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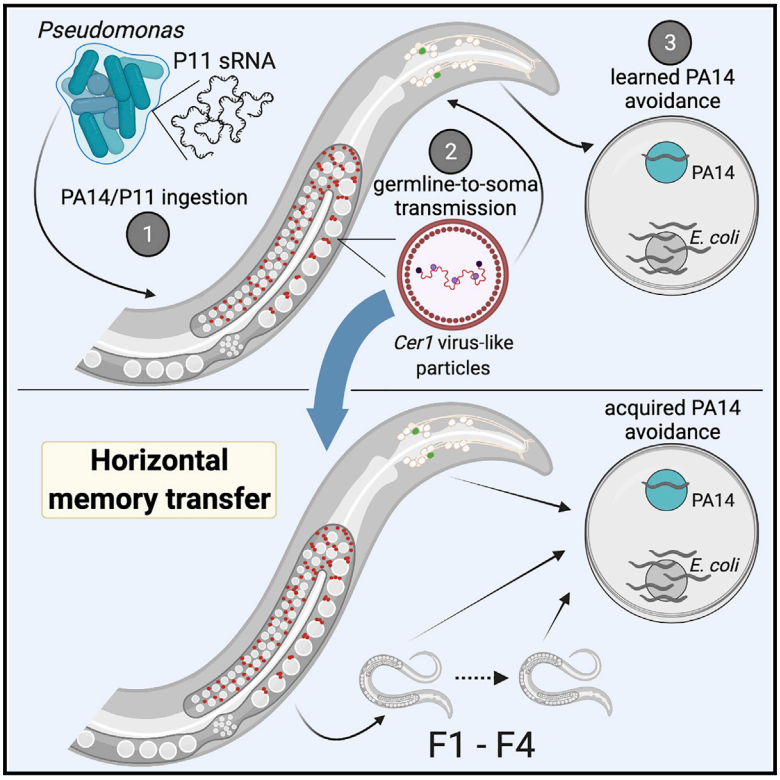
The role of the *Cer1* transposon in horizontal transfer of transgenerational memory

Graphical abstract



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In brief

Cer1 retrotransposon particles mediate inter-tissue and inter-worm transfer of learned pathogen-avoidance behavior in *Caenorhabditis elegans*, providing an example of how a retrotransposon is used for a beneficial adaptive response in the host.

Highlights

- Small-RNA-induced pathogen avoidance memory is transferred horizontally to naive animals
- Horizontally acquired avoidance memory is inherited transgenerationally by progeny
- Learned avoidance and horizontal memory transfer require the *Cer1* retrotransposon
- *Cer1* expression in wild strains correlates with sRNA-induced pathogen avoidance

Moore et al., 2021, Cell *184*, 4697–4712
September 2, 2021
https://doi.org/10.1016/j.cell.2021.07.022

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The role of the *Cer1* transposon in horizontal transfer of transgenerational memory

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https://doi.org/10.1016/j.cell.2021.07.022

SUMMARY

Animals face both external and internal dangers: pathogens threaten from the environment, and unstable genomic elements threaten from within. *C. elegans* protects itself from pathogens by "reading" bacterial small RNAs, using this information to both induce avoidance and transmit memories for four generations. Here, we found that memories can be transferred from either lysed animals or from conditioned media to naive animals via *Cer1* retrotransposon-encoded virus-like particles. Moreover, *Cer1* functions internally at the step of transmission of information from the germline to neurons and is required for learned avoidance. The presence of the *Cer1* retrotransposon in wild *C. elegans* strains correlates with the ability to learn and inherit small-RNA-induced pathogen avoidance. Together, these results suggest that *C. elegans* has co-opted a potentially dangerous retrotransposon to instead protect itself and its progeny from a common pathogen through its inter-tissue signaling ability, hijacking this genomic element for its own adaptive immunity benefit.

INTRODUCTION

The transmission of information across generations through non-genetic means, or transgenerational epigenetic inheritance (TEI), was long thought to be impossible due to the Weismann barrier between the germline and somatic cells, which preserves immortal germ cells in their pristine state. However, recent data from worms (Burton et al., 2020; Houri-Zeevi et al., 2020; Palominos et al., 2017; Pereira et al., 2020; Perez and Lehner, 2019; Rechavi et al., 2014; Singh and Aballay, 2019; Webster et al., 2018), flies (Bozler et al., 2019), and mice (Dias and Ressler, 2014) suggest that inheritance of stress responses may help animals survive in harsh environments. We recently discovered that *C. elegans* passes small RNA-mediated learned *Pseudomonas aeruginosa* avoidance behavior on to several generations of progeny through a molecular mechanism that requires an intact germline and neuronal signaling (Kaletsky et al., 2020a). This process requires uptake of a *P. aeruginosa* small RNA called P11, processing through the RNA interference pathway, piRNA regulation and P granule function in the germline, downregulation of a neuronal gene with homology to a specific bacterial small RNA, and gene expression changes in the ASI sensory neuron (Kaletsky et al., 2020a). This small RNA-mediated process enables mothers and four generations of her progeny to avoid pathogenic *P. aeruginosa*.

The question of whether animals can transmit memories to one another has a storied and controversial history (McConnell et al., 1959; Shomrat and Levin, 2013), but recent work in *Aplysia* suggests that RNA from the CNS of trained animals can induce a form of non-associative long-term memory when injected into naive animals (Bédécarrats et al., 2018). Whether these horizontally transferred memories could be transmitted transgenerationally, thereby breaking the Weismann barrier, or in a natural context has not yet been addressed.

Here, we find that whole-worm lysates and conditioned media (CM) from the grandprogeny of trained *C. elegans* can transmit memory of learned avoidance and transgenerational inheritance of that avoidance behavior to naive animals and their four generations of progeny through virus-like particles (VLPs) encoded by the *Cer1* retrotransposon. In addition to its role in horizontal memory transfer, *Cer1* is required within individuals for small-RNA-mediated learned pathogen avoidance and transgenerational epigenetic inheritance through its ability to convey information from the germline to neurons. *Cer1*'s expression in wild strains correlates with their ability to carry out these behaviors, a beneficial role for *Cer1* that contrasts with its previously reported deleterious effects (Dennis et al., 2012). Thus, *Cer1* function may provide *C. elegans* long-lasting protection from pathogens in their natural environments.

RESULTS

Transgenerational memories are horizontally transferred to naive worms

C. elegans is initially attracted to *P. aeruginosa* (Shtonda and Avery, 2006) but learns to avoid this pathogen after exposure

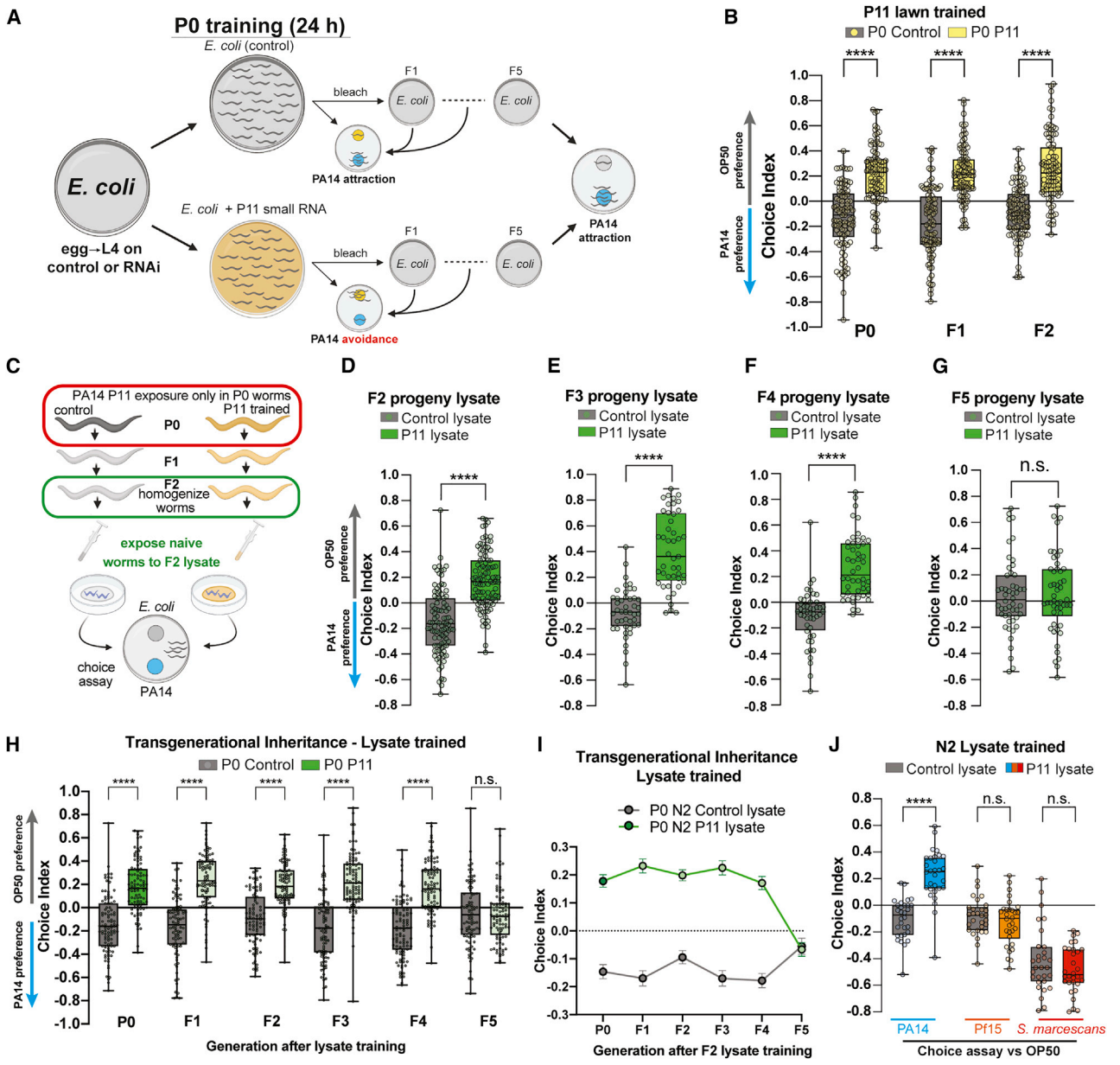

Figure 1. Horizontal transmission of transgenerational PA14 avoidance learning

(A) Worms were trained on non-pathogenic OP50 or *E. coli* expressing the PA14 P11 small RNA or a control. Choice assays to OP50 versus PA14 bacteria were then performed. Trained animals were bleached to maintain subsequent generations without additional PA14 or P11 exposure. Choice index = (number of worms on OP50 − number of worms on PA14) / (total number of worms).

(B) Worms exposed to a the P11 small RNA (for 24 h) learn to avoid PA14. F1 and F2 progeny of P11-trained P0s inherit PA14-avoidance behavior compared to controls.

(C) Schematic of protocol for horizontal memory transfer experiments. F2 progeny from control or P11-trained grandmothers are homogenized, and naive worms are exposed to the F2 lysate for 24 h before testing for learned avoidance behavior.

(D–G) PA14-avoidance behavior in naive animals trained with worm lysate from F2s (D), F3s (E), F4s (F), or F5 animals (G) derived from P0-control or P11-trained animals. F2 thorough F4 worm lysates confer PA14-avoidance behavior (D–F), while F5 worm lysate does not (G).

(H and I) (H) The avoidance behavior acquired by naive worms trained with F2 lysate is inherited in progeny through the F4 generation compared to controls. (I) The mean choice index for each generation is shown ± SEM.

(J) Naive worms were trained with lysate from F2s grand-progeny of control or P11-trained grandmothers, as in (C). After lysate exposure, worms were split into groups and tested in three different choice assays: OP50 versus PA14 (left), OP50 versus Pf15 (middle), or OP50 versus *S. marcescens* (right).

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(Zhang et al., 2005) (Figure 1A). Worms learn to avoid *P. aeruginosa* (PA14) through several independent mechanisms involving bacterial small RNAs, metabolites, and additional pathogenesis factors (Kaletsky et al., 2020a; Meisel et al., 2014; Singh and Aballay, 2019; Troemel et al., 2006) (Figure S1A). However, small RNA-mediated learned avoidance is the only pathway that leads to transgenerational memory inheritance, a process in which the worms "read" PA14's P11 small RNA and use it to alter neuronal function, resulting in learned avoidance (Kaletsky et al., 2020a) (Figure S1A).

To test whether transgenerational learned avoidance can be horizontally transferred to naive worms, we used mechanical homogenization to prepare crude lysates from wild-type grandprogeny (F2) of P11- or control-trained grandmothers (Figures 1B and 1C). Naive animals were exposed to the lysate on *E. coli* plates for 24 h, then tested for *P. aeruginosa* avoidance learning. We found that lysate from F2s of P11-trained, but not control-trained, grandmothers was sufficient to induce naive worms to avoid *P. aeruginosa* (Figures 1C and 1D), indicating horizontal transmission of memory.

We previously observed that training of mothers with either *P. aeruginosa* or P11 small RNA induces a memory of learned avoidance that lasts through the F4 generation (Kaletsky et al., 2020a; Moore et al., 2019). While the lysates from F2, F3, and F4 progeny can induce learning in naive animals (Figures 1D–1F), lysate from the F5 generation—which does not show inheritance of learned behavior from either *P. aeruginosa* or P11 training—is not able to transfer learned avoidance (Figure 1G; Figures S1B–S1G). Furthermore, progeny of F2 lysate-trained P0 animals inherited this learned avoidance behavior, lasting through the F4 generation after training (Figures 1H and 1I), indicating that transgenerational inheritance can occur after horizontal transfer of memory.

We also previously established that training animals on *P. aeruginosa* or P11 small RNA induces avoidance specifically against *P. aeruginosa*, rather than to other bacteria (Kaletsky et al., 2020b; Moore et al., 2019). To test whether the horizontally acquired memory is specific to *P. aeruginosa*, lysate-trained animals were tested for changes in preference to *Pseudomonas fluorescens* (Pf15) or *Serratia marcescens*. While worms exposed to lysate from grandprogeny of P11 small RNA-trained grandmothers learned to avoid *P. aeruginosa* compared to controls, lysate training did not alter the worms' attraction to either *P. fluorescens* (Pf15) or *S. marcescens* (Figure 1J). These results indicate that the horizontally transferred memories are specifically encoded for *P. aeruginosa* avoidance and are likely not caused by a non-specific response that induces broad neuronal changes in preference.

VLPs in lysate transmit transgenerational memories to naive worms

RNA has been implicated in the transfer of memory from the CNS of trained *Aplysia* to naive animals (Bédécarrats et al., 2018), and we previously showed that the mechanism by which *C. elegans* learns the identity of pathogenic *Pseudomonas* requires bacterial small RNAs (Kaletsky et al., 2020a). Therefore, we tested whether there is transfer of memory from (1) free, total RNA isolated from F2s of trained animals or (2) RNase-treated lysate. We found that Trizol-isolated, free, total RNA from trained F2s (Figure 2A) prevented avoidance learning from lysate, while RNase treatment of the trained F2 lysate did not prevent memory transfer from F2 lysate (Figure 2B).

While on its face these results might suggest RNA is not involved, another possibility is that the RNA information could be protected; for example, a similar RNase treatment of Arc VLPs still allows transfer of Arc mRNA between neurons (Pastuzyn et al., 2018). To determine if purified VLPs might carry the memory of P11 training, we tested density-fractionated lysates from F2s of P11-trained grandmothers for their ability to induce avoidance. Only the densest fraction (#6), which should contain heavier particles including VLPs, induced *P. aeruginosa* avoidance behavior in naive animals (Figure 2C).

To determine whether VLPs were present in the fraction that induced learning behavior, we performed electron microscopy on the densest fraction (Figure 2D) and identified ~90 nm VLPs. Not only was this fraction able to induce behavior in the trained generation, but the memory was also retained in progeny through the F4 generation (Figure 2E; Figure S2A). RNase treatment of the VLP fraction did not prevent the induction of avoidance learning (Figure 2F), supporting the model that VLPs protect cargo RNA. We isolated RNA from the VLP fraction and found that this protected RNA from the RNase-treated and purified lysate was mostly <200 nt, with limited larger size species present, suggesting that a small or fragmented RNA could carry the memory signal (Figure S2B).

Cer1 is required for small RNA-induced pathogen avoidance learning and transgenerational memory

The VLPs we observed by electron microscopy (EM) were similar in size to VLPs made by the *Cer1* retrotransposon (Dennis et al., 2012). *Cer1* has homology to the Ty3/Gypsy family of retrotransposons (Figure 3A) and forms VLPs that are detectable by EM and present in the germline of N2 animals at 20°C (Figure 3B). Therefore, we investigated whether *Cer1* might be involved in learned pathogen avoidance and its inheritance. The *Cer1* GAG protein was detected in the densest, VLP-containing fraction (fraction #6), which induced learned avoidance (Figure 2C) in wild-type worms (Figures 3C and 3D). A point mutation (G6369A) in *Cer1* abolishes its detection by immunofluorescence (Figure 3B) or by western blot (Figure 3D), suggesting that this mutation prevents expression of *Cer1* gene products. *Cer1* mutant mothers were still able to learn on a *P. aeruginosa* lawn (Figure S3A), consistent with intact routes of lawn learning, such as innate immunity and metabolites; however, loss of *Cer1* abolishes the F1 inheritance of *P. aeruginosa* avoidance behavior (Figure 3E), which functions

Each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 30–99 plates per condition. At least three biological replicates were performed for all experiments. Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. Unpaired, two-tailed Student's t test (D–G), One-way analysis of variance (ANOVA) (B, H, and J), Tukey's multiple comparison test. ****p < 0.0001, NS, not significant. See also Figure S1 and Table S1 for exact sample sizes (n) and p values.

