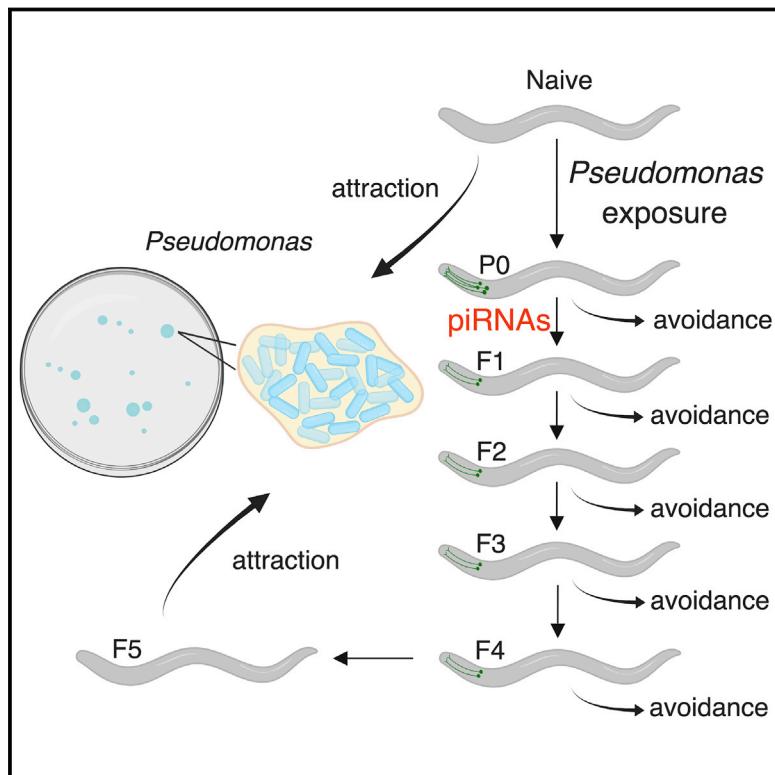


Piwi/PRG-1 Argonaute and TGF- β Mediate Transgenerational Learned Pathogenic Avoidance

Graphical Abstract



Authors

Rebecca S. Moore, Rachel Kaletsky,
Coleen T. Murphy

Correspondence

ctmurphy@princeton.edu

In Brief

Worms can transmit avoidance behavior to their young for four generations, providing progeny with a mechanism to survive dangerous conditions.

Highlights

- *C. elegans* transmit learned avoidance of *P. aeruginosa* for four generations
- ASI and the TGF- β ligand *daf-7* are required for transgenerational PA14 avoidance
- Piwi/PRG-1 is required for transgenerational inheritance of *P. aeruginosa* avoidance
- Transgenerational avoidance of *P. aeruginosa* provides fitness benefits to offspring



Piwi/PRG-1 Argonaute and TGF- β Mediate Transgenerational Learned Pathogenic Avoidance

Rebecca S. Moore,¹ Rachel Kaletsky,¹ and Coleen T. Murphy^{1,2,*}

¹Department of Molecular Biology & LSI Genomics, Princeton University, Princeton, NJ 08544, USA

²Lead Contact

*Correspondence: ctmurphy@princeton.edu

<https://doi.org/10.1016/j.cell.2019.05.024>

SUMMARY

The ability to inherit learned information from parents could be evolutionarily beneficial, enabling progeny to better survive dangerous conditions. We discovered that, after *C. elegans* have learned to avoid the pathogenic bacteria *Pseudomonas aeruginosa* (PA14), they pass this learned behavior on to their progeny, through either the male or female germline, persisting through the fourth generation. Expression of the TGF- β ligand DAF-7 in the ASI sensory neurons correlates with and is required for this transgenerational avoidance behavior. Additionally, the Piwi Argonaute homolog PRG-1 and its downstream molecular components are required for transgenerational inheritance of both avoidance behavior and ASI *daf-7* expression. Animals whose parents have learned to avoid PA14 display a PA14 avoidance-based survival advantage that is also *prg-1* dependent, suggesting an adaptive response. Transgenerational epigenetic inheritance of pathogenic learning may optimize progeny decisions to increase survival in fluctuating environmental conditions.

INTRODUCTION

The physical and behavioral characteristics of plants and animals are greatly influenced by the environment. Plasticity in response to environmental changes can be encoded independently of changes to the DNA through a suite of molecular mechanisms collectively known as epigenetics. Epigenetic encoding mechanisms include genome-associated changes, such as DNA and histone modifications and small RNA regulation, and genome-independent changes, including maternal provisioning and microbiome effects. Remarkably, epigenetic changes and their subsequent effects on gene expression are not limited to a single organism's lifetime but also can be transmitted across multiple generations in a phenomenon known as transgenerational epigenetic inheritance (TEI). TEI has been shown to play roles in silencing of repetitive elements (such as transposons) (Ghildiyal and Zamore, 2009) and in physiological responses to cellular stresses, such as starvation (Rechavi et al., 2014), heat (Klosin et al., 2017), and osmotic stress (Burton et al., 2017). Despite advances in characterizing TEI and its molecular com-

ponents (Perez and Lehner, 2019), it is less well understood whether TEI can communicate information encoding the behavior of an animal, and, if so, which epigenetic mechanisms underlie those behaviors.

Caenorhabditis elegans has emerged as a premier organism to study TEI, due to its exceptionally short generation times, large broods, and evolutionarily conserved epigenetic mechanisms that encode and transmit transgenerational information. Moreover, worms do not provide maternal care, and parental microbiomes are not transferred to progeny in laboratory settings—two factors that can confound mechanistic studies of TEI in mammals.

RNAi was first discovered in *C. elegans* over two decades ago (Fire et al., 1998) and was later found to be functionally conserved in many species (Grimson et al., 2008), including mammals (Wianny and Zernicka-Goetz, 2000). Subsequently, it was observed that gene silencing induced by double-stranded RNA (dsRNA) could be inherited for 3–20+ generations in *C. elegans* (Vastenhouw et al., 2006). Small interfering RNAs, including exogenous and primary endogenous small interfering RNAs (siRNAs), and Piwi-associated RNAs (piRNAs) trigger an RNA amplification mechanism that guides the production of secondary endo-siRNAs (22G RNAs) (Pak and Fire, 2007) via RNA-dependent RNA polymerases (RdRPs) (Sijen et al., 2001). 22G RNAs are carried by Argonautes to the nucleus (Guang et al., 2008), where they guide the deposition of repressive histone methylation marks that establish gene silencing (Berkhout et al., 2011; Burton et al., 2011; Gu et al., 2012; Mao et al., 2015). Chromatin methylation of H3K9, H3K27, and H3K4 are required for TEI-related phenotypes in worms, flies, and mice (Perez and Lehner, 2019).

The molecular characterization of TEI pathways has provided the framework for understanding how information is encoded and transmitted across several generations, but important questions remain unanswered. For example, while antiviral immunity and physiological stress responses can be transmitted transgenerationally (Rechavi et al., 2011; Vassoler et al., 2013; Tauffenberger and Parker, 2014; Rechavi et al., 2014; Kishimoto et al., 2017; Perez et al., 2017; Burton et al., 2017), it is not known whether complex neurological behaviors, such as learning and memory, can be inherited across generations. Traumatic stress (Gapp et al., 2014) and odor-specific conditioned fear responses (Dias and Ressler, 2014) can be inherited for up to two generations, but the underlying molecular epigenetic mechanisms encoding this behavior remain unknown. Moreover, it is unclear how the currently reported TEI phenomena provide an



evolutionary advantage to organisms in the wild. For example, parental mutations of the COMPASS complex of chromatin modifiers extend *C. elegans* lifespan in both mutant parents and wild-type cross-progeny through the third generation (Greer et al., 2011), but it is less clear how these effects might relate to the fitness of wild-type animals in a natural setting. Finally, learned behaviors that might help animals adapt to the environment have not been previously reported to be transgenerationally inherited in *C. elegans*.

C. elegans inhabit naturally diverse environments (Schulenburg and Félix, 2017) and must constantly discern between nutritious and infectious bacterial food sources (Shtonda and Avery, 2006). In fact, ~30% of *C. elegans*' natural microbial environment is composed of *Pseudomonas* species (Samuel et al., 2016), some of which are pathogens. Although *Pseudomonas aeruginosa* (PA14) kills *C. elegans* within hours or days of exposure (Tan et al., 1999), initially, worms are attracted to PA14—in fact, they prefer it to the non-pathogenic lab *E. coli* (OP50)—but, within hours of exposure, they learn to avoid this pathogen (Zhang et al., 2005). Worms can even form long-term, CREB-dependent memories of the pathogenic experience when exposed as larvae and tested as adults, but this behavior is not transmitted across generations (Jin et al., 2016).

Here, we have found that worms not only learn to avoid PA14 but can also pass on this avoidance behavior to progeny for up to four generations, through transforming growth factor β (TGF- β) signaling in sensory neurons and the Piwi Argonaute small RNA pathway. Worms that inherit the TEI-encoded behavior are able to avoid pathogenic bacteria, providing them with a survival advantage compared to their naive counterparts. TEI of pathogenic avoidance may allow worms to navigate a complex environment, improving their ability to obtain adequate nutrition while avoiding illness.

RESULTS

Learned Pathogenic Avoidance Is Transgenerationally Inherited

C. elegans are exposed to and consume a variety of bacterial food sources in their natural environment (Schulenburg and Félix, 2017). Several of these bacteria are pathogens that reduce *C. elegans* lifespan and progeny production (Tan et al., 1999; Dirksen et al., 2016). Naive *C. elegans* initially prefer pathogenic *Pseudomonas aeruginosa* (PA14) to a laboratory strain of nonpathogenic *E. coli* (OP50). Upon brief (4 h) exposure to PA14, however, worms switch their preference and instead avoid PA14 (Zhang et al., 2005). We wondered whether *C. elegans* can pass this learned avoidance behavior to their naive progeny (Figure 1A). To test this hypothesis, we exposed wild-type L4 (pre-adult) hermaphrodites to PA14 for 4 h (Zhang et al., 2005) and bleached the mothers to obtain F1 eggs, but we found that this short exposure does not induce transgenerational effects in the adult progeny (Figures 1A and 1B). By contrast, when we exposed the mothers to PA14 for 24 h, their adult F1 progeny exhibited avoidance, despite never previously having encountered PA14 (Figure 1C).

A previous study reported that brief (30 min) pre-exposure to the odor of PA14 or a chemical, 2-aminoacetophenone (2AA),

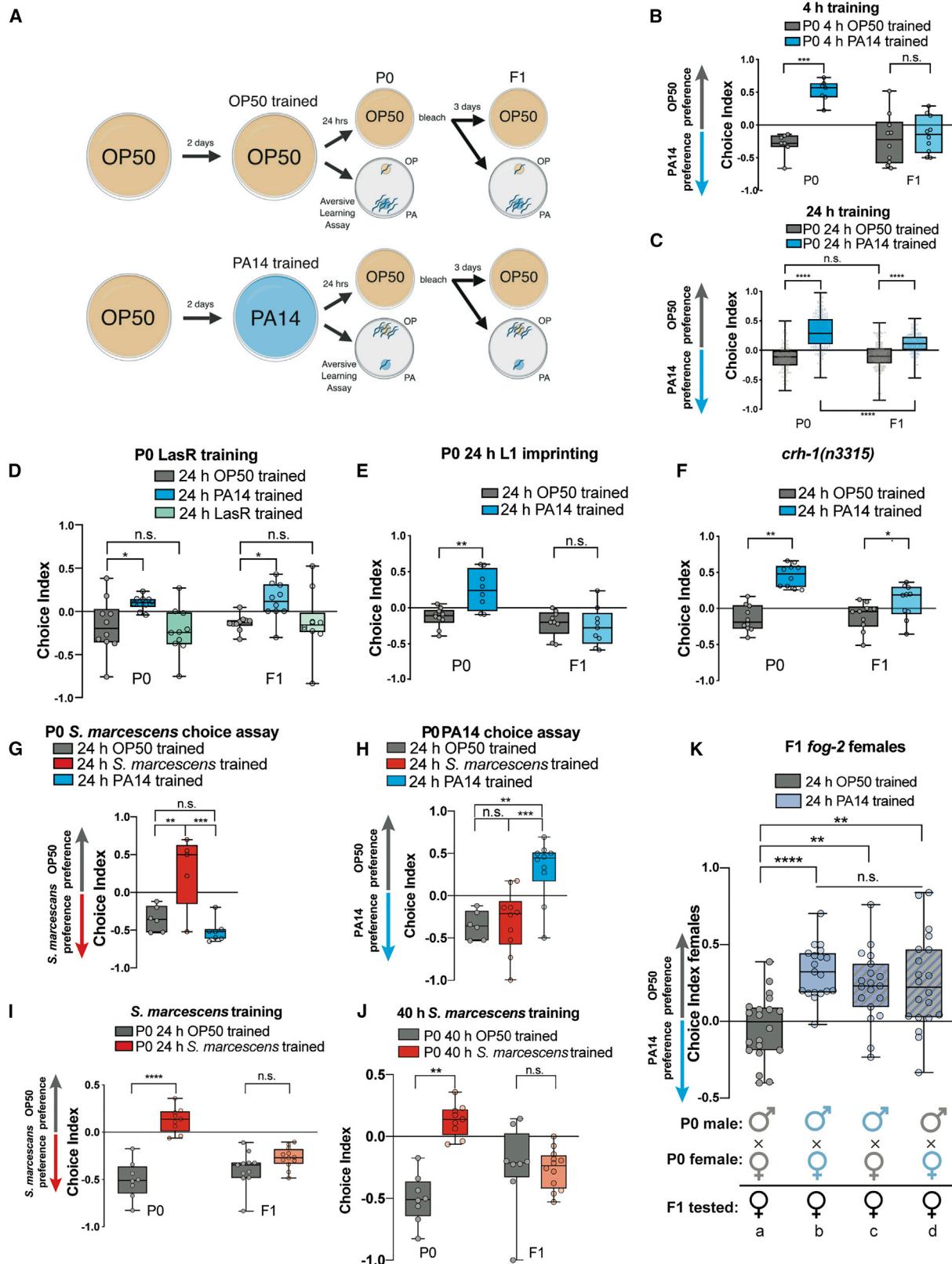
can cause temporary (4 h) PA14 avoidance (Ooi and Prahlad, 2017). We wondered whether prolonged exposure to odor alone would be sufficient to induce transgenerational memory. However, neither mothers nor progeny of mothers exposed to PA14 odor or 2AA for 24 h avoided PA14 (Figures S1A and S1B), suggesting that physical contact is required for both pathogenic learning and for the transgenerational inheritance of this behavior. We next asked whether virulence is required for these effects. Prolonged PA14 exposure kills *C. elegans* within 60 h (Tan et al., 1999), cutting short their normal 2- to 3-week lifespan (Figure S1C). The PA14 quorum-sensing mutant LasR is markedly less virulent to *C. elegans*, such that LasR-exposed worms remain alive after ~70 h of exposure, when all of the wild-type PA14-exposed animals have died (Tan et al., 1999). We found that wild-type worms exposed to LasR do not learn to avoid PA14 (Figure 1D), and progeny of LasR-trained mothers also fail to avoid PA14 (Figure 1D). Together, these results suggest that pathogenic learning and transgenerational inheritance of pathogen avoidance require both physical contact and infection with PA14.

Early developmental stage larvae (L1) are capable of learning to avoid PA14 (Jin et al., 2016). This "imprinting" results in the maintenance of PA14 avoidance in early adulthood but is not transmitted to progeny of imprinted mothers (Jin et al., 2016). Because imprinting training is 12 h (Jin et al., 2016), we asked whether longer L1 training would cause both adult aversion to PA14 and TEI of pathogen avoidance behavior. Although 24 h of larval training was sufficient to elicit parental avoidance of PA14, progeny of parents trained as L1s did not avoid PA14 (Figure 1E). Furthermore, the transcription factor CREB (*crh-1*), which is required for L1 imprinting of PA14 avoidance (Jin et al., 2016) and for many forms of long-term memory (Kauffman et al., 2010), is not necessary for transgenerational pathogenic avoidance, further distinguishing these processes (Figure 1F). Therefore, the mechanism of TEI is distinct from other forms of CREB-dependent, aversive long-term memory, including larval imprinting of PA14 avoidance.

