C. elegans interprets bacterial non-coding RNAs to learn pathogenic avoidance

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Rachel Kaletsky[1,2,3], Rebecca S. Moore[1,3], Geoffrey D. Vrla[1], Lance R. Parsons[2], Zemer Gitai[1] & Coleen T. Murphy[1,2]✉

Caenorhabditis elegans must distinguish pathogens from nutritious food sources among the many bacteria to which it is exposed in its environment[1]. Here we show that a single exposure to purified small RNAs isolated from pathogenic *Pseudomonas aeruginosa* (PA14) is sufficient to induce pathogen avoidance in the treated worms and in four subsequent generations of progeny. The RNA interference (RNAi) and PIWI-interacting RNA (piRNA) pathways, the germline and the ASI neuron are all required for avoidance behaviour induced by bacterial small RNAs, and for the transgenerational inheritance of this behaviour. A single *P. aeruginosa* non-coding RNA, P11, is both necessary and sufficient to convey learned avoidance of PA14, and its *C. elegans* target, *maco-1*, is required for avoidance. Our results suggest that this non-coding-RNA-dependent mechanism evolved to survey the microbial environment of the worm, use this information to make appropriate behavioural decisions and pass this information on to its progeny.

The natural habitat of *C. elegans* contains many bacterial species; about a third of these are in the genus *Pseudomonas*, and can be beneficial or detrimental[1]. Despite their natural attraction to pathogenic *P. aeruginosa* (PA14), *C. elegans* learns to avoid this pathogen after becoming ill[2]. It was previously discovered[3] that worms pass this learned avoidance of PA14 to their progeny, but the nature of the signal that conveys the identity of the pathogen was unknown.

sRNAs from PA14 induce avoidance

To identify this signal, we trained worms for 24 h on a non-pathogenic *Escherichia coli* strain, OP50, spiked with components isolated from pathogenic PA14 or OP50, and then tested avoidance behaviour using a standard OP50 versus PA14 bacteria lawn-choice assay (Fig. 1a). Although bacterial metabolites can alter worm behaviour[4], training worms with PA14 supernatant did not induce avoidance learning (Fig. 1b). Next, we tested nucleic acid components (total DNA, total RNA, large RNA (>200 nt), small RNA (<200 nt) and RNase- and DNase-treated fractions) from pathogenic (25 °C, plate-grown) PA14, adding the purified samples to lawns of OP50 for 24 h (Fig. 1a, c, d, Extended Data Fig. 1a, b). Total RNA, small RNA (sRNA) and DNase-treated sRNA samples from PA14 (Fig. 1c, d) induced avoidance of PA14, whereas DNA and large RNA samples had no effect at the concentrations we tested (Fig. 1b, Extended Data Fig. 1a–c).

Worms trained on heat-killed OP50 bacteria supplemented with purified PA14 sRNAs also learned avoidance (Fig. 1e). sRNAs isolated from the less-virulent *ΔlasR* mutant of PA14 did not induce PA14 avoidance (Extended Data Fig. 1d). Together, these results suggest that sRNAs that are present in pathogenic bacteria—rather than changes to bacterial metabolism once inside the worm—are responsible for learned pathogenic avoidance.

sRNA-induced avoidance is species-specific

Exposure to *Serratia marcescens* also induces avoidance in *C. elegans*[2]; however, this avoidance is not passed on to the next generation[3], and PA14-induced transgenerational avoidance is species-specific[3]. Treatment with sRNA isolated from *S. marcescens* does not induce avoidance of *S. marcescens* or PA14 (Fig. 1f, g), and sRNA from PA14 does not induce avoidance of *S. marcescens* (Fig. 1f). Therefore, similar to the transgenerational inheritance of avoidance, sRNA-induced avoidance is a response that is induced by specific bacteria.

sRNA avoidance is immunity-independent

The fact that purified sRNAs are sufficient to induce avoidance of *P. aeruginosa* in the absence of intact pathogen suggests that sRNA-induced avoidance does not require virulence. Worms treated with sRNAs are healthy and their offspring develop normally (Fig. 1h, Extended Data Fig. 1e). Moreover, the innate immunity regulator *pmk-1* (ref. [5]) is not required for avoidance (Extended Data Fig. 1f, g), consistent with the dispensability of *pmk-1* for aversive behaviour[6,7]. Additionally, the *pmk-1*-independent innate immune response (*irg-1p::gfp*)[8] is not induced by sRNA from PA14 (Extended Data Fig. 1h, i). Thus, mothers learn avoidance through the innate immune response (lawn exposure and/or metabolites)[9], which causes half of the avoidance behaviour exhibited by lawn-trained worms (Fig. 1i, j, Extended Data Fig. 1j). In a separate mechanism that we identify here, pathogen-derived bacterial sRNA induces the other half of the learned avoidance behaviour of the mother (Fig. 1i,j, Extended Data Fig. 1j).

PA14 sRNA induces *daf-7* in ASI neurons

The TGF-β ligand DAF-7 is induced in ASI and ASJ neurons when exposed to PA14[4] (Extended Data Fig. 1k), and *daf-7* expression

[1]Department of Molecular Biology, Princeton University, Princeton, NJ, USA. [2]LSI Genomics, Princeton University, Princeton, NJ, USA. [3]These authors contributed equally: Rachel Kaletsky, Rebecca S. Moore. ✉e-mail: ctmurphy@princeton.edu

