]. To examine male-hermaphrodite EV interactions during mating, we paired fluorescently labeled transgenic adult males with unlabeled adult hermaphrodites (Figure 1A). Male sperm transfer was visualized with MitoTracker dye and ciliary EVs were tracked via the PKD-2::GFP EV cargo protein. After mating, we scored the hermaphrodite uterus for the presence of MitoTracker labeled male sperm. In all inseminated hermaphrodites, we observed highly localized deposition of the male-specific PKD-2-carrying EVs along the hermaphrodite vulva (Figure 1B-C). These data demonstrate that the male directly transferred PKD-2::GFP-carrying EVs to the hermaphrodite during mating. The location of the male-deposited EVs at the hermaphrodite's vulva is consistent with the position of male

tail during mating and suggests that EVs were released in the timeframe between successful location of the vulva and retraction of spicules post-copulation. This timeframe represents the closest contact between the male tail and the vulva area of the hermaphrodite, suggesting that the vulva may provide mechanical or chemical cues to stimulate ciliary EV release from the male.

Living *C. elegans* males release EVs when mounted between an agarose-padded microscopy slide and bare glass coverslip [3]. We hypothesized that the bare coverslip might mechanically stimulate the release of the PKD-2::GFP carrying EVs. To test this hypothesis, we developed the double agarose sandwich imaging protocol to reduce mechanical stimulation. For that, males were mounted and cushioned between two soft agarose layers – one attached to the slide, and another one attached to the coverslip (see Supplemental method for details). This double agarose sandwich protocol drastically and significantly reduced the number of the EV release events (Figure 1D-E), indicating that soft surface-mounted condition did not stimulate PKD-2::GFP EV release. Replacement of the padded coverslip with a new bare coverslip resulted in abundant EV release (Figure 1D-E), confirming our hypothesis that mechanical stimulation from the bare coverslip triggers the PKD-2 EV release.

Anatomically, the male tail has ventrally and dorsally positioned EV-releasing ray cilia (Figure 1D) [3, 8]. Our analysis of the high-resolution images revealed that the EVs were almost always released from the sensory rays that open to the side adjacent to the bare coverslip (Figure 1D, F). All *pkd-2*-expressing neurons of the male tail released EVs upon the touch, including the ray B-type neurons (RnB 1-5, 7-9) (Figure 1D) and the hook B-type neuron (HOB) (Figure S1). Statistical analysis of frequencies of the EV release events indicated that EV release corresponded with the position of the ciliary pore against the bare surface of the coverslip (Figure 1F and Table S1). These data suggest that the mechanical stimulation of the tip of the cilium protruding from rays and the hook triggers release of the PKD-2::GFP-carrying EVs.

Our findings of very localized EV deposition around hermaphrodite's vulva suggest that the EV release is a regulated event rather than a constitutive process. The mechanoresponsive nature of the EV release suggests a similar cue may function during male-hermaphrodite mating *in vivo*. The success of vulva location depends on proper localization of PKD-2 to the sensory cilia of male-specific EV-releasing neurons [7, 9]. Transcriptional profiling of these EV-releasing neurons revealed enrichment with transcripts encoding a large variety of adhesive membrane proteins [10]. These observations taken together suggest that the male-derived PKD-2-carrying EVs might adhere to and bind receptors on the hermaphrodite's vulva to ensure localized deposition and retention of the EVs post-copulation (Graphic Abstract). In this scenario, an EV-labeled vulva may attract or repel potential mates depending on EV cargo-content. Another significant number of transcripts enriched in EV-releasing neurons were implicated in cellular stress or innate immune responses, including antimicrobial peptide EV cargo. Intriguingly, human urinary EVs contain antimicrobial peptides that have bactericidal activity. Thus, an alternative hypothesis is that ciliary EVs might be protective against sexually transmitted diseases during the mating process in *C. elegans*. Thus, our study opens a new venue for exploring ciliary EV function in inter-organismal communication and in reproductive biology.

Cells package beneficial or toxic signaling cargo using tiny extracellular vesicles (EVs) to promote health or disease, such as tumor metastasis or neurodegeneration [1]. Understanding the fundamental biology of EV-based signaling in vivo is essential to uncover their physiological and pathological functions and to provide a basis for developing therapeutic applications. A major challenge in the EV field is precisely tracing EV cargo carriers from their cellular origin to cellular target. Since the polycystins are evolutionary conserved ciliary EV cargoes, our findings suggest that similar mechanisms for EV release and targeting may occur in other animals and provide insight to existing models for EV signaling in mammals, with direct implications for human health and disease.