Learned Avoidance Behavior and TEI Is Pathogen Specific

Pseudomonas aeruginosa exposure induces behavioral responses in mothers and progeny that alter animals' chemotactic preferences. We next asked whether these behavioral changes are specific to *Pseudomonas* exposure and choice, or whether generalized changes occur that alter attraction and avoidance of other known *C. elegans* pathogens. Like *Pseudomonas*, naive worms prefer *Serratia marcescens* (Sm) to OP50, and training for 24 h induces learned Sm avoidance (Pujol et al., 2001) (Figure 1G). However, PA14-trained worms specifically avoid PA14 and remain attracted to Sm (Figure 1G), suggesting that the learned avoidance is specific to PA14. Conversely, Sm-trained worms exhibit behavioral responses that are specific to Sm: Sm-trained worms avoid Sm but remain attracted to PA14, similar to naive animals (Figure 1H).

Since exposure to *S. marcescens* induces pathogen-specific changes in learned avoidance behavior, similar to PA14 training, we tested whether that learned avoidance is transmitted to their progeny; however, the progeny of Sm-trained mothers did not



(legend on next page)

avoid Sm (Figure 1I). *S. marcescens* kills *C. elegans* more slowly than does PA14 (Pujol et al., 2001); therefore, we tested whether longer (40 h) *S. marcescens* training is necessary for the transmission of learned avoidance. However, 40 h Sm-trained mothers did not pass on the learned avoidance behavior to their progeny, either (Figure 1J). Together, these results suggest that inheritance of learned avoidance behavior is not a universal response to pathogen exposure but rather may be specific to *Pseudomonas aeruginosa*.

Both Male and Female Germlines Transmit TEI Avoidance Information

Transgenerational inheritance has been shown to be transmitted through the male germline in mice (Dias and Ressler, 2014; Gapp et al., 2014); however, the contribution of the mammalian female germline has been less well studied due to technical limitations. Therefore, we took advantage of the tractability of *C. elegans* (Burton et al., 2017; Klosin et al., 2017) to determine through which germline the learned pathogenic avoidance is transmitted. *C. elegans* is a hermaphroditic species and typically reproduces through self-fertilization, since males are rare in nature (<0.01% of the population), but males can mate with hermaphrodites to produce cross-progeny, as well (Ward and Carrel, 1979).

To determine the contributions of the male and female germlines in transmission of pathogen avoidance learning, we asked whether sperm or oocytes from trained parents could induce the learned avoidance behavior in the next generation (F1) using *fog-2* animals; *fog-2* males are normal, but *fog-2* hermaphrodites are defective in spermatogenesis, resulting in true females (Schedl and Kimble, 1988). Following PA14 or OP50 exposure, males and females were washed thoroughly to remove bacteria and were mated in the depicted experimental combinations (Figure 1K) for 24 h on OP50-seeded plates containing streptomycin to prevent additional PA14 exposure. Since naive P0 males already avoid PA14 (Figure S1D), likely due to sexually dimorphic neuronal gene expression (Hilbert and Kim, 2017) and differences in male mating and foraging behavior (Ryan et al., 2014), we tested the F1 female cross-progeny for acquisition of the inherited learned avoidance behavior (Figure 1K). As expected, the

F1 progeny of OP50-trained males × OP50-trained females (1) preferred PA14, similar to naive animals, while the F1 progeny of PA14-trained males × PA14-trained females (2) exhibited transmission of learned avoidance, demonstrating that mating alone does not diminish the behavior. Interestingly, both PA14-trained males (PA14-trained males × OP50-trained females, [3]) and PA14-trained females (OP50-trained males × PA14-trained females, [4]) transmitted learned avoidance behavior to the next generation, suggesting a role for both sperm and oocytes in intergenerational inheritance of pathogenic avoidance.

TEI of Pathogen Avoidance Persists for Four Generations

During training, progeny may have been exposed to the bacterial pathogen while still inside the mother, in which case the F1 avoidance effect could not be considered truly transgenerational but rather should be restricted to one generation. True epigenetic inheritance persists beyond the first generation, and the duration of this persistence often depends on the specific phenotype; therefore, we tested how many generations the learned pathogenic avoidance of PA14 is transmitted. We found that training of mothers (P0) induces pathogenic avoidance in the four subsequent generations, F1–F4 (Figures 2A and 2B). Fifth-generation (F5) descendants no longer avoid PA14 but instead are attracted to PA14, resuming naive behavior (Figures 2A and 2B).

Neuronal Gene Expression Changes upon *Pseudomonas* Pathogenic Learning and Transgenerational Inheritance of Avoidance

To better understand the molecular mechanisms underlying transgenerational inheritance of pathogen avoidance learning, we compared the transcriptional profiles of wild-type mothers (P0; Figure S2; Table S1) and progeny (F1; Figure S3; Table S2), in which the mothers had been treated with OP50 or PA14. Both mothers exposed to PA14 (P0) and their progeny (F1) exhibit a large set of gene expression differences from OP50-trained controls that include immune responses

Figure 1. Pathogenic Avoidance Can Be Inherited and Is Transmitted through Both Germlines

- (A) Adult pathogen training protocol. Choice Index = (# of worms on OP50 – # of worms on PA14)/(total # of worms).
 - (B) 4 h of training on PA14 is sufficient to elicit maternal avoidance (P0) of PA14 but is not sufficient for progeny avoidance (F1). At least 3 biological replicates were performed.
 - (C) 24 h of training on PA14 induces both pathogen avoidance and transgenerational inheritance of pathogen avoidance (F1); n = 177 replicate assays.
 - (D) 24 h of training on avirulent *Pseudomonas* (LasR) does not induce maternal pathogenic learning or progeny avoidance of PA14. At least 3 biological replicates were performed.
 - (E) L1-imprinted animals (24 h) exhibit adult (day 1) aversion to PA14, but progeny of imprinted mothers do not. At least 3 biological replicates were performed.
 - (F) CREB (*crh-1*) is not required for maternal pathogenic learning or progeny avoidance of PA14. At least 3 biological replicates were performed.
 - (G) 24 h of training on *S. marcescens* (red) is sufficient to elicit maternal avoidance of *S. marcescens*. However, 24 h of training on PA14 (blue) does not induce maternal avoidance of *S. marcescens*. At least 3 biological replicates were performed.
 - (H) PA14 training (blue) induces maternal avoidance of PA14, while *S. marcescens* (red) training does not induce avoidance of PA14. At least 3 biological replicates were performed.
 - (I and J) 24 h (I) or 40 h (J) of training on *S. marcescens* is sufficient to elicit maternal (P0) avoidance of *S. marcescens* but does not induce progeny avoidance (F1). At least 3 biological replicates were performed.
 - (K) Progeny of (b) PA14-trained mated parents (PA14-trained males × PA14-trained females) inherit pathogen avoidance, while progeny of (a) OP50-trained mated parents (OP50-trained males × OP50-trained females) prefer PA14. Progeny in which only one parent was trained on PA14 (c, PA14-trained male × OP50-trained female, or, d, OP50-trained male × PA14-trained female) inherit pathogen avoidance. 2 biological replicates were performed.
- One-way ANOVA, Tukey's multiple comparison test, mean \pm SEM n \geq 7 choice assay plates containing 50–200 worms. *p \leq 0.05, **p \leq 0.01, ***p \leq 0.001, ****p $<$ 0.0001; ns, not significant. See also Figures S1 and S2.

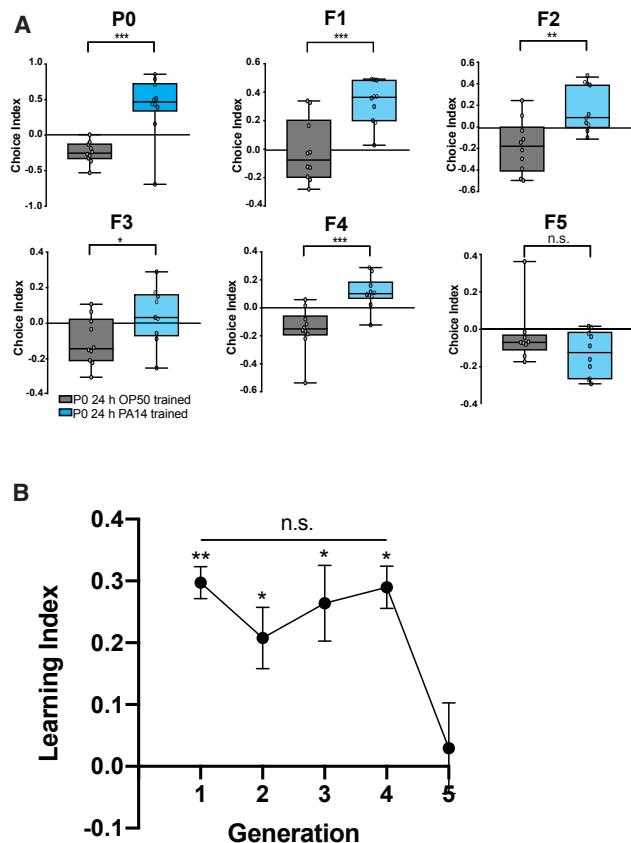


Figure 2. Learned Pathogenic Avoidance Lasts through the F4 Generation

(A) Untrained (naive) progeny of PA14-trained mothers avoid PA14 from generation F1–F4; the 5th generation returns to normal PA14 attraction. Student's t test, mean \pm SEM n = 10 per generation. At least 3 biological replicates were performed.

(B) Learning index (naive choice index – trained choice index) of generation F1–F5. One-way ANOVA, Tukey's multiple comparison test, mean \pm SEM n \geq 7. *p \leq 0.05, **p \leq 0.01, ***p \leq 0.001; ns, not significant.

to infection, changes in metabolism, and epigenetic and post-transcriptional regulation of gene expression. Consistent with our observed changes in behavior, the upregulated GO categories (Figure S3C) were primarily neuronal (ion transport, chemical synapse, neurotransmitter transport, neuropeptide signaling, membrane potential, and behavior); in fact, 65%–70% of the genes upregulated by PA14 in P0 and F1 animals, respectively, are expressed in adult neurons (Figure S3E). These include the TGF- β ligand *daf-7*, GPCRs, neuropeptides, insulin-like peptides (*ins-11*, *ins-20*), MAPK (*sek-1*), cholinergic signaling, potassium channels, and other receptors, and *egl-30* G_{aq} (Table S1). The genes downregulated in the F1 progeny from mothers exposed to PA14 compared to OP50 included several types of metabolism, regulation of gene expression, RNA processing, response to dsRNA, chromatin modifications, and epigenetic gene expression regulation (Figure S3D; Tables S1 and S2). Together, these data suggest that, in addition to metabolic, immune, and epigenetic changes, the primary upre-

gulated genes in progeny of trained mothers may affect neuron function and behavior.

DAF-7/TGF- β Expression and the ASI Neuron Are Required for Transgenerational Inheritance of Avoidance

Sensing and subsequent avoidance of *Pseudomonas* require the activity of the nervous system (Zhang et al., 2005; Ha et al., 2010; Meisel et al., 2014), and our transcriptional data suggested that neuronal components change upon pathogenic training and learning. Meisel et al. previously showed that the TGF- β ligand DAF-7 is basally expressed in ASI sensory neurons, but, upon PA14 exposure, *daf-7* expression increases in the ASI and is induced in the ASJ neurons (Meisel et al., 2014), consistent with our RNA sequencing (RNA-seq) data (Figure S2). (DAF-7 signaling in the ASJ controls expression of TGF- β signaling in downstream RIM/RIC interneurons, which in turn control reversals through downstream motor neurons [Greer et al., 2008]). We confirmed these observations in mothers exposed to PA14 for 24 h (Figures 3A–3D). We then asked whether transgenerational avoidance behavior training induces higher expression of *daf-7p::gfp* in the ASI or ASJ in progeny of trained mothers. Naive F1 progeny of PA14-trained mothers maintained a high level of *daf-7p::gfp* expression in the ASI (Figures 3E–3G), while *daf-7p::gfp* expression in the ASJ returned to basal levels (Figure 3F). As we found for pathogenic learning, training with avirulent LasR *Pseudomonas* did not increase *daf-7p::gfp* expression in the ASI nor did it induce expression of *daf-7p::gfp* in the ASJ of mothers or their progeny (Figures 3C and 3G), suggesting that virulence is required for transgenerational *daf-7p::gfp* expression changes. With 4 h of exposure to PA14, *daf-7p::gfp* expression in the ASJ was induced to higher levels in the progeny of PA14 trained mothers compared to OP50 trained mothers (Figure 3H), but because the choice assay measures an animal's food preference within a short time frame (<1 h), our *daf-7p::gfp* expression data suggest that the elevation of basal *daf-7* expression in the ASI of progeny, rather than its delayed induction in the ASJ, is likely to mediate transgenerational avoidance behavior. Indeed, genetically ablated ASI worms avoid PA14 after P0 training, similar to wild-type animals (Figure 3I), but the F1 progeny of pathogen-trained ASI mutants do not inherit their learned avoidance of PA14 (Figure 3J), indicating that the ASI is required for pathogen avoidance in the F1s. Moreover, loss of *daf-7* causes no defects in the mother's ability to avoid PA14 or to learn avoidance after 24 h of training (Figure 3K) but abolishes the ability of their F1 progeny to avoid PA14 (Figure 3L). To more definitively rule out a role for *daf-7* in the P0 generation, we used RNAi to knock down *daf-7* exclusively in the F1 generation following parental PA14 training. We found that reduction of *daf-7* in P0 animals had no effect on P0 learned PA14 avoidance (Figure 3M). However, when only the F1 progeny of trained mothers were treated with *daf-7* RNAi, the avoidance behavior was lost (Figure 3N), suggesting that *daf-7* acts in the F1, rather than the P0, to mediate avoidance.

Next, we asked how many generations the increased expression of *daf-7* in the ASI persists after pathogenic training. *daf-7p::gfp* remained elevated in the ASI of progeny of PA14 trained mothers for four generations and then returned to basal

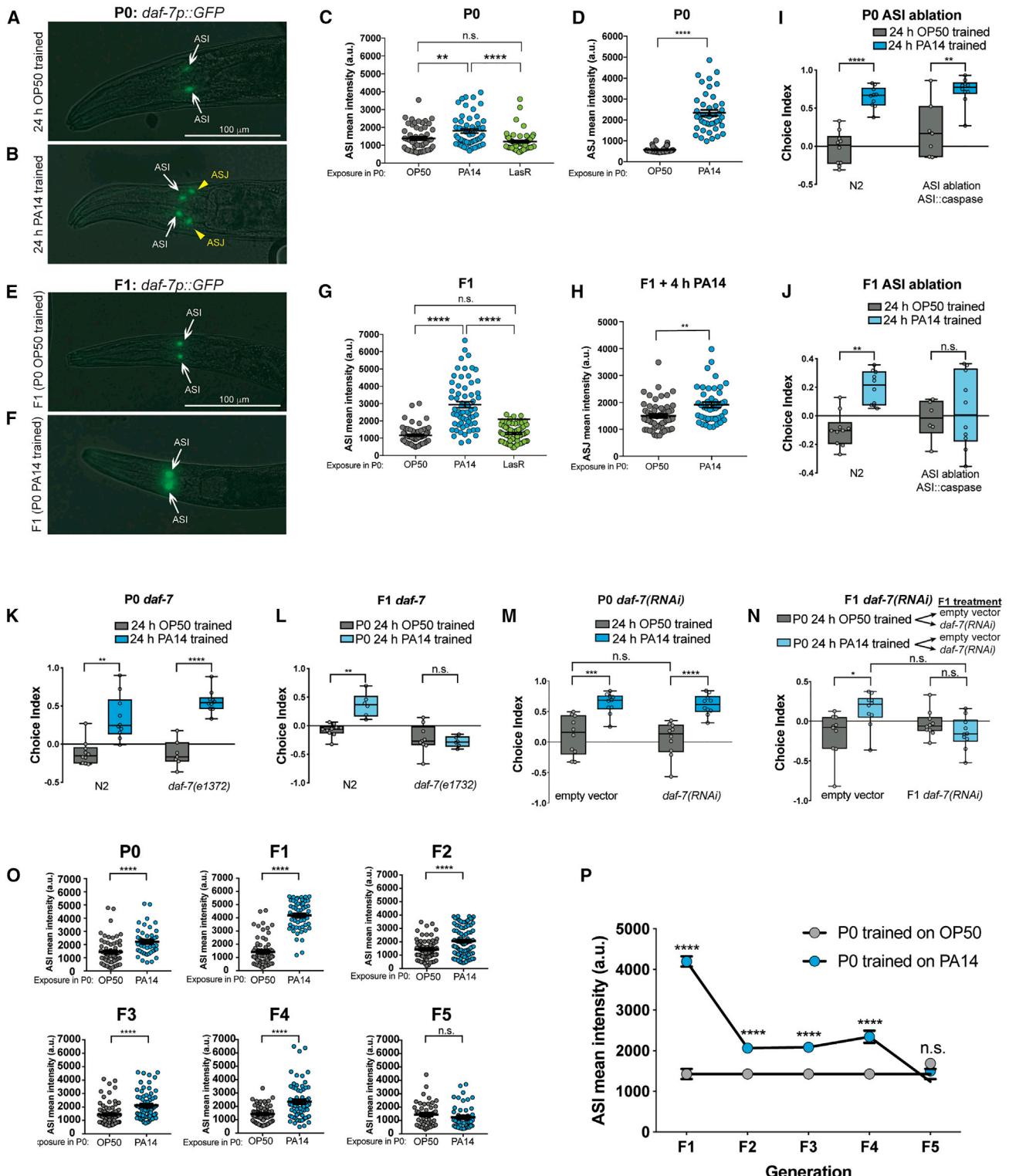


Figure 3. DAF-7/TGF- β Expression and the ASI Neuron Are Required for Transgenerational Inheritance of Avoidance

(A) *daf-7p::qfp* is expressed in the ASI neuron of naive animals (white arrow).