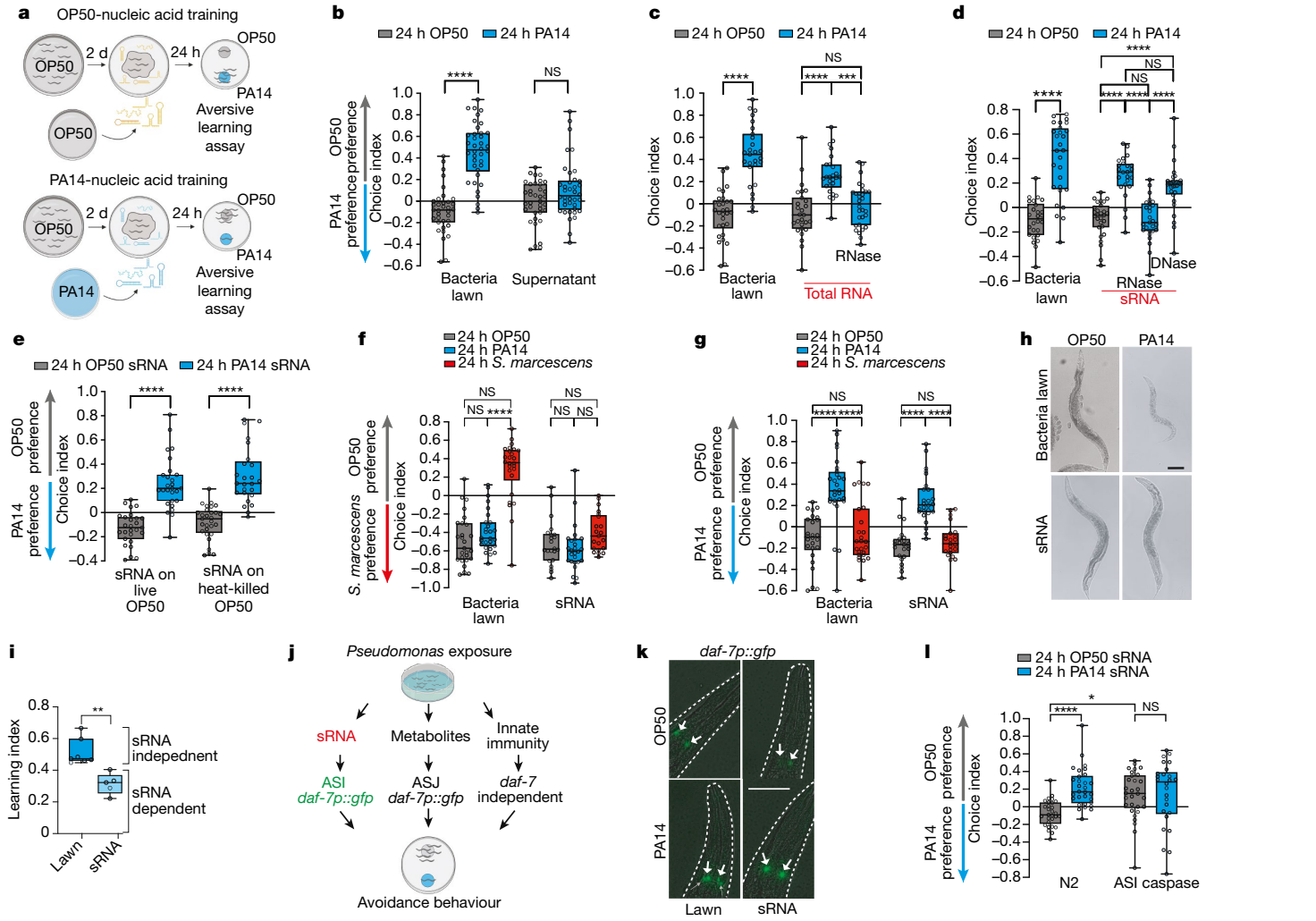

Fig. 1 | sRNA of PA14 is sufficient to induce *C. elegans* pathogen avoidance. **a**, Worms were trained on non-pathogenic OP50, PA14 lawns, or OP50 lawns spiked with bacterial components. Choice assays to OP50 versus PA14 bacteria were then performed. Choice index = (number of worms on OP50 − number of worms on PA14)/(total number of worms). **b**, Worms exposed to a PA14 lawn (for 24 h) learn to avoid PA14, but PA14 supernatant does not elicit PA14 avoidance. **c**, Training with purified PA14 total RNA confers PA14 avoidance; RNase treatment abolishes this effect. **d**, Purified PA14 sRNA (<200 nt) induce PA14 avoidance; RNase treatment abolishes this effect, but DNase treatment does not. **e**, Heat-killing bacteria does not abolish sRNA learning. **f**, Trained worms were tested in an OP50 versus *S. marcescens* choice assay. The *S. marcescens* lawn training induces avoidance of *S. marcescens*, but exposure to *S. marcescens* sRNA does not. PA14 sRNA training does not affect OP50 versus *S. marcescens* preference. **g**, PA14 lawn and PA14 sRNA training induce PA14 avoidance, whereas *S. marcescens* bacteria and sRNA training do not affect PA14 preference. **h**, Unlike PA14, sRNA exposure does not cause illness. Scale bar, 100 µm. **i**, P_0 learning index. sRNA-induced learning is about half of that of PA14 lawns. Learning index = average PA14 choice index − average OP50 choice index. Each data point represents the learning index from an independent experiment containing about 7–10 choice assay plates with an average of 115 worms per plate. Unpaired, two-tailed Student's *t*-test. **j**, Worms learn to avoid PA14 through several independent mechanisms. **k**, *daf-7p::gfp* expression in ASI neurons increases upon PA14 lawn or sRNA exposure (white arrows); expression in ASJ neurons is induced only in PA14 lawn-trained worms (grey arrows). Scale bar, 50 µm. **l**, Genetic ablation of ASI neurons abolishes PA14 sRNA-induced learning. Each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. Biological replicates: 3 (**c**–**h**, **k**, **m**), 4 (**b**) or 6 (**i**). Box plots: centre line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. One-way (**c**, **d**) and two-way (**b**, **e**, **f**, **l**) analysis of variance (ANOVA), Tukey's multiple comparison test. $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{****}P < 0.0001$, NS, not significant. Estimation plots are provided in the Supplementary Information; see Supplementary Table 4 for exact sample sizes (*n*) and *P* values.

increases in the ASI neurons of the progeny of PA14-trained mothers[3]. *daf-7p::gfp*[3,4]—which exhibits fluorescence in ASJ neurons in response to bacterial metabolites[4] and fluorescence in ASI neurons in the progeny of PA14 lawn-trained mothers[3]—is induced by exposure to PA14 sRNA solely in the ASI neurons of trained mothers, resembling expression in the F_1–F_4 generations after PA14 lawn training[3] (Fig. 1k, Extended Data Fig. 1l). sRNA from *ΔlasR* PA14 did not increase levels of *daf-7* in ASI neurons (Extended Data Fig. 1m). Worms with genetically ablated ASI neurons exhibit high levels of naive avoidance of PA14, but exposure to sRNA does not induce further avoidance (Fig. 1l); this suggests that the ASI neuron is required for PA14 avoidance mediated by bacterial sRNA (Fig. 1k), as it is required in the F_1 generation for transgenerational inheritance of avoidance[3].

sRNA avoidance requires the RNAi pathway

We next wondered whether avoidance induced by bacterial sRNA requires the RNAi pathway[10]. Although neither the double-stranded (ds)RNA transporter SID-2[11] nor the dsRNA endoribonuclease Dicer