LEGEND

Figure 1. Ciliary extracellular vesicles (EVs) produced by mechanical stimulation of male-specific neurons can be directionally transferred to hermaphrodite targets during *C. elegans* mating. (A) Experimental design for tracking the PKD-2::GFP-carrying EVs and sperm during mating. (B) Orthogonal projections of confocal optical sections through the inseminated hermaphrodite's body. Body is outlined with solid white; PKD-2 carrying EVs, sperm, and intestine are outlined with green, magenta, and white dashed lines, respectively. Autofluorescence from *C. elegans* intestinal gut granules is marked with asterisks; it is visible through both channels. (C) Imaris 3D surface rendering of the confocal optical sections from (B). (D) Diagram of male tail shows identities of sensory rays: rays 1/5/7/9 open to a dorsal side, rays 2/4/8 open to a ventral side of the male tail. Ray 3 is lateral and opens to a side of the male tail. Ray 6 is a closed ray that does not release EVs, nor does ray 6 B-type neurons express *pkd-2*. Confocal optical sections show ray specific EV release (indicated with arrows and bolded numbers) from male tails that were brought in immediate contact with a bare coverslip via either a dorsal or a ventral side. An agarose-padded coverslip drastically reduced release of the EVs. (E) Quantitative analysis of the EV release from male tail upon different mechanical stimulation (n=39). Each male was first imaged being covered with an agarose-padded coverslip, then the padded coverslip was replaced with a bare coverslip and imaging was repeated for that male. The box plots show median values, top and bottom hinges correspond to the first and third quartiles (the 25th and 75th percentiles), and the whiskers extend to the smallest and the largest value within 1.5 distance of the interquartile range. (F) Quantitative analysis of the propensity of dorsal and ventral rays to release the PKD-2 EVs when positioned on a side that is either adjacent or opposite to the coverslip (n=14). * p-value <0.01 in the Kruskal-Wallis test for (E) and in the two proportion Z-test for (F).

Graphic Abstract. Wang *et al.* report that ciliary extracellular vesicles (EVs) carrying polycystin-2 are triggered by mechanical stimulation of the male's sensory cilia and are targeted at vulva of his mating partner. This is the first report of directional transfer of sensory-evoked EVs from one animal to another.

SUPPLEMENTAL INFORMATION: Supplemental information includes one table, one supplemental figure, and experimental procedures.

ACKNOWLEDGEMENTS: This work was supported by grants from Kansas PKD Research and Translation Core Center, P30 DK 106912 to J.W. and National Institutes of Health (NIH) awards DK059418 and DK116606 to M.M.B. We thank Noriko Kane-Goldmith for assistance with confocal microscopy, Gloria Androwski and Helen Ushakov for excellent technical assistance, Barr labmates and the Rutgers *C. elegans* community for feedback and constructive criticism throughout this project, and WormBase. We also thank the *Caenorhabditis* Genetics Center (CGC) for strains. The CGC is supported by the National Institutes of Health - Office of Research Infrastructure Programs (P40OD010440).

DECLARATION OF INTERESTS: The authors declare no conflicts of interests.

REFERENCES:

1. Mathieu, M., Martin-Jaular, L., Lavieu, G., and Théry, C. (2019). Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat. Cell Biol. *21*, 9–17.

2. Wood, C.R., Huang, K., Diener, D.R., and Rosenbaum, J.L. (2013). The cilium secretes bioactive ectosomes. Curr. Biol. *23*, 906–911.

3. Wang, J., Silva, M., Haas, L.A., Morsci, N.S., Nguyen, K.C.Q., Hall, D.H., and Barr, M.M. (2014). *C. elegans* ciliated sensory neurons release extracellular vesicles that function in animal communication. Curr. Biol. *24*, 519–525.

4. Luxmi, R., Kumar, D., Mains, R.E., King, S.M., and Eipper, B.A. (2019). Cilia-based peptidergic signaling. PLoS Biol. *17(1)*, e3000566.