(B and D) PA14 training induces *daf-7p::gfp* expression in the ASJ neuron (yellow arrowhead, B; D, fluorescence quantitation). Student's t test, mean \pm SEM n \geq 36 neurons per training condition, 3 biological replicates.

(legend continued on next page)

levels in the fifth generation (Figures 3O and 3P), similar to the trajectory of transgenerational avoidance (Figure 2B). Together, our results suggest that *daf-7* expression in the ASI neurons of the progeny of PA14-trained mothers is required for transgenerational avoidance behavior, and progeny who inherit PA14 avoidance behavior maintain high ASI *daf-7p::gfp* expression levels.

COMPASS Complex Histone Modifiers Are Required for Pathogenic Learning

To identify possible regulators of epigenetic effects, we investigated the role of candidate TEI regulators. Transient mutation of the COMPASS histone modification complex components induces a transgenerational longevity effect that, like TEI of pathogen avoidance, lasts for four generations (Greer et al., 2011). However, we found that mutants of the PRC1/COMPASS complex H3K4me3 methyltransferases (*set-2*, *wdr-5.1*), the H3K4me3 demethylase *rbr-2*, and mutants of *set-32*, a putative H3K9me3 methyltransferase that links histone modifications and siRNAs (Woodhouse et al., 2018), were already defective in either their naive P0 attraction for PA14 or in aversive pathogenic learning (Figures 4A–4C), so their roles in subsequent transgenerational effects are unclear (Figures S4A–S4D and S5A–S5C). To further investigate possible epigenetic differences, we compared the global H3K4me3 and H3K9me3 methylation levels of OP50 and PA14-treated P0 mothers; although the worms exposed to PA14 are sick and their germlines are diminished, the global methylation level is not obviously altered (Figures S4H and S4I), suggesting that global histone methylation is unlikely to be the primary mechanism by which avoidance behavior is inherited.

Small RNA Regulators Are Required for Naive Preference and Pathogenic Learning

Regulators of small RNAs (siRNAs), such as the nuclear argonaute HRDE-1/Ago (Buckley et al., 2012), have also been implicated in TEI (Rechavi et al., 2014). Similar to the histone modification mutants (Figures 4A–4C), *hrde-1* mutants had normal attraction to PA14 but were defective for pathogenic aversive learning (Figure 4D), and thus their transgenerational

inheritance of learning could not be tested (Figures S4E, S5D, and S5E). We also tested *nrde-4*, which functions downstream of *hrde-1* to link siRNAs to H3K9me3 (Berkhout et al., 2011), for its role in TEI. *nrde-4* mutants were defective in naive attraction to PA14 (Figures 4E and S4F), and thus their transgenerational inheritance of learning could not be tested. Therefore, we instead asked whether previously identified direct (chromatin immunoprecipitation sequencing [ChIP-seq]) HRDE-1 targets (Buckley et al., 2012) overlapped with the differential gene expression changes we found in the P0 mothers (Figure S2), and we determined the overlap with genes that we previously found to be enriched in neurons (Kaletsky et al., 2016). Strikingly, a large fraction of the PA14-induced genes (3,793/5,828, or 65%) and NRDE-2/4 independent HRDE-1 targets (251/414, 61%) are neuronal (Figure 4F), suggesting that HRDE-1 may regulate neuronal genes in response to PA14 exposure.

Exposure to *Pseudomonas* Induces Changes in piRNA Abundance

Because *nrde-4* and *hrde-1* have defects in naive attraction to and learned avoidance of PA14, respectively, we could not easily assess whether these small RNA regulators play a role in transgenic inheritance of learned avoidance. To directly determine whether P0 treatment with PA14 affects small RNA expression as a result of PA14 treatment, we isolated RNA from PA14-treated and control mothers and identified small RNAs (21–26 nt) that are significantly changed upon PA14 treatment (Figure 5A; Table S3). As expected, several microRNAs, including those previously associated with responses to PA14 (*mir-233*, *let-7*, *mir-355*, *mir-75*, *mir-63*, *mir-84*, *mir-241*, *mir-251*, and *mir-252*) (Kudlow et al., 2012; Liu et al., 2013; Dai et al., 2015; Ren and Ambros, 2015; Ma et al., 2017), were significantly differentially expressed (Figure 5B). More strikingly, a large group of non-coding, small Piwi-interacting RNAs (piRNAs) were significantly changed upon exposure to PA14 (Figure 5C; Table S3), confirming a change in piRNA abundance in response to environmental changes (Belicard et al., 2018). The majority of piRNAs were downregulated in response to PA14 exposure (466) compared to those with increased expression (254) (Figure 5C).

(C) PA14 training increases *daf-7p::gfp* expression in the ASI compared to training with OP50 or LasR. One-way ANOVA, Tukey's multiple comparisons test, mean \pm SEM n \geq 54 neurons per training, 2 biological replicates.

(E–G) Naive progeny of PA14-trained mothers (F) have increased expression of *daf-7p::gfp* in the ASI compared to progeny of OP50- (E) or LasR-trained (G) progeny. One-way ANOVA, Tukey's multiple comparisons test, mean \pm SEM n \geq 61 neurons per training condition, 3 biological replicates.

(H) Progeny (F1) of PA14-trained mothers have higher *daf-7p::gfp* expression in the ASJ after 4 h of PA14 training. Student's t test, mean \pm SEM n \geq 44 neurons per training condition.

(I and J) The ASI is not required for maternal (P0) pathogenic learning (I) but is required for progeny (F1) avoidance of PA14 (J).

(K) Like wild-type (N2), *daf-7* mutants (P0) have normal naive preference and can learn to avoid PA14 after training, but naive progeny (F1) of *daf-7(pk720)* PA14-trained progeny do not avoid PA14 (L).

(M) PA14 training elicits maternal avoidance of neuron RNAi-sensitive (LC108) worms treated with control vector or *daf-7* RNAi bacteria.

(N) P0 worms were trained on OP50 or PA14 without any RNAi exposure and then F1 progeny were exposed to control or *daf-7* RNAi to test the requirement of *daf-7* exclusively in F1s. Progeny of PA14-trained mothers treated with *daf-7* RNAi lose the TEI avoidance behavior. One-way ANOVA, Tukey's multiple comparison test, mean \pm SEM n \geq 7 choice assay plates with 50–200 worms.

(O and P) PA14 training increases *daf-7p::gfp* expression in the ASI of progeny of PA14-trained mothers for four generations (F1–F4; O) before returning to low levels in the 5th generation (F5; O and P).

Student's t test, mean \pm SEM n \geq 44 neurons per condition, 2 biological replicates. *p \leq 0.05, **p \leq 0.01, ***p \leq 0.001, ****p $<$ 0.0001; ns, not significant. See also Figure S3.

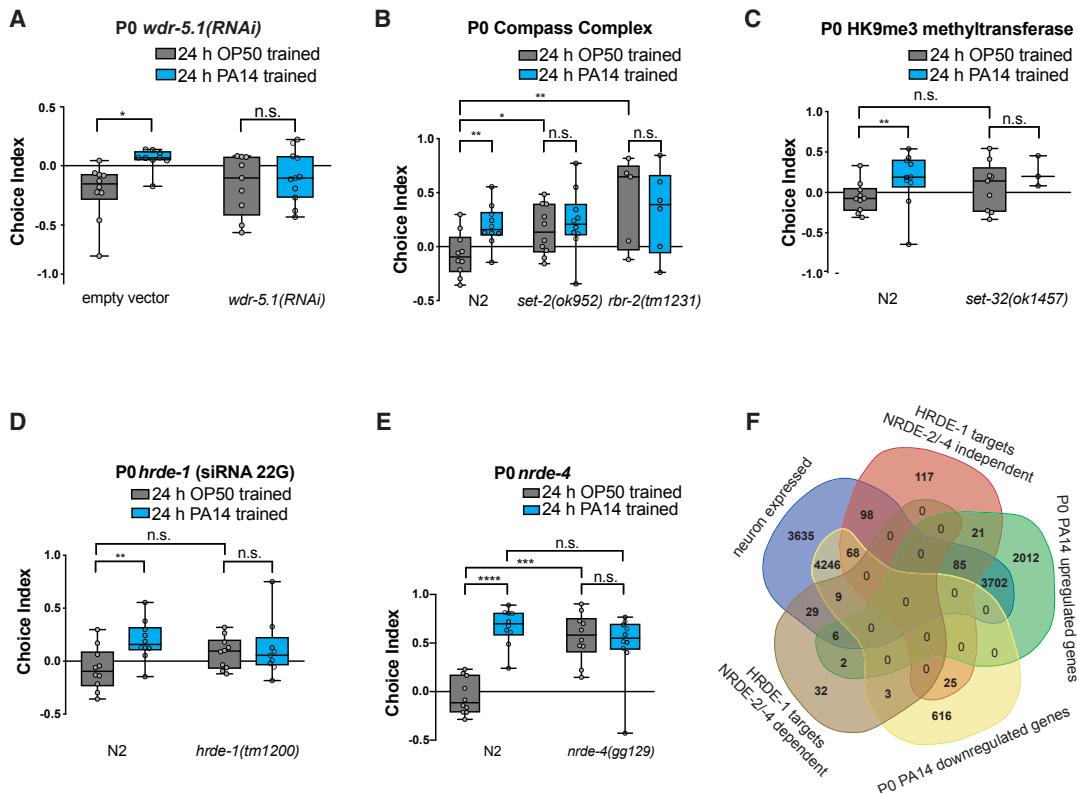


Figure 4. COMPASS Complex Histone Modifiers and Small RNA Regulators Are Required for Pathogenic Learning

- (A) *wdr-5.1(RNAi)* animals have normal naive preference but are defective for pathogenic learning.
- (B) *set-2(ok952)* and *rbr-2(tm1231)* mutants are defective for naive preference.
- (C) *set-32(ok1457)* mutants have normal naive preference, but are defective for pathogenic learning. One-way ANOVA, Tukey's multiple comparison test, mean \pm SEM $n \geq 7$ choice assay plates with 50–200 worms. * $p \leq 0.05$, ** $p \leq 0.01$; ns, not significant.
- (D) *hrde-1(tm1200)* mutants have normal naive preference but are defective for pathogenic learning.
- (E) *nrde-4(gg129)* mutants have high naive preference and do not appear to learn. One-way ANOVA, Tukey's multiple comparison test, mean \pm SEM $n \geq 7$ choice assay plates with 50–200 worms. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$; ns, not significant.
- (F) NRDE-2/4-dependent and -independent HRDE-1 targets (Buckley et al., 2012) were examined for overlap with PA14-upregulated and downregulated genes in P0 animals (false discovery rate [FDR] <5%), as well as adult neuron expression (Kaletsky et al., 2016). See also Figures S4 and S5.

PRG-1/Piwi Is Required for TEI of Pathogenic Avoidance and Induced *daf-7* ASI Expression in the Progeny of Trained Mothers

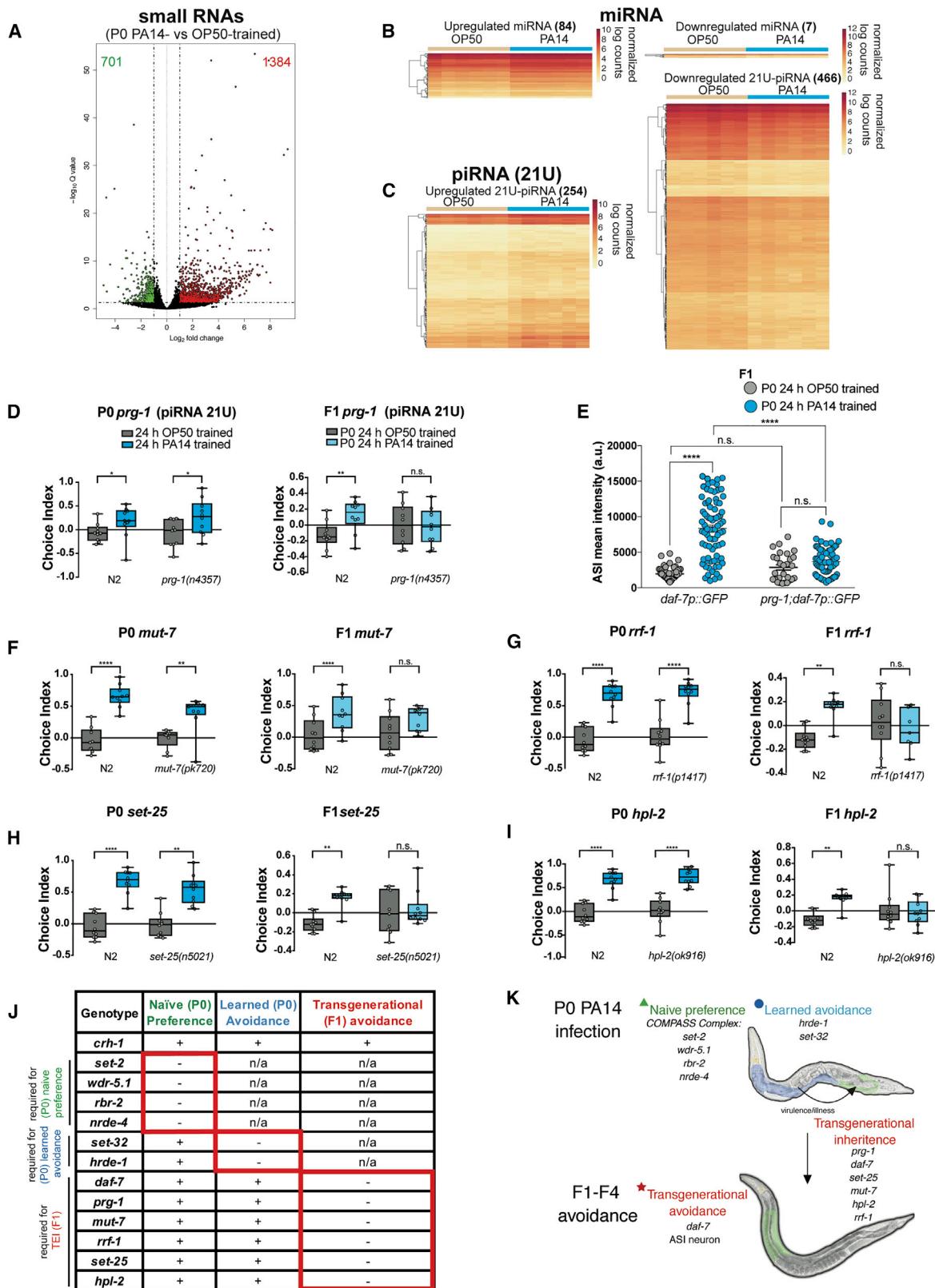
The differential expression of piRNAs upon PA14 treatment suggested that Piwi may play a role in transgenerational inheritance of pathogenic learning. PRG-1 is the *C. elegans* ortholog of the Piwi Argonaute and has been implicated in transgenerational inheritance of transposon silencing (Brennecke et al., 2008; Grentzinger et al., 2012) and germline immortality (Simon et al., 2014; Heestand et al., 2018) but has not previously been implicated in response to PA14. Indeed, mutants of *prg-1/Piwi* display normal naive choice preference and normal pathogenic learning after PA14 training (Figure 5D). However, the progeny of trained *prg-1/Piwi* mothers were defective in their avoidance of PA14 (Figure 5D; Figure S4G). These data suggest that PRG-1/Piwi is specifically required for the inheritance of learned pathogenic avoidance behavior.