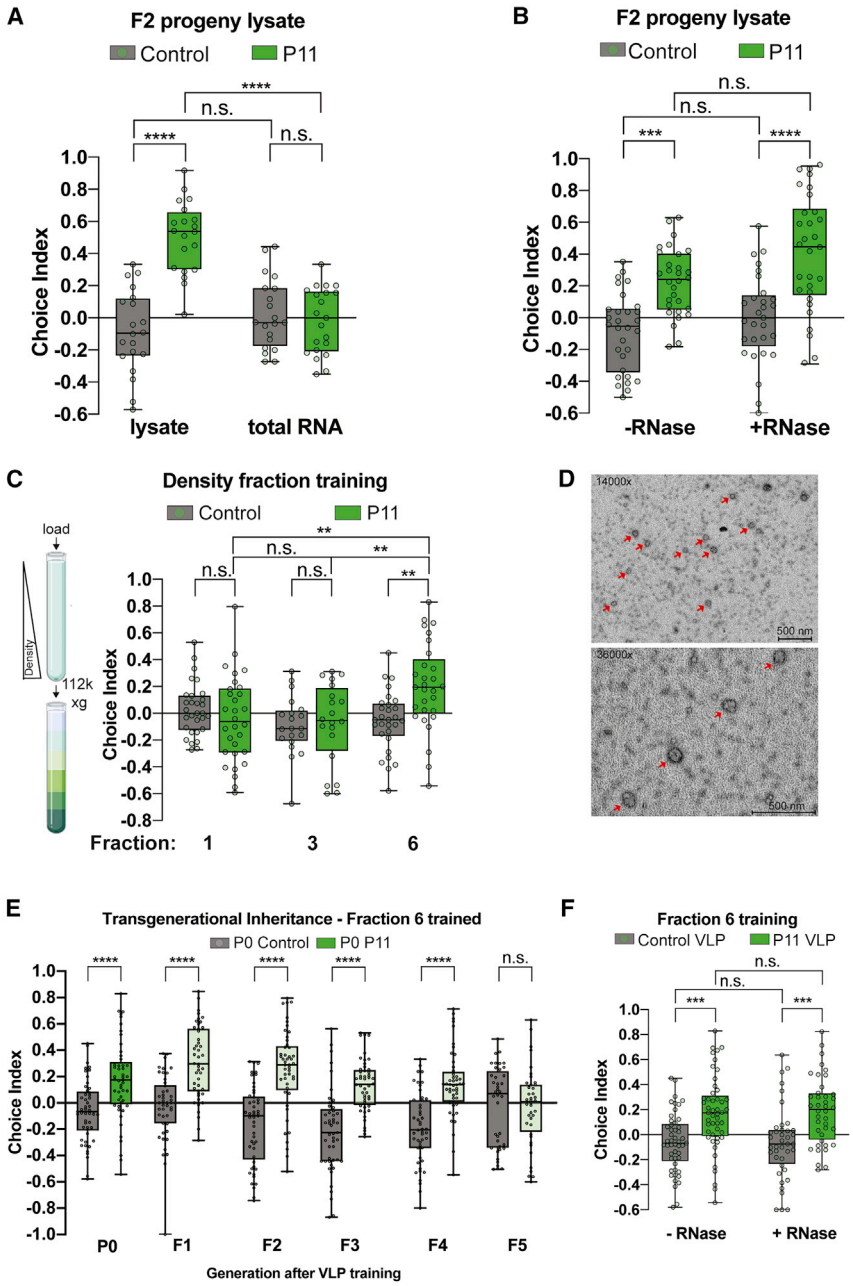

Figure 2. Transgenerational memories are transferred to naive worms via extracellular vesicles or VLPs

(A) Training with total RNA isolated from F2 progeny descended from control or P11-trained grandmothers does not confer PA14 avoidance learning compared to F2 lysate training.

(B) Memory conferred by F2 worm lysate training is resistant to RNase treatment. Lysate was exposed to RNase prior to naive worm exposure.

(C) F2 worm lysates were fractionated using density-based centrifugation. Fractions 1, 3, and 6 from the gradient were used to train naive worms, followed by PA14 choice assays to measure learned behavior.

(D) Negative-stain electron microscopy was performed on fraction 6.

(E) Transgenerational inheritance of PA14 avoidance in progeny of worms exposed to fraction 6 (derived from F2s from control or P11-trained grandmothers).

(F) PA14-avoidance behavior induced by fraction 6 is resistant to RNase treatment. Fraction 6 was exposed to RNase prior to naive worm exposure.

Each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 20–49 plates per condition. At least three biological replicates for all experiments. Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. One-way (E) or two-way (A–C and F) analysis of variance (ANOVA), Tukey's multiple comparison test. **p ≤ 0.01, ***p ≤ 0.001, ****p < 0.0001, NS, not significant. See also Figure S2 and Table S1 for exact sample sizes (n) and p values.

through the separate small RNA-mediated pathway (Kaletsky et al., 2020a). Reduction of *Cer1* via RNAi also abrogated *P. aeruginosa*-mediated pathogen avoidance inheritance (Figure 3F; Figure S3B). Loss of *Cer1* by mutation or RNAi also completely abrogated the ability of mothers trained on *E. coli*+P11 to learn *P. aeruginosa* avoidance (Figures 3G and 3H). Unlike *Cer1*, loss of a different Ty3/Gypsy retrotransposon, *Cer4*, had no effect on learning or transgenerational memory induced by PA14 lawn or *E. coli*+P11 training of N2 mothers (Figures S3C–S3F).

Since *Cer1* is required for small RNA-mediated learned avoidance behavior, we next asked whether Cer1 acts directly in neurons. We examined Cer1 expression in neurons using immunofluorescence staining of worms following control or P11 exposure, and while Cer1 was detected in the germline, it was not detected in neurons (Figure 3I). Furthermore, neuronal expression of Cer1 in the *Cer1* mutant background did not rescue PA14 learned avoidance (Figures S3G and S3H). These results show that Cer1 expression in neurons is not likely to regulate avoidance learning. Moreover, P11 exposure does not increase the level of Cer1 expressed in the worm (Figure S3I).

Upon training with *P. aeruginosa* or P11 small RNA, *daf-7p::gfp* expression increases in the ASI sensory neuron (Kaletsky et al., 2020a; Meisel et al., 2014; Moore et al., 2019). Loss of *Cer1* prevents this increase in expression, indicating that *Cer1* acts upstream of the regulation of *daf-7* expression in the ASI neuron (Figure 3J). Together, these results suggest that *Cer1* is required for small RNA-mediated pathogen avoidance in mothers and their progeny, *Cer1* acts upstream of neurons in the small RNA-mediated learning pathway, and Cer1 protein is present in the VLP fraction

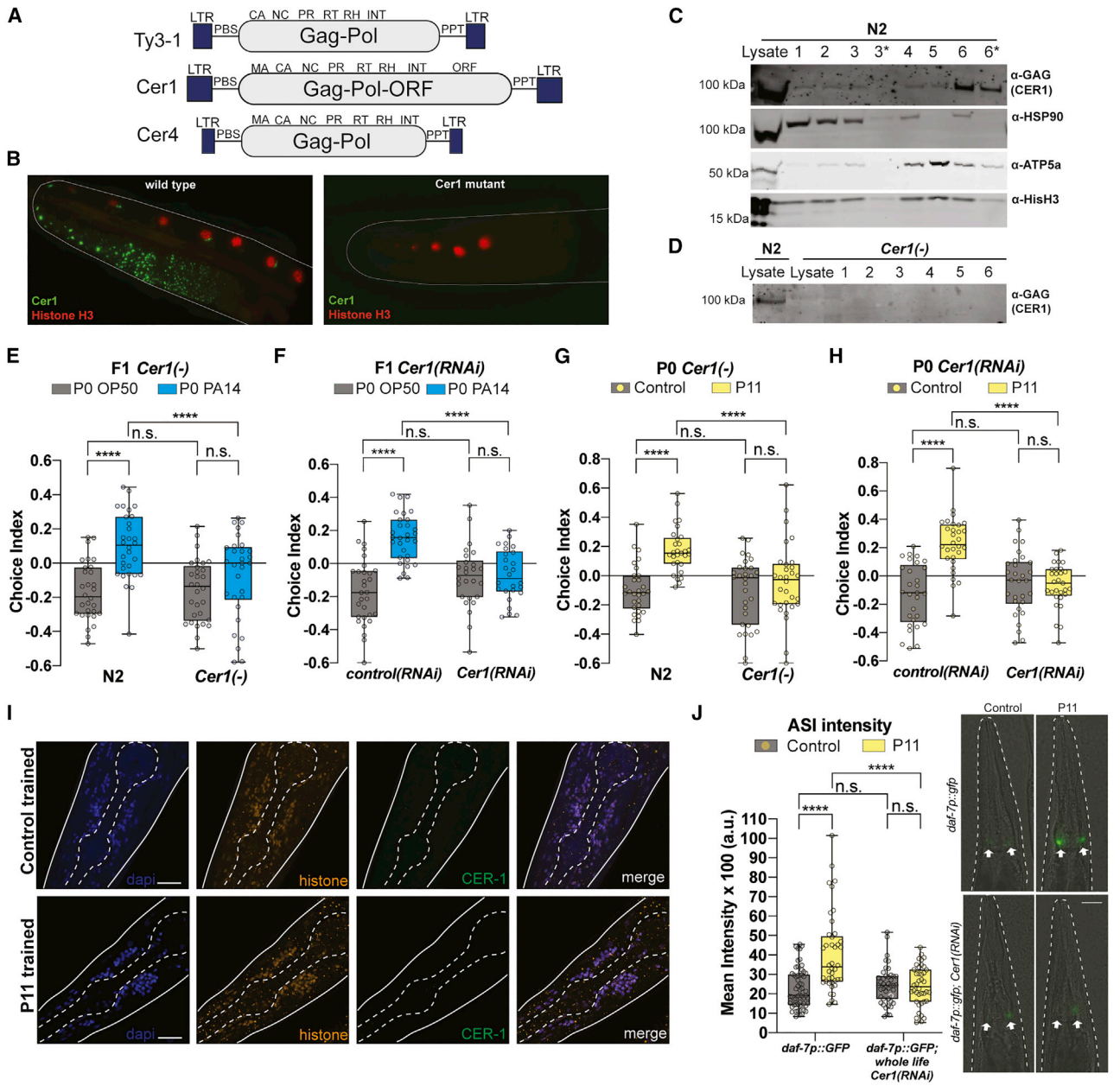

Figure 3. *Cer1* is required for P11-acquired learning and vertically inherited memory

(A) Schematic of *C. elegans Cer1*, *Cer4*, and *S. cerevisiae* Ty3-1. (LTR, long terminal repeat; PBS, primer binding site; MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; RH = RNaseH; INT, tntegrase; PPT, PolyPurine. Tract, ORF, open reading frame).

(B) Immunofluorescence of wild-type or *Cer1* mutant worm germlines stained for Histone H3 (control) or Cer1 GAG.

(C) Cer1 GAG is detected prominently in fraction 6 by western blot, while other cellular markers are enriched in lighter fractions. Each number represents a fraction from the gradient. The 3* and 6* fractions were further concentrated by ultracentrifugation before western blot.

(D) Western blot of Cer1 GAG in wild type and *Cer1* mutant animals. GAG is absent from all fractions in the *Cer1(gk870313)* mutants (G6369A substitution).

(E and F) *Cer1(gk870313)* mutants (E) and *Cer1* RNAi-treated worms (F) are defective for transgenerational inheritance of avoidance behavior when P0 mothers are trained using PA14 bacteria.

(G and H) *Cer1(gk870313)* mutants (G) and *Cer1* RNAi-treated worms (H) are defective for transgenerational inheritance of avoidance behavior when P0 mothers are trained on the P11 small RNA.

(I) Control or P11-trained worms express Cer1 in the germline, but it is not detectable in neurons.

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from worm lysates that induces horizontal transfer of learned memories.

Cer1 is required in both donor and recipient for horizontal transfer of memory

To determine whether *Cer1* is required for not only vertical memory transmission to progeny but also for horizonal memory transfer, we prepared worm lysates and VLP-containing fractions from wild-type and *Cer1* mutant F2s from control or P11-trained grandmothers (Figure 4A). Consistent with the requirement for *Cer1* in horizontal memory acquisition, neither the lysate nor the analogous density-purified fraction (fraction #6) isolated from *Cer1* mutants derived from P11-trained grandmothers were able to induce avoidance of *P. aeruginosa* (Figures 4B and 4C). Immunogold staining for Cer1 GAG protein also demonstrated the presence of Cer1 in the VLP fraction from wild-type worms (Figures 4D–4F). Cer1-positive VLPs were not detected in fraction #6 from a *C. elegans* wild isolate (CB4856) that lacks full-length *Cer1* in its genome (Figures 4D–4F). These results suggest that *Cer1* VLPs are required for the horizontal transfer of transgenerational epigenetic memories to naive worms.

We previously showed that the intestine-expressed double-stranded RNA transporter SID-2 (McEwan et al., 2012) is required for P11-mediated small RNA learning (Kaletsky et al., 2020a). Since *Cer1* is required for both vertical and horizontal transfer of pathogen avoidance learning, we next asked whether SID-2 is required in recipient worms or whether *Cer1*-mediated horizontal memory transfer bypasses this requirement. We exposed *sid-2* mutants to the lysate from wild-type donors (Figures 4G and 4H); both wild-type and *sid-2* mutant worms acquired PA14 avoidance memory, which was inherited through the F4 generation of progeny (Figure S4). These results demonstrate that SID-2 is not required for uptake of the memory signal from donor worms.

We next asked whether a germline is required in recipient worms or if treating with *Cer1*-containing lysate bypasses this requirement in recipient animals (for example, by direct uptake by neurons). However, germline-less *glp-1(e2141)* mutants failed to learn *P. aeruginosa* avoidance upon F2 lysate training (Figure 4I). Similarly, *Cer1* mutants trained with wild-type F2 lysates were unable to learn *P. aeruginosa* avoidance (Figure 4J); this was also true of a wild isolate that lacks a copy of *Cer1* in its genome (Figure S4B). These results show that both *Cer1* and a functional germline are required in recipient animals for horizontal memory transfer through Cer1 VLPs.

Cer1 is required for transmission of germline state of avoidance learning

Our results show that *Cer1* is required in mothers for small RNA-mediated learned avoidance and in their progeny for the inheritance of this behavior. Previously, we found that the process of inducing transgenerational inheritance of pathogen avoidance requires uptake of small, non-coding RNAs from *Pseudomonas*, processing of this small RNA in the intestine and germline, and transmission of an unknown signal that is conveyed to the ASI neurons to influence avoidance behavior (Kaletsky et al., 2020a).

To identify the mechanism of *Cer1*'s function in learned pathogen avoidance and its inheritance, we wanted to determine the step at which it is required:the initiation of the transgenerational signal, maintenance of this signal in the germline from generation to generation, or a subsequent, post-germline step that results in execution of avoidance behavior (transmission of the signal from germline to neurons or neuronal function) (Figure 5A). The step at which *Cer1* acts in the pathway was not clear from our experiments because a mutant or *Cer1* RNAi for several generations would not distinguish a lasting and permanent effect of *Cer1* activation from a transient effect that only affects one step of the transgenerational learned pathogen avoidance process. However, these steps can be distinguished through a simple experiment: knockdown of the gene of interest in the F1 generation after P0 training, followed by control RNAi in generations F2-F5. Knockdown of a gene involved in initiation (P0) would have no effect if reduced only in the F1 generation (Figure 5A, blue line, "initiation"), F1 knockdown of a gene involved in germline maintenance or propagation would permanently eliminate learned behavior (orange line, "maintenance/ propagation"), and F1 knockdown of a gene that only functions in transmission of the signal or functions in neurons would eliminate the behavior for a generation or two but should return once the RNAi knockdown is ended (green line, "behavior").

Knockdown of *sid-2*, the RNA transporter that is expressed in the intestine (McEwan et al., 2012), only in F1 does not affect behavior in any generation, likely because its role is to facilitate uptake of bacterial small RNAs from the gut, which is critical in initiation (P0) but is not needed in later generations (Figure 5B; Figure S5A). By contrast, knockdown of the piRNA Piwi/Argonaute PRG-1 in the F1 generation not only eliminates behavior in F1, but also causes a permanent loss of avoidance behavior (Figure 5C; Figure S5B). These results are consistent with previous data suggesting that *prg-1* is required for maintenance or propagation of avoidance behavior and that loss of *prg-1* erases transgenerational memory (Ashe et al., 2012). The TGF-beta ligand DAF-7 is expressed in the ASI neuron and is required to execute the avoidance behavior (Kaletsky et al., 2020a; Moore et al., 2019). Reduction of *daf-7* by RNAi in the F1 generation following maternal *P. aeruginosa* (Figure S5C) or *E. coli*+P11 (Figure 5D) training abrogated avoidance behavior in the same generation (F1). However, progeny raised on control RNAi recovered their avoidance behavior in the F2–F4 generations (Figure 5D; Figure S5C), demonstrating that the encoded memory was retained even when *daf-7* expression was reduced, and that avoidance behavior could return. This shows that *daf-7* is

(J) *daf-7p::gfp* expression in ASI neurons (white arrows) increases upon P11 small RNA exposure compared to controls. *daf-7p::gfp* expression does not increase upon P11 exposure in *Cer1* RNAi-treated worms. Scale bar, 25 μm.