5. Nager, A.R., Goldstein, J.S., Herranz-Pérez, V., Portran, D., Ye, F., Garcia-Verdugo, J.M., and Nachury, M. V. (2017). An Actin Network Dispatches Ciliary GPCRs into Extracellular Vesicles to Modulate Signaling. Cell *168*, 252-263.e14.

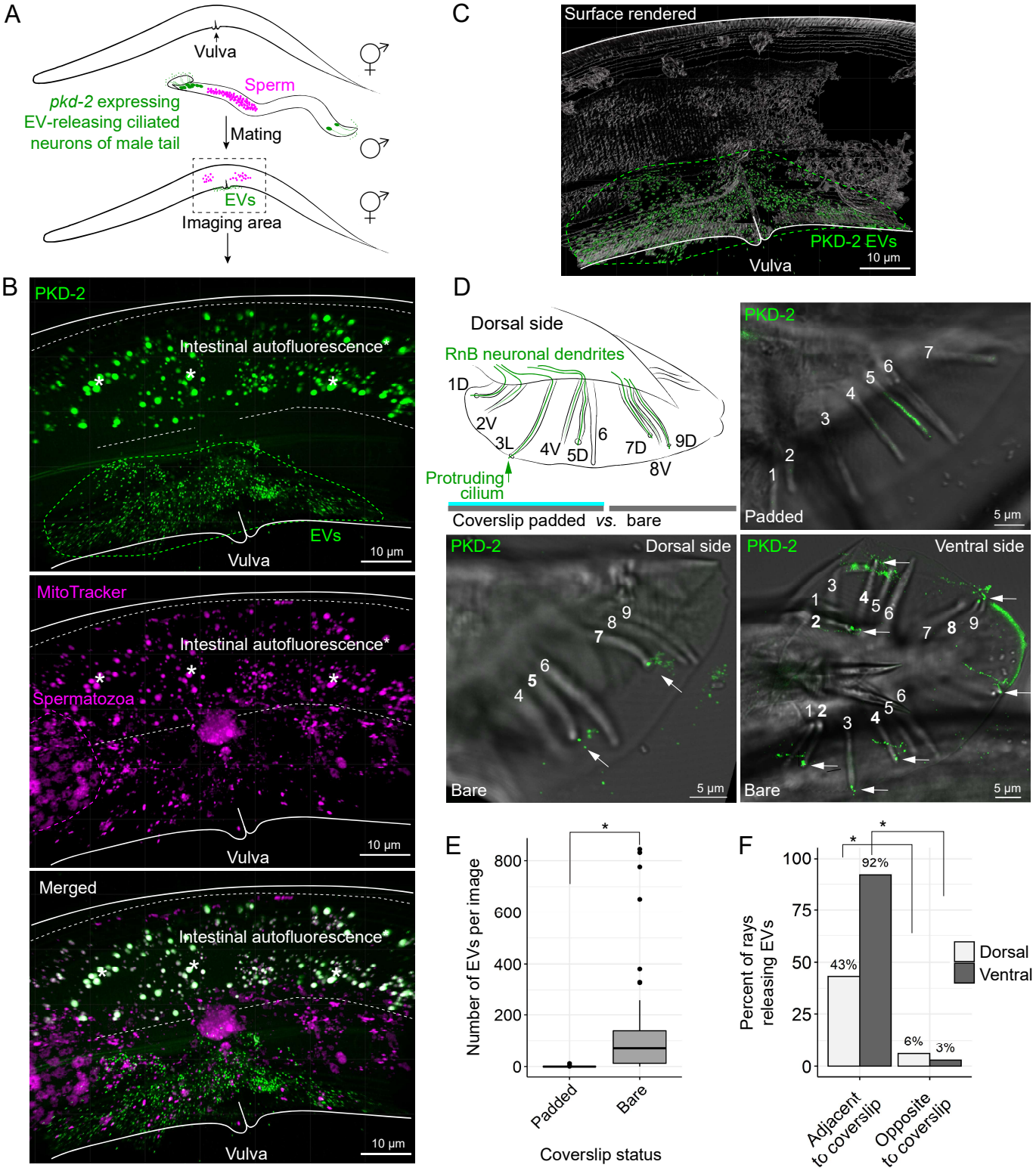
6. Phua, S.C., Chiba, S., Suzuki, M., Su, E., Roberson, E.C., Pusapati, G. V., Setou, M., Rohatgi, R., Reiter, J.F., Ikegami, K., *et al.* (2017). Dynamic Remodeling of Membrane Composition Drives Cell Cycle through Primary Cilia Excision. Cell *168*, 264-279.e15.