To test whether PRG-1 activity is required for the regulation of DAF-7, the TGF- β ligand whose expression correlates with

avoidance behavior in post-P0 generations and is required for TEI of pathogenic learning (Figure 3), we examined the expression level of *daf-7p::gfp* in progeny of PA14-trained *prg-1* mutants. While control *daf-7p::gfp* F1 animals exhibited increased GFP fluorescence in the ASI neuron after P0 training with PA14 compared to OP50 exposure, loss of *prg-1* completely abrogated this fluorescence change (Figure 5E). This result suggests that Piwi/PRG-1 Argonaute activity is required for the induction of *daf-7* expression in the ASI neurons of F1 animals, which in turn is required for transgenerational pathogenic avoidance behavior.

The PRG-1/Piwi 22G siRNA Pathway Is Required for Transgenerational Inheritance of Pathogenic Aversive Learning

Next, we tested components of the nuclear RNAi pathway downstream of *prg-1* that have been implicated in transgenerational epigenetic inheritance. RdRPs amplify primary siRNAs and are essential for the production of secondary siRNAs that execute



(legend on next page)

TEI. To test the contribution of RdRPs to TEI of aversive learning, we tested *mut-7*, an RNase D homolog and part of the Mutator complex (Ketting et al., 1999), and *rrf-1*, an RdRP required for 22G siRNA biogenesis (Aoki et al., 2007). Like *prg-1* mutants, *mut-7* and *rrf-1* have normal P0 naive attraction to and learned avoidance of PA14 but are specifically defective in F1 avoidance of PA14 (Figures 5F and 5G), suggesting that they are part of the *prg-1*-mediated inheritance mechanism of learned avoidance.

Once secondary siRNAs are made, they are able to direct chromatin modifications via histone modifying enzymes. SET-25 is a histone H3K9 methyltransferase (Towbin et al., 2012) that acts downstream of PRG-1 and has been implicated in the establishment of TEI (Ashe et al., 2012; Klosin et al., 2017). HPL-2, an H3K9me3 reader and homolog of mammalian HP1 (Couteau et al., 2002), is involved in transgene and piRNA-mediated gene silencing in the gonad (Ashe et al., 2012). Similar to *prg-1* and the RdRP mutants, *set-25* and *hpl-2* mutants have normal P0 naive attraction to and learned avoidance of PA14 but are specifically defective in F1 avoidance of PA14 (Figures 5H and 5I). Together, these data suggest that *prg-1*/piRNAs and its downstream nuclear RNAi pathway function in the transgenerational inheritance of learned pathogen avoidance (Figures 5J and 5K).

Inheritance of Learned Avoidance of *Pseudomonas* Confers Survival Advantages to Progeny

Because we observed pathogenic avoidance behavior in wild-type animals rather than in mutants or an artificial situation, the transgenerational pathogenic avoidance paradigm may represent a natural context for such behaviors. Providing worms with the opportunity to avoid PA14 (by placing them on a small PA14 lawn, where there is some opportunity to avoid the pathogen, instead of a fully covered plate) significantly increases their survival (Figure 6A; 76%; $p < 0.0001$). Perhaps more importantly, we find that naive F1 progeny of PA14-trained mothers survive significantly longer ($p = 0.0013$) on a small PA14 lawn compared to progeny of OP50-trained controls (Figure 6B). The increased survival of progeny of trained mothers is dependent on the ability to avoid PA14, since obligate PA14 exposure (full PA14 lawn) confers no survival difference (Figure 6C). Finally, *prg-1* mutant F1s do not benefit from P0 training on PA14 (Figure 6D), even

when presented with the opportunity to avoid *Pseudomonas*. Thus, the PRG-1-dependent transgenerational inheritance of learned pathogenic avoidance is required for this enhanced survival.

PA14 exposure provides both parents and several generations of progeny with the ability to avoid this pathogen later, promoting survival. Why, then, are these advantageous behavioral responses not hard-wired into *C. elegans*' naive behavioral repertoire? Could it in fact be beneficial for the progeny of PA14-exposed parents to reacquire PA14 attraction later? To explore the hypothesis that loss of learned avoidance could be beneficial, we measured behavioral responses to PA14 grown under varying temperature conditions that alter bacterial virulence, as might be experienced in the wild. PA14 is pathogenic to worms when grown at elevated temperatures (25°C) but is less pathogenic when raised at lower temperatures (15°C) (Figure 6E). Similarly, animals escape from a lawn of pathogenic PA14 (grown at 25°C), but not from less virulent PA14 (grown at 15°C), similar to their behavior on OP50 (Figure 6F). Despite differences in the potential quality and pathogenicity of the food source, naive worms similarly prefer PA14 raised at 25°C and 15°C compared to OP50 (Figure 6G). Strikingly, while worms trained on 15°C-grown non-pathogenic bacteria do not avoid PA14, underscoring the importance of virulence in inducing avoidance, worms trained on pathogenic (25°C-grown) PA14 avoid the less-pathogenic (15°C-grown) PA14 (Figure 6H). This result suggests that pathogenic *Pseudomonas* training causes worms to avoid all *Pseudomonas*, including non-pathogenic bacteria that may provide adequate nutrition. Therefore, “forgetting” the learned avoidance after a few generations may be necessary to allow the worms to once again be attracted to nutritious, non-pathogenic *Pseudomonas*.

DISCUSSION

Animals exposed to pathogens employ several strategies to maintain fitness in the face of imminent death. *C. elegans* exposed to PA14 immediately engage innate immune responses (Troemel et al., 2006) and subsequently induce behavioral avoidance (Zhang et al., 2005). In fact, the ability to escape PA14 after direct contact promotes survival (Figure 6A). Transgenerational

Figure 5. The PRG-1/Piwi Pathway Is Required for Transgenerational Inheritance of Pathogenic Aversive Learning

(A) PA14-upregulated (1364, red) and -downregulated (701, green) small RNAs were differentially expressed using DESeq2. Volcano plot shown at FDR <5% and \log_2 fold change $>$ and <1 .
 (B and C) Regularized expression counts from differentially expressed microRNAs (miRNAs) (B) and piRNAs (C) in P0 animals.
 (D) *prg-1(n4357)* mutants are defective for transgenerational inheritance of pathogenic avoidance.
 (E) Elevated *daf-7p::gfp* expression is abrogated in progeny of *prg-1* PA14-trained mutants.
 (F–I) *mut-7(pk720)* (F), *rrf-1(pk1426)* (G), *set-25(n5021)* (H), and *hpl-2(ok916)* (I) P0s have normal naive preference and can learn to avoid PA14 after training; however, naive progeny are defective for inherited avoidance. One-way ANOVA, Tukey's multiple comparison test, mean \pm SEM $n \geq 7$ choice assay plates with 50–200 worms. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$; ns, not significant.
 (J and K) Naive *C. elegans* prefer PA14, which requires the activity of the COMPASS complex (SET-2, WDR-5.1, and RBR-2) and NRDE-4 (J). After infection by PA14, *C. elegans* learn to avoid PA14, which requires HRDE-1 and SET-32. piRNA pathway components (PRG-1, MUT-7, RRF-3, RRF-1) and histone modification regulators (SET-25 and HPL-2) are required to transmit transgenerational inheritance of pathogenic avoidance from the P0-F1. (K) *daf-7* expression is induced in the ASI neuron, and *daf-7* and ASI are required for avoidance in the F1 generation. *daf-7* levels in the ASI may set the avoidance response ability of that generation, rendering the animals “primed” for increased expression of *daf-7* in the ASJ and subsequent avoidance upon PA14 encounter. (yellow, neurons; blue, intestine; green, germline). Table: genes are characterized by their requirement for normal naive preference for PA14 (green), learned avoidance (blue), and transgenerational epigenetic inheritance (red).
 See also Figure S6.

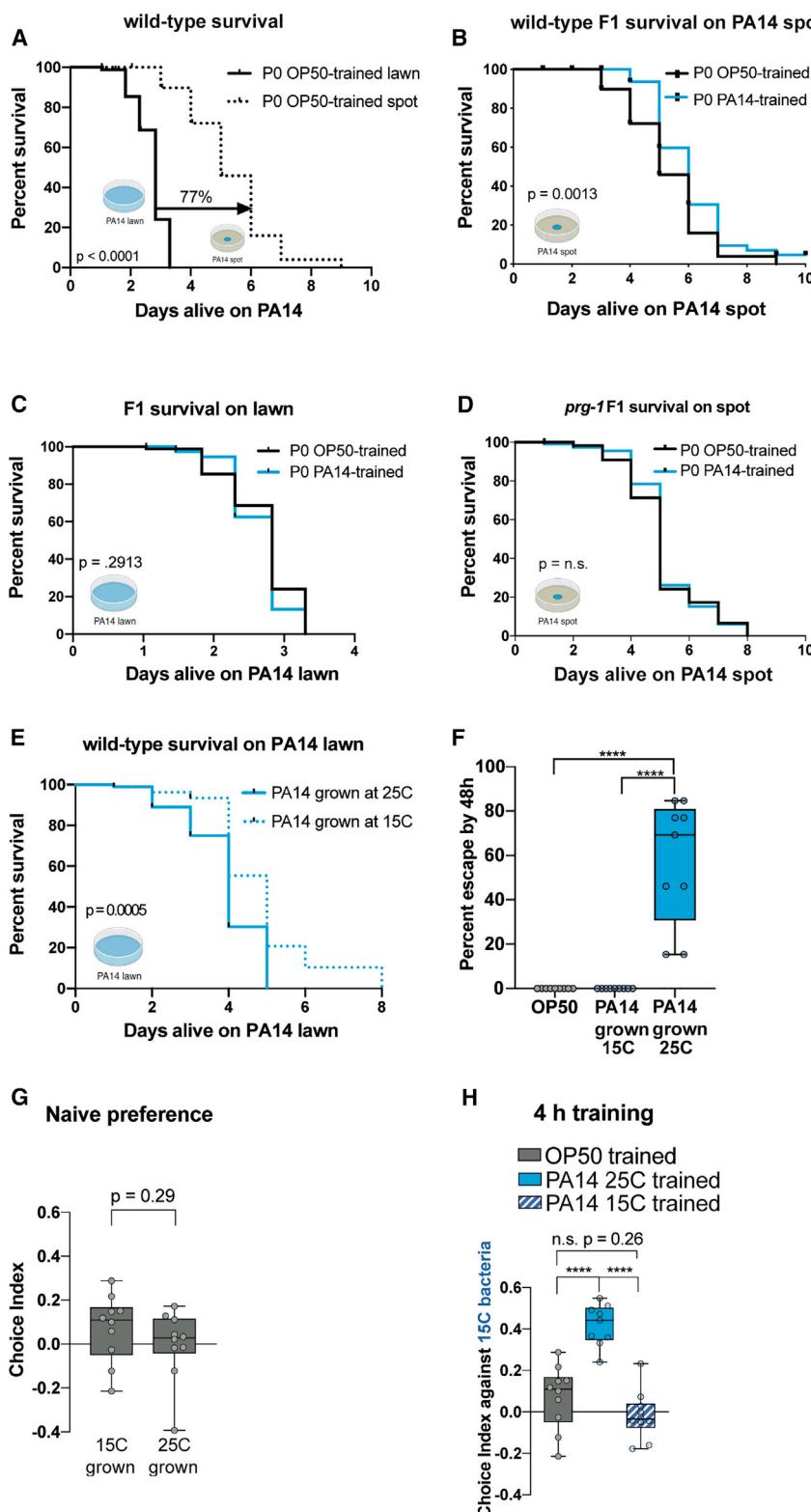


Figure 6. Inheritance of Learned Avoidance of *Pseudomonas* Confers Survival Advantages to Progeny

(A) Survival was measured on PA14 spots versus lawns. The ability to escape PA14 provides a survival advantage, compared to animals on a lawn of PA14 ($p < 0.0001$, 77% increase).

(B) Progeny of PA14-trained mothers have a survival advantage on an escapable spot of PA14, compared to progeny of OP50-trained mothers ($p = 0.0013$).

(C) No difference in survival of OP50- or PA14-trained mothers was observed when worms were unable to escape PA14.

(D) *prg-1* mutants do not exhibit the survival benefit on escapable lawns, unlike wild-type (B).

(E) PA14 grown at 15°C is less pathogenic than PA14 grown at 25°C. Bacteria was grown on plates at the indicated temperatures, and the survival assay was performed for all conditions at 20°C.

(F) At 20°C, *C. elegans* leave pathogenic (25°C grown) PA14 at greater rates than from OP50 or less pathogenic (15°C grown) PA14.

(G) Untrained (naive) animals are attracted to PA14 regardless of the pathogenicity of PA14 (15°C grown versus 25°C grown).

(H) 4 h of training on virulent PA14 (25°C grown) is sufficient to cause maternal avoidance of less virulent (15°C grown). However, 4 h of training on less virulent PA14 is not sufficient to elicit avoidance.

All survival assays (A-E): log-rank (Mantel-Cox) test. $n = 80$ worms. One-way ANOVA, Tukey's multiple comparison test, mean \pm SEM $n \geq 7$ choice assay plates with 50–200 worms. $***p < 0.0001$; ns, not significant.

epigenetic inheritance of learned PA14 avoidance may serve to prepare progeny and generations of grandprogeny for likely environmental PA14 exposure, so that they, too, can escape the pathogen. Our findings suggest that exposure to the pathogen PA14 engages a piRNA-dependent transgenerational inheritance network that confers multigenerational behavioral and gene expression changes; these changes may provide an adaptive survival advantage to progeny. The PA14-induced TEI behavior is dependent on PA14 virulence, lasts through the F4 generation, can be transmitted by either the male or female germline, and is distinct from previously described pathogenic learning behaviors in *C. elegans*. We also found that the learned TEI behavior correlates with elevated *daf-7p::gfp* expression in the ASI neuron on the same generational timescale as the behavior, and that the learned ability to avoid PA14 results in a survival advantage in the progeny of trained parents—all phenotypes that are dependent on PIWI/PRG-1 and its downstream molecular components to execute transgenerational epigenetic inheritance.

By analyzing mutants for their roles in naive P0 attraction to PA14, P0 learned avoidance of PA14 after 24 h exposure, and the transgenerational inheritance of learned avoidance in the F1 progeny, we identified the molecular processes required for each step (Figures 5J and 5K). In contrast to its previously identified role in L1 imprinting in the P0 generation (Jin et al., 2016), CREB is not required for any of the steps (Figure 1F), likely because our testing of behavior occurs immediately after training in the P0s, rather than a long-term, spaced-training induced memory. Components of the COMPASS complex (SET-2, WDR-5.1, and RBR-2) are required for naive preference for PA14, whereas components of the nuclear RNAi pathway (SET-32 and HRDE-1) are required for P0 pathogenic learning. Despite the fact that NRDE-4 can act with HRDE-1, NRDE-4's requirement for normal naive preference suggests that the NRDE-2/-4-independent HRDE-1 pathway may regulate pathogenic learning (Figure 4E). Finally, the transgenerational inheritance of pathogenic learning requires the activity of PRG-1/Piwi Argonaute, MUT-7/RNase D, and RRF-1/RdRP (Figure S6), which have been previously shown to work together to regulate 22G siRNA amplification (Phillips et al., 2012). These 22G siRNAs then guide the deposition and maintenance of histone marks through the activity of SET-25/H3K9 histone methyltransferase (Towbin et al., 2012) and the HPL-2/HP1 H3K9me3 reader (Couteau et al., 2002), which are also required for TEI of pathogen avoidance (Figures 5J and 5K; Figure S6).