For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 29–30 plates per condition. For imaging experiments n = 48–52 neurons imaged. At least three biological replicates for all experiments. Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. Two-way (E–H and L) analysis of variance (ANOVA), Tukey's multiple comparison test. ****$p < 0.0001$, NS, not significant. See also Figure S3 and Table S1 for exact sample sizes (n) and p values.

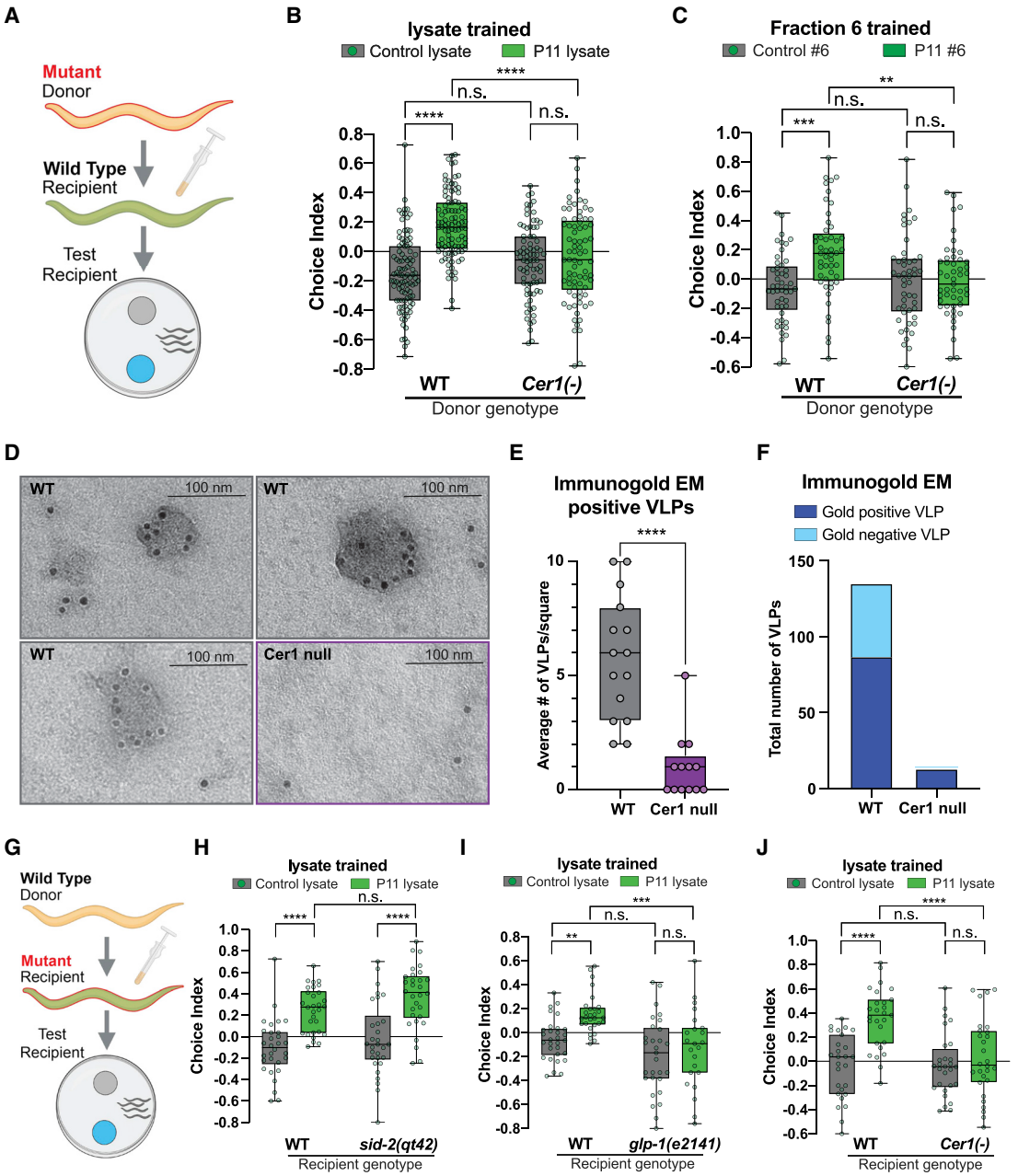

Figure 4. Cer1 is required for horizontal transmission of learned avoidance

(A) Experimental design for (B) and (C), where *Cer1* mutant worms were used to prepare the F2 donor lysate that was then used to train wild-type worms for subsequent behavioral analysis.

(B and C) F2 lysate (B) or the purified EV/VLP fraction 6 (C) from *Cer1* mutant worms does not induce horizontal memory transfer compared to wild-type F2 lysate. Each F2 worm lysate (wild-type or *Cer1* mutant) were the grand-progeny from control or P11-trained grandmothers. Lysate from wild-type or *Cer1* mutant F2 was used to train naive wild-type animals.

(D) Anti-Cer1 GAG immunogold staining for EM was performed on the purified, concentrated, and permeabilized EV/VLP fraction 6 from N2 wild-type worms or a *C. elegans* strain (CB4856) that does not genomically encode *Cer1*.

(E) EM images were quantified for the number immunogold-positive EV/VLP-like structures. Unpaired, two-tailed Student's t test.

(F) The total number of immunogold + or − EV/VLP-like structures is shown. Images were blindly scored.

(G) Experimental design for panels.

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not required for germline maintenance of transgenerational memory but is instead involved in the execution of avoidance behavior in each generation. These *sid-2*, *prg-1*, and *daf-7* RNAi initiation versus maintenance versus execution behavior results, respectively, agree with their previously determined roles in intestine (McEwan et al., 2012), germline (Batista et al., 2008), and neurons (Kaletsky et al., 2020a; Moore et al., 2019; Ren et al., 1996).

Cer1 capsids are present in the germline, and their presence depends on *prg-1* and P granules in worms (Dennis et al., 2012); in yeast, Ty3 VLP formation is similarly dependent on P-bodies (Beliakova-Bethell et al. 2006). Therefore, we first hypothesized that *Cer1* might function at the step of maintaining the transgenerational signal in the germline, similar to *prg-1* (Figure 5C). However, while *Cer1(RNAi)* treatment in the F1 progeny of wild-type mothers trained with *E. coli* expressing P11 (Figure 5E; Figure S4D, right) or with *P. aeruginosa* (Figure S5D, left) caused loss of avoidance behavior, the avoidance memory recovered in subsequent generations maintained on control RNAi allowing *Cer1* re-expression (F2–F4; Figure 5E). These results resembled *daf-7* knockdown and recovery rather than the permanent loss of learned avoidance that *prg-1* knockdown causes, suggesting that *Cer1* acts in the execution of avoidance behavior rather than at the step of maintenance of the transgenerational signal. This further suggested that *Cer1*'s role in learned pathogen avoidance might not be restricted to germline function, despite the fact that it is primarily expressed in the germline (Dennis et al., 2012), but rather it may act at a step between germline and neuron function.

To test the notion that *Cer1* might act in a post-germline, dynamic, transient step, we carried out RNAi starting in adulthood. First, knockdown of *Cer1* in trained P0 adults (Figure 5F) blocked avoidance learning as well as whole-life RNAi treatment (Figure 3H), showing that *Cer1* can be knocked down effectively in adults. Similarly, loss of Cer1 only in adults prevents the induction of *daf-7p::gfp* expression in the ASI (Figure 5G). Knockdown of *Cer1* in trained P0 adults followed by treatment on control RNAi in F1 allowed the re-emergence of avoidance behavior (Figures S6A–S6D), further establishing that *Cer1* is not involved in establishment of the transgenerational signal. Knockdown of *Cer1* only in adults of the F2 generation abrogated behavior (Figure 5H; Figure S6E), despite the F1 animals having demonstrated inheritance of avoidance (Figures S6B–S6D). Together, these results suggest that the process is dynamic: if the transgenerational inheritance of avoidance had been set by regulation of neuronal gene expression levels in the embryonic state, then knockdown of *Cer1* should not have affected behavior. Instead, we see that *Cer1*, which acts upstream of *daf-7* in the ASI, dynamically regulates behavior in adult animals.

Together, these results show that loss of *Cer1* does not erase transgenerational memory but rather is required downstream of the memory maintenance machinery in order to execute avoidance behavior. Thus, its role is unlikely to be solely in the germline but more likely in the communication of the status of avoidance state information from the germline to the neurons in every generation. This germline-to-soma signaling (Figure 5I) ultimately affects neuronal activity and behavior to avoid a common pathogen, and it also improves their survival on that pathogen (Moore et al., 2019). Together, these functions might provide an evolutionary benefit from the insertion and activity of a retrotransposon that was previously thought to be solely deleterious.

The ability of wild strains of *C. elegans* to carry out small RNA-induced pathogen avoidance learning, and transgenerational memory correlates with *Cer1* expression

Roughly 15% of the *C. elegans* genome consists of transposon genetic material (Laricchia et al., 2017). The Ty3/Gypsy family retrotransposon *Cer1* is one of these elements and is inserted into the genomes of roughly 70% of wild *C. elegans* strains (Palopoli et al., 2008), although the sites of these insertions differ—some are present in the *plg-1* locus, which regulates "plugging" upon mating, while others are present elsewhere (Laricchia et al., 2017) (Figure 6A). Similarly, some *Cer1* insertions are only remnants of the active transposon, with only LTRs (long terminal repeats) detectable (Figure 6A). Therefore, we wondered whether the presence of full-length *Cer1* in the genomes of strains isolated from the wild is required for the ability to learn and remember pathogen avoidance.

An intact copy of *Cer1* is present in the wild strain JU1580, as shown by the complete coverage of the coding sequences and LTRs by *de novo* assembly (Cook et al., 2017) (Figure S6F). We found that like N2, JU1580 animals learn to avoid *P. aeruginosa* both through exposure to the pathogen (Figure 6B, left) and small RNAs (Figure 6B, right), as well as by exposure to *E. coli*+P11 (P11 training) (Figure 6C). Furthermore, trained JU1580 mothers can pass this information on to their progeny for four generations, just as N2 does (Figures 6D and 6E). These results suggest that the mechanisms underlying transgenerational inheritance of learned pathogen avoidance via small RNAs are conserved.

In contrast to our findings with JU1580, another *C. elegans* strain, CB4856 ("Hawaiian"), is unable to learn to avoid *P. aeruginosa* after lawn (Figure 6F) or *E. coli*+P11 training (Figure 6G) or to pass this information on to its progeny (F1). It was previously shown that Hawaiian does not have *Cer1* inserted into its genome (Palopoli et al., 2008) (Figure S6G), but this is not the only difference between N2 and Hawaiian. CB4856 and N2 differentially survive on *P. aeruginosa*, and this difference is mediated by the *npr-1* gene, which regulates leaving behavior in response to oxygen levels. However, the genomic region of *npr-1* in JU1580 has the "wild" SNP of *npr-1*, as Hawaiian

(H–J) Where wild-type worms were used to prepare the F2 donor lysate that was then used to train *sid-2* (H), germline-less *glp-1* (I), or *Cer1* (J), mutant worms or controls for subsequent behavioral analysis. Each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 23–99 plates per condition. At least three biological replicates for all experiments. Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. Two-way (B, C, and H–J) analysis of variance (ANOVA), Tukey's multiple comparison test. **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$, NS, not significant.

See also Figure S4 and Table S1 for exact sample sizes (n) and p values.

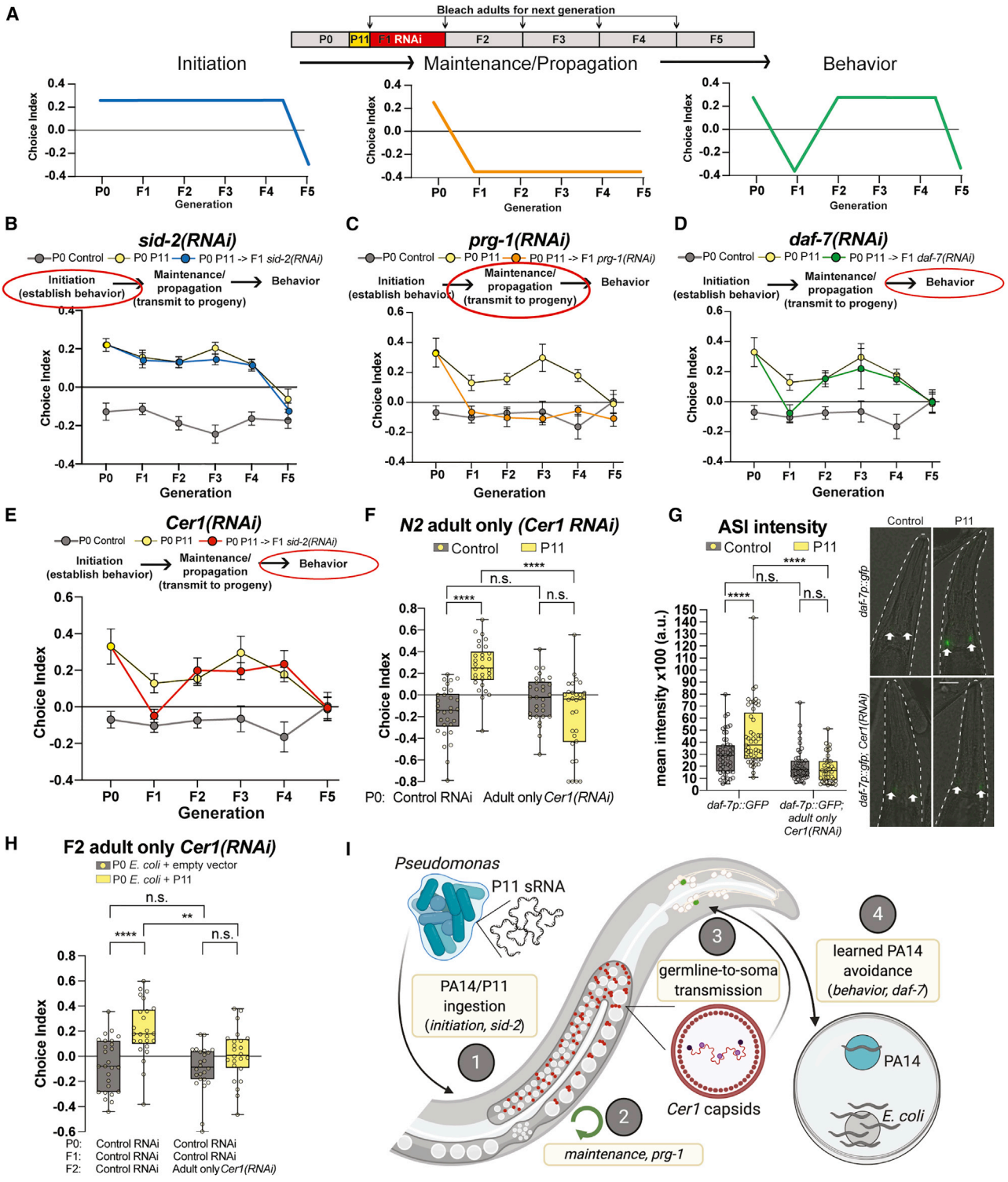

Figure 5. *Cer1* is required for execution of PA14 small RNA-mediated transgenerational inheritance of avoidance behavior

(A) Schematic of F1 RNAi treatment following control or P11 exposure in P0 mothers. Reducing F1 expression of a gene required for initiation of transgenerational inheritance should have no effect on behavior (blue), while reduced F1 expression of a TEI maintenance gene should eliminate memory in the F1 and subsequent generation (orange). F1 knockdown of a gene required for the execution of behavior should affect F1 behavior but not that of subsequent generations (green). (B–E) Wild-type mothers were trained with control or P11-expressing *E. coli*. F1 progeny were then treated with either *sid-2* (B), *prg-1* (C), *daf-7* (D), or *Cer1* (E) RNAi. Subsequent generations of progeny were maintained on normal food and examined for PA14-avoidance behavior.

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does (Cook et al., 2017), ruling out *npr-1* as the source of the difference in pathogenic learning ability. Similarly, the *maco-1* gene, which is downregulated upon exposure to *P. aeruginosa* and is required for learned *P. aeruginosa* avoidance behavior (Kaletsky et al., 2020a), is identical between N2 and Hawaiian in the 17 nucleotides of homology to P11 (Figure S6H), suggesting that Hawaiian's inability to learn and pass on learned avoidance is not due to a lack of sequence matching between P11 small RNA and its *maco-1* target.

To determine whether the presence of *Cer1* correlates with the ability to learn pathogen avoidance more widely in nature, we examined the expression of *Cer1* RNA via real-time PCR (Figure 6A) and the presence of *Cer1* GAG protein via immunofluorescence (Figure 6H) in N2, JU1580, Hawaiian, and an additional nine wild strains of *C. elegans*, and then we tested the ability of these wild strains to carry out P11-mediated learned avoidance of *P. aeruginosa*. Like N2 and JU1580, the wild strains DH424 and KR314 expressed *Cer1* RNA and Cer1 GAG protein and were able to learn *P. aeruginosa* avoidance after P11 training (Figures 6A, 6H, and 6I). Other strains behaved like Hawaiian, as they were unable to learn P11-induced avoidance and were defective for attraction to *P. aeruginosa* (Figures 6A); none of these strains had *Cer1* inserted into the genome or expressed *Cer1* at appreciable levels (Figures 6A and 6H) (although the twelfth strain, ED3040, has *Cer1* inserted into its genome and expresses *Cer1*, it is defective for normal attraction to *P. aeruginosa* and does not exhibit increased avoidance upon training). Finally, treatment of the *Cer1*-expressing wild strain KR314 with *Cer1* RNAi abolished its P11-mediated learning (Figure 6J). Thus, the presence and expression of *Cer1* in wild strains of *C. elegans* largely correlates with ability to learn to avoid *P. aeruginosa* after small RNA-mediated training.