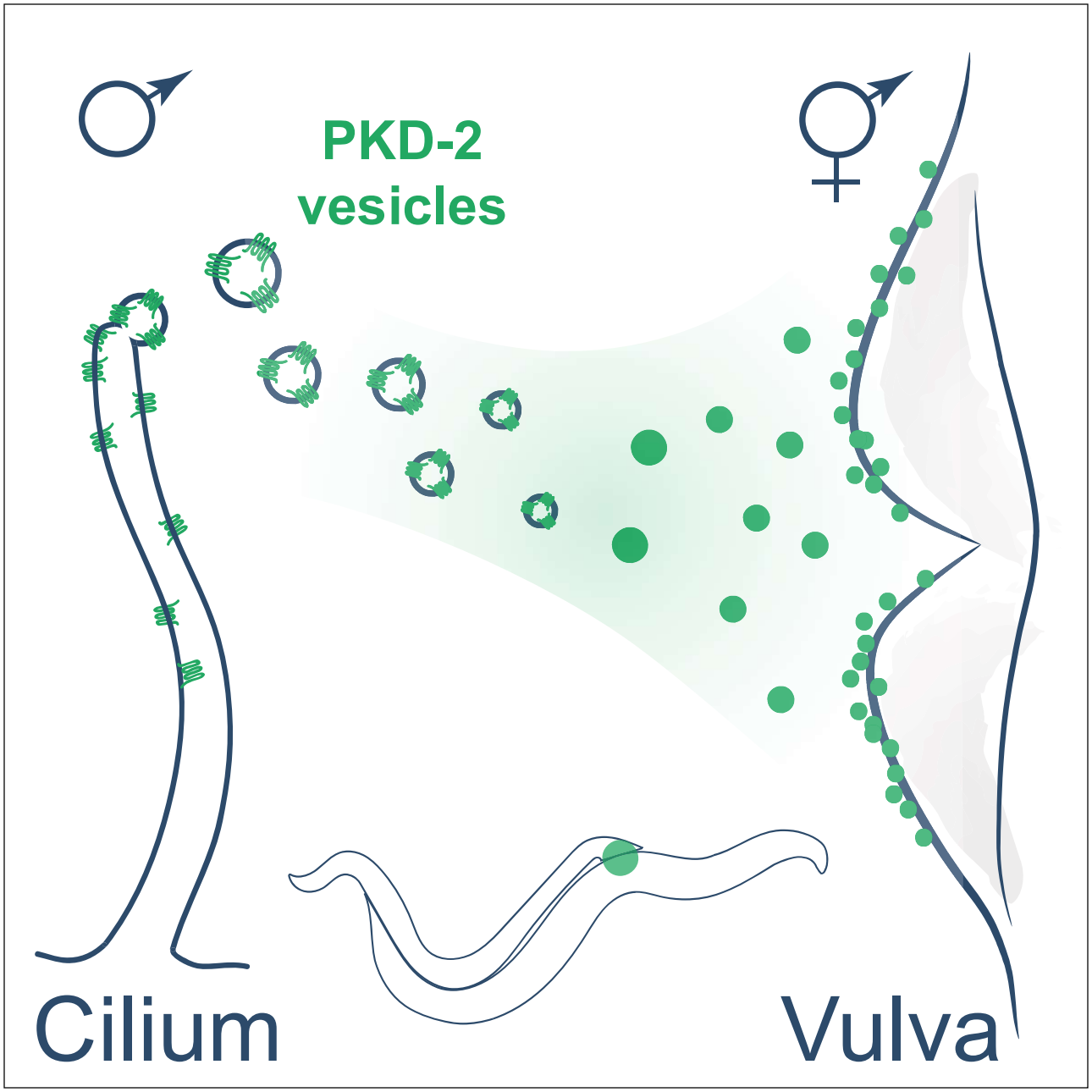
7. Barr, M.M., García, L.R., and Portman, D.S. (2018). Sexual dimorphism and sex differences in *Caenorhabditis elegans* neuronal development and behavior. Genetics *208*, 909–935.