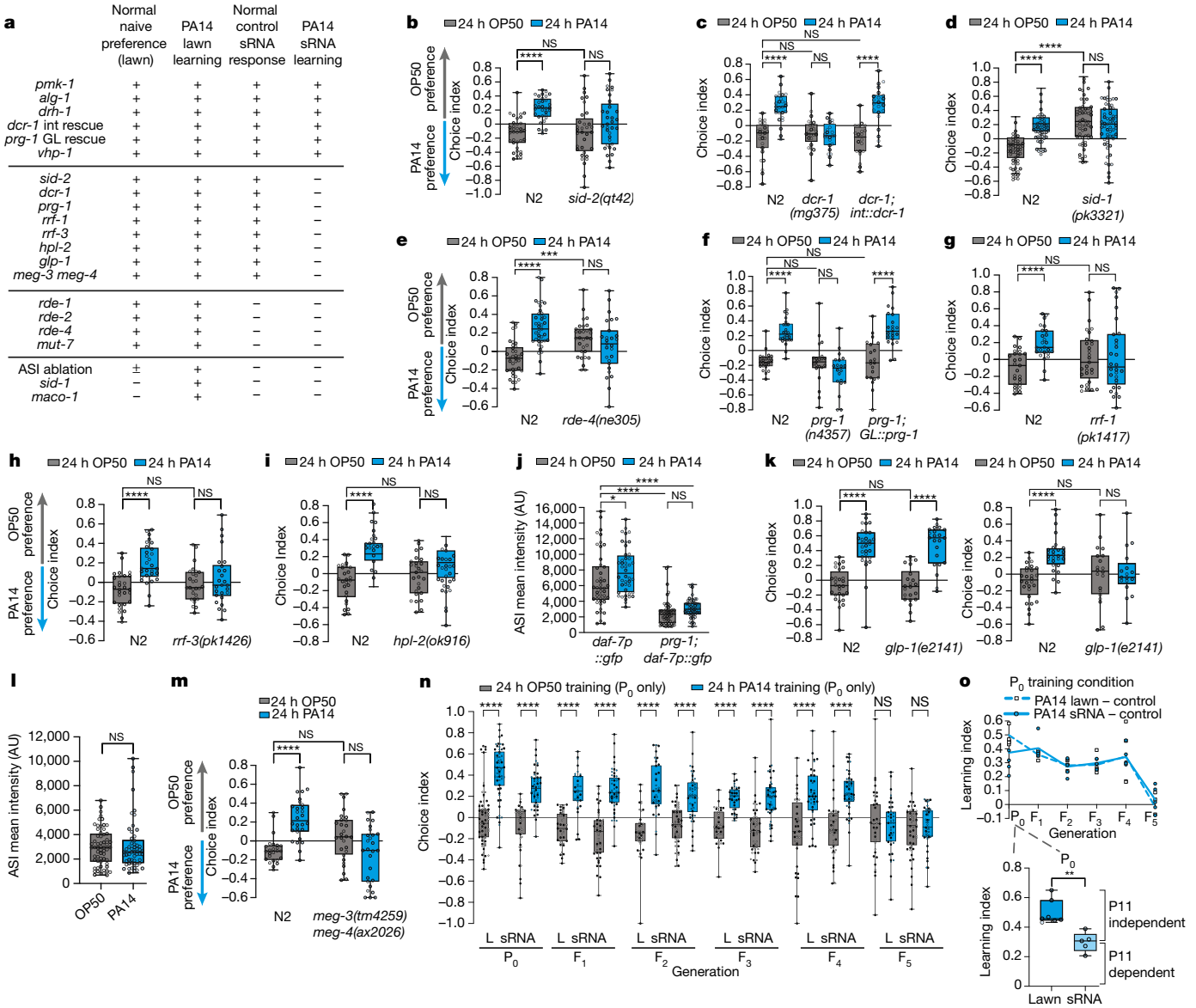

Fig. 2 | dsRNA transport, processing machinery, the PRG-1 piRNA pathway, and the germline are required for avoidance induced by bacterial sRNA. **a**, Summary of genes required for naive preference, PA14 lawn learning and sRNA learning. +, functions similarly to wild type; –, defective (required for) naive choice or learning; ±, statistically borderline between the two. Int, intestinal rescue; GL, germline rescue. **b**, dsRNA transporter *sid-2* is required for sRNA-induced learning. **c**, Intestinal rescue of *dcr-1* restores sRNA-mediated learning. **d**, **e**, *sid-1(pk3321)* dsRNA transporter (**d**) and *rde-4(ne305)* dsRNA binding protein (**e**) mutants exhibit an abnormal sRNA response. **f**, Germline *prg-1* rescue restores sRNA-induced learning. **g**–**i**, RNA-dependent RNA polymerase *rrf-1(pk1417)* (**g**), *rrf-3(pk1426)* (**h**), and heterochromatin protein *hpl-2(ok916)* (**i**) mutants are deficient in PA14 sRNA-induced avoidance. **j**, *prg-1(n4357)* mutants do not induce *daf-7p::gfp* expression in ASI neurons upon PA14 training ($n = 46, 44, 58$ and 55 neurons, from left to right). AU, arbitrary units. **k**, Germline-less *glp-1(e2141)* mutants learn to avoid PA14 after lawn training (left), but are defective in sRNA-induced learning (right). **l**, *glp-1* mutants do not induce *daf-7p::gfp* expression in ASI neurons upon PA14 exposure ($n = 60$ (OP50), 54 (PA14) neurons, two-sided Student's *t*-test). **m**, Germline P-granule mutants exhibit defective sRNA-induced learning. **n**, Untrained progeny of PA14 lawn (L) and sRNA-trained mothers avoid PA14 from generation F_1 to F_4; generation F_5 resumes PA14 attraction. **o**, Top, learning index for each generation, mean ± s.e.m. Bottom, P_0. Two-sided Student's *t*-test. Each data point represents the learning index from an independent experiment of about 7–10 choice assay plates; each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. **b**–**k**, **m**, Two-way ANOVA, Tukey's multiple comparison test. Biological replicates: 3 (**c**, **f**–**m**), 4 (**b**, **e**), 5 (sRNA in **n**), 6 (**d**, lawn in **n**). Box plots: centre line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{****}P < 0.0001$, NS, not significant. Estimation plots are provided in the Supplementary Information; see Supplementary Table 4 for exact sample sizes (*n*) and *P* values.

(DCR-1)[12,13] is required for the avoidance induced by lawn exposure (Extended Data Fig. 2a, b), avoidance induced by sRNA specifically requires SID-2 and DCR-1[12,13] (Fig. 2a–c, Extended Data Fig. 2c).

SID-2 is expressed in the intestine[11,14] and is required for learning induced by sRNA (Fig. 2b), whereas other components—including DCR-1—are expressed more broadly[15]. Intestinal rescue of *dcr-1* restored sRNA-mediated learning (Fig. 2a, c, Extended Data Fig. 2d), which suggests that the uptake and processing of bacterial sRNA by SID-2 and DCR-1, respectively, are initiated in the intestine.