Cer1 is required for horizontally transferred memory from cCM

Since *Cer1* is both highly prevalent in *C. elegans* wild isolates and capable of horizontal memory transfer from worm lysate, we wondered whether worms can secrete *Cer1* into the environment, acting like an extracellular vesicle or virus rather than functioning solely intercellularly. To explore this possibility, we obtained Day 1 adult F2s derived from control or P11-trained grandmothers (Figure S7A) and cultured them in liquid with OP50 for 24 h (Figure 7A). We then filtered out the worms and bacteria and applied the CM to a fresh spot of OP50 to train naive worms. After 24 h of exposure to CM, worms were tested for PA14-avoidance behavior (Figures 7A and 7B). Strikingly, CM from worms that possess the memory of PA14 avoidance can transmit that memory to a naive population of worms, and CM-mediated horizontal memory transfer is *Cer1*-dependent (P0, Figure 7B). As we observed for Trizol-treated lysate fractions (Figure 2A), pre-treatment with detergent (1% Triton) prevented the conferral of behavior from conditioned media, suggesting that the information is protected by a lipid, as in a VLP (Figure S7B). Notably, *C. elegans* is able to distinguish this signal from other RNA that is abundant in the worms' environment, despite its comparatively low abundance (Figure S7C). Consistent with CM-mediated acquisition of PA14-avoidance behavior, CM-treated worms upregulated *daf-7* expression in the ASI neurons (Figure 7C). Worms that acquired PA14-avoidance behavior from CM were also able to transmit the memory transgenerationally to their progeny, as both F1 and F2 animals similarly avoid PA14 (Figure 7B).

Cer1 is required for CM-mediated horizontal memory transfer, suggesting that *Cer1*-containing VLPs are involved in transmitting the memory information. To rule out the contribution of other secreted factors that may be present in CM, we tested whether memory transfer requires pheromones. Pheromones are chemical signals that *C. elegans* secrete to communicate information about sex, mating potential, age, and competitors (Chasnov et al., 2007; Shi et al., 2017). To test whether pheromones are required for horizontal memory transfer, we examined *daf-22* mutants, which are defective in pheromone production (Golden and Riddle, 1985). *Daf-22* mutants learned to avoid PA14 when trained on P11-expressing bacteria, and their progeny inherited the memory transgenerationally (Figures S7D and S7E). Lysate (Figure S7F) or CM from *daf-22* mutant F1s was also able to train naive worms to avoid PA14 (Figure 7D), demonstrating that pheromones are not required for horizontal memory transfer.

We next determined the requirement for extracellular vesicles (EVs), which are membrane-wrapped structures that encapsulate a wide array of signaling molecule cargo and are abundantly secreted by *C. elegans*. EV biogenesis requires the p38 mitogen-activated MAPK (PMK-1) (Wang et al., 2015), so we tested whether *pmk-1* mutants can transmit PA14 learned avoidance through CM. Similar to *daf-22* mutants, *pmk-1* mutant animals can learn to avoid PA14, vertically inherit the memory, and transfer the memory through lysate and the CM (Figure 7E; Figures S7G–S7J). While PMK-1 is a major regulator of EV biogenesis in *C. elegans*, extracellular vesicle biogenesis can occur through alternative MAPK-independent pathways in other organisms (van Niel et al., 2018); therefore we cannot entirely rule out the contribution of EVs in horizontal memory transfer. However, since our behavioral results were entirely dependent on the presence of *Cer1*, our data suggest that neither pheromones nor EVs are the secreted factor that carry the memory and further support the direct involvement of secreted *Cer1* VLPs in horizontal memory transfer.

(F) Worms were treated with *Cer1* RNAi only during adulthood (from L4 to Day 1) before training on control or P11-expressing *E. coli*. *Cer1* is required specifically during adulthood for learned avoidance behavior in mothers.

(G) *daf-7p::gfp* expression does not increase upon P11 exposure in *Cer1* adult-only RNAi-treated worms. Scale bar, 25 μm.

(H) F2 progeny of control or P11 grandmothers were treated with *Cer1* adult-only RNAi. *Cer1* is required in adults in F2 worms that have inherited PA14-avoidance behavior.

(I) Model of germline-to-soma communication of PA14 avoidance through *Cer1*.

For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 24–30 plates per condition. For imaging experiments n = 41–51 neurons. At least three biological replicates for all experiments. Boxplots: center line, median; box range, 25–75th percentiles; whiskers denote minimum-maximum values. Two-way (F–H) analysis of variance (ANOVA), Tukey's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, NS, not significant. See also Figure S5 and S6 and Table S1 for exact sample sizes (n) and p values.

A

Wild-isolate	Plugging	LTRs in genome	Cer1 in genome	Cer1 by rtPCR	Cer1 by IF	Normal attraction	P11-induced learning
N2	-	+	+	+	+	+	+
JU1580	+	+	+	+	+	+	+
DH424	-	+	+	+	+	+	+
KR314	+	+	+	+	+	+	+
ED3040	+	+	+	+	+	-	-
MY2	+	+	+	+	-	+	-
ED3077	+	+	+	+/-	-	+	-
CB4856	+	+	-	-	-	-	-
JU363	+	+	-	-	-	-	-
JU322	+	-	-	-	-	+	-
ED3054	+	-	-	-	-	+	-
ED3073	n/a	-	-	-	-	+	-

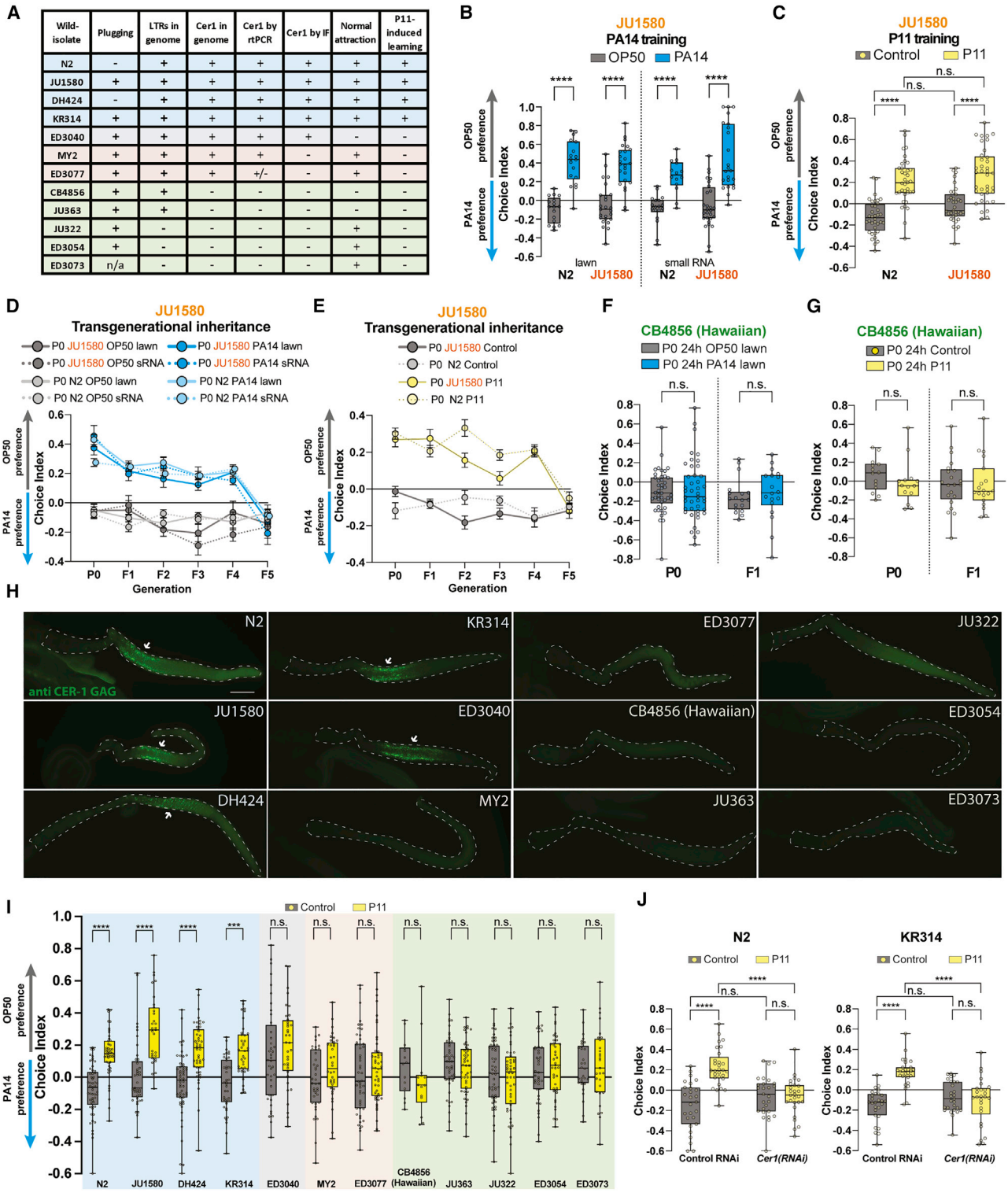

Figure 6. *Cer1* expression correlates with PA14 avoidance learning in *C. elegans* wild isolates

(A) *C. elegans* wild isolates were characterized for plugging (Palopoli et al., 2008), presence, and expression of Cer1, naive PA14 attraction, and P11 small RNA-induced learning.

(B) *C. elegans* wild-isolate JU1580 mothers exposed to PA14 lawns (left) or small RNAs (right) learn to avoid PA14 in a choice assay.

(C) JU1580 mothers exposed to *E. coli* expressing P11 learn to avoid PA14 after training compared to controls.

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To demonstrate that the *Cer1*-dependent memory signal is not a small soluble factor, we purified and concentrated the CM using standard virus purification and ultracentrifugation techniques. The filtered CM was pelleted through a sucrose cushion, and the re-suspended pellet was used to train naive worms. Similar to the VLP fraction purified from worm lysate, RNase treatment of the CM yielded a small but protected population of RNA consisting mostly of species <200 nt but with RNA up to 1,000 nt (Figure S7K). Consistent with our previous results, the VLP fraction from the CM from wild-type worms induced PA14-avoidance behavior, while CM from *Cer1* mutants did not (Figure 7F).

DISCUSSION

Here we have shown that information conveying pathogenic exposure status can be transferred from trained to naive *C. elegans*, via VLPs of Ty3/Gypsy *Cer1* retrotransposon. Additionally, the transfer of this information induces memory that lasts for four additional generations, similar to training on *P. aeruginosa* or its small RNA, P11. Our results provide a molecular mechanism by which memory transmission might occur: the *Cer1* retrotransposon expresses VLPs that can confer memory of learned pathogen avoidance to other individuals when secreted or when the animal is lysed and, within an individual, from germline to neurons. Thus, memories of learned avoidance of pathogens can be transferred between individuals and can induce transgenerational inheritance of the learned information. The fact that worms secrete a *Cer1*-dependent signal into the environment that carries information about pathogen status suggests that Cer1 VLPs function as a form of communication to confer pathogen avoidance to naive relatives, with recipients restricted to those who also carry *Cer1* in its genome. Thus, *C. elegans* has hijacked a potentially deleterious retrotransposon element for its own advantage, using the virus to protect its kin from infectious agents.

The idea that memory can be transferred between individuals is old but controversial. Reports of horizontally transferred memory in planarians (McConnell et al., 1959) seemed to contradict both the concept of memory storage occurring only at synapses and the strict protection of the germline from somatic changes proposed by Weismann in the late 1800s. These findings were more recently supported by an independent study in Planaria that used an automated system to reduce bias (Shomrat and Levin, 2013). However, planaria divide asexually, and thus the concept of a Weismann barrier might be less strict. Furthermore, no molecular mechanism for this type of memory transfer has been determined. Another example of memory transfer between individuals is from recent work in *Aplysia*, in which the RNA extracted from the CNS of trained animals injected into naive animals was able to increase sensitization in a DNA methylation-dependent manner (Bédécarrats et al., 2018), an example of an epigenetic mechanism of memory storage, but whether this could happen in the wild or influence the behavior of progeny is unknown. Our results in *C. elegans* suggest that the *Cer1* retrotransposon enables the transfer of a memory of a pathogen from germline to nervous system, between generations, and from animal to animal.

The fact that *Cer1*'s presence in wild strains of *C. elegans* correlates with the ability to learn and transgenerationally inherit pathogen avoidance suggests that *Cer1* itself may have enabled the acquisition of this behavior. *C. elegans* dies within 2–3 days in the presence of *P. aeruginosa*, killing mothers before they have finished reproducing, which would deleteriously affect their fitness. *Cer1* was previously noted to reduce fecundity in non-pathogenic conditions (Dennis et al., 2012), but here we found that the presence of *Cer1* enables the worms to learn to avoid *Pseudomonas*. If naive animals are able to take up *Cer1* VLPs from animals who are infected, either through secretion of VLPs or by lysis, it would allow them to acquire learned avoidance without experiencing illness themselves (Figure 7G), effectively vaccinating them against future *P. aeruginosa* exposure by inducing avoidance behavior. Furthermore, as infected mothers often "bag" (die of matricide), the ability of other worms to take up *Cer1* VLPs might provide them with the ability to avoid the pathogen. The ability to avoid pathogens for multiple generations could provide *C. elegans* that have acquired *Cer1* an advantage in environments rife with pathogens, perhaps the first demonstration of the physiological relevance of such memory transfer.

Here, we have shown that rather than being solely deleterious (Dennis et al., 2012), the presence of the *Cer1* retrotransposon in fact may have been co-opted by *C. elegans* to help it survive in an environment that requires frequent encounters with pathogens. The ability of the *Cer1* retrotransposon to confer a benefit to the host is surprising, considering the classical nature of transposons in genomes. Transposons are highly abundant in animal genomes and generally regarded as pernicious, mutagenic genetic elements whose mobility can lead to disease and the erosion of host fitness. Transposons incur damage to hosts on several fronts: through misregulation of host processes (such as interfering with host transcription), processing of mRNAs,

(D) Like wild-type N2 *C. elegans*, progeny of JU1580 PA14 lawn- or small RNA-trained mothers continue to avoid PA14 through the F4 generation. Fifth generation progeny return to naive preference.
(E) Progeny of JU1580 *E. coli*+P11-trained mothers continue to avoid PA14 for four generations (F1–F4) before attraction to PA14 resumes in the 5th generation.
(F and G) *C. elegans* Hawaiian mothers exposed to PA14 bacteria lawns (F) or *E.* coli*+P11* (G) do not learn to avoid PA14, and progeny of trained mothers do not inherit avoidance behavior.
(H) Immunofluorescence of *Cer1* GAG was visualized in *C. elegans* wild isolates.
(I) PA14-avoidance behavior in wild-isolate mothers trained on control bacteria or P11-expressing *E. coli*.
(J) Whole-life RNAi knockdown of *Cer1* in N2 (left) and KR314 (right) eliminates P11-induced PA14 learned avoidance.
Each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 14–50 plates per condition. At least three biological replicates for all experiments. Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. One-way (B, F, and G) or two-way (C and J) analysis of variance (ANOVA), Tukey's multiple comparison test. ***$p \leq 0.001$, ****$p < 0.0001$, NS, not significant. See also Figure S7 and Table S1 for exact sample sizes (n) and p values.