Piwi/PRG-1-dependent piRNA pathways are potent mediators of TEI-related phenomena across a variety of metazoan species. In the germline of flies, worms, and mice, piRNAs regulate the stable silencing of germline transposons and are indispensable for germline development (Ghildiyal and Zamore, 2009). While piRNA biogenesis and secondary amplification mechanisms vary, the output of these Piwi-piRNA pathways converges on germline silencing through gene expression regulation (Ghildiyal and Zamore, 2009). Our results support the emerging model that, in addition to the conserved, long-lasting silencing mediated by piRNAs (Ashe et al., 2012; Bagijn et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012), this class of small RNAs is also involved in regulating transient transgenera-

tional phenotypes (Brennecke et al., 2008; Grentzinger et al., 2012; Ashe et al., 2012), including pathogen avoidance behavior, as we have shown here.

Our results support a role for both sperm and oocytes in the transmission of learned pathogenic avoidance. There is substantial evidence supporting the role of mammalian sperm in TEI (Donkin and Barres, 2018), with less direct evidence that oocytes can transmit transgenerational information; this is due to the difficulties of separating transgenerational mechanisms from environmental (i.e., *in utero*) influences in females, rather than the relative contributions of sperm and oocytes to TEI phenotypes. *C. elegans* hermaphrodites have higher nutritional needs and must make critical food choice decisions to support not only themselves but also the development of their progeny. Furthermore, males of androdioecious species, such as *C. elegans*, are rare in nature (Ward and Carrel, 1979), and males prioritize exploration over food (Ryan et al., 2014). If hermaphrodites depended exclusively on male sperm for TEI of pathogenic learning, they might have no way of preparing progeny for potential PA14 exposure. By contrast, males that encounter PA14 and subsequently mate with naive hermaphrodites may prepare progeny for potentially hazardous local PA14 environments. In effect, the ability of both parents to epigenetically warn progeny of the presence of pathogenic *Pseudomonas* could be an ideal survival strategy.

If PA14 avoidance promotes *C. elegans* fitness, why is this behavior not hard-wired, and what is the advantage of losing the TEI avoidance behavior after several generations? The *Pseudomonas* genus, which includes several non-pathogenic *Pseudomonas* species, makes up a large fraction—perhaps a third—of *C. elegans*' natural environment (Samuel et al., 2016). While some *Pseudomonas* are detrimental to fitness, consumption of other *Pseudomonas* species increases *C. elegans* progeny production (Dirksen et al., 2016), suggesting that several *Pseudomonas* may provide a substantial source of nutrition. This appears true even among the same species of *Pseudomonas*, in that PA14 can exhibit variable pathogenicity depending on environmental conditions, such as temperature, and that virulent PA14-exposed worms cannot distinguish between harmful and potentially nutritious PA14 (Figures 6E–6H). Thus, parental and learned avoidance behavior in subsequent generations may only be beneficial to worms that may continue to encounter PA14 in their local environment. Therefore, naive avoidance and indefinite transgenerational inheritance of avoidance of all *Pseudomonas* species might be a poor long-term strategy that could deprive animals of adequate nutrition. Instead, temporary avoidance of pathogenic *Pseudomonas* might drive the worms' progeny to escape pathogenic food sources, while eventually allowing the return to consumption of potentially nutritious *Pseudomonas*. While germline silencing of transposons can last indefinitely, transgenerational epigenetic inheritance of transient phenotypes occurs over shorter generational timescales, typically lasting 1–4 generations in flies, worms, and mice (Perez and Lehner, 2019). While our work does not define the molecular events that govern how long a TEI phenotype lasts, it does provide biological insight into why both the acquisition and loss of a behavior several generations later would be beneficial. Together, our results demonstrate

that transgenerational avoidance of pathogenic bacteria provides a biological context for TEI in *C. elegans*, where animals must distinguish between both beneficial and detrimental food sources to ensure survival of self and progeny in a dynamic environment.

STAR★METHODS

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

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
 - *C. elegans* and bacterial strains and cultivation
- **METHOD DETAILS**
 - Pathogen training
 - Aversive learning assay
 - Odor + 2-aminoacetophenone (2AA) training
 - L1 imprinting
 - PA14 survival assay
 - *fog-2* mating assay
 - RNA Interference
 - Antibodies
 - Whole-mount immunofluorescence
 - Imaging and fluorescence quantitation
 - RNA isolation
 - RNA-seq data analysis
 - Gene Ontology analysis
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
- **DATA AND SOFTWARE AVAILABILITY**

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.cell.2019.05.024>.

ACKNOWLEDGMENTS

We thank the *C. elegans* Genetics Center for strains, the Genomics Core Facility at Princeton University, J. Wiggins, and J. Miller for RNA-seq library preparation and sequencing, L. Parsons for assistance with data analysis, V. Cota for gonad dissections, W. Keyes, J. Ashraf, and R. Clausen for assistance counting aversive learning assays, Z. Gitai for bacteria strains, and the Murphy lab for discussion. C.T.M. is the Director of the Glenn Center for Aging Research at Princeton and an HHMI-Simons Faculty Scholar. R.S.M. was supported by T32GM007388 (NIGMS), and further support was provided by an NIH DP1 Pioneer Award to C.T.M. (NIGMS 5DP1GM119167), The Glenn Foundation for Medical Research (GMFR CNV1001899), and the HHMI Faculty Scholar Program (HHMI-Simons AWD1005048).

AUTHOR CONTRIBUTIONS

Conceptualization, R.S.M., R.K., and C.T.M.; Methodology, R.S.M., R.K., and C.T.M.; Investigation, R.S.M. and R.K.; Writing – Review & Editing, R.S.M., R.K., and C.T.M.; Funding Acquisition, C.T.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: February 14, 2019

Revised: April 4, 2019

Accepted: May 13, 2019

Published: June 6, 2019

REFERENCES

- Aoki, K., Moriguchi, H., Yoshioka, T., Okawa, K., and Tabara, H. (2007). In vitro analyses of the production and activity of secondary small interfering RNAs in *C. elegans*. *EMBO J.* **26**, 5007–5019.
- Ashe, A., Sapetschnig, A., Weick, E.-M., Mitchell, J., Bagijn, M.P., Cording, A.C., Doebley, A.-L., Goldstein, L.D., Lehrbach, N.J., Le Pen, J., et al. (2012). piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* **150**, 88–99.
- Bagijn, M.P., Goldstein, L.D., Sapetschnig, A., Weick, E.-M., Bouasker, S., Lehrbach, N.J., Simard, M.J., and Miska, E.A. (2012). Function, targets, and evolution of *Caenorhabditis elegans* piRNAs. *Science* **337**, 574–578.
- Belicard, T., Jareosettasin, P., and Sarkies, P. (2018). The piRNA pathway responds to environmental signals to establish intergenerational adaptation to stress. *BMC Biol.* **16**, 103.
- Brennecke, J., Malone, C.D., Aravin, A.A., Sachidanandam, R., Stark, A., and Hannon, G.J. (2008). An epigenetic role for maternally inherited piRNAs in transposon silencing. *Science* **322**, 1387–1392.
- Buckley, B.A., Burkhart, K.B., Gu, S.G., Spracklin, G., Kershner, A., Fritz, H., Kimble, J., Fire, A., and Kennedy, S. (2012). A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. *Nature* **489**, 447–451.
- Burkhart, K.B., Guang, S., Buckley, B.A., Wong, L., Bochner, A.F., and Kennedy, S. (2011). A pre-mRNA-associating factor links endogenous siRNAs to chromatin regulation. *PLoS Genet.* **7**, e1002249.
- Burton, N.O., Burkhart, K.B., and Kennedy, S. (2011). Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **108**, 19683–19688.
- Burton, N.O., Furuta, T., Webster, A.K., Kaplan, R.E.W., Baugh, L.R., Arur, S., and Horvitz, H.R. (2017). Insulin-like signalling to the maternal germline controls progeny response to osmotic stress. *Nat. Cell Biol.* **19**, 252–257.
- Couteau, F., Guerry, F., Muller, F., and Palladino, F. (2002). A heterochromatin protein 1 homologue in *Caenorhabditis elegans* acts in germline and vulval development. *EMBO Rep.* **3**, 235–241.
- Dai, L.-L., Gao, J.-X., Zou, C.-G., Ma, Y.-C., and Zhang, K.-Q. (2015). mir-233 modulates the unfolded protein response in *C. elegans* during *Pseudomonas aeruginosa* infection. *PLoS Pathog.* **11**, e1004606.
- Dias, B.G., and Ressler, K.J. (2014). Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nat. Neurosci.* **17**, 89–96.
- Dirksen, P., Marsh, S.A., Braker, I., Heitland, N., Wagner, S., Nakad, R., Mader, S., Petersen, C., Kowallik, V., Rosenstiel, P., et al. (2016). The native microbiome of the nematode *Caenorhabditis elegans*: gateway to a new host-microbiome model. *BMC Biol.* **14**, 38.
- Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21.
- Donkin, I., and Barrès, R. (2018). Sperm epigenetics and influence of environmental factors. *Mol. Metab.* **14**, 1–11.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.
- Gapp, K., Jawaid, A., Sarkies, P., Bohacek, J., Pelczar, P., Prados, J., Farinelli, L., Miska, E., and Mansuy, I.M. (2014). Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nat. Neurosci.* **17**, 667–669.
- Ghildiyal, M., and Zamore, P.D. (2009). Small silencing RNAs: an expanding universe. *Nat. Rev. Genet.* **10**, 94–108.

- Greer, E.R., Pérez, C.L., Van Gilst, M.R., Lee, B.H., and Ashrafi, K. (2008). Neural and molecular dissection of a *C. elegans* sensory circuit that regulates fat and feeding. *Cell Metab.* 8, 118–131.
- Greer, E.L., Maures, T.J., Ucar, D., Hauswirth, A.G., Mancini, E., Lim, J.P., Benayoun, B.A., Shi, Y., and Brunet, A. (2011). Transgenerational epigenetic inheritance of longevity in *Caenorhabditis elegans*. *Nature* 479, 365–371.
- Grentzinger, T., Armenise, C., Brun, C., Mugat, B., Serrano, V., Pelisson, A., and Chambeillon, S. (2012). piRNA-mediated transgenerational inheritance of an acquired trait. *Genome Res.* 22, 1877–1888.
- Grimson, A., Srivastava, M., Fahey, B., Woodcroft, B.J., Chiang, H.R., King, N., Degnan, B.M., Rokhsar, D.S., and Bartel, D.P. (2008). Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. *Nature* 455, 1193–1197.
- Gu, S.G., Pak, J., Guang, S., Maniar, J.M., Kennedy, S., and Fire, A. (2012). Amplification of siRNA in *Caenorhabditis elegans* generates a transgenerational sequence-targeted histone H3 lysine 9 methylation footprint. *Nat. Genet.* 44, 157–164.
- Guang, S., Bochner, A.F., Pavlec, D.M., Burkhart, K.B., Harding, S., Lachowiec, J., and Kennedy, S. (2008). An Argonaute transports siRNAs from the cytoplasm to the nucleus. *Science* 321, 537–541.
- Ha, H.I., Hendricks, M., Shen, Y., Gabel, C.V., Fang-Yen, C., Qin, Y., Colón-Ramos, D., Shen, K., Samuel, A.D.T., and Zhang, Y. (2010). Functional organization of a neural network for aversive olfactory learning in *Caenorhabditis elegans*. *Neuron* 68, 1173–1186.
- Heestand, B., Simon, M., Frenk, S., Titov, D., and Ahmed, S. (2018). Transgenerational Sterility of Piwi Mutants Represents a Dynamic Form of Adult Reproductive Diapause. *Cell Rep.* 23, 156–171.
- Hilbert, Z.A., and Kim, D.H. (2017). Sexually dimorphic control of gene expression in sensory neurons regulates decision-making behavior in *C. elegans*. *eLife* 6. Published online January 24, 2017. 10.7554/eLife.21166.
- Jin, X., Pokala, N., and Bargmann, C.I. (2016). Distinct Circuits for the Formation and Retrieval of an Imprinted Olfactory Memory. *Cell* 164, 632–643.
- Kaletsky, R., Lakhina, V., Arey, R., Williams, A., Landis, J., Ashraf, J., and Murphy, C.T. (2016). The *C. elegans* adult neuronal IIS/FOXO transcriptome reveals adult phenotype regulators. *Nature* 529, 92–96.
- Kauffman, A.L., Ashraf, J.M., Corces-Zimmerman, M.R., Landis, J.N., and Murphy, C.T. (2010). Insulin signaling and dietary restriction differentially influence the decline of learning and memory with age. *PLoS Biol.* 8, e1000372.
- Ketting, R.F., Haverkamp, T.H., van Luenen, H.G.A., and Plasterk, R.H. (1999). Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* 99, 133–141.
- Kishimoto, S., Uno, M., Okabe, E., Nono, M., and Nishida, E. (2017). Environmental stresses induce transgenerationally inheritable survival advantages via germline-to-soma communication in *Caenorhabditis elegans*. *Nat. Commun.* 8, 14031.
- Klosin, A., Casas, E., Hidalgo-Carcedo, C., Vavouri, T., and Lehner, B. (2017). Transgenerational transmission of environmental information in *C. elegans*. *Science* 356, 320–323.
- Kudlow, B.A., Zhang, L., and Han, M. (2012). Systematic analysis of tissue-restricted miRISCs reveals a broad role for microRNAs in suppressing basal activity of the *C. elegans* pathogen response. *Mol. Cell* 46, 530–541.
- Liu, F., He, C.-X., Luo, L.-J., Zou, Q.-L., Zhao, Y.-X., Saini, R., Han, S.-F., Knöller, H.-J., Wang, L.-S., and Ge, B.-X. (2013). Nuclear hormone receptor regulation of microRNAs controls innate immune responses in *C. elegans*. *PLoS Pathog.* 9, e1003545.
- Luteijn, M.J., van Bergeijk, P., Kaaaij, L.J.T., Almeida, M.V., Roovers, E.F., Berzikov, E., and Ketting, R.F. (2012). Extremely stable Piwi-induced gene silencing in *Caenorhabditis elegans*. *EMBO J.* 31, 3422–3430.
- Ma, Y.-C., Zhang, L., Dai, L.-L., Khan, R.U., and Zou, C.-G. (2017). mir-67 regulates *P. aeruginosa* avoidance behavior in *C. elegans*. *Biochem. Biophys. Res. Commun.* 494, 120–125.
- Mao, H., Zhu, C., Zong, D., Weng, C., Yang, X., Huang, H., Liu, D., Feng, X., and Guang, S. (2015). The Nrde Pathway Mediates Small-RNA-Directed Histone H3 Lysine 27 Trimethylation in *Caenorhabditis elegans*. *Curr. Biol.* 25, 2398–2403.
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. Journal* 17, 10.
- Meisel, J.D., Panda, O., Mahanti, P., Schroeder, F.C., and Kim, D.H. (2014). Chemosensation of bacterial secondary metabolites modulates neuroendocrine signaling and behavior of *C. elegans*. *Cell* 159, 267–280.
- Ooi, F.K., and Prahad, V. (2017). Olfactory experience primes the heat shock transcription factor HSF-1 to enhance the expression of molecular chaperones in *C. elegans*. *Sci. Signal.* 10, eaan4893.
- Pak, J., and Fire, A. (2007). Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. *Science* 315, 241–244.
- Perez, M.F., and Lehner, B. (2019). Intergenerational and transgenerational epigenetic inheritance in animals. *Nat. Cell Biol.* 21, 143–151.
- Perez, M.F., Francesconi, M., Hidalgo-Carcedo, C., and Lehner, B. (2017). Maternal age generates phenotypic variation in *Caenorhabditis elegans*. *Nature* 552, 106–109.
- Phillips, C.M., Montgomery, T.A., Breen, P.C., and Ruvkun, G. (2012). MUT-16 promotes formation of perinuclear mutator foci required for RNA silencing in the *C. elegans* germline. *Genes Dev.* 26, 1433–1444.
- Pujol, N., Link, E.M., Liu, L.X., Kurz, C.L., Alloing, G., Tan, M.-W., Ray, K.P., Solari, R., Johnson, C.D., and Ewbank, J.J. (2001). A reverse genetic analysis of components of the Toll signaling pathway in *Caenorhabditis elegans*. *Curr. Biol.* 11, 809–821.
- Rechavi, O., Minevich, G., and Hobert, O. (2011). Transgenerational inheritance of an acquired small RNA-based antiviral response in *C. elegans*. *Cell* 147, 1248–1256.
- Rechavi, O., Houri-Ze'evi, L., Anava, S., Goh, W.S.S., Kerk, S.Y., Hannon, G.J., and Hobert, O. (2014). Starvation-induced transgenerational inheritance of small RNAs in *C. elegans*. *Cell* 158, 277–287.
- Reimand, J., Arak, T., Adler, P., Kolberg, L., Reisberg, S., Peterson, H., and Vilo, J. (2016). g:Profiler-a web server for functional interpretation of gene lists (2016 update). *Nucleic Acids Res.* 44, W83–W89.
- Ren, Z., and Ambros, V.R. (2015). *Caenorhabditis elegans* microRNAs of the let-7 family act in innate immune response circuits and confer robust developmental timing against pathogen stress. *Proc. Natl. Acad. Sci. USA* 112, E2366–E2375.
- Ryan, D.A., Miller, R.M., Lee, K., Neal, S.J., Fagan, K.A., Sengupta, P., and Portman, D.S. (2014). Sex, age, and hunger regulate behavioral prioritization through dynamic modulation of chemoreceptor expression. *Curr. Biol.* 24, 2509–2517.
- Samuel, B.S., Rowedder, H., Braendle, C., Félix, M.-A., and Ruvkun, G. (2016). *Caenorhabditis elegans* responses to bacteria from its natural habitats. *Proc. Natl. Acad. Sci. USA* 113, E3941–E3949.
- Schedl, T., and Kimble, J. (1988). fog-2, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. *Genetics* 119, 43–61.
- Schulenburg, H., and Félix, M.-A. (2017). The Natural Biotic Environment of *Caenorhabditis elegans*. *Genetics* 206, 55–86.
- Shirayama, M., Seth, M., Lee, H.-C., Gu, W., Ishidate, T., Conte, D., Jr., and Mello, C.C. (2012). piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* 150, 65–77.
- Shtonda, B.B., and Avery, L. (2006). Dietary choice behavior in *Caenorhabditis elegans*. *J. Exp. Biol.* 209, 89–102.
- Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H.A., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* 107, 465–476.
- Simon, M., Sarkies, P., Ikegami, K., Doebley, A.-L., Goldstein, L.D., Mitchell, J., Sakaguchi, A., Miska, E.A., and Ahmed, S. (2014). Reduced insulin/IGF-1 signaling restores germ cell immortality to *Caenorhabditis elegans* Piwi mutants. *Cell Rep.* 7, 762–773.