Aberrant sRNA responses in RNAi mutants

Although DCR-1 and SID-2 are required for PA14 sRNA-mediated learning, we identified a class of RNAi mutants with aberrant PA14 and/or

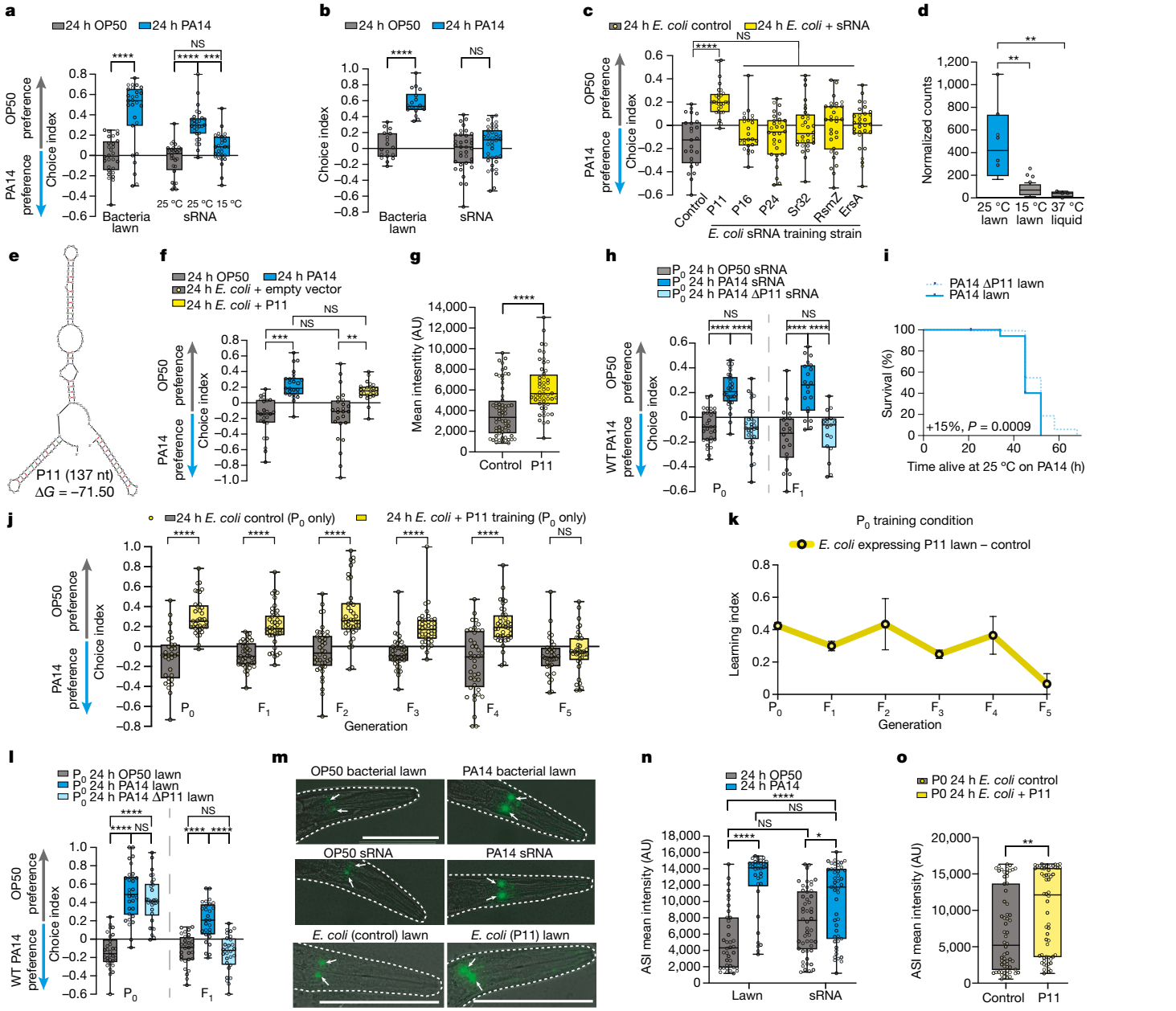

Fig. 3 | P11 is required and sufficient for learned avoidance behaviour. **a**, **b**, sRNAs isolated from 15-°C-grown (**a**) or liquid-grown PA14 (**b**) do not induce PA14 avoidance, whereas PA14 lawns and sRNA from 25-°C plate-grown PA14 do. **c**, PA14 preference after training on *E. coli* expressing PA14 sRNAs. **d**, DESeq2-normalized counts of P11 expression (25 °C versus 15 °C, adjusted $P = 0.006$, 6.8× fold change; 25 °C versus liquid, adjusted $P = 0.002$, 13.7× fold change; $n = 6$ (25 °C) or 4 (15 °C, liquid) biological replicates). **e**, Predicted secondary structure of P11 ($\Delta G = -71.50$)[43]. **f**, sRNAs isolated from PA14 (blue) or *E. coli*–P11 (yellow) induce PA14 avoidance. **g**, *E. coli*–P11 training induces *daf-7p::gfp* expression in ASI neurons ($n = 65$ (control) or 54 (P11) neurons, two-sided Student's *t*-test). **h**, PA14 ΔP11 sRNA does not induce PA14 avoidance in mothers (P_0, left) or their progeny (F_1, right). WT, wild type. **i**, Deletion of P11 reduces PA14 pathogenicity. log–rank (Mantel–Cox) test, $n = 120$ worms per condition. **j**, **k**, Mothers exposed to lawns of *E. coli*–P11 avoid PA14. Untrained progeny of P11-trained mothers avoid PA14 from generation F_1 to F_4; the F_5 generation resume PA14 attraction. The learning index for each generation (**k**), mean ± s.e.m. **l**, Mothers trained on PA14 ΔP11 bacteria avoid PA14 (left), but do not transmit avoidance (right). **m**–**o**, *daf-7p::gfp* expression in ASI neurons (white arrows) (**m**) remains elevated in F_1 progeny of PA14- and PA14-sRNA-trained (**n**) ($n = 38$, 34, 50 and 47 neurons, from left to right) and P11-trained (**o**) ($n = 62$ (control) or 58 (P11) neurons, two-sided Student's *t*-test) mothers. Scale bars, 100 µm. For all learning assays, each dot represents an individual choice assay plate (average of 115 worms per plate) from all replicates. Biological replicates: 2 (**i**), 3 (**a**, **c**, **d**, **f**–**h**, **l**–**o**), 4 (**b**, **j**, **k**). Box plots: centre line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{****}P < 0.0001$, NS, not significant. Estimation plots are provided in the Supplementary Information; see Supplementary Table 4 for exact sample sizes (n) and P values.

sRNA responses. *sid-1* (ref. [16]) mutants, which are defective in systemic RNAi, exhibit high levels of naive avoidance of PA14 (Extended Data Fig. 2e), but also learn to avoid PA14 with lawn training; however, *sid-1* is required for learning induced by sRNA (Fig. 2d). By contrast, the RNAi-defective (rde) mutants *rde-1* (an *AGO3* homologue)[17], *rde-2* (also known as *mut-8*)[18], *rde-4* (ref. [19]) and *mut-7* (ref. [20]) have normal levels of naive attraction to PA14 and avoidance after PA14 lawn training, but have an abnormal response to control (OP50) sRNA and show no