8. Sulston, J.E., Albertson, D.G., and Thomson, J.N. (1980). The *Caenorhabditis elegans* male: Postembryonic development of nongonadal structures. Dev. Biol. *78*, 542–576.

9. Barr, M.M., and Sternberg, P.W. (1999). A polycystic kidney-disease gene homologue required for male mating behaviour in *C. elegans*. Nature *401*, 386–389.

10. Wang, J., Kaletsky, R., Silva, M., Williams, A., Haas, L.A., Androwski, R.J., Landis, J.N., Patrick, C., Rashid, A., Santiago-Martinez, D., *et al.* (2015). Cell-Specific Transcriptional Profiling of Ciliated Sensory Neurons Reveals Regulators of Behavior and Extracellular Vesicle Biogenesis. Curr. Biol. *25*, 3232–3238.





SUPPLEMENTAL INFORMATION

Sensory-evoked extracellular vesicle release and targeting

Juan Wang, Inna A. Nikonorova, Amanda Gu, Paul W. Sternberg, Maureen M. Barr

Table of contents

Materials and methods

Strains

All *C. elegans* strains were maintained under standard conditions as described in [1]. The mating assay used uncoordinated hermaphrodites of the CB369 (*unc-51* (e369)) strain to serve as easy mating targets for males. The males were of the PT443 strain (*myIs1*[P*pkd-2*::PKD2::GFP+P*unc-122*::GFP] I; *pkd-2(sy606)* IV; *him-5(e1490)* V) that carries a loss-of-function *pkd-2* allele together with an integrated rescuing array encoding GFP-labeled PKD-2 protein [2].

Scoring of the PKD-2 EV release was performed using the PT3112 strain (*pha-1(e2123)* III; *him-5(e1490)* V, *myIs4*[P*pkd-2*::PKD2::GFP+P*unc-122*::GFP] V; *myEx888*[CIL-7::tagRFP + pBX1]).

Mating assay tracking sperm and EV transfer

Male worms were pre-stained by soaking in 10 µM MitoTracker Ted CMXRos dye (ThermoFisher Scientific #M7512) buffered with the isosmotic M9 buffer (22 mM KH_2PO_4, 43 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$) for 7 hours in dark to label their spermatozoa [3]. Prior to introduction to the hermaphrodites, males were allowed to crawl on a clean plate to remove any excess of the dye. Mating was conducted on an agar plate with a bacterial lawn 1 cm in diameter generated with 15 ul of overnight OP50 *E. coli* culture. Ten unstained L4 *unc-51 (e369)* hermaphrodites were placed together with fifty pre-stained males and allowed to mate for 24 hours in the dark at 22^{o}C. Following mating, hermaphrodites were mounted in a 0.5 ul drop of 10 mM levamisole (prepared in the M9 buffer) placed on a 5% agarose pad (prepared with ultra-pure water and agarose, Sigma #A9539) for further imaging.

Mounting of males for quantitative scoring of the EV release

For regular imaging of the EV release, males were mounted on agarose padded glass slides in the same way as described above for the hermaphrodites [4]. In order to diminish mechanical stimulation and test the hypothesis about mechanoresponsive EV release, we developed the double agarose sandwich protocolo. Coverslips underwent a special procedure to layer them with a thinnest possible agarose pad. Specifically, 20 µl of 5% agarose gel was dropped on a pre-heated to 90^{o}C coverslip and pressed with another coverslip to make the agarose pad as thin as possible. The coverslip-agarose sandwich was allowed to cool to ambient temperatures and carefully separated so that only one piece of the coverslip was left covered with the agarose gel lining. Imaging of the male worms mounted with agarose-padded coverslips and slides was performed in 1 mM levamisole and within 30 minutes. After each imaging session, the agarose padded coverslip was replaced with a bare coverslip to conduct a second imaging session on the same males to score their response to a bare coverslip.

Fluorescent imaging and quantification

Images were acquired using Zeiss LSM880 confocal microscope with Airyscan high-resolution detector. Image processing included Airyscan processing performed with the accompanying Zeiss software ZEN 2 (Blue version). Surface rendering of the hermaphrodite images was

obtained with Imaris software. PKD-2::GFP-carrying EVs were quantified using ZEN Blue imaging analysis software.