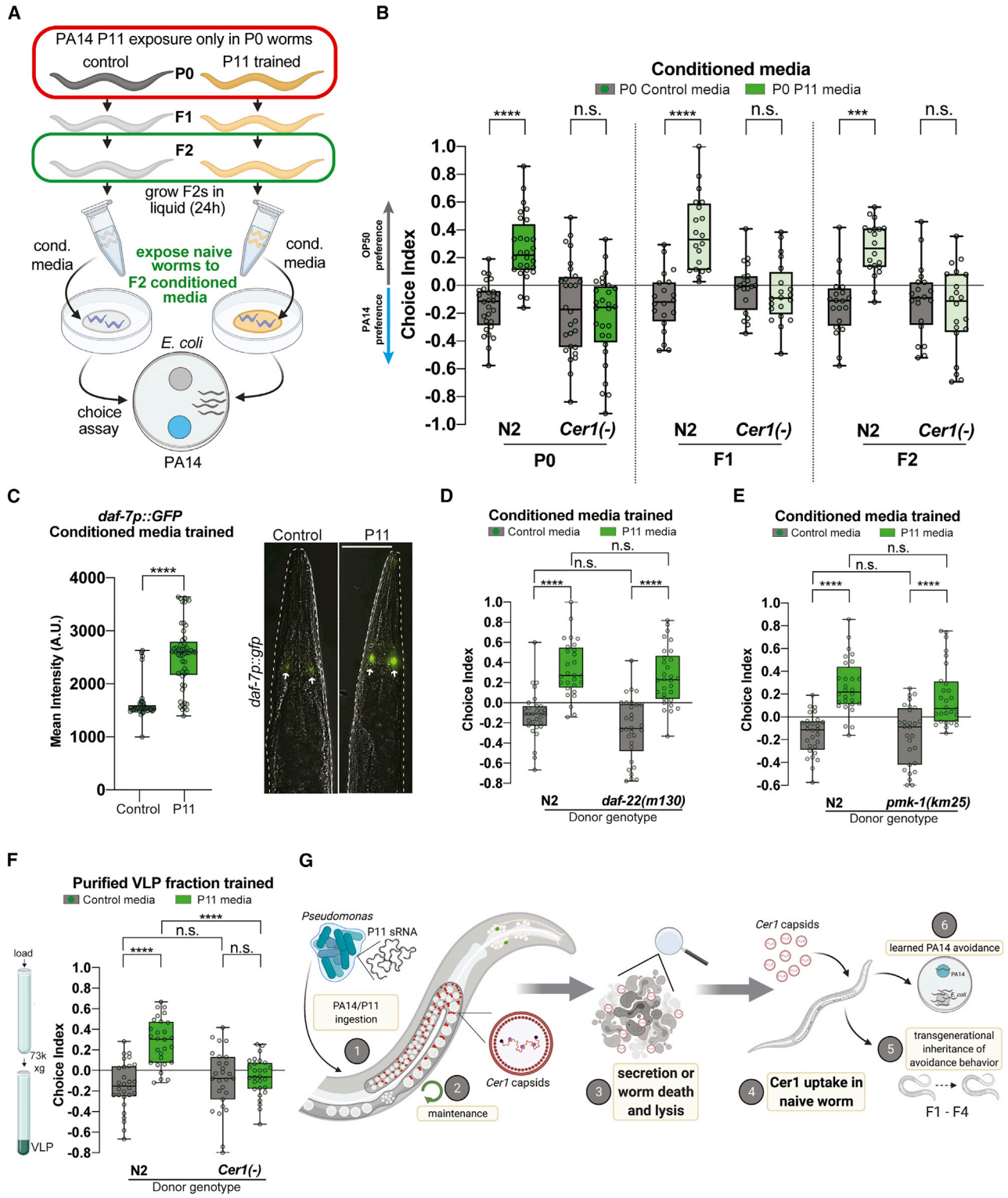

Figure 7. *Cer1*-dependent horizontal memory transfer via secreted VLPs

(A) Schematic of protocol for horizontal memory transfer experiments using CM. Adult F2 progeny from control or P11-trained grandmothers are cultured in S-Basal with OP50 for 24 h. No worms exploded or died during this time. The CM was filtered and plated on fresh OP50 spots. Naive worms are then exposed to the F2 CM for 24 h before testing for learned avoidance behavior.

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and chromatin structure (Elbarbary et al., 2016), or through disruption of the host genome through transposition. Consistent with other transposons, the presence of *Cer1* was previously only noted to be deleterious, as its expression decreases fecundity and lifespan in non-pathogenic conditions (Dennis et al., 2012). The finding that *Cer1* is required for learned and transgenerationally inherited PA14-avoidance behavior shows that ancient retrotransposons can be co-opted and repurposed to benefit the worm, an example of transposon-host mutualism (Feschotte and Gilbert, 2012). Since retrotransposition in *C. elegans* has never observed under laboratory conditions (Bessereau, 2006; Laricchia et al., 2017), it is likely that *Cer1* mediates this acquired worm behavior independent of its potential for novel genome insertion as a retrotransposon.

While the domestication of transposons underlies some of the most critical transitions in animal evolution (Agrawal et al., 1998; Dupressoir et al., 2012; Hiom et al., 1998; Sheen and Levis, 1994; Smit and Riggs, 1996; Tudor et al., 1992), the requirement for *Cer1* in transgenerational learned behavior is unique in that *Cer1* is an active transposon and that *Cer1* confers a behavioral ability, avoidance, on the animals. An interesting parallel arises with comparison to recent studies of Arc (of Ty3/Gypsy family origin), which showed that Arc VLPs can transmit cellular genetic material across neurons in a process that underlies synaptic plasticity in fly and mammalian brains (Ashley et al., 2018; Lyford et al., 1995; Pastuzyn et al., 2018). While *C. elegans* lacks a direct *Arc* ortholog, *Cer1* is also a member of the Ty3/Gypsy family and similarly forms capsids (Dennis et al., 2012). *Cer1*'s role in pathogen avoidance, and specifically in the avoidance behavior step—rather than in generation or maintenance of the transgenerational memory—was surprising, given the fact that *Cer1* produces VLPs in the germline; however, VLPs are also present in non-germline cells at lower abundance, perhaps suggesting at least a transient presence outside of the germline (Dennis et al., 2012). Although it is possible that *Cer1* acts like Arc, transmitting information between neurons, a more parsimonious explanation, given the abundance of *Cer1* VLPs in the germline and our genetic evidence placing it upstream of *daf-7* regulation in the ASI neuron, is that germline *Cer1* VLPs carry host cargo to neurons, where subsequent changes in expression and activity modulate behavior (Figure 5C).

Our data suggest that *Cer1* functions in a dynamic germline-to-neuron signaling mechanism that may represent the co-option of retrotransposon function to improve *C. elegans*' survival and its progeny's survival in pathogenic environments. *Cer1* appears to provide *C. elegans* immediate protection from abundant pathogenic *Pseudomonas* species in its environment but also confers lasting generational benefits by communicating an adaptive immune signal of learned avoidance to its descendants. Moreover, the ability to provide memories of pathogen avoidance to neighboring worms might allow greater survival of its kin.

Limitations of the study

This study demonstrates that learned memories can be transmitted horizontally from trained to naive *C. elegans* via the Cer1 Ty3 retrotransposon. However, there are limitations to the current study that require further investigation. While we observed Cer1 protein present in VLPs, we do not have the resolution to determine whether Cer1 is forming a capsid structure inside of the membrane or upon secretion. Additional studies will be needed to examine the precise structure of Cer1 in these VLPs, as well as identifying the memory-containing cargo. The future challenge will be to determine how the Cer1-dependent VLP signal is transported across membranes and tissues within the worm and between worms. These studies are limited by the vanishingly small amount of RNA present in the samples (despite the high volumes of worms obtained for experimental inputs), the unknown size and modifications of the RNA memory signal (which can preclude incorporation into RNA sequencing libraries), the high level of rRNA and small nucleolar RNA present during high-throughput sequencing (up to 95% of the sample, Figure S7K), and the presence of RNA contamination from the worms' OP50 bacteria food source (Figure S7C).

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
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  - Materials availability
  - Data and code availability

(B) CM from wild-type F2 worms confers learned PA14-avoidance behavior on a naive population of wild-type worms (P0, left). The F1 (center) and F2 (right) progeny of the wild-type trained worms inherit learned PA14 avoidance. CM from F2 *Cer1* mutant worms does not induce learning (P0) or transgenerational inheritance (F1, F2) when used to train wild-type naive worms.

(C) Training with F2 CM induced *daf-7p::gfp* expression in the ASI neurons. Scale bar, 25 μm. Unpaired, two-tailed Student's t test.

(D and E) F2 CM from pheromone defective *daf-22(m130)* mutants (D) or EV-defective *pmk-1(km25)* mutants (E) was used to train a population of naive wild-type worms. Both *daf-22(m130)* and *pmk-1(km25)* are not required for horizontal memory transfer via CM.

(F) VLPs were purified from the F2 CM by ultracentrifugation through a 25% sucrose cushion. The pellet was resuspended in PBS, plated on fresh OP50 spots, and used to train naive worms. The wild-type VLP-purified fraction induced learned PA14 avoidance behavior in naive worms, while purified media from *Cer1* mutant worms did not.

(G) In a model of horizontal memory transfer, PA14-avoidance memory occurs when naive worms are exposed to *Cer1*-dependent VLPss from an animal that has already inherited the memory. Uptake of *Cer1* induces memory directly in that animal and in four generations of its progeny.

For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 20–30 plates per condition. For imaging experiments n = 45–52 neurons. At least three biological replicates for all experiments. Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. One-way (B) or two-way (D–F) analysis of variance (ANOVA), Tukey's multiple comparison test. ***p $\leq$ 0.001, ****p < 0.0001, NS, not significant.

See also Figure S7 and Table S1 for exact sample sizes (n) and p values.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cell.2021.07.022.

ACKNOWLEDGMENTS

We thank the *C. elegans* Genetics Center for strains, Princeton's Imaging and Analysis Center; J. Priess (FHCC) for providing anti-*Cer1* GAG antibody hybridoma, S. Petry for equipment; BioRender.com for model figure design software, J. Shepherd and the laboratory of C.T.M. for discussion, and R. Clausen for assistance. C.T.M. is the Director of the Glenn Center for Aging Research at Princeton and an HHMI-Simons Faculty Scholar. This work was supported by the HHMI-Simons Faculty Scholar Program (AWD1005048), a Pioneer Award to C.T.M. (NIGMS DP1GM119167), T32GM007388 (NIGMS) to R.S.M., and a Pioneer Award to Z.G. (DP1A1124669).

AUTHOR CONTRIBUTIONS

Conceptualization, R.S.M., R.K., and C.T.M.; methodology, R.S.M., R.K., C.L., and C.T.M.; investigation, R.S.M., R.K., C.L., V.C., E.B., and L.R.P.; writing, R.S.M., R.K., and C.T.M.; funding acquisition, C.T.M. and Z.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

Received: December 31, 2020
Revised: May 21, 2021
Accepted: July 19, 2021
Published: August 6, 2021

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-*Cer1*-GAG	Dennis et al., 2012	Anti-Cer1-GAG
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Flour 488	Invitrogen	A-11008
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Flour 555	Invitrogen	A32732
rabbit polyclonal anti-Histone H3	Abcam	ab1791
mouse monoclonal anti-ATP5A	Abcam	ab14748
mouse monoclonal anti-Hsp90	Abcam	ab13492
Goat anti-Mouse IgG (H+L), Superclonal Recombinant Secondary Antibody, Alexa Fluor 488	Invitrogen	A28175
Goat Anti-Rabbit IgG Polyclonal Antibody (IRDye 680)	Li-COR Biosciences	926-68071
Goat Anti-Rabbit IgG Polyclonal Antibody (IRDye 800)	Li-COR Biosciences	926-32210
Goat Anti-Mouse IgG (whole molecule)-gold antibody	Millipore Sigma	G7652
Bacterial and Virus Strains		
P. aeruginosa: PA14	Tan et al., 1999	PA14
S. marcescens: 274	ATCC	247
P. fluorescens 15: Pf15	Mohamed Donia, Princeton University	Pf15
E. coli expressing P11	Kaletsky and Moore et al., 2020a	P11
E. coli expressing empty vector	Kaletsky and Moore et al., 2020a	Control
Chemicals, Peptides, and Recombinant Proteins		
Sodium azide	Fisher Scientific	S2271-25
Potassium Hydroxide	Fisher Scientific	P258-212
Sodium Hypochlorite	Thermo Fisher Scientific	SS290-1
Peptone	Fisher Scientific	DF0118-07-2
Sodium Chloride	Sigma-Aldrich	S9888
Bacto-Agar	Fisher Scientific	DF0140-07-4
L-Arabinose	Bioworld	40100301-3
Potassium Phosphate monobasic	Fisher Scientific	7778-77-0
Potassium Phosphate dibasic	Millipore Sigma	7758-11-4
Magnesium Sulfate Anhydrous	Fisher Scientific	7487-88-9
Calcium Chloride	Fisher Scientific	AC349615000
Yeast Extract	Fisher Scientific	DF0127-17-9
Tryptone	Biosciences	211705
Sodium Phosphate Dibasic Anhydrous	Fisher Scientific	7558-79-4
Cholesterol	Fisher Scientific	C314-500
Carbenicillin	Sigma-Aldrich	C4316
Tetracycline	Sigma-Aldrich	87128
Trizol LS Reagent	Fisher Scientific	10-296-010
Levamisole solution	Vector Laboratories	SP-500

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
10x phosphate buffered saline (PBS)	Bio-Rad	1610780
Triton	Millipore Sigma	9002-93-1
Poly-Prep Slides, poly-L-lysine coated glass slides	Sigma-Aldrich	P0425-72EA
Methanol	Thermo Fisher Scientific	A452SK-1
Chloroform	Sigma-Aldrich	319988
OptiPrep Density Gradient Medium	Millipore Sigma	D1556
SW55 Ti Swinging-Bucket Rotor	Beckman Coulter	N/A
SW32.1Ti	Beckman Coulter	N/A
SW32.1T1 adaptor	Beckman Coulter	N/A
Open-Top Thinwall Ultra-Clear Tube	Beckman Coulter	344057
RNase A	Omega BioTek	AC117
RNase inhibitor	Invitrogen	AM2696
10X Bolt Sample Reducing Agent	Invitrogen	B0009
4X Bolt LDS Sample Buffer	Invitrogen	B0007
20X Bolt™ MOPS SDS Running Buffer	Invitrogen	B0001
4%-12% Bis-Tris gel	Invitrogen	NW04125BOX
PVDF Membrane	Millipore Sigma	IPVH00010
Milk nonfat Powdered	VWR	M203-10 g-10pk
Glow-Discharged Grids	Electron Microscopy Sciences	CF400-Cu
Uranyl Formate	Electron Microscopy Sciences	16984-59-1
2 mL Dounce All-Glass Tissue Grinder	DWK Life Sciences	8853000-0002
Q5 High Fidelity 2X Master Mix	New England Biolabs	M0494S
Bovine Serum Albumin solution	Millipore Sigma	A0336
Gateway pDONR 221 vector	Thermo Fisher Scientific	12536017
Gateway BP Clonase II Enzyme mix	Thermo Fisher Scientific	11789020
Critical Commercial Assays		
mirVana, miRNA Isolation Kit with phenol	Thermo Fisher Scientific	AM1560
Quant-iT Protein Assay Kit	Invitrogen	Q33211
Superscript III First Synthesis System	Invitrogen	18080-051
Experimental Models: Organisms/Strains		
C. elegans: Strain N2 var. Bristol: wild-type	*Caenorhabditis* Genetics Center	WB Strain: N2
C. elegans: Strain *ksIs2 [daf-7p::GFP + rol-6(su1006)]*	*Caenorhabditis* Genetics Center	WB Strain: FK181
C. elegans: Strain *glp-1(e2141)III*	*Caenorhabditis* Genetics Center	WB Strain: CB4037
C. elegans: Strain CB4856	*Caenorhabditis* Genetics Center	WB Strain: CB4856
C. elegans: Strain JU1580	*Caenorhabditis* Genetics Center	WB Strain: JU1580
C. elegans: Strain KR314	*Caenorhabditis* Genetics Center	WB Strain: KR314
C. elegans: Strain DH424	*Caenorhabditis* Genetics Center	WB Strain: DH424
C. elegans: Strain MY2	*Caenorhabditis* Genetics Center	WB Strain: MY2
C. elegans: Strain JU363	*Caenorhabditis* Genetics Center	WB Strain: JU363
C. elegans: Strain JU322	*Caenorhabditis* Genetics Center	WB Strain: JU322
C. elegans: Strain ED3077	*Caenorhabditis* Genetics Center	WB Strain: ED3077
C. elegans: Strain ED3040	*Caenorhabditis* Genetics Center	WB Strain: ED3040
C. elegans: Strain ED3054	*Caenorhabditis* Genetics Center	WB Strain: ED3054
C. elegans: Strain ED3073	*Caenorhabditis* Genetics Center	WB Strain: ED3073
C. elegans: Strain VC40895	*Caenorhabditis* Genetics Center	WB Strain: VC40895
C. elegans: Strain *pmk-1(km25)*	*Caenorhabditis* Genetics Center	WB Strain: KU25
C. elegans: Strain *daf-22(m130)*	*Caenorhabditis* Genetics Center	WB Strain: DR476

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
C. elegans: Strain *Cer1(gk870313)*	This Study	CQ655
C. elegans: Strain *sid-2(qt42)*	*Caenorhabditis* Genetics Center	WB Strain: VC1119
C. elegans: Strain *Cer1(gk870313); unc-119p::Cer1::unc-54 3'UTR*	This Study	CQ670
Oligonucleotides		
Cer1 coding region Set 1: 5'-CGGATGAGCAACAAGTAGCAATT-3'	Adapted from Palopoli et al., 2008	N/A
Cer1 coding region Set 1: 5'-AGCAGCCTTGTTTGGAGAAAAC-3'	Adapted from Palopoli et al., 2008	N/A
Cer1 coding region Set 2: 5'-CCGCTAGAAATAGTAGCTTGT GATC-3'	This Study	N/A
Cer1 coding region Set 2: 5'-ATGCATAATCGTTTTGTTAAATCTTT CCAC-3'	This Study	N/A
Cer1 LTRs: 5'-ATGACTGACGCACTCTCACG-3'	This Study	N/A
Cer1 LTRs: 5'-CCAATCCAGTAAACGAGACCA-3'	This Study	N/A
Cer1 neuronal rescue 5'-GGGGACAAGTTTGTACAAAAAAGC AGGCTT CATGGAGGTGAACGAGGG ACAGG-3'	This Study	N/A
Cer1 neuronal rescue 3'-GGGGACCACTTTGTA CAAGAAAGCTGGGTTTCATTTTTTAC GCCCGTA TGGAGTGAAAT-5'	This Study	N/A
Recombinant DNA		
Plasmid: pL4440 RNAi control	*C. elegans* RNAi Collection (Ahringer)	Control
Plasmid: pL4440-*daf-7* RNAi	*C. elegans* RNAi Collection (Ahringer)	*daf-7*
Plasmid: pL4440-*sid-2* RNAi	*C. elegans* RNAi Collection (Ahringer)	*sid-2*
Plasmid: pL4440-*Cer1* RNAi	*C. elegans* RNAi Collection (Ahringer)	*Cer1*
Plasmid: pL4440-*prg-1* RNAi	*C. elegans* RNAi Collection (Ahringer)	*prg-1*
Plasmid: pL4440-*Cer4* RNAi	*C. elegans* RNAi Collection (Ahringer)	*Cer4*
Software and Algorithms		
Prism 8	GraphPad Prism	https://www.graphpad.com/scientific-software/prism/
NIS-Elements AR	Nikon Instruments	https://www.microscope.healthcare.nikon.com/products/software/nis-elements/nis-elements-advanced-research
LiCOR-Image Studio	Li-COR	https://www.licor.com/bio/image-studio-lite/d5

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Coleen T. Murphy (ctmurphy@princeton.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C. elegans and bacterial strain cultivation

Worm strains were provided by the *C. elegans* Genetics Center (CGC): N2 (wild type), FK181, CB4856 (Hawaiian), JU1580, KR314, DH424, MY2, JU363, JU323, ED3077, ED3040, ED3054, ED3073 and, CB4037, DR476, KU25, VC1119, and VC40895 (*gk870313*) (*Cer1* mutant). VC40895 was outcrossed 6 times to generate CQ655. CQ655 was used for all the *Cer1* mutant experiments reported in this paper. CQ655 was used to make CQ670 (see below).