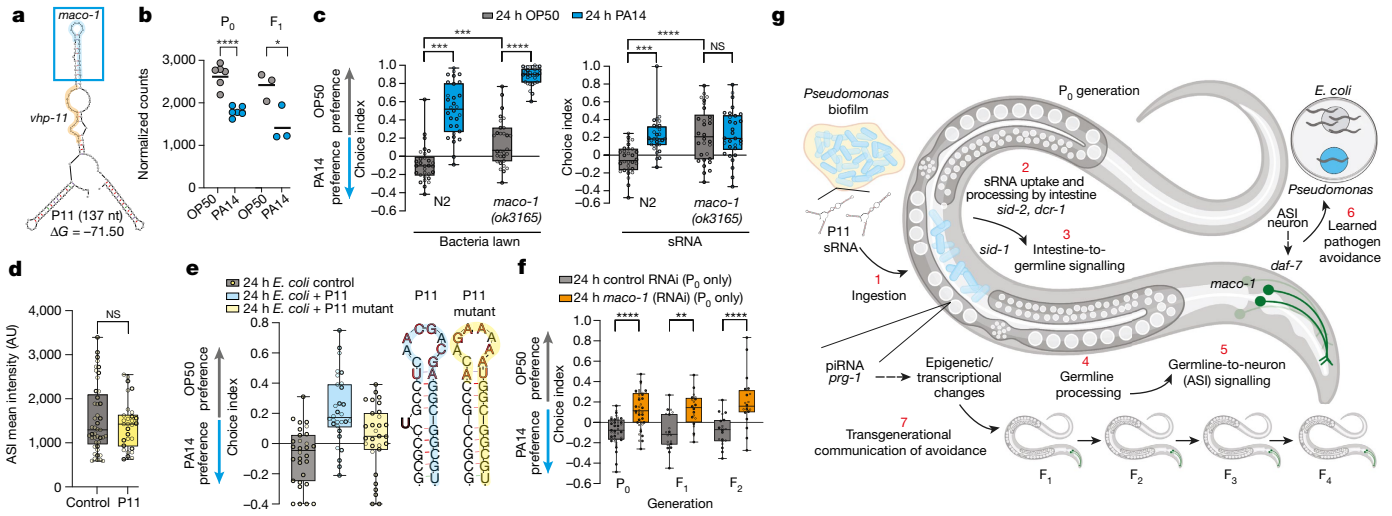

Fig. 4 | P11 induces avoidance through its target *maco-1*. **a**, Predicted secondary structure of wild-type P11 with regions of worm gene homology. **b**, *maco-1* expression is reduced in PA14-exposed mothers and their naive F_1 progeny[3] (mean, DESeq2; P_0 adjusted $P = 4.6 \times 10^{-9}$, $n = 6$ replicates per condition; F_1 adjusted $P = 0.014$, $n = 4$ replicates per condition). **c**, Left, *maco-1* mutants exhibit high levels of naive PA14 avoidance, but learning is intact when trained on PA14 lawns. Right, *maco-1* mutants do not increase PA14 avoidance when trained with PA14 sRNA. **d**, *maco-1* mutants do not increase *daf-7p::gfp* expression in ASI neurons upon *E. coli*–P11 training ($n = 45$ (control) or 30 (P11) neurons, two-sided Student's *t*-test). **e**, Wild-type worms do not avoid PA14 when trained with a P11 mutant in which the *maco-1* homology site is altered. Wild-type bases in bold were mutated to disrupt *maco-1* homology but retain predicted P11 structure ($\Delta G = -71.30$)[43]. **f**, Mothers trained on *maco-1* RNAi for 24 h exhibited PA14 avoidance that persisted for at least two generations. **g**, Model of P11-induced transgenerational learned avoidance. For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) with data shown from all replicates. Biological replicates: 3 (**c**, **e**, **f**), 4 (**d**). Box plots: centre line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P < 0.0001$, NS, not significant. Estimation plots are provided in the Supplementary Information; see Supplementary Table 4 for exact sample sizes (n) and P values.

further avoidance upon training with PA14 sRNA (Fig. 2e, Extended Data Fig. 2f, i). This indicates that these mutants are defective for normal sRNA responses (Fig. 2a). These results are consistent with the previously reported roles of neuronal *rde-4* in chemotaxis, and the requirement for the rde genes in response to *E. coli*[21,22].

sRNA learning does not use viral or microRNA pathways

Caenorhabditis elegans processes exogenous small interfering (si)RNAs and endogenous microRNAs using different Argonaute homologues. Mutants for the microRNA-specific Argonaute *alg-1* (a homologue of *AGO1*)[23] were competent for learning induced by PA14 sRNA (Fig. 2a, Extended Data Fig. 2m, n). Moreover, microRNA processing is unaffected in the *dcr-1(mg375)* mutant[26], which is unresponsive to sRNA from PA14 (Fig. 2c). Similarly, mutants of the virus-responsive component of RNAi *drh-1* were also competent for sRNA-induced learning (Fig. 2a, Extended Data Fig. 2o, p). Thus, the intracellular pathogen response to viral infection is not involved in learned avoidance induced by sRNA in *C. elegans*[24,25]. Together, these data suggest that the siRNA pathway—and not the microRNA or viral processing pathways—mediates avoidance learning induced by bacterial sRNA.

Role of the PIWI-interacting RNA pathway

PRG-1 (a homologue of PIWI) and its downstream components are required for the inheritance of learned pathogenic avoidance[3], but are not required for maternal learning of pathogen avoidance upon PA14 lawn training[3] (Extended Data Fig. 3a–e). We were therefore surprised that *prg-1*-mutant mothers were defective in the sRNA-induced avoidance response (Fig. 2a, f, Extended Data Fig. 3a). Furthermore, mutants of *rrf-1* (ref. [27]) and *rrf-3* (ref. [28]) (which encode RNA-dependent RNA polymerases) and *hpl-2* (ref. [29]) (which encodes a heterochromatin regulator) phenocopied the sRNA learning defect of *prg-1* mutants (Fig. 2a, g–i, Extended Data Fig. 3c–e). Consistently, *prg-1* mutants exposed to PA14 lawns failed to upregulate *daf-7* in the ASI neurons, but did induce *daf-7* expression in the ASJ neurons (Fig. 2j, Extended Data Fig. 3f).

sRNA avoidance requires a germline

Similar to wild-type worms, germline-less *glp-1(e2141)*[30] mutants learn to avoid PA14 after training on PA14 (Fig. 2k, left) and upregulate *daf-7* expression in the ASJ neurons (Extended Data Fig. 3g, h), which demonstrates that learning via innate immune pathways does not require a functional germline. However, *glp-1* mutants fail to exhibit sRNA-induced avoidance of PA14 (Fig. 2a, k right), and did not increase *daf-7p::gfp* in ASI neurons (Fig. 2l). Worms with defective germline granules (*meg-3 meg-4* mutants)[31] are also unable to induce avoidance in response to PA14 sRNA (Fig. 2a, m), despite having normal naive preferences and lawn learning (Extended Data Fig. 3i). Finally, germline-specific expression of *prg-1* fully rescued the impairment of sRNA-induced PA14 avoidance in *prg-1* mutant worms (Fig. 2f). Thus, although a functional germline is dispensable for avoidance induced by innate immunity, it is required for learned avoidance mediated by sRNA, and the piRNA pathway acts in the germline to do so. Furthermore, our data suggest that bacteria-derived sRNA does not act directly in neurons, but rather through an indirect mechanism that first requires uptake by the intestine, followed by piRNA processing and P granule function in the germline to communicate to the ASI neurons.

sRNA memory is transgenerationally inherited

It was previously shown that PA14 training induces heritable avoidance behaviour that persists through to the F_4 generation[3]. A single 24-h exposure of *C. elegans* to PA14 sRNA induced avoidance of PA14 not only in mothers but also in the subsequent four generations (Fig. 2n, o), replicating the transgenerationally inherited avoidance induced by pathogenic bacteria[3]. This avoidance persisted despite the fact that neither the mothers nor their progeny had ever directly encountered

PA14. Transgenerational inheritance of avoidance for both bacterial lawn and sRNA exposure required SID-1, SID-2 and DCR-1 (Extended Data Fig. 4a–c). The similar magnitude of the behaviour in progeny of worms trained on live bacteria or sRNA suggests that the sRNA response is sufficient to fully explain the transgenerational change in behaviour (Fig. 2o).