Statistical analysis

Non-parametric Kruskal-Wallis test was used to test the hypothesis that the double agarose sandwich mounting protocol results in significantly different number of EVs released from cilia of a male tail.

Two-proportion Z-test was used to test the hypothesis that frequencies of EV release events from male ray cilia facing or not facing the bare coverslip are significantly different (Table 1). Lateral ray 3 was also producing EVs but was excluded from the analysis as its position relative to coverslip could not be established unequivocally.

Supplemental references:

1. Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71–94.

2. Bae, Y.K., Lyman-Gingerich, J., Barr, M.M., and Knobel, K.M. (2008). Identification of genes involved in the ciliary trafficking of *C. elegans* PKD-2. Dev. Dyn. *237*, 2021–2029.

3. Wang, Y., Zhang, Y., Chen, L., Liang, Q., Yin, X.M., Miao, L., Kang, B.H., and Xue, Di. (2016). Kinetics and specificity of paternal mitochondrial elimination in *Caenorhabditis elegans*. Nat. Commun. *7*.

4. Wang, J., Silva, M., Haas, L.A., Morsci, N.S., Nguyen, K.C.Q., Hall, D.H., and Barr, M.M. (2014). *C. elegans* ciliated sensory neurons release extracellular vesicles that function in animal communication. Curr. Biol. *24*, 519–525.

Figure S1. Release of the PKD-2::GFP-labeled EVs from the HOB neuronal cilium of the male tail.

Optical sections through *C. elegans* male tail positioned ventrally to the bare coverslip. The hook structure carrying cilium of the HOB sensory neuron is labeled with arrow, PKD-2::GFP-carrying EVs released from the HOB cilium are labeled with arrowheads.

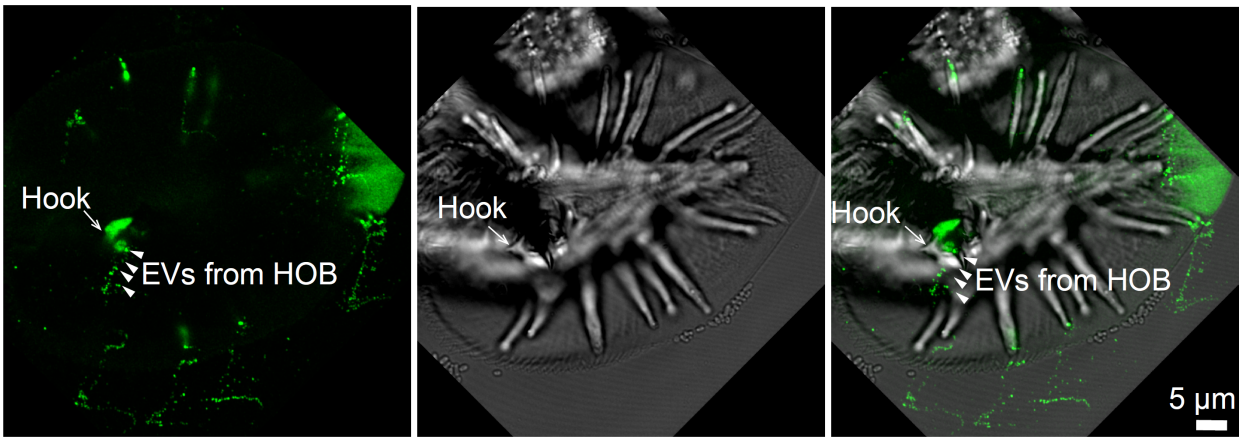


Table S1. Statistical analysis of the frequencies of PKD-2::GFP EV releasing events from sensory rays of the male tail when positioned either adjacent or opposite to the coverslip.

Table S1.	Side of male tail adjacent to coverslip			Side of male tail opposite to coverslip			Two proportion Z-test	
Ray identity	Total number of rays examined	Rays that release EVs	%	Total number of rays examined	Rays that release EVs	%	Z-value	*p*-value
Ventral & dorsal rays	52	28	54%	46	2	4%	5.3	$p < 0.0001$
Ventral rays	12	11	92%	30	1	3%	5.7	$p < 0.0001$
Dorsal rays	40	17	43%	16	1	6%	2.6	$p < 0.01$