Bacterial strains: *P. aeruginosa* PA14 was a gift from Z. Gitai. *P. fluorescens* 15 (Pf15) was a gift from M. Donia, OP50 was provided by the CGC, and *Serratia marcescens* (ATCC 274) was provided by the ATCC. Control (*L4440*), *Cer1*, *Cer4*, *daf-7*, and *prg-1* RNAi clones were obtained from the Ahringer library and sequenced verified before use. *E. coli* expressing P11 was made as previously described (Kaletsky et al., 2020a).

METHOD DETAILS

General worm maintenance

Worm strains were maintained at 20°C on High Growth Media (HG) plates (3 g/L NaCl, 20 g/L Bacto-peptone, 30 g/L Bacto-agar in distilled water, with 4 mL/L cholesterol (5 mg/mL in ethanol), 1 mL/L 1M $CaCl_2$, 1 mL/L 1M $MgSO_4$, and 25 mL/L 1M potassium phosphate buffer (pH 6.0) added to molten agar after autoclaving) on *E. coli* OP50 using standard methods.

RNAi treatment

For all experiments using RNAi treated worms, worms were cultured on HG plates (supplemented with 1 mL/L 1M IPTG, and 1 mL/L 100 mg/mL carbenicillin). 1 h before use of RNAi plates, 200 μL of 0.1M IPTG was spotted onto seeded RNAi plates and left to dry at room temperature.

General bacteria cultivation

OP50 and *P. aeruginosa* PA14 were cultured overnight in autoclaved and cooled Luria Broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl in distilled water) shaking (250 rpm) at 37°C. *E. coli* strains expressing PA14 small RNAs were cultured overnight shaking (250 rpm) at 37°C in Luria Broth supplemented with final concentrations of 0.02% arabinose w/v and 100 μg/mL carbenicillin. *E. coli* RNAi strains were cultured overnight shaking (250 rpm) at 37°C in Luria Broth supplemented with filter sterilized (final concentration) 12.5 μg/mL tetracycline and 100 μg/mL carbenicillin. 10x concentrated OP50 (used in conditioned media preparation) was resuspended in autoclaved S-basal (5.85 g/L NaCl, 1 g/L K_2HPO_4, 6 g/L KH_2PO_4) in MilliQ H_2O, supplemented with 1 mL/L cholesterol (5 mg/mL in ethanol).

Training plate/worm preparation

Worm preparation: Eggs from young adult hermaphrodites were obtained by bleaching and subsequently placed onto HG plates seeded with *E. coli* OP50 or HG RNAi plates seeded with RNAi and incubated at 20°C for 2 days. Synchronized L4 worms were used in all training experiments.

Bacteria lawn training plate preparation: Overnight cultures of bacteria (prepared as described above) were diluted in LB to an Optical Density (OD_{600}) = 1 and used to fully cover Nematode Growth Media (NGM) (3 g/L NaCl, 2.5 g/L Bacto-peptone, 17 g/L Bacto-agar in distilled water, with 1 mL/L cholesterol (5 mg/mL in ethanol), 1 mL/L 1M CaCl2, 1 mL/L 1M MgSO4, and 25 mL/L 1M potassium phosphate buffer (pH 6.0) added to molten agar after autoclaving) plates. For preparation of *E. coli* expressing PA14 P11 RNA, bacteria were seeded on NGM or HG plates supplemented with 0.02% arabinose and 100 μg/mL carbenicillin (final concentration). All plates were incubated for 2 days at 25°C unless specified otherwise (in separate incubators for control/pathogen seeded plates). On the day of training (i.e., 2 days post bleaching), plates were left to cool on a benchtop for 1 h to equilibrate to room temperature before the addition of worms. Additionally, for *E. coli* strains expressing PA14 small RNAs, 200 μL of 0.01% arabinose was spotted onto seeded training plates 1 h prior to use.

Worm preparation for training

Synchronized L4 worms were washed off plates using M9 and left to pellet on the bench top for approximately 5 min. 5 μL of worms were placed onto small RNA-spotted training plates, while 10 μL or 40 μL of worms were plated onto OP50 or *E. coli* expressing PA14 small RNAs, or pathogen-seeded training plates, respectively. Worms were incubated on training plates at 20°C in separate containers for 24 h. After 24 h, worms were washed off plates using M9 and washed an additional 3 times to remove excess bacteria. Worms were tested in an aversive learning assay described below.

Aversive learning assay

Overnight bacterial cultures were diluted in LB to an Optical Density (OD_{600}) = 1, and 25 μL of each bacterial suspension was spotted onto one side of a 60 mm NGM plate and incubated for 2 days at 25°C. After 2 days assay plates were left at room temperature for 1 h before use. Immediately before use, 1 μL of 1M sodium azide was spotted onto each respective bacteria spot to be used as a paralyzing agent during choice assay. To start the assay (modified from (Zhang et al., 2005)), worms were washed off training plates in M9 allowed to pellet by gravity, and washed 2 additional times in M9. 5 μL of worms were spotted at the bottom of the assay plate, using a wide orifice tip, midway between the bacterial lawns. Aversive learning assays were incubated at room temperature for 1 h before manually counting the number of worms on each lawn. Plating a large number of worms (> 200) on choice assay plates was avoided, since excess worms clump at bacterial spots making it difficult to distinguish animals, and high densities of worms can alter behavior. A detailed protocol is described in (Moore et al., 2021).

In experiments in which each generation was treated with RNAi: Animals were washed off plates with M9 at Day 1 of adulthood. A subset of the pooled animals was subjected to an aversive learning assay, while the remaining worms were bleached to obtain eggs, which were then placed onto HG or HG RNAi plates and left at 20°C for 3 days before the next generation was tested.

Adult only RNAi: At the L4-stage, worms were washed off of OP50 plates with M9 and left to pellet on the bench. Worm pellets were washed two more times in M9. Worms were then pipetted with a large orifice pipette tip onto RNAi plates. Worms were left on RNAi plates for 24 h at 20°C. Following 24 h of RNAi exposure, worms were washed off RNAi plates with M9 and used the appropriate experiment, either training or behavior testing.

C. elegans total RNA isolation

F2 worms from trained grandmothers were washed off of plates using M9. Three additional M9 washed were performed to remove excess bacteria, and the supernatant was discarded. 1 mL of Trizol LS was added per 100 μL of worm pellet. Worms were lysed in Trizol by incubation at 65°C for 10 min with occasional vortexing. RNA was extracted with chloroform, and the aqueous phase was used as input for RNA purification using the mirVana miRNA isolation kit according to the manufacturer's instructions for total RNA. Approximately 100 μg of total RNA from either control or P11 grandmother-trained F2 worms was used per training plate. This amount of RNA was chosen as it correlates to the same input of worms used for training with worm lysate (see Preparation of Worm Lysates). Purified RNA was used immediately by dropping RNA onto pre-seeded spots of OP50 on NGM plates. Plates were allowed to air dry before the addition of naive worms for training. Worms were trained on RNA-seeded plates for 24 h at 20°C and subsequently tested for PA14 aversive learning using a standard choice assay.

Analysis of JU1580 genomic sequences

Fastq files from SRA (accession numbers SRR9322509, SRR9322510, SRR9322511, SRR9322512, SRR9322514) were uploaded to Galaxy (Afgan et al., 2018) for analysis. De novo assembly of Illumina reads was performed using SPAdes (Bankevich et al., 2012) (Galaxy wrapper version 3.12.0), and contigs were aligned to the *C. elegans* N2 strain genome (WBcel235) using minimap2 (Li, 2018). For structural variant detection, alignment of raw fastq reads to *C. elegans* was performed using BWA (Li and Durbin, 2009), followed by analysis using Lumpy (Layer et al., 2014).

Preparation of worm lysates

Day 1 F2 progeny from control or P11-trained grandmothers were collected from plates and washed 3 times in M9. The worm pellet was washed with DPBS, and the pellet was resuspended in DPBS. Worms were homogenized using an all-glass Dounce tissue grinder, and homogenization was monitored using a microscope. Different worm lysates within an experiment were normalized to the starting amount of worms. For training naive worms with lysates from F2 animals, the normalized lysate was diluted 1:3 with DPBS, such that 400 μL of lysate was obtained for every 100 μL of starting worm pellet. 200 μL of lysate was immediately pipetted directly onto the bacterial spot of 10 cm NGM plate (seeded with 200 μL of an OP50 spot in the center of the plate, 2 days prior to the experiment). Worm lysates were allowed to air dry, and plates with lysates were monitored to ensure no worms were alive following homogenization. Naive Day 1 worms were then transferred to lysate-seeded plates for 24 h of training at 20°C, followed by testing for learned avoidance using the standard OP50 v. PA14 choice assay.

Cer1-enriched fraction isolation from worm lysate

Homogenates were prepared as described (Preparation of worm lysates) and cleared from debris by a 750 x g centrifugation at 4°C for 5 min. Homogenization and clearing steps were repeated twice. The homogenates were then passed twice through a 0.22 μm filter. For each sample, the homogenate protein concentration was measured using Quant-iT Protein Assay Kit. Per experiment, if needed, the homogenates were diluted in DPBS in order to load similar concentrations. From each sample, a small aliquot was kept as a "load" sample, and 830 μL was layered on top of an Iodixanol gradient. For each gradient- 5%, 11%, 17%, 24% and 30% Iodixanol solutions were made by mixing solution A (0.1 M NaCl, 0.5 mM EDTA, 50 mM Tris HCl, pH 7.4) with solution B [50% Iodixanol solution, 0.5 mM EDTA, and 50 mM Tris HCl, pH 7.4]. The gradient was made in a 5 mL, Open-Top Thinwall Ultra-Clear Tube from equal volumes (830 μL) of each Iodixanol solution that were allowed to diffuse by an overnight incubation at 4°C. Samples were then centrifuged at 112,000 x g (4°C) for 2 h, using SW55 Ti Swinging-Bucket Rotor. Six fractions of equal volumes were collected. In addition to fraction 6, fractions 1 and 3 were chosen for further analysis because they appeared de-enriched

for Cer1 and more enriched for other cellular components based on the western blot (Figure 3C). The Cer1-enriched fraction (fraction 6), as well as fraction 3, were diluted in DPBS and centrifuged at 335,000 x g (4°C) for 30 min. Each pellet was then resuspended in DPBS and used for western blots, naive worm training, or electron microscopy. For each experiment, the enrichment of Cer1 in fraction 6 was verified by western blot. For fractions treated with RNase, 1:1000 RNaseA was added following resuspension in PBS after the final spin, and samples were incubated for 15 min at room temperature. For behavior experiments with RNase-treated samples, the reaction was terminated by adding RNase inhibitor (1 unit final).

Negative-stain Electron Microscopy

5 μl of sample from fraction 6 that was purified from worm lysates was applied to glow-discharged grids, washed once with ultrapure water, and stained with 0.75% uranyl formate. Images were collected with a Talos F200X Transmission Electron Microscope with CCD camera at 200 keV.

Immunogold labeling

Immunogold labeling protocol was adjusted from (Gulati et al., 2019); 5 μl of samples were applied to glow-discharged grids, washed once with ultrapure water. Grids were then washed three times with 0.1 M Tris HCl, pH 7.4, and samples were permeabilized by adding 0.1% Triton X-100 and incubating for 10 min at room temperature. Blocking was performed for 30 min at room temperature in blocking buffer [0.1% (v/v) Tween 20, 0.3% (v/v) bovine serum albumin, IgG free], followed by 60 min incubation with anti-Cer1 GAG antibody in blocking buffer (1:25 dilution). Grids were then washed five times with wash buffer [0.1% (v/v) Tween 20, 0.03% (v/v) bovine serum albumin, IgG free], and were next incubated with goat Anti-Mouse IgG (whole molecule)-Gold antibody (10 nm colloidal gold) (1:20 dilution) for 60 min. Each grid was then washed five times in wash buffer, and then washed three times in distilled water. Staining and imaging were performed as described in *Negative-stain Electron Microscopy.*

Conditioned media collection

Conditioned media was prepared by obtaining F2 progeny from control or P11-trained grandmothers. Day 1 adult worms were washed off of maintenance plates using M9. The worm pellet was washed 1x in M9, and the supernatant was removed. For every 50 μL of worm pellet, 300 μL of S-Basal media supplemented with 10X OP50 was added. 1.5 mL of the worm and S-Basal/OP50 solution was transferred to each well of a 6-well plate and allowed to incubate for 24 h at 20°C. After 24 h, plates were inspected for worm health. We confirmed that no worms had exploded, bagged, or died. Conditioned media was harvested using wide-orifice pipette tips and pooled into 15 mL or 50 mL conical tubes. The media was cleared from bacteria and progeny by a 6500 x g centrifugation at room temperature for 10 min. The media were then passed through a 0.22 μm filter. Unconcentrated conditioned media was used immediately to train worms. 200 μL of conditioned media was placed directly on the OP50 spot on a pre-prepared 10 cm plate seeded with 250 μL OP50 the previous day. The conditioned media was allowed to dry at room temperature before the addition of 5 μL of L4 worms pipetted from a washed worm pellet using wide-orifice tips. Worms were trained for 24 h at 20°C before testing PA14 avoidance behavior or bleaching to obtain progeny.

To purify and concentrate VLPs from the conditioned media, the filtered media was treated with 1:1000 RNaseA and samples were incubated for 15 min at room temperature. 5-9 mL of conditioned media was then layered on top of a 25% sucrose cushion (5 mL, diluted in S-basal) in a 17 mL, Open-Top Thinwall Ultra-Clear Tube and was then centrifuged at 20,000 rpm (72,900 x g) (4°C) for 2.5 h, using a SW32Ti (with SW32.1Ti adapters) Swinging-Bucket Rotor. Pellets were then washed in DPBS and centrifuged again at 20,000 rpm (4°C) for 30 min. Final pellets were resuspended in 400-600 μL of S-basal, and subsequently 200 μL was placed onto a pre-seeded OP50 spot for worm training, as performed for the unconcentrated conditioned media.

Western blot

For western blot analysis, samples were mixed with 10X Bolt Sample Reducing Agent and 4X Bolt LDS Sample Buffer. Samples were then heated at 70°C for 10 min before loading on a gradient-PAGE (4% −12%) Bis-Tris gel. After their separation, samples were transferred to a PVDF membrane and blocked with 5% milk in TBST (10X TBST: 200 mM Tris- HCl, pH 7.5, 1.5 M NaCl, 1% Tween20). Membranes were incubated with one of the following primary antibodies: anti-Cer1 GAG (1:150 dilution), rabbit polyclonal anti-Histone H3 (1:1000 dilution), mouse monoclonal anti- ATP5A (1:1000 dilution), mouse monoclonal anti- Hsp90 (1:1000 dilution). After washing with 1x TBST, membranes were incubated with the corresponding fluorescent secondary antibody (either goat anti-rabbit IgG, or goat anti- mouse IgG). Membranes were then washed with 1x TBST and imaged on ODYSSEY CLx.

Signals were quantified using LI-COR Image Studio; background was reduced from each signal, and each signal was normalized to the alpha-tubulin loading control.

Imaging and fluorescence quantification

All *daf-7p::gfp* images were taken on a Nikon Eclipse Ti microscope. Differential inference contrast (DIC) images of whole worms following OP50, or PA14 lawn or small RNA training, were imaged at 20 × . *Z*-stack multi-channel (DIC and GFP) of day-1 adult GFP-transgenic worms were imaged every 1 μm at 60 × magnification; Maximum intensity projections and 3D reconstructions of head neurons were built with Nikon NIS-Elements. To quantify *daf-7p::gfp* levels, worms were prepared and treated as described in 'Worm preparation for training'. Worms were mounted on agar pads and immobilized using 1 mM levamisole. GFP was imaged

at 60 × magnification and quantified using NIS-Elements software. Average pixel intensity was measured in each worm by drawing a Bezier outline of the neuron cell body for 2 ASI head neurons.