Our data support a model in which mothers respond acutely through the innate immune response[9] to pathogen exposure and metabolites, which induces *daf-7* expression in the ASJ neuron[4], causing half of the avoidance behaviour exhibited by trained worms (Figs. 1i, 2o inset, Extended Data Fig. 1j). Separately, pathogenic bacterial sRNA is taken up by mothers and processed through the canonical RNAi pathway via DCR-1 activity in the intestine. Subsequent germline signalling involving the piRNA pathway and epigenetic modifiers[3] induce *daf-7* expression in the ASI neuron, which ultimately regulates the avoidance of PA14. Only sRNA is required for the transgenerational inheritance of the avoidance behaviour signal (Figs. 2n, o, 4g).

PA14 P11 sRNA induces avoidance of PA14

Caenorhabditis elegans uses information from the sRNA of pathogenic PA14 to induce avoidance, despite never having been infected; we wondered whether this was due to a specific sRNA. sRNA isolated from PA14 grown under pathogenic conditions (25 °C, plate-grown), but not from less-virulent PA14 grown at 15 °C or liquid-grown PA14, induced avoidance of PA14 (Fig. 3a, b). Differential expression analysis (Extended Data Fig. 5a–d) revealed 18 and 22 annotated sRNAs[32] that were significantly more highly expressed in PA14 grown at 25 °C compared to that grown at 15 °C or liquid-grown, respectively (DESeq2, adjusted $P < 0.05$, Supplementary Tables 1, 2). Six sRNAs were upregulated specifically in samples grown at 25 °C: P11, P16, P24, PA14sr-032, ErsA and PrrB (also known as RsmZ) (Supplementary Tables 1, 2).

Training worms on *E. coli* that individually expressed one of the six PA14 sRNAs showed that, of these, only *E. coli* expressing P11 (*E. coli*–P11) induced PA14-avoidance behaviour similar to that of worms treated with pathogenic PA14 bacteria (Fig. 3c, Extended Data Fig. 6a). Worms do not avoid *E. coli*–P11, which eliminates P11 itself as the detected component (Extended Data Fig. 6b). The function of P11 (Fig. 3d, e)—a 137-nt non-coding RNA (ncRNA) specific to the *Pseudomonas* genus[33]—is unknown, but its *Pseudomonas stutzeri* orthologue nfiR is required for growth under nitrogen-stress conditions[34]. Worms treated with sRNA isolated from *E. coli*–P11 also displayed PA14 avoidance at a similar magnitude to those treated with sRNA from PA14 (Fig. 3f). Treatment with *E. coli*–P11 induced *daf-7* expression in the ASI neuron (Fig. 3g), as with sRNA from PA14 (Fig. 1k). By contrast, mothers treated with sRNA isolated from PA14 that lacks P11 (PA14 ΔP11) (Fig. 3h, Extended Data Fig. 6c–e) did not acquire PA14 avoidance, nor did their progeny (Fig. 3h). Together, these results demonstrate that P11 is both necessary and sufficient for learning PA14 avoidance, despite the fact that exposure to P11 ncRNA does not make worms ill or affect reproduction (Extended Data Fig. 6f, g). Thus, *C. elegans* may have evolved the ability to detect P11, which is involved in PA14 pathogenesis (Fig. 3i) and upregulated in *P. aeruginosa* grown in virulent conditions (Fig. 3d), as a biomarker of pathogenic PA14.

P11 induces transgenerational memory

Exposing mothers to *E. coli*–P11 induced transgenerational inheritance through the F_4 generation, similar to that observed with PA14 lawns and PA14 sRNA (Fig. 3j, k). Inherited learned avoidance required P11, as a PA14 ΔP11 lawn failed to induce avoidance in progeny (Fig. 3l). Consistent with sRNA being required for transgenerational avoidance, exposing mothers to sRNA from PA14 (Fig. 3m, n) and *E. coli*–P11 (Fig. 3m, o) induced *daf-7* expression in the ASI neurons of their progeny, similar to PA14 lawn exposure[4] (Fig. 3m, n).

MACO-1 is required for sRNA avoidance

A behavioural response to a specific bacterial sRNA implies the existence of a matching *C. elegans* sequence. We analysed the *C. elegans* genome, including mRNAs and non-coding RNAs, for homology to P11 (Supplementary Table 3). *maco-1*, the *C. elegans* homologue of the mammalian neuronal gene macoilin[35], contains the longest perfect match (17 nt) to P11 (Fig. 4a, Extended Data Fig. 7a). *maco-1* functions in chemotaxis, thermotaxis[36], oxygen sensing, and neuronal excitability[37]. It is expressed in neurons, including the ASI, where it is required for dauer formation[38]. Upon exposure to PA14, *maco-1* expression decreases in mothers and their progeny (Fig. 4b). As with ASI ablation mutants, *maco-1(ok3165)*-null mutants exhibit high levels of naive avoidance of PA14 (Fig. 4c) and do not increase PA14 avoidance upon treatment with sRNA (Fig. 4c right), and nor do their progeny (Extended Data Fig. 7b, c). By contrast, *vhp-1*—which contains a 16-nt match to P11 (Fig. 4a)—increased expression upon exposure to PA14, and a null allele (*vhp-1(sa366)*) did not affect PA14- or sRNA-induced avoidance or inheritance (Fig. 2a, Extended Data Fig. 7d–g). The innate immune pathways of *maco-1* are intact, as *maco-1* mutants can learn to avoid PA14 lawns (Fig. 4c left), but this does not persist in progeny (Extended Data Fig. 7b, h). Expression of *daf-7p::gfp* in the ASI neurons of *maco-1* mutants is not increased upon treatment with *E. coli*–P11 (Fig. 4d); moreover, training on a P11 mutant that disrupts the perfect match to *maco-1* but conserves P11 secondary structure induced no avoidance (Fig. 4e). These results suggest that MACO-1 regulates *daf-7* expression in the ASI neuron specifically in response to P11.

Wild-type mothers exposed to *E. coli* expressing *maco-1* dsRNA for 24 h learned to avoid PA14 (Fig. 4f). As with *E. coli*–P11, this single exposure to *maco-1* RNAi in the P_0 generation was sufficient to recapitulate the transgenerational memory of PA14 in naive progeny. Therefore, *maco-1* is specifically required for the response to sRNA from PA14, probably through its sequence matching P11.