- Siryaporn, A., Kim, M.K., Shen, Y., Stone, H.A., and Gitai, Z. (2015). Colonization, competition, and dispersal of pathogens in fluid flow networks. *Curr. Biol.* 25, 1201–1207.
- Supek, F., Bošnjak, M., Škunca, N., and Šmuc, T. (2011). REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS ONE* 6, e21800.
- Tan, M.W., Mahajan-Miklos, S., and Ausubel, F.M. (1999). Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. *Proc. Natl. Acad. Sci. USA* 96, 715–720.
- Tauffenberger, A., and Parker, J.A. (2014). Heritable transmission of stress resistance by high dietary glucose in *Caenorhabditis elegans*. *PLoS Genet.* 10, e1004346.
- Towbin, B.D., González-Aguilera, C., Sack, R., Gaidatzis, D., Kalck, V., Meister, P., Askjaer, P., and Gasser, S.M. (2012). Step-wise methylation of histone H3K9 positions heterochromatin at the nuclear periphery. *Cell* 150, 934–947.
- Troemel, E.R., Chu, S.W., Reinke, V., Lee, S.S., Ausubel, F.M., and Kim, D.H. (2006). p38 MAPK regulates expression of immune response genes and contributes to longevity in *C. elegans*. *PLoS Genet.* 2, e183.
- Vassoler, F.M., White, S.L., Schmidt, H.D., Sadri-Vakili, G., and Pierce, R.C. (2013). Epigenetic inheritance of a cocaine-resistance phenotype. *Nat. Neurosci.* 16, 42–47.
- Vastenhouw, N.L., Brunschwig, K., Okihara, K.L., Müller, F., Tijsterman, M., and Plasterk, R.H.A. (2006). Long-term gene silencing by RNAi: Gene expression. *Nature* 442, 882.
- Ward, S., and Carrel, J.S. (1979). Fertilization and sperm competition in the nematode *Caenorhabditis elegans*. *Dev. Biol.* 73, 304–321.
- Wianny, F., and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. *Nat. Cell Biol.* 2, 70–75.
- Woodhouse, R.M., Buchmann, G., Hoe, M., Harney, D.J., Low, J.K.K., Laranje, M., Boag, P.R., and Ashe, A. (2018). Chromatin Modifiers SET-25 and SET-32 Are Required for Establishment but Not Long-Term Maintenance of Transgenerational Epigenetic Inheritance. *Cell Rep.* 25, 2259–2272.
- Zhang, Y., Lu, H., and Bargmann, C.I. (2005). Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. *Nature* 438, 179–184.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Histone H3 (tri methyl K4) antibody	Abcam	Cat# Ab8580; RRID: AB_306649
Anti-Histone H3 (tri methyl K9) antibody	Abcam	Cat# Ab8898; RRID: AB_306848
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Flour 488	Fisher Scientific	Cat# A-11008; RRID: AB_143165
Bacterial and Virus Strains		
<i>E. coli</i> : OP50	Caenorhabditis Genetics Center	OP50
<i>E. coli</i> : OP50-streptomycin resistant	Caenorhabditis Genetics Center	OP50-1
<i>E. coli</i> : HT115	Caenorhabditis Genetics Center	HT115
<i>P. aeruginosa</i> : PA14	Z. Gitai	PA14
<i>P. aeruginosa</i> : LasR	Siryaporn et al., 2015	LasR
<i>S. marcescens</i> : 274	ATCC	ATCC 274
Chemicals, Peptides, and Recombinant Proteins		
2-aminoacetophenone hydrochloride 99%	Sigma-Aldrich	A38207-1G; CAS: 5468-37-1
Streptomycin sulfate	Fisher Scientific	BP910-50; CAS: 3810-74-0
TRIzol LS Reagent	Fisher Scientific	10-296-010
Sodium azide	Fisher Scientific	S2271-25
Levamisole solution	Vector Laboratories	SP-5000
10x phosphate buffered saline (PBS)	Bio-Rad	1610780
Triton		
Poly-Prep Slides, poly-L-lysine coated glass slides	Sigma-Aldrich	P0425-72EA
DAPI	Sigma-Aldrich	10236276001
RNA 5' Polyphosphatase	Lucigen	RP8092H
Critical Commercial Assays		
mirVana, miRNA Isolation Kit with phenol	Thermo Fisher Scientific	AM1560
PrepX PolyA mRNA Isolation Kit, 96 Samples (400047)	Takara Bio	640098
PrepX RNA-Seq for Illumina Library Kit, 24 Samples (400039)	Takara Bio	640096
HiSeq Rapid SR Cluster kit v2	Illumina	GD-402-4002
HiSeq Rapid SBS kit v2	Illumina	FC-402-4022
Deposited Data		
RNA-sequencing raw data	This study	NCBI BioProject PRJNA509938
Experimental Models: Organisms/Strains		
<i>C. elegans</i> : Strain N2 var. Bristol: wild-type	Caenorhabditis Genetics Center	WB Strain: N2
<i>C. elegans</i> : Strain SX922: <i>prg-1(n4357)</i>	Caenorhabditis Genetics Center	WB Strain: SX922
<i>C. elegans</i> : Strain RB1025: <i>set-2(ok952)</i>	Caenorhabditis Genetics Center	WB Strain: RB1025
<i>C. elegans</i> : Strain FK181: [(<i>Pdraf-7::GFP</i> + <i>rol-6(su10060)</i>)]	Caenorhabditis Genetics Center	WB Strain: FK181
<i>C. elegans</i> : Strain MT9973: <i>crh-1(n3315)</i>	Caenorhabditis Genetics Center	WB Strain: MT9973
<i>C. elegans</i> : Strain PY7505: <i>oyls84</i> [<i>gpa-4p::TU#813</i> + <i>gcy-27p::TU#814</i> + <i>gcy-27p::GFP</i> + <i>unc-122p::DsRed</i>]	Caenorhabditis Genetics Center	WB Strain: PY7505
<i>C. elegans</i> : Strain LC108: <i>uls69</i> [(<i>pCFJ90</i>) <i>myo-2p::mCherry</i> + <i>unc-119p::sid-1</i>], NL1820: <i>mut-7(pk720)</i>	Caenorhabditis Genetics Center	WB Strain: LC108
<i>C. elegans</i> : Strain MAH23: <i>rrf-1(p1417)</i>	Caenorhabditis Genetics Center	WB Strain: MAH23

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>C. elegans</i> : Strain MT17463: <i>set-25(n5021)</i>	Caenorhabditis Genetics Center	WB Strain: MT17463
<i>C. elegans</i> : Strain RB995: <i>hpl-2(ok916)</i>	Caenorhabditis Genetics Center	WB Strain: RB995
<i>C. elegans</i> : Strain YY453: <i>nrde-4(gg129)</i>	Caenorhabditis Genetics Center	WB Strain: YY453
<i>C. elegans</i> : Strain CB4108: <i>fog-2(q71)</i>	Caenorhabditis Genetics Center	WB Strain: CB4108
<i>C. elegans</i> : Strain YY453: <i>nrde-4(gg129)</i>	Caenorhabditis Genetics Center	WB Strain: YY453
<i>C. elegans</i> : Strain <i>hrde-1(tm1200)</i>	National Bioresource Project for the Experimental Organism "C. elegans"	WB Strain: WBVar00250211
<i>C. elegans</i> : Strain CQ605 (<i>prg-1(n4357)</i> ; <i>ksls2</i> [<i>Pdaf-7::GFP</i> + <i>rol-6(su1006)</i>]) (SX922 with FK181)	This study	CQ605
Oligonucleotides		
<i>prg-1</i> Forward sequencing primer	This study	
GCCAGCGTACGCAAGTTCAC		
<i>prg-1</i> Reverse sequencing primer	This study	
AGCATCCAGTACCAACGTCG		
Recombinant DNA		
Plasmid: pL4440 RNAi control	Ahringer Library	
Plasmid: pL4440- <i>daf-7</i> RNAi	Ahringer Library	
Plasmid: pL4440- <i>wdr-5</i> . RNAi	Ahringer Library	
Plasmid: pL4440- <i>set-2</i> RNAi	Ahringer Library	
Plasmid: pL4440- <i>set-32</i> RNAi	Ahringer Library	
Plasmid: pL4440- <i>hrde-1</i> RNAi	Ahringer Library	
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
FASTQC	Babraham Bioinformatics	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Cutadapt v1.6	Martin 2011	https://cutadapt.readthedocs.io/en/stable/#
STAR	Dobin et al., 2013	https://github.com/alexdobin/STAR
g:Profiler	Reimand et al., 2016	https://biit.cs.ut.ee/gprofiler/
REVIGO	Supek et al., 2011	http://revigo.irb.hr

CONTACT FOR REAGENT AND RESOURCE SHARING

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).

EXPERIMENTAL MODEL AND SUBJECT DETAILS***C. elegans* and bacterial strains and cultivation**

Strains were provided by the CGC. SX922: *prg-1(n4357)*, RB1025: *set-2(ok952)*, ZR1: *rbr-2(tm1231)*, VC967: *set-32(ok1457)*, FK181: *ksls2* [*Pdaf-7::GFP* + *rol-6(su1006)*], MT9973: *crh-1(n3315)*, PY7505: *oyls84* [*gpa-4p::TU#813* + *gcy-27p::TU#814* + *gcy-27p::GFP* + *unc-122p::DsRed*], LC108: *uls69* [*pCFJ90* *myo-2p::mCherry* + *unc-119p::sid-1*], NL1820: *mut-7(pk720)*, MAH23: *rrf-1(p1417)*, MT17463: *set-25(n5021)*, RB995: *hpl-2(ok916)*, YY453: *nrde-4(gg129)*, CB4108: *fog-2(q71)*. Strains were provided by the National Bioresource *hrde-1(tm1200)*. Strain CQ605 (*prg-1(n4357)*; *ksls2* [*Pdaf-7::GFP* + *rol-6(su1006)*]) was made by mating SX922 with

FK181. OP50 and OP50-1 were provided by the CGC. *S. marcescens* (ATCC 274) was purchased from ATCC. PA14 and LasR (Siryaporn et al., 2015) were gifts from Z. Gitai. Worm strains were maintained at 15°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 CaCl₂, 1 mL/L 1M MgSO₄, and 25 mL/L 1M potassium phosphate buffer (pH 6.0) added to molten agar after autoclaving) on *E. coli* OP50 using standard methods.

METHOD DETAILS

Pathogen training

Eggs from young adult hermaphrodites were obtained by bleaching (alkaline-bleach solution (e.g., 5.5 mL water, 1.5 mL 5N KOH, 3.0 mL sodium hypochlorite), followed by repeated washing of collected eggs in M9 buffer (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 5 g/L NaCl and 1 mL/L 1M MgSO₄ in distilled water), and were placed on to High Growth (HG) plates, and raised at 20°C for 2 days. Training plates were prepared by inoculating overnight cultures of OP50, PA14, or *S. marcescens* in LB at 37°C. Overnight cultures were diluted in LB to an Optical Density (OD₆₀₀) = 1 and used to seed Nematode Growth Media (NGM) (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. Plates were incubated at 25°C in separate incubators for 2 days. For 15°C experiments, PA14 was prepared by centrifuging 5 mL overnight cultures for 10 minutes at 5000 rpm. The supernatant was removed, and the remaining pellet was resuspended in 5 mL of fresh LB. Washed bacteria were used to inoculate (1:500) fresh LB to grow at 15°C for 2 days. Cultures were diluted in LB to an OD₆₀₀ = 1 and used to seed NGM plates. Plates were incubated at 15°C for 2 days. On day of training (i.e., 2 days post bleaching), all training plates were left to cool on a bench top for < 1 hr. 10 µL of pooled L4 worms were plated onto OP50 seeded training plates, while 40 µL of worms were plated onto pathogen seeded training plates. Worms were incubated on training plates at 20°C in separate containers for 24h. After 24h, worms were washed off plates in M9 3x. Some worms were used for an aversive learning assay, while the majority of worms were bleached and eggs were placed onto HG plates at 20°C for 3 days. For experiments involving RNAi the standard HGM agar was supplemented with 1 mL/L 1M IPTG (isopropyl β-D-1-thiogalactopyranoside) and 1 mL/L 100 mg/mL carbenicillin; animals were trained using pL4440 empty vector as control RNAi in HT115 bacteria.

Aversive learning assay

Overnight bacterial cultures were diluted in LB to an Optical Density (OD₆₀₀) = 1, and 25 µL of each bacterial suspension was seeded on a 60 mm NGM plate and incubated at 25°C for 2 days or 15°C for 2 days. After two days, assay plates were left at room temperature for 1 hr before use. Immediately before use, 1 µL of 1M sodium azide was spotted onto each bacterial lawn 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, 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. Assays were incubated at room temperature for 1 hr before counting the number of worms on each lawn.

In experiments in which F1 and subsequent generations are used: All animals tested are washed off HG plates with M9 at Day 1. Some of the pooled animals are subjected to an aversive learning assay, while the majority of worms are bleached onto HG plates left at 20°C for 3 days and used to test F2s.

Odor + 2-aminoacetophenone (2AA) training

3 mL of fresh overnight bacterial cultures (OD₆₀₀ OP50 = 1.4-1.7, OD₆₀₀ PA14 = 3.3-3.5), water, or 2AA (catalog no. A38207, Sigma-Aldrich) (1 mM, diluted in water) were placed into 2 lids of a 35 mm Petri dish, respectively, which was then placed in the lid of an inverted 10 cm NGM Petri dish that had been prepared as described for pathogen training. Odor training assays were left in the dark at room temperature for 24h. All training conditions were maintained in separate containers.