Whole-mount immunofluorescence

Antibody staining of *C. elegans* gonads was performed according to (Shaham, 2006). Day 1 hermaphrodites were suspended in M9 on a glass slide and gonads were dissected. Slides were freeze-cracked on dry-ice, fixed for 5 min in cold MeOH/5 min in EtOH, and washed 3x in PBST. Primary antibodies used: anti-*Cer1*-GAG (1:50), and anti-Histone H3 (Abcam, 1:200). Secondary antibodies used: goat anti-mouse AlexaFluor 488-labeled IgG (1:500), goat-anti rabbit AlexaFluor 555-labeled IgG (1:500). Both primary and secondary antibodies were incubated overnight at 4°C. Images were taken at 40x or 100x on a Nikon A1 confocal. Levels were set for the N2 positive control, and each germline was imaged at the same settings. Images were processed through NIS-Elements. Each image is a 12-22 image stack superimposed as a maximum projection.

Neuron expression of Cer1

The *Cer1* open reading frame was cloned into the pDONR221 entry vector using BP recombination. The final destination vector was generated by recombining the *unc-119* promoter, the *Cer1* ORF, and the *unc-54* 3¿UTR. The *unc-119p::Cer1::unc-54 3¿UTR* plasmid was injected into the Cer1(*gk870313*) mutant strain CQ655 at 10 ng/μl with 1 ng/μl of *myo2p::gfp* to create strain CQ670. Rescue of *Cer1* expression was confirmed by western blot.

QUANTIFICATION AND STATISTICAL ANALYSIS

For the comparison of choice indices between two genotypes or treatment conditions (i.e., Control versus P11 trained animals), two-way ANOVA with Tukey's multiple comparison test was used. For the comparison of choice indices in which only one genotype was tested (i.e., wild-type only), unpaired t tests were performed. For the comparison of learning indices between generations (i.e., wild-type only), one way ANOVA with Tukey's multiple comparison test was used. For quantification of neuron intensity, two-way ANOVA with Tukey's multiple comparison test was used.

Populations of worms were raised together under identical conditions and were randomly distributed into treatment conditions. Trained worms were pooled and randomly chosen for choice assays. For all choice assays, each dot represents an individual choice assay plate (about 10–300 worms per plate) with all data shown from at least 3 independent replicates (Table S1). Plates were excluded that contained less than 10 total worms per plate.

In all box-and-whiskey plots: the box extends from the 25th to the 75th percentile, with whiskers from the minimum to the maximum values. All figures in the Article and Supplementary Information pooled data from independent experiments. Statistics were generated using Prism 8; software used for genomic analysis are described in the Methods Details section of the STAR methods. Counting of worms on choice assay plates was performed blind with respect to worm genotype and training condition. Additional statistical details of experiments, including sample size can be found in figure legends and Table S1.

Supplemental figures

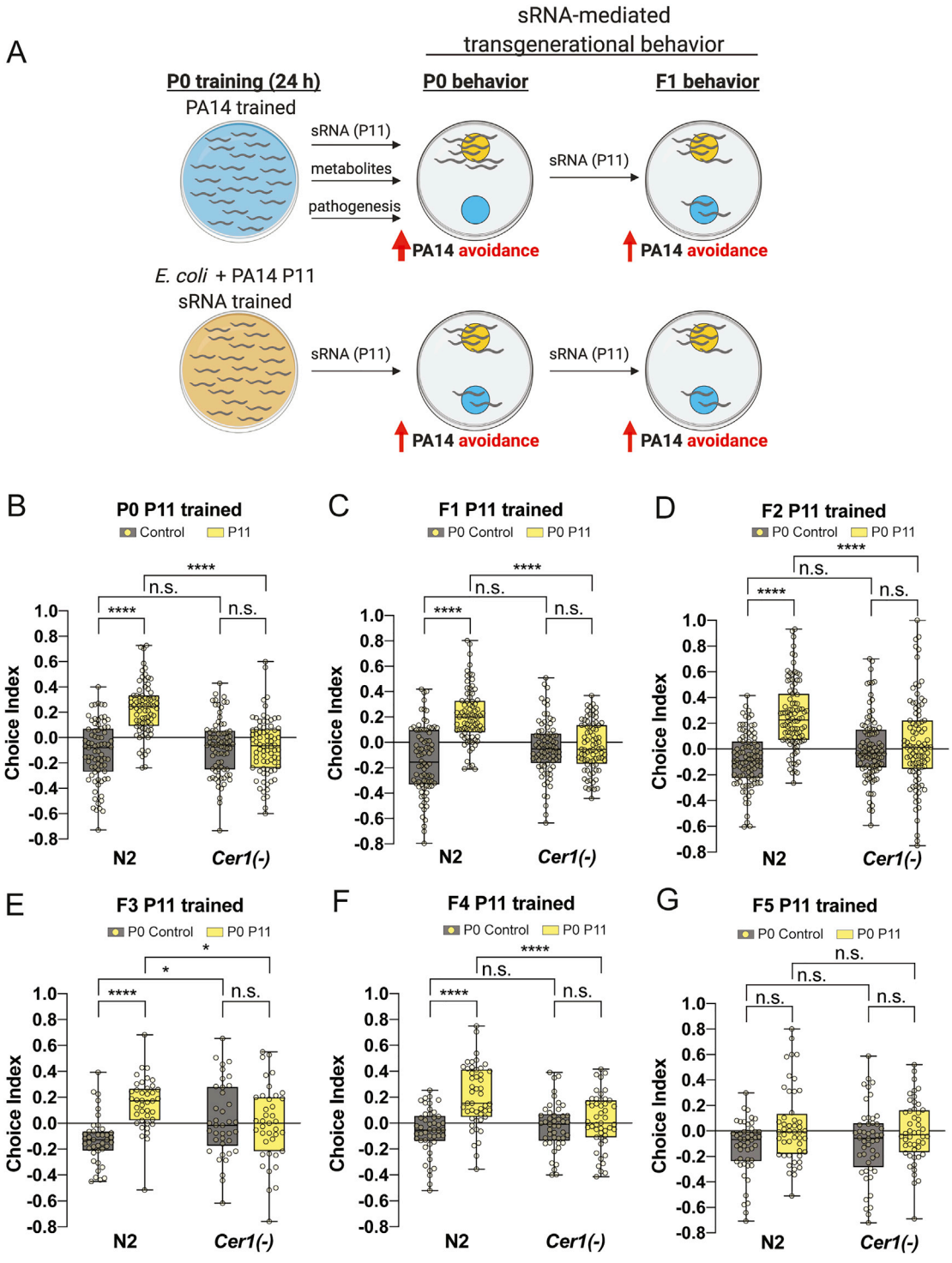

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Figure S1. Learned and inherited P11-induced PA14-avoidance behavior in donor worms used for lysate training of naive animals, related to Figure 1 and Table S1

(A) Modes of PA14 and P11-induced learning and inheritance. Naive *C. elegans* prefer PA14 if given a choice between OP50 (*E. coli*) and PA14. After exposure to PA14 for 24 h, worms learn to avoid PA14 via three cues: (1) small RNAs (specifically P11), (2) metabolites, and (3) innate immune pathways. This avoidance behavior can be transgenerationally inherited in naive progeny for four generations before resetting in the 5th. Only small RNAs are required for transgenerational inheritance of pathogen avoidance.

(B) Mothers trained on P11-expressing *E. coli* learn to avoid PA14 compared to controls. F1 (C), F2 (D), F3 (E), and F4 (F) progeny inherit PA14 avoidance memory from their ancestors. P0-F2 control data is also displayed in Figure 1B. G, PA14 avoidance memory is reset in the F5 generation. B–G *Cer1* mutant mothers cannot learn to avoid PA14 upon P11 exposure., and the F1-F4 progeny

(C–F) also do not exhibit transgenerational memory. Each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 38-96 plates per condition. At least 3 biological replicates for all experiments. Boxplots: center line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. Two-way (B–G) analysis of variance (ANOVA), Tukey's multiple comparison test. * $p < 0.05$, **** $p < 0.0001$, NS, not significant. Table S1 for exact sample sizes (*n*) and *P value*s.

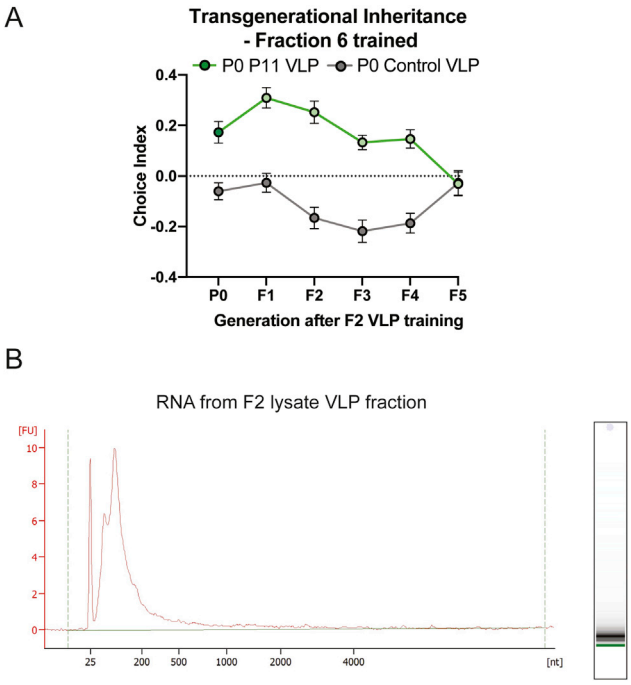

Figure S2. RNA isolated from the VLP fraction of worm lysate, related to Figure 2 and Table S1

(A) Transgenerational inheritance of PA14 avoidance in progeny of worms exposed to fraction 6 (derived from F2s from control or P11-trained grandmothers); the mean choice index for each generation is shown ± SEM.

(B) Dounce homogenized worm lysate was purified by ultracentrifugation through a density gradient. Fraction 6 was harvested from the gradient, and the re-suspended pellet was treated with RNase to degrade any free RNA not protected by a VLP or other extracellular vesicle. The RNase was inactivated, and the fraction was pelleted to concentrate the sample. The pellet was resuspended, and a portion was tested for the ability to confer PA14-avoidance behavior (control versus P11 samples), and remaining sample was lysed in Trizol for total RNA purification and then analyzed via Bioanalyzer using an RNA pico chip.

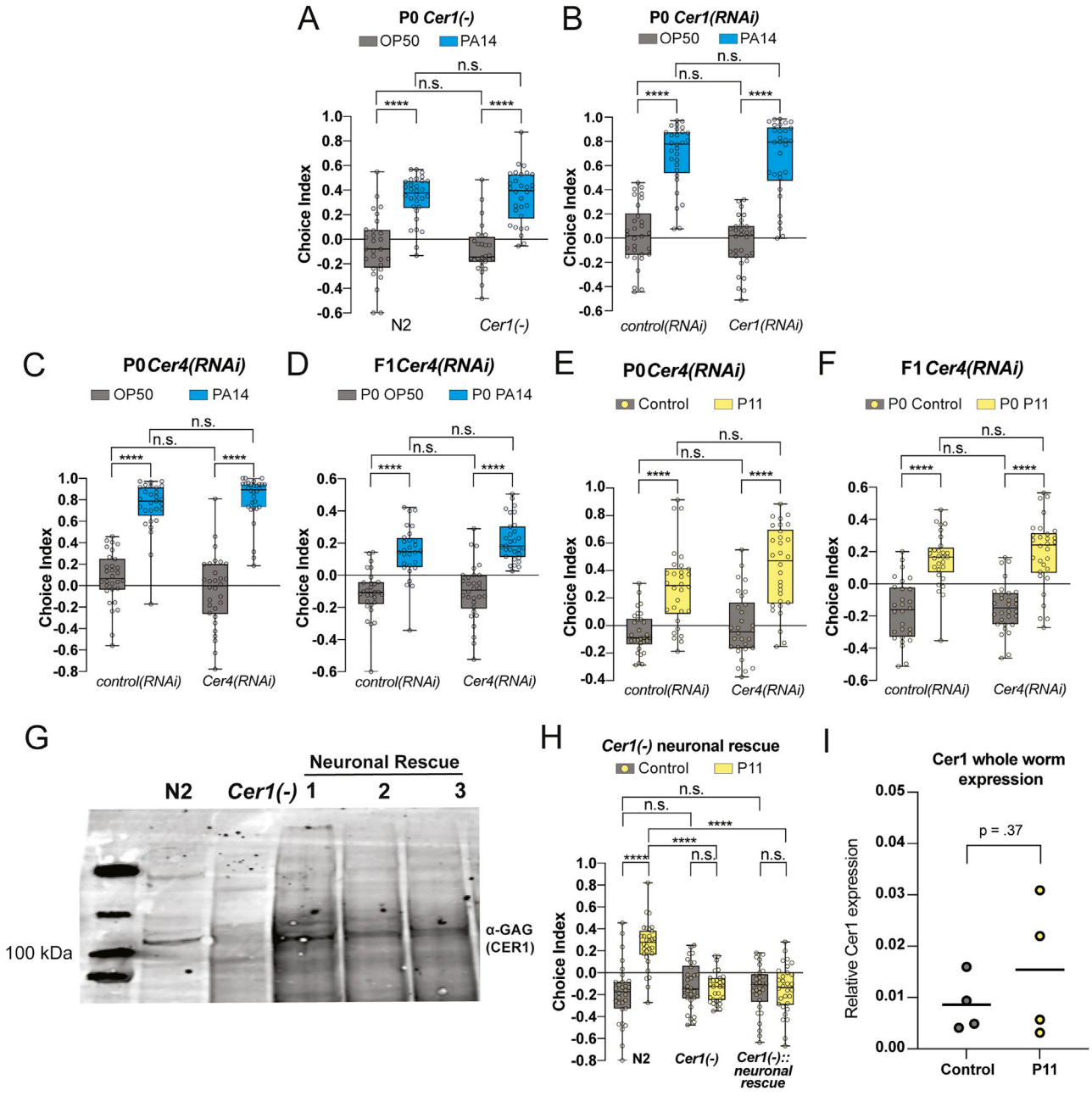

Figure S3. PA14-avoidance behavior in worms with *Cer1(RNAi)* or *Cer4(RNAi)* knockdown, related to Figure 3 and Table S1

(A and B) *Cer1* mutants (A) or worms treated with *Cer1(RNAi)* (B) can learn PA14 avoidance when exposed to a PA14 bacteria lawn, likely through the mechanisms described in Figure S1A.

(C and D) (C) *Cer4* is not required for PA14 lawn-induced learning or transgenerational memory inheritance (D).

(E and F) P11 small RNA-induced learning (E) and transgenerational memory (F) is intact in worms treated with *Cer4(RNAi).*

(G) Western blot of Cer1 from lysates of wild type worms, Cer1 mutant worms, and 3 independent transgenic lines expressing Cer1 in neurons in the Cer1 mutant background.

(H) Neuronal *Cer1* expression in the *Cer1*-mutant background (*Cer1*(*gk870313*); *unc-119p::Cer1::unc-54 3¿UTR*) does not rescue P11-mediated avoidance learning.

(I) Cer1 expression levels were quantified by western blot following control or P11 training. Exposure to P11 does not change Cer1 protein abundance. Four biological replicates were performed. Unpaired, two-tailed Student's t test. For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 28-30 plates per condition. At least three biological replicates for all experiments. Boxplots: center line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. Two-way (A–F, H) analysis of variance (ANOVA), Tukey's multiple comparison test. ****p < 0.0001, NS, not significant. Table S1 for exact sample sizes (*n*) and *P value*s.

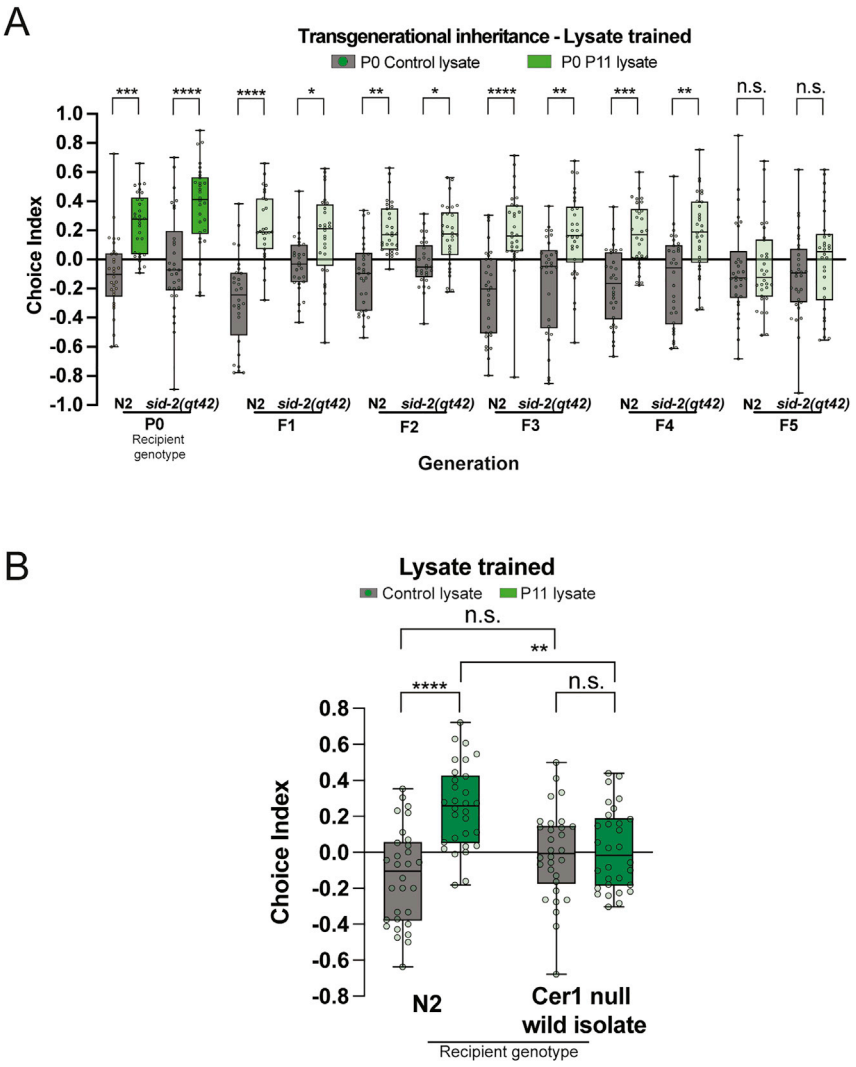

Figure S4. *sid-2* is not required in recipient worms for horizontal memory transfer and subsequent transgenerational inheritance, related to Figure 4 and Table S1

(A) F2 lysate from wild-type worms was used to train wild-type or *sid-2* recipient naive worms. A subset of P0 worms were tested for PA14-avoidance behavior, and the remaining animals were bleached to propagate progeny on normal OP50 food without subsequent training. Both wild-type and *sid-2* mutant worms learned PA14-avoidance behavior upon lysate training, and the memory was inherited in both wild type and *sid-2* through the F4 generation.