Together, our data suggest that the ingestion of PA14 and subsequent uptake and DCR-1-mediated processing of the P11 in the intestine, followed by further processing and piRNA regulation in the germline, leads to the downregulation of *maco-1*, which in turn is required for upregulation of *daf-7* expression in the ASI neurons and subsequent maternal avoidance behaviour (Fig. 4g).

Discussion

Here we have identified a trans-kingdom signalling system that uses components of the RNAi pathway to 'read' bacterial sRNAs. Bacterial sRNAs are an ideal biomarker message, because they are dynamically regulated and reflect the pathogenic state of bacteria. This sRNA-sensing pathway depends on processing through the germline and subsequent communication to neurons, and is independent of metabolites and innate immunity pathways[4]. Although trans-kingdom signalling has been reported in which the sRNAs of a pathogen hijack the host immune system to avoid detection[39,40], here we report that an animal host uses a pathogen-produced sRNA to mount a behavioural defence response.

The single exposure of *C. elegans* to specific bacterial sRNA causes an avoidance response that is propagated for four additional generations. Although other, previously described trans-kingdom signalling systems benefit the pathogen, *C. elegans* identifies the sRNA of pathogens to protect itself and its progeny, and to induce a search for less-pathogenic food sources. This phenomenon may explain why both systemic and transgenerational epigenetic inheritance responses to exogenous RNA exist: to modify the behaviour of the worm in response to encounters with naturally abundant pathogenic bacterial species that are identified by the worm through unique and decipherable sRNA signatures. This process is distinct from responses that require prolonged, multi-generational bacterial exposure to induce dauer formation[41] or

infection adaptation[42]. Rapid and lasting behavioural changes may be more efficient and reproductively advantageous, forcing worms to escape and explore new and potentially safe environments. Passing this avoidance behaviour on to future generations may spare progeny from ever experiencing a prolonged exposure to the pathogen, despite its abundance in the environment. Such a species-specific and plastic response may provide worms with a powerful survival mechanism that is fast-acting and rapidly reversible, a first line of defence against pathogens. This trans-kingdom communication paradigm may represent an adaptive immune memory that prepares future generations for encounters with harmful environmental conditions, allowing them to properly respond to a pathogenic threat without ever experiencing infection and illness.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-020-2699-5.

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Methods

No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

Cultivation of *C. elegans* strains

The following worm strains were provided by the *C. elegans* Genetics Center (CGC). KU25: *pmk-1(km25)*, AU133: *agIs17[Pmyo-2::mCherry + Pirg-1::GFP]*, PY7505: oyIs84 [*gpa-4p*::TU#813 + *gcy-27p*::TU#814 + *gcy-27p*::GFP + *unc-122p*::DsRed], NL3321: *sid-1(pk3321)*, HC271: ccIs4251 [(pSAK2) myo-3p::GFP::LacZ::NLS + (pSAK4) myo-3p::mitochondrial GFP + dpy-20(+)] I. qtIs3 [myo-2p::GFP dsRNA hairpin]. mIs11 [myo-2p::GFP + pes-10p::GFP + gut-promoter::GFP], YY470: *dcr-1(mg375)*, WM27: *rde-1(ne219)*, NL3531: *rde-2(pk1657)*, WM49: *rde-4(ne301)*, COP2012: *alg-1(knu867)*, RB2519: *drh-1(ok3495)*, CQ636 *dcr-1(mg375); vha-6p::dcr-1::dcr-1 3′UTR + [Pmyo-2p::mCherry]*, MAH23: *rrf-1(p1417)*, NL2099: *rrf-3(ok1426)*, NL917: *mut-7(pk204)*, SX922: *prg-1(n4357)*, RB995: *hpl-2(ok916)*, CQ605 *prg-1(n4357); ksIs2 [Pdaf-7p::GFP + rol-6(su1006)]*, CF1903: *glp-1(e2141)*, CQ640: *glp-1(e2141); ksIs2 [Pdaf-7p::GFP + rol-6(su1006)]* (strain was made by mating CF1903 with FK181), YL243: *unc-119(ed) III; vrls79 [pie-1p::GFP::prg-1 + unc-119(+)]*, CQ655: *prg-1(n4357); unc-119(ed) III; vrls79 [pie-1p::GFP::prg-1 + unc-119(+)]* (strain was made by mating SX922 with YL243), JH3225: *meg-3(tm4259) meg-4(ax2026)*, FK181: *ksIs2 [Pdaf-7p::GFP + rol-6(su1006)]*, RB2329: *maco-1(ok3165)*, CQ654: *ksIs2 [Pdaf-7p::GFP + rol-6(su1006)]; maco-1(ok3165)* (this strain was made by mating FK181 with RB2329), JT366: *vhp-1(sa366)*. Hermaphrodites were used in all experiments.

Bacterial strains. The PA14 and *P. aeruginosa ΔlasR* were gifts from Z. Gitai. OP50 was provided by the CGC. *S. marcescens* (ATCC 274) was provided by the ATCC. Control (L4440) and *maco-1* RNAi were obtained from the Ahringer library and sequence-verified before use.

General worm maintenance. Worm strains were maintained at 15 °C on high growth medium (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 1 M $CaCl_2$, 1 ml/l 1 M $MgSO_4$ and 25 ml/l 1 M potassium phosphate buffer (pH 6.0) added to molten agar after autoclaving) on OP50 using standard methods.

General bacteria cultivation. OP50, PA14, *S. marcescens* and *P. aeruginosa ΔlasR* were cultured overnight in Luria broth (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl in distilled water), shaking (250 rpm) at 37 °C. *Escherichia coli* strains expressing PA14 sRNA were cultured overnight in Luria broth supplemented with 0.02% arabinose w/v and 100 μg/ml carbenicillin. Strains expressing RNAi were cultured overnight in Luria broth supplemented with 100 μg/ml carbenicillin and 12.5 μg/ml tetracycline.

Training plate and worm preparation

Worm preparation. Eggs from young adult hermaphrodite worms were obtained by bleaching and subsequently placed onto HG plates and incubated at 20 °C for 2 days. Synchronized L4 worms were used in all training experiments. For experiments involving CF1903 (*glp-1(e2141)*), eggs from mutant and wild-type adult hermaphrodite worms were obtained by bleaching and placed onto HG plates and left at 25 °C for 2 days. Germline loss was confirmed in adult *glp-1(e2141)* worms raised at 25 °C.