L1 imprinting

Eggs from young hermaphrodites were obtained by bleaching and placed directly onto OP50 or pathogen prepared training plates (5 µL of eggs were placed onto OP50 training plates, and 20 µL of eggs were placed onto PA14 training plates). Plates were incubated at 20°C for 24h. After 24h, worms were washed off training plates using M9 + 50 mg/mL streptomycin. Worms were washed 2 times and plated onto HGM+300 mg/mL streptomycin plates seeded with OP50-1 (streptomycin resistant OP50). Worms were left to mature to Day 1 adults and used in an aversive learning assay. Subsequent generations were prepared by bleaching pooled animals onto HG plates.

PA14 survival assay

OP50 and PA14 were grown in liquid culture and diluted as described above. For full lawn assays. 750 µL of diluted OP50 or PA14 was spread to completely cover a 10 cm NGM plate. For PA14 spot assays, 100 µL of diluted PA14 was placed in the center of a 10 cm NGM plate, resulting in a 2 cm spot in the center of the plate. Plates were incubated for 2 days at 25°C or 15°C to allow bacterial growth. Plates were equilibrated to 20°C before the addition of Day 1 worms to plates. Assays were performed at 20°C.

PA14-lawn assays were counted every 6-8 h. PA14-spot assays were counted every 24h. Every 48h, worms in both assays were moved onto new plates. For spot assays, animals were transferred to similar locations on new plates.

fog-2 mating assay

L4 males or females were picked onto PA14 or OP50 training plates, prepared as described above. 24 h after training, males and females were washed 2 times using M9 + 300 ug/mL streptomycin. Worms were then pipetted (in a ratio of 2:1 males to females) onto NGM + 300 ug/mL streptomycin plates, seeded the day before with 300 μ L of OP50-1 (streptomycin resistant) bacteria in the following combinations: OP50 males x OP50 females, OP50 males x PA14 females, PA14 males x PA14 females, PA14 males x OP50 females. Worms were left to mate for 24 h at 20°C. Following mating, worms were bleached and eggs were transferred onto OP50-seeded HG plates. Early Day 1 animals were tested in an aversive learning assay as previously described. Females and males were counted separately.

RNA Interference

RNAi experiments were conducted using the standard feeding RNAi method. Bacterial clones expressing the control (empty vector, pL4440) construct and the dsRNA targeting *C. elegans* genes were obtained from the Ahringer RNAi library. All RNAi clones were sequenced prior to use. RNAi-induced knockdown was conducted by bleaching progeny of HT115 pL4440 or PA14 trained animals onto RNAi seeded plates.

Antibodies

The primary H3K4me3 antibody (8580), and the primary H3K9me3 antibody (8898) were obtained from Abcam. The secondary antibody (A-11008) was obtained from Invitrogen.

Whole-mount immunofluorescence

Worms were dissected on positively-charged glass slides in 8 μ L of 0.5 mM levamisole in M9 to paralyze worms followed by freeze-cracking on dry ice for 20 minutes. The worms were fixed by washing slides in cold methanol for 10 minutes followed by 5 minutes in acetone. The slides were then washed three times in PBST (PBS pH 7.4, 0.5% Triton X-100, 1mM EDTA) for 10 minutes. Following application of antibody, worms were covered with a 20 x 20-mm piece of parafilm and then incubated overnight at room temperature in a humidified-dark chamber. Primary antibodies used were H3K4me3 (Abcam 8580) or H3K9me3 (Abcam 8898) at 1:200 dilution. The next day slides were washed three times in PBST for 10 minutes. Secondary antibodies were added at 1:500 dilution (A-11008) and incubated overnight as before. The next day slides were again washed in PBST. Then 8 μ L of vectasheild with DAPI was added to each slide and the coverslip was sealed with fingernail polish. Slides were visualized by Z stack multi-channel (DIC, GFP) every 1 mm at 60X magnification. Images taken after whole-mount immunofluorescence are unable to be quantified because each condition analyzed must be dissected and stained on different slides, and staining varies slide to slide.

Imaging and fluorescence quantitation

Z stack multi-channel (DIC, GFP) of day 1 adult GFP transgenic worms were imaged every 1 μ m at 60X magnification; Maximum Intensity Projections and 3D reconstructions of head neurons, or germlines were built with Nikon *NIS-Elements*. To quantify *daf-7p*::GFP levels, worms were prepared and treated as described for pathogen training. Worms were mounted on agar pads and immobilized using 1 mM levamisole. GFP was imaged at 60X 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 and/or 2 ASJ head neurons.

RNA isolation

Adult worms (P0) and eggs (F1) were collected in M9 and washed several times to remove excess bacteria. Worm pellets were crushed in liquid nitrogen and transferred to an appropriate volume of Trizol LS. Total RNA was extracted from Trizol using the mirVana miRNA isolation kit (ThermoFisher). mRNA libraries for directional RNA sequencing were prepared using the SMARTer Apollo System and were sequenced (180-nt single-end) on the Illumina HiSeq 2000 platform. RNA samples for small RNA-seq were treated with 5'polyphosphatase (Lucigen) and prepared using the SMARTer Apollo system with modifications for small RNA library preparation. Briefly, sample fragmentation and initial bead size selection steps were omitted, and small RNA-containing libraries were Blue Pippin size selected (21-26nt insert size) prior to 75-nt single-end sequencing.

RNA-seq data analysis

FASTQC was used to assess read quality scores. Universal adaptor sequences were trimmed from small RNA library sequences using Cutadapt v1.6 (Martin, 2011). Reads were mapped to the *C. elegans* genome (UCSC Feb 2013, ce11/ws245) using STAR (Dobin et al., 2013). Count matrices were generated using htseq-counts (mode: union). DESeq2 was used for differential expression analysis. Genes at FDR < 0.5 were considered significantly differentially expressed. Regularized log transformations were applied to the data to generate heatmaps. Sequences are deposited at NCBI BioProject PRJNA509938.

Gene Ontology analysis

g:Profiler (Reimand et al., 2016) was used to identify significantly enriched gene ontology (GO) terms from upregulated or downregulated gene lists (DESeq2 genes FDR < 1%). Biological Process GO terms were plotted using REVIGO (Supek et al., 2011). The size of the circle in each GO term plot reflects the overall size of the GO term category such that more general GO terms appear larger, while more specific GO terms are smaller. The color of the circle represents the significance of the GO term in the dataset.

QUANTIFICATION AND STATISTICAL ANALYSIS

Lifespan assays were assessed using Kaplan-Meier log rank tests. For the comparison of choice indices between two genotypes (i.e., *crh-1(n3315)* versus *wildtype*), one-way ANOVA with Tukey's multiple comparison test were used. For the comparison of choice indices between two generations in which only one genotype was tested (i.e., *wildtype* only), unpaired t tests were performed. For the comparison of learning indices between generations (i.e., in *wildtype* only), one-way ANOVA with Tukey's multiple comparison test were used. For quantification of neuron intensity one-way ANOVA with Tukey's multiple comparison test were used. Experiments were repeated on separate days with separate populations, to confirm that results were reproducible. Prism 8 software was used for all statistical analyses; software and further statistical details used for RNA-seq analyses are described in the [Method Details](#) section of the [STAR Methods](#). Additional, statistical details of experiments, including sample size (with n representing the number of chemotaxis assays performed for behavior, number of individual neurons imaged, and number of worm used in survival assays) can be found in the figure legends.

DATA AND SOFTWARE AVAILABILITY

Raw RNA-seq datasets are publically available through NCBI BioProject PRJNA509938 (<https://www.ncbi.nlm.nih.gov/bioproject>)

Supplemental Figures

Cell

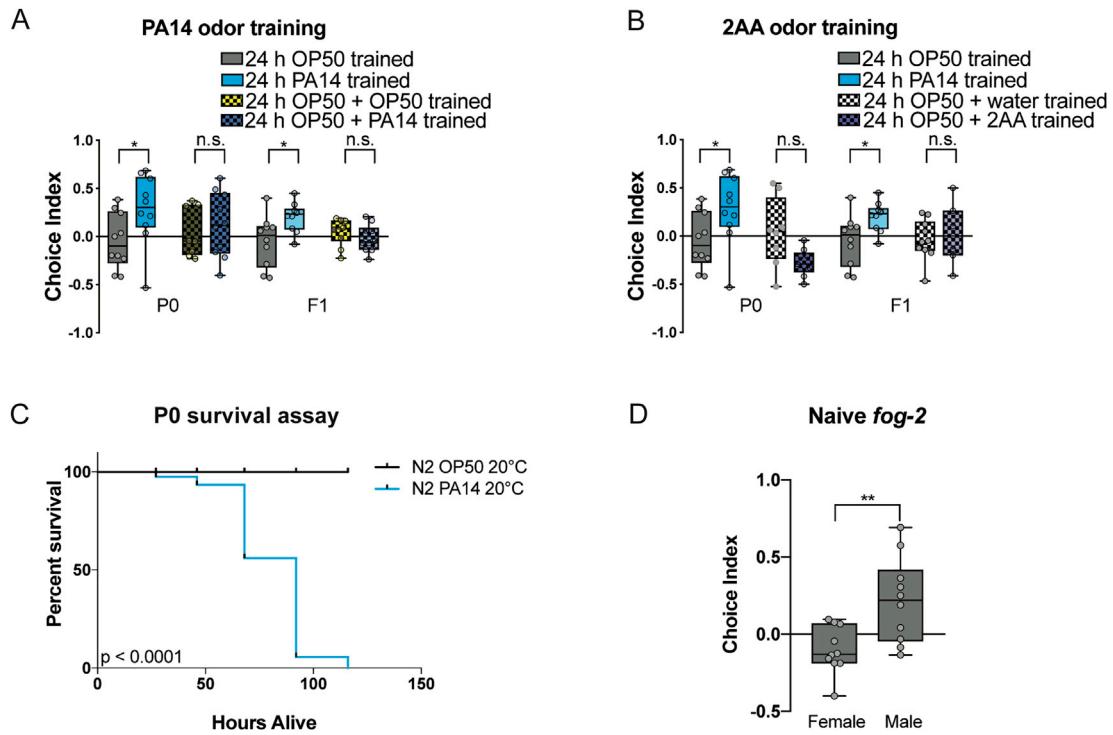


Figure S1. Transgenerational Inheritance of Pathogen Avoidance Requires Direct Contact with PA14, Related to Figure 1

(A) PA14 odor is not sufficient to induce aversive pathogenic learning. (B) 2-aminoacetophenone (2AA) is not sufficient to induce aversive pathogenic learning. One-Way ANOVA, Tukey's multiple comparison test, mean \pm SEM n \geq 6-10 choice assay plates with 50-200 worms. * $p \leq 0.05$, ** $p \leq 0.01$, ns = not significant. At least 2 biological replicates were performed for all aversive learning assays. (C) PA14 is lethal at 20°C. $p < 0.0001$, Log-rank (Mantel-Cox) test. n \geq 78-81 worms per condition. (D) Adult male worms naively avoid PA14. One-Way ANOVA, Tukey's multiple comparison test, mean \pm SEM n \geq 6-10 choice assay plates with 50-200 worms. * $p \leq 0.05$, ** $p \leq 0.01$, ns = not significant.

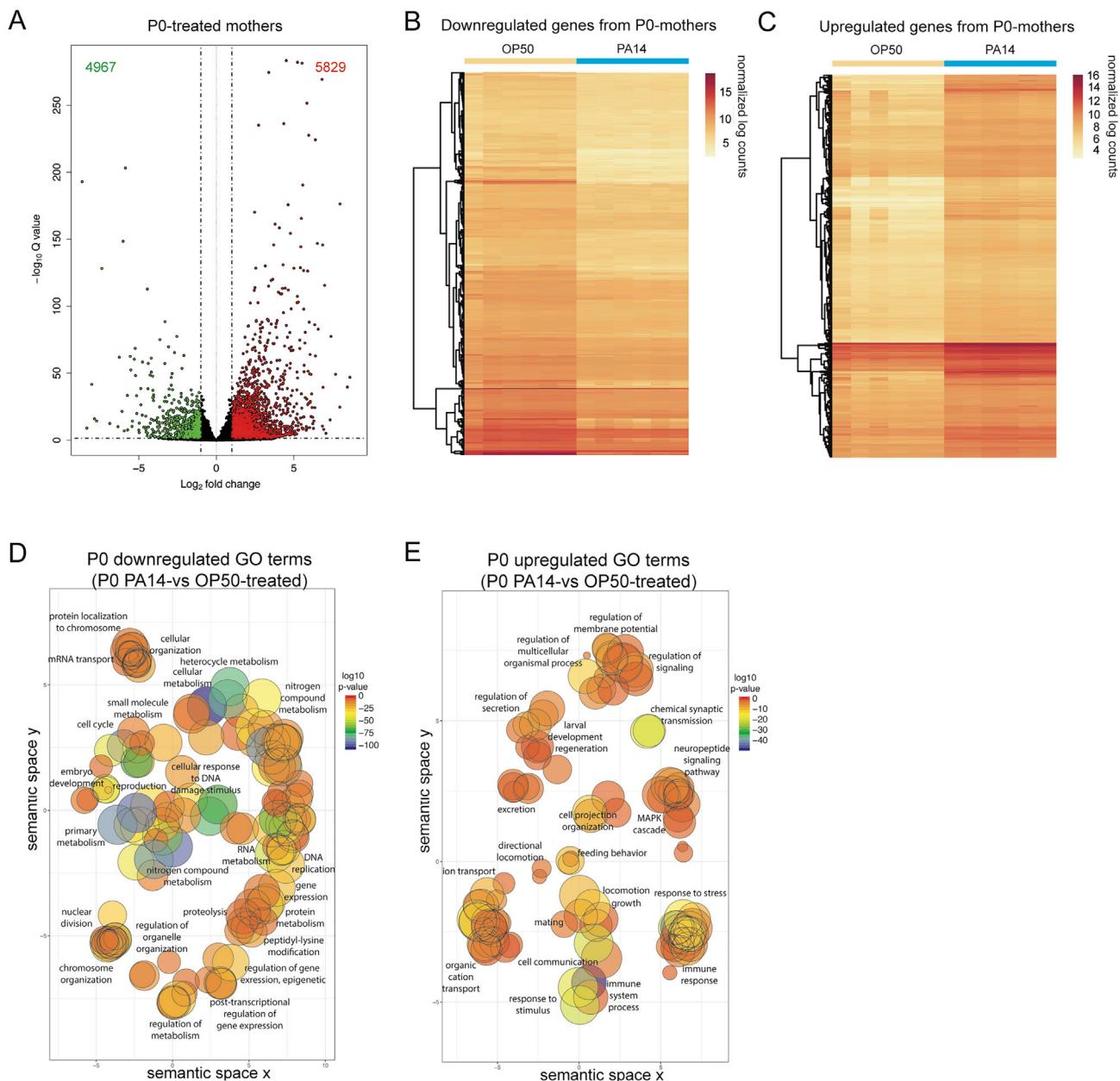
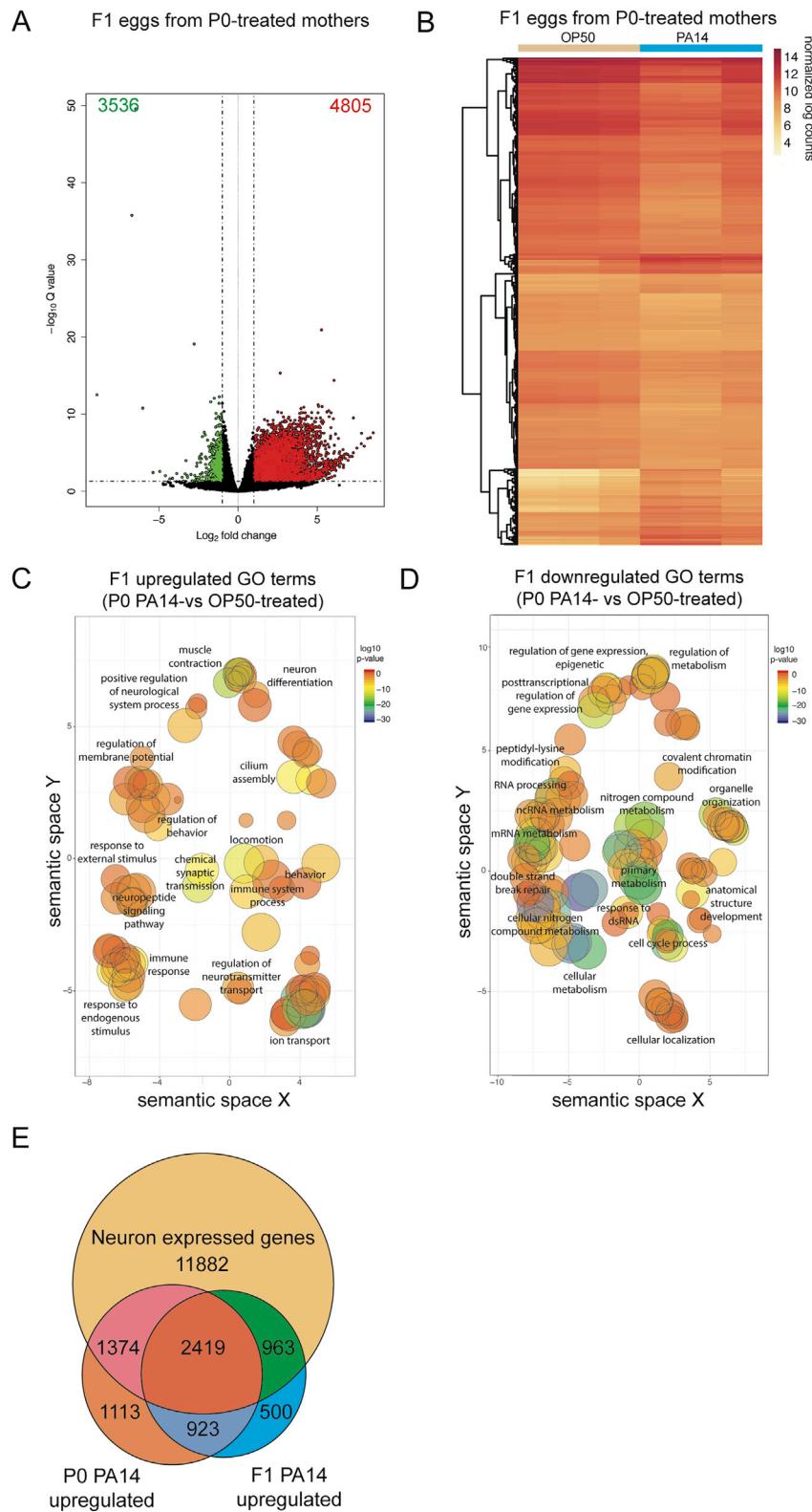