(B) The *C. elegans* wild isolate JU322 does not encode *Cer1*. JU322 *Cer1* null worms were used as the recipients for N2 control- or P11-F2 lysate training. While N2 worms were able to acquire PA14 avoidance upon F2 lysate exposure, *Cer1* null worms were not. Each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 27-31 plates per condition. Three biological replicates were performed. Boxplots: center line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. (A) One-way analysis of variance (ANOVA) or (B) two-way ANOVA, Tukey's multiple comparison test. * $p < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $p < 0.0001$, NS, not significant. Table S1 for exact sample sizes (*n*) and *P values*.

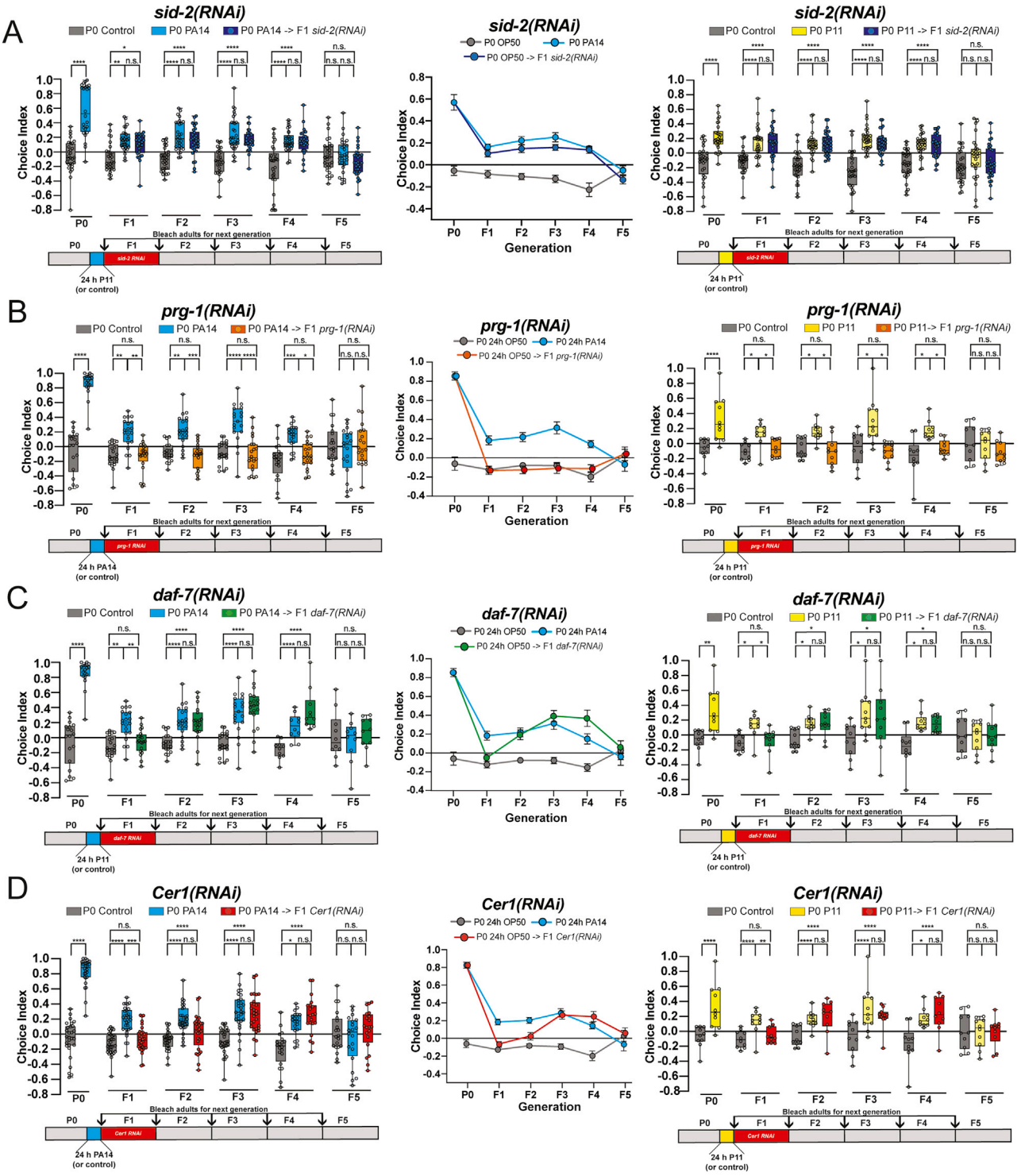

Figure S5. Transgenerational behavior effects of F2 generation-RNAi knockdown of *sid-2*, *prg-1*, *daf-7*, or *Cer1*, related to Figure 5 and Table S1

(A–D) Wild-type mothers were trained with control, PA14 (left panels and line graphs) or P11-expressing *E. coli* (right panels). F1 progeny were then treated with either *sid-2* (A), *prg-1* (B), *daf-7* (C), or *Cer1* (D) RNAi. Subsequent generations of progeny were maintained on normal food and examined for PA14-avoidance

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behavior. Each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 18-31 plates per condition. At least three biological replicates for all experiments. Boxplots: center line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. One-way (A–D) analysis of variance (ANOVA), Tukey's multiple comparison test. * $p < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $p < 0.0001$, NS, not significant. Table S1 for exact sample sizes (*n*) and *P value*s.

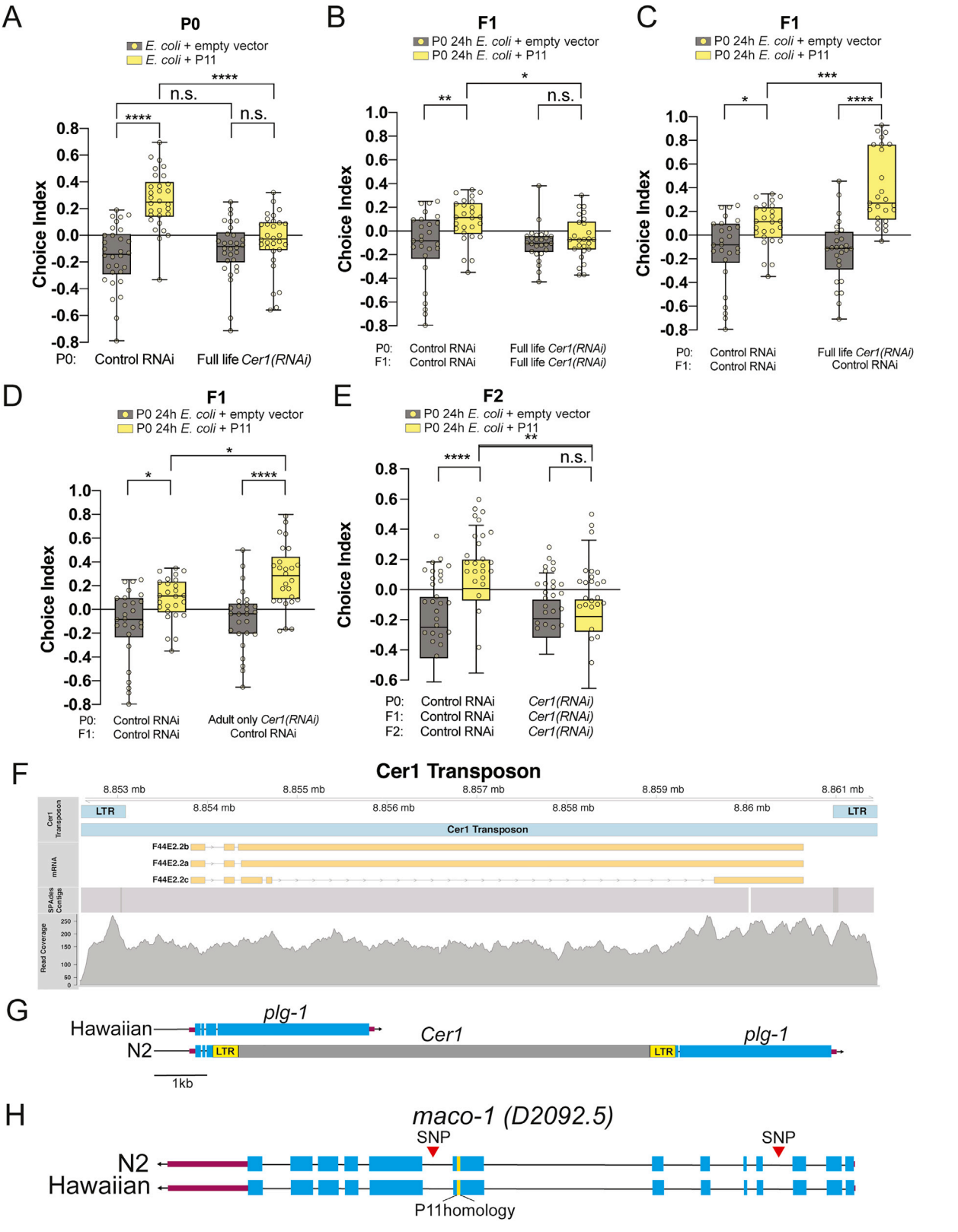

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Figure S6. Adult-only RNAi knockdown of *Cer1* and genomic analysis of *Cer1*, *maco-1*, and *plg-1* loci, related to Figures 5 and 6 and Table S1

(A) Naive mothers were treated from egg with *Cer1* or control RNAi. At the L4 stage, worms were trained on control or P11-expressing *E. coli* and tested for PA14-avoidance behavior.

(B) Progeny obtained from the trained mothers in (A) continued to be treated with whole-life control or *Cer1* RNAi.

(C) Progeny obtained from the trained mothers in (A) were treated only with control RNAi from egg to adulthood, then tested for PA14 -behavior.

(D) Progeny obtained from the trained mothers in (Figure 4F) were treated only with control RNAi from egg to adulthood, then tested for PA14-avoidance behavior.

(E) The F2 grandprogeny from (A) and (B) continued to be treated with whole-life control or *Cer1* RNAi before PA14-avoidance behavior testing in adulthood.

(F) Full-length *Cer1* is present in JU1580, but not in the *plg-1* locus.

(G) Cer1 is inserted in the *plg-1* locus of N2, but not Hawaiian.

(H) The P11 mRNA target *maco-1* is intact in Hawaiian worms. Each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 24-31 plates per condition. At least three biological replicates for all experiments. Boxplots: center line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. Two-way (A–E) analysis of variance (ANOVA), Tukey's multiple comparison test. * $p < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $p < 0.0001$, NS, not significant. Table S1 for exact sample sizes (*n*) and *P value*s.

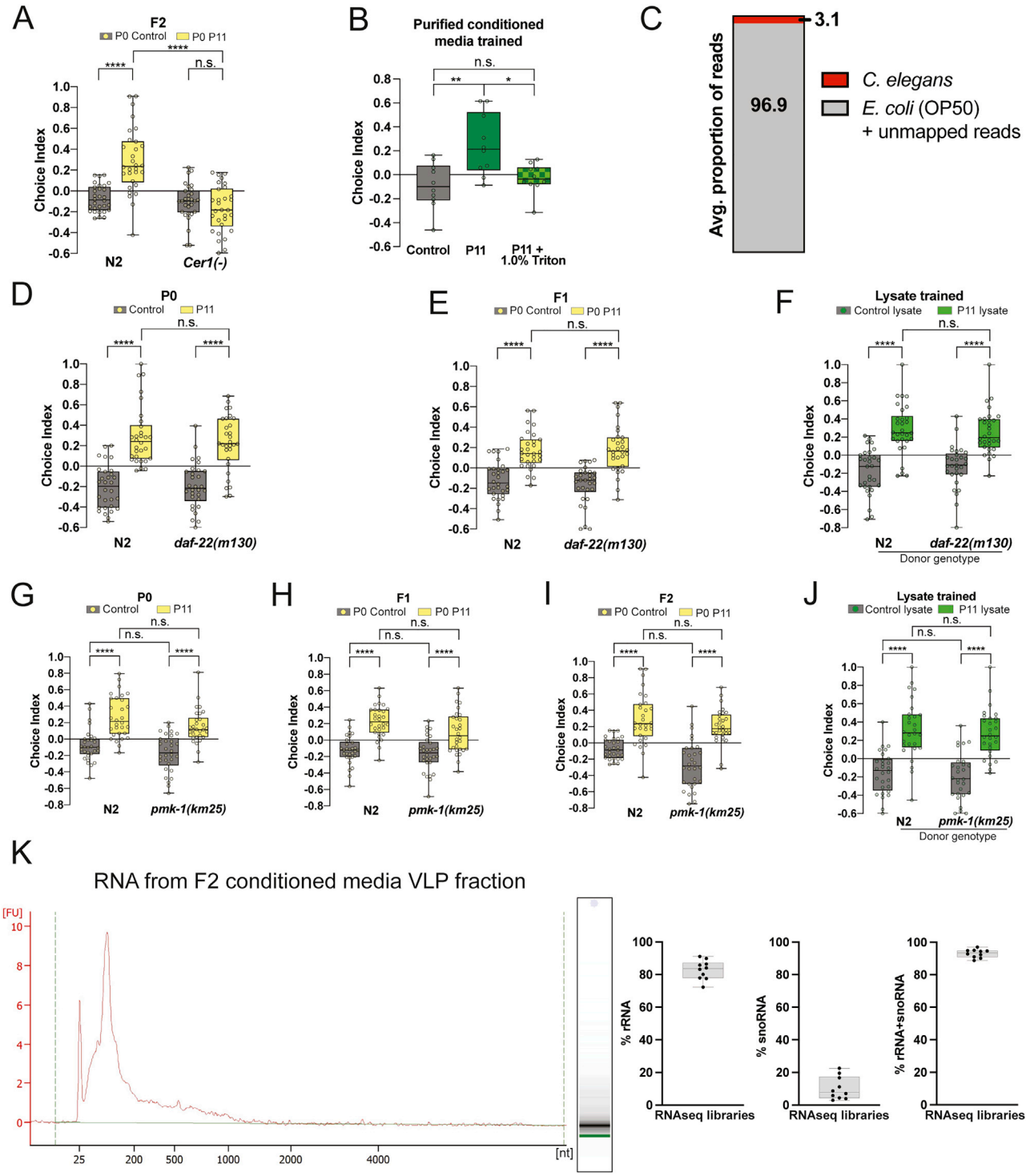

Figure S7. Confirmation of learning and transgenerational inheritance in donor worms used for CM training and horizontal memory transfer, related to Figure 7 and Table S1

(A) Wild-type and *Cer1* mutants were trained on control or P11-expressing *E. coli*. The F2 progeny were raised on OP50 plates and tested for PA14 avoidance memory as adults. Wild-type worms learn and inherit PA14 avoidance memory, while *Cer1* mutants do not. These populations were used to condition media for experiments in Figure 7B.

(B) Filtered F2 CM was treated with a final concentration of 1% Triton X-100. All of the CM samples were then purified and concentrated through a sucrose cushion. The resuspended pellet was used to train wild-type worms, and the resulting PA14-avoidance behavior was measured.

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(C) The majority of RNA sequences obtained from purified CM are from *E. coli* OP50.
(D and E) Wild-type and *daf-22(m130)* mutants were trained on control or P11-expressing bacteria (D) and propagated to the F1 generation (E). Wild-type and *daf-22(m130)* mutants learn PA14 avoidance in mothers (D) and F1s inherit the memory (E).
(F) The lysate from the F2 worms derived from the populations in E is able to horizontally transfer PA14 avoidance memory to naive wild-type worms, similar to the effect of CM (Figure 7D).
(G–I) Wild-type and *pmk-1(km25)* mutants were trained on control or P11-expressing bacteria (G) and propagated to the F1 (H) and F2 (I) generations. Wild-type and *daf-22(m130)* mutants learn PA14 avoidance in mothers (G) and both F1s and F2s inherit the memory.
(J) The lysate from the F2 worms derived from the populations in (I) is able to horizontally transfer PA14 avoidance memory to naive wild-type worms, similar to the effect of CM (Figure 7E). Each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. For choice assays n = 28-31 plates per condition. three biological replicates were performed for all experiments. Boxplots: center line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. One-way (B) or two-way (A, D–J) analysis of variance (ANOVA), Tukey's multiple comparison test, * $p < 0.05$, ** $P \pounds 0.01$, **** $p < 0.0001$, NS, not significant. Table S1 for exact sample sizes (*n*) and *P value*s.
(K) RNA was isolated from RNase-treated CM that was purified through a sucrose cushion.