Figure S2. Analysis of Differentially Expressed Genes from P0 Animals Treated with PA14 versus OP50, Related to Figure 1

(A-C) mRNAs differentially regulated in P0 animals after PA14 treatment. (A) Volcano plot and (B,C) heatmaps of PA14-upregulated (5829, red) and downregulated (4967, green). (A) Volcano plot of mRNAs shown at FDR < 5% and \log_2 fold change > and < 1. (B, C) Heatmaps of normalized log expression counts are shown for genes with FDR < 1% and base mean expression > 500. PA14-downregulated (D) and upregulated (E) genes (FDR < 1%) were analyzed for enriched gene ontology (GO) terms using g:Profiler, and significant GO terms were plotted using REVIGO.



(legend on next page)

Figure S3. Analysis of Differentially Expressed Genes from F1 Animals Treated with PA14 versus OP50, Related to Figure 2

(A) F1 eggs were collected from PA14- or OP50-treated mothers. PA14-upregulated (4805, red) and downregulated (3536, green) mRNAs shown at FDR < 5% and \log_2 fold change > and < 1. (B) Heatmap of differentially expressed genes from F1 animals from OP50- or PA14-treated mothers. Normalized log expression counts are shown for all genes with FDR < 1% and base mean expression > 500. (C) PA14-upregulated and (D) downregulated genes (FDR < 1%) were analyzed for enriched gene ontology (GO) terms using g:Profiler, and significant GO terms were plotted using REVIGO. (E) PA14-upregulated genes in P0 and F1 animals (FDR < 5%) were examined for adult neuron expression (Kaletsky et al., 2016).

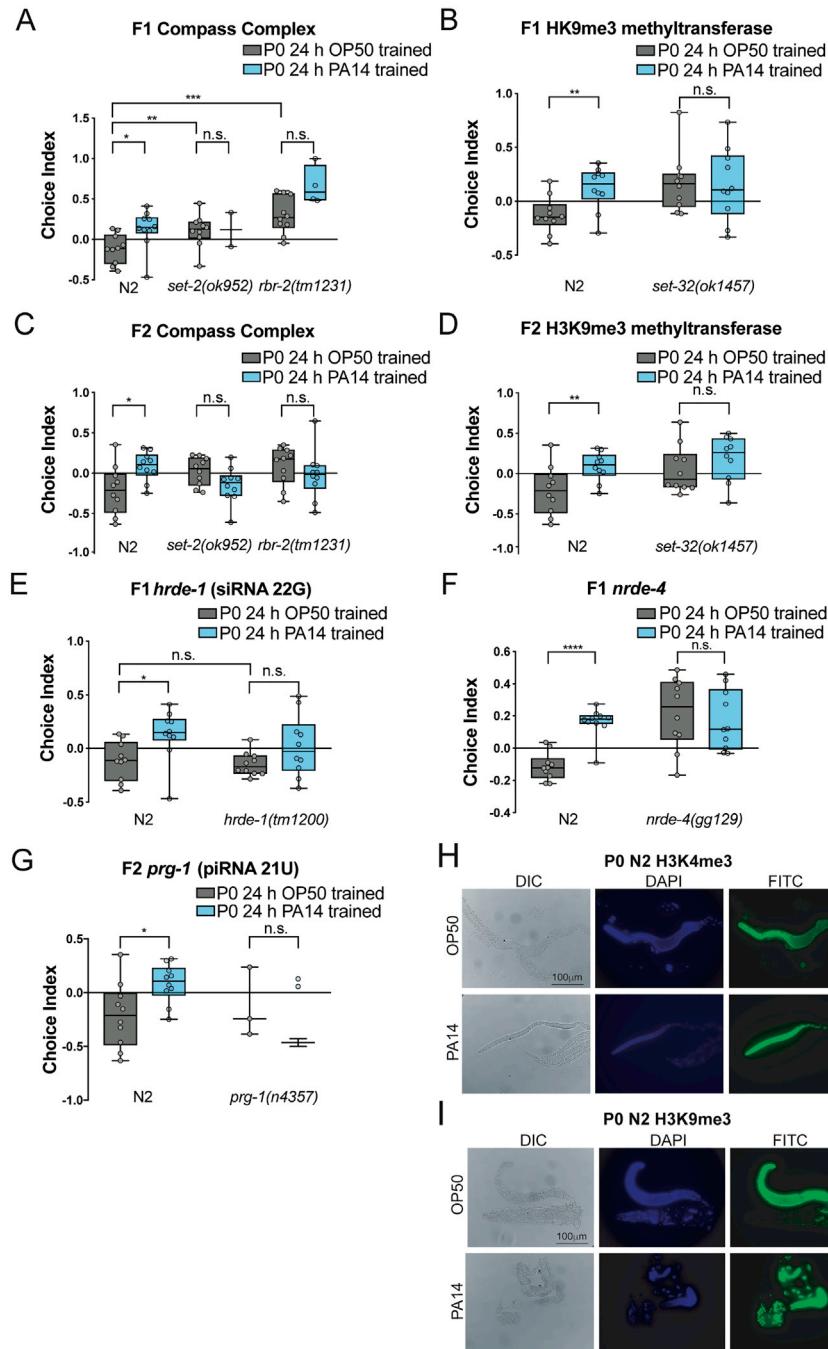


Figure S4. Mutants that Do Not Learn Avoidance Behavior Exhibit Similar Behavior in F1 and F2 following P0 Training, Related to Figure 4

(A) F1 progeny of PA14-trained *set-2(ok952)*, *rbr-2(tm1231)*, and (B) *set-32(ok1457)* are defective in pathogenic aversive learning. (C) F2 progeny of PA14-trained *set-2*, *rbr-2*, and (D) *set-32* are defective in pathogenic aversive learning. (E) F1 progeny of PA14-trained *hrde-1(tm1200)* are defective in pathogenic aversive learning. (F) F1 progeny of PA14-trained *nrde-4(gg129)* have high naive preference and are defective in pathogenic aversive learning. (G) F2 progeny from PA14-trained *prg-1* grandmothers are defective in pathogenic aversive learning. One-Way ANOVA, Tukey's multiple comparison test, mean \pm SEM n \geq 6-10 choice assay plates with 50-200 worms per plate. *p \leq 0.05, **p \leq 0.01, ns = not significant. At least 2 biological replicates were performed for all aversive learning assays. Global H3K4me3 (H) and H3K9me3 (I) levels remain unchanged after exposure to PA14.

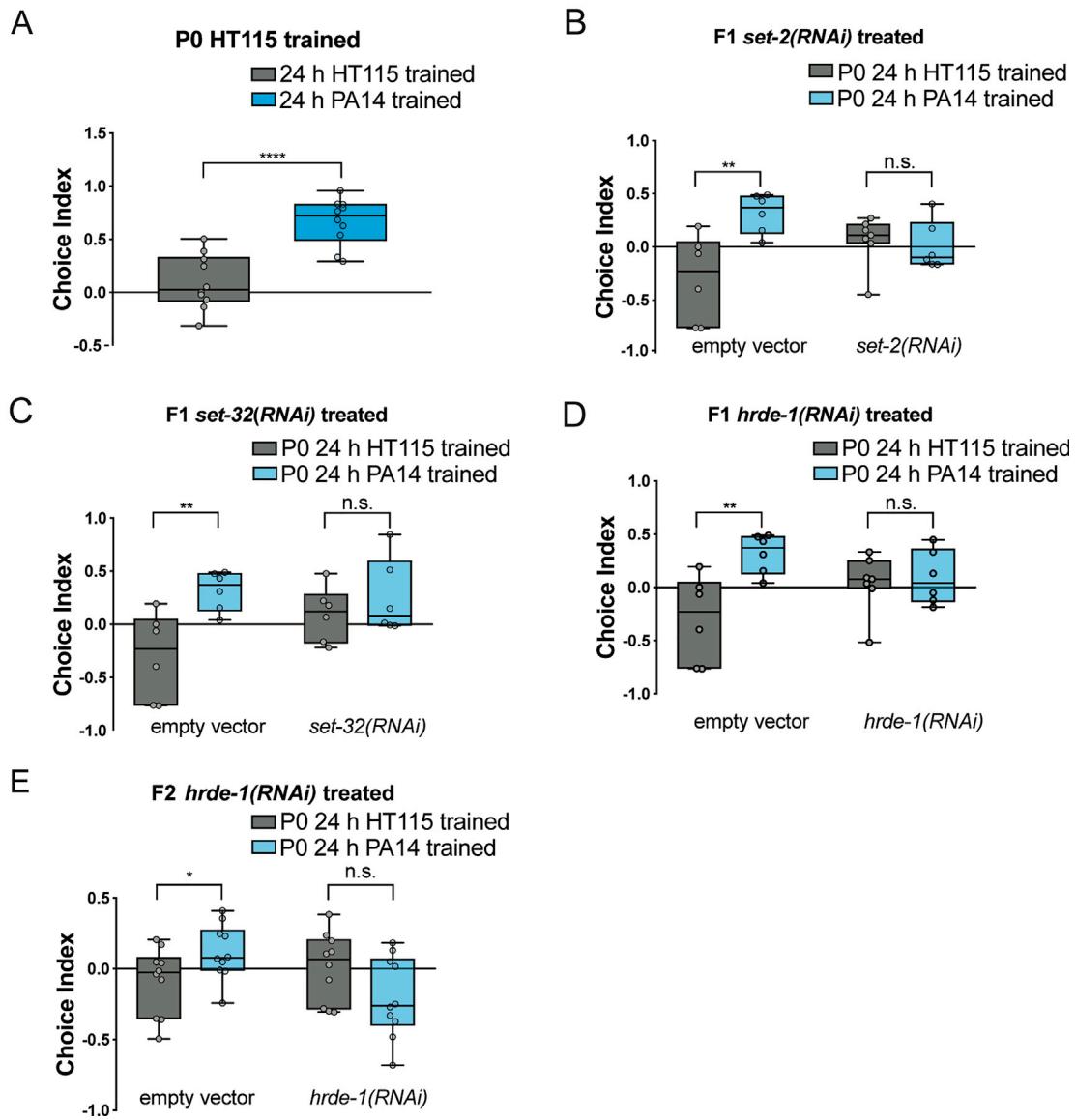


Figure S5. RNAi-Treated Progeny of PA14-Trained Mothers Still Exhibit Aversive Learning Defects in the F1 and F2 Generations, Related to Figure 4

(A) 24 h of PA14 training in wild-type animals results in aversive learning compared to 24h of training with *E. coli* HT115. F1 progeny of wild-type PA14-trained mothers treated with *set-2(RNAi)* (B), *set-32(RNAi)* (C), *hrde-1(RNAi)* (D) were defective in pathogenic learning. (E) F2 *hrde-1(RNAi)* of PA14-trained mothers are defective in pathogenic learning. One-Way ANOVA, Tukey's multiple comparison test, mean \pm SEM n = 10 choice assay plates with 50-200 worms per plate. *p \leq 0.05, **p \leq 0.01, ***p \leq 0.001, ****p < 0.0001, ns = not significant. At least 2 biological replicates were performed for all aversive learning assays.

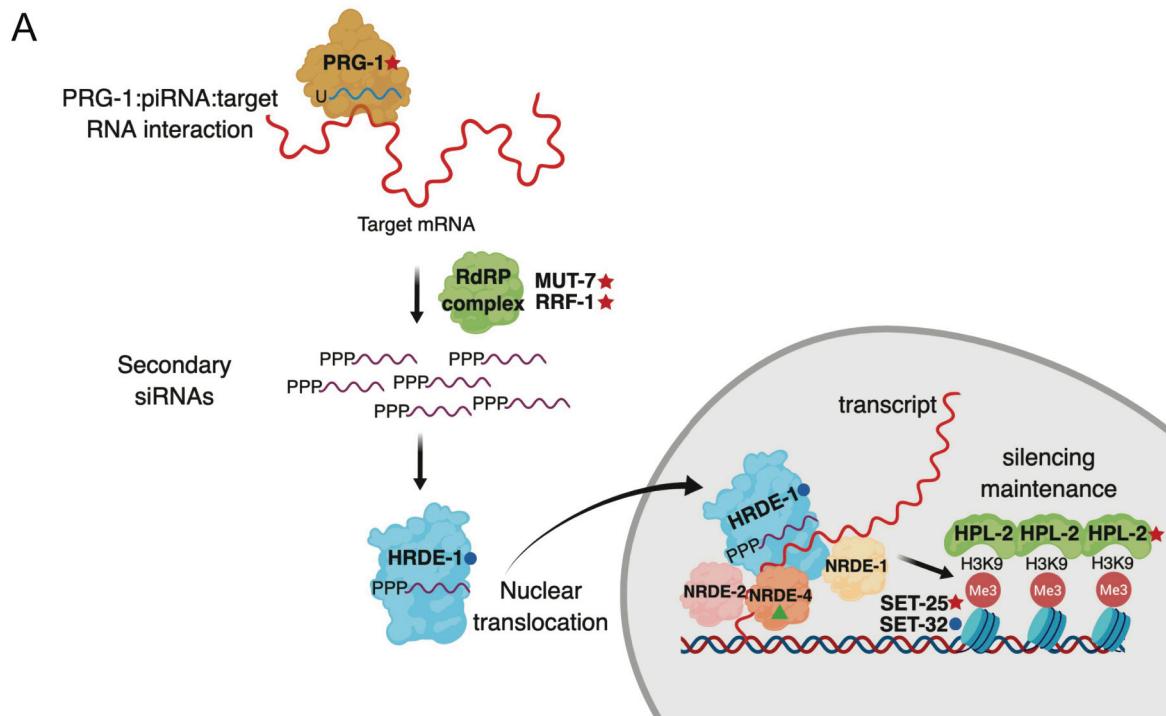


Figure S6. Molecular Model of Known TEI Components, Related to Figure 5

Summary of genes implicated in naive preference, learning, and TEI avoidance based upon previous descriptions of protein function.