Bacteria lawn (25 °C) training plate preparation. Overnight cultures of bacteria (prepared as described in 'General bacteria cultivation') were diluted in LB to an optical density at 600 nm (OD_{600}) = 1 and used to fully cover nematode growth medium (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 1 M $CaCl_2$, 1 ml/l 1 M $MgSO_4$ and 25 ml/l 1 M potassium phosphate buffer (pH 6.0) added to molten agar after autoclaving) plates. For preparation of *E. coli* expressing PA14 small RNA, bacteria were seeded on NGM plates supplemented with 0.02% arabinose and 100 μg/ml carbenicillin. All plates were incubated for 2 days at 25 °C, unless specified otherwise (in separate incubators for control and pathogen-seeded plates). On the day of training (that is, 2 days after bleaching), plates were left to cool on a benchtop for 1 h to equilibrate to room temperature before the addition of worms. Additionally, for *E. coli* strains expressing PA14 sRNA, 200 μl of 0.01% arabinose was spotted onto seeded training plates 1 h before use.

Bacteria lawn (15 °C) training plate preparation. PA14 was prepared by centrifuging 5-ml overnight cultures for 10 min at 5,000*g*. 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 OD_{600} = 1 and used to seed NGM plates. Plates were incubated at 15 °C for 2 days.

DNA, supernatant and sRNA training plate preparation. In brief, 200 μl of OP50 was spotted in the centre of a 10-cm NGM plate. Plates were incubated at 25 °C for 2 days.

Heat-killed bacteria training plate preparation. One day before plate use, overnight bacteria cultures of OP50 were centrifuged at 5,000*g* for 10 min. Following centrifugation, pellets were resuspended in 1/10 volume of LB. Resuspended bacteria pellets were heat-shocked at 95 °C for 1 h. Heat-shocked bacterial suspensions were left to cool at room temperature for 1 h, and 200 μl of heat-killed bacteria was spotted in the middle of a 10-cm NGM plate supplemented with 100 μg/ml carbenicillin. Plates were incubated at 25 °C for 1 day, before use. No bacterial growth was observed on heat-killed bacteria plates both before worm training and 24 h after worm training.

DNA preparation and training. Overnight cultures were pelleted at 5,000*g* for 10 min at room temperature. DNA was prepared from pelleted bacteria according using the Qiagen DNeasy Blood and Tissue kit and subsequently used fresh. Ten ng of bacterial DNA was placed onto *E. coli* spots and left to completely dry at room temperature for approximately 1 h before the addition of worms for training.

Supernatant. Overnight bacterial cultures (undiluted) were pelleted at 5,000*g* for 10 min at room temperature. Supernatant was collected and filtered using a 0.22-μm syringe filter. For worm training plates, 1 ml of filtered supernatant was put onto OP50 spots and left to completely dry at room temperature for approximately 1 h before the addition of worms for training.

Preparation of bacteria for RNA isolation

Bacteria for RNA collection were grown for 2 days on plates at 25 °C. Bacterial lawns were collected from the surface of NGM plates using a cell scraper. In brief, 1 ml of M9 buffer was applied to the surface of the bacterial lawn, and the bacterial suspension following scraping was transferred to a 15-ml conical tube. PA14, *ΔlasR*, *S. marcescens* or *E. coli* expressing PA14 sRNA strains from 10 plates or OP50 from 15 plates was pooled in each tube and pelleted at 5,000*g* for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 1 ml of Trizol LS for every 100 μl of bacterial pellet recovered. The pellet was resuspended by vortexing and subsequently frozen at −80 °C until RNA isolation.

Bacteria RNA isolation

To isolate RNA from bacterial pellets, Trizol lysates were incubated at 65 °C for 10 min with occasional vortexing. Debris was pelleted at

7,000*g* for 5 min at 4 °C. The supernatant was transferred to new tubes containing 1/5 the volume of chloroform. Samples were mixed thoroughly by inverting and centrifuged at 12,000*g* for 10 min at 4 °C. The aqueous phase was used at input for RNA purification using the mirVana miRNA isolation kit according to the manufacturer's instructions for total RNA, large RNA (>200 nt) or small RNA (<200 nt) isolation. Purified RNA was used immediately or frozen at −80 °C until further use.

RNase-treated samples. For RNase treatment of purified RNA, samples containing 100 µg of RNA were treated with 2.5 µl of an RNase A (500 U/ml) and RNase T (20,000 U/ml) cocktail for every 50 µl of RNA (RNase Cocktail Enzyme Mix, Ambion). Samples were incubated at room temperature for 20 min before adding to worm training plates seeded with OP50. RNase degradation was confirmed using an Agilent 2100 Bioanalyzer. In total, 100 µg of purified small RNA (measured before RNase degradation) treated with RNase was spotted onto plates before use for worm training.

DNase-treated samples. For DNase treatment, samples containing 100 µg of purified RNA were treated with 2 U of DNase I per 10 µg of RNA using the Invitrogen DNA-free kit according to the manufacturer's instructions. In total, 100 µg of purified small RNA treated with DNase was spotted onto plates before use for worm training.

Total RNA, large RNA and sRNA on heat-killed bacteria. In brief, 240 µg of total RNA, 100 µg of sRNA or large RNA, or RNase- and DNase-treated sRNA was placed directly onto OP50 spots and left to completely dry at room temperature (for about 1 h) before use on day of experiment for worm training.

Worm preparation for training

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

Aversive learning assay

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

In experiments in which F_1 and subsequent generations are used: day 1 worms after from parental (P_0) training were bleached and eggs were placed onto HG plates and left for 3 days at 20 °C. All worms tested were washed off HG plates with M9 at day 1. Some of the pooled worms were subjected to an aversive learning assay, and the majority of worms were bleached to obtain eggs, which were then placed onto HG plates left at 20 °C for 3 days and used to test the F_2 generation.

Imaging and fluorescence quantification

All images were taken on a Nikon Eclipse Ti microscope. Differential inference contrast (DIC) images of whole worms following OP50, or PA14 lawn or sRNA training, were imaged at 20×.

Z-stack multi-channel (DIC and GFP) of day-1 adult GFP-transgenic worms were imaged every 1 µm at 60× magnification; Maximum intensity projections and 3D reconstructions of head neurons were built with Nikon NIS-Elements. To quantify *daf-7p::gfp* levels, worms were prepared and treated as described in 'Worm preparation for training'. Worms were mounted on agar pads and immobilized using 1 mM levamisole. GFP was imaged at 60× magnification and quantified using NIS-Elements software. Average pixel intensity was measured in each worm by drawing a Bezier outline of the neuron cell body for 2 ASI head neurons and/or 2 ASJ head neurons.

For *irg-1p::gfp* quantification, whole worms were prepared as described in 'Worm preparation for training' and imaged at 20× magnification. Average pixel intensity was measured in each worm by drawing a Bezier outline the entire worm.

Brood size assay

L4-stage mothers were trained for 24 h on control (empty vector) or P11-expressing *E. coli* as described in 'Worm preparation for training'. After 24 h, 15 individual worms for each condition were transferred to NGM plates seeded with OP50. Worms were transferred to fresh plates every 24 h. Progeny containing plates were incubated at 20 °C for 2 days before progeny were counted. Worms were moved daily until progeny production ceased.

Progeny development assay

Mothers were trained on OP50 or PA14 sRNAs as described in 'Worm preparation for training'. After 24 h of training, mothers were bleached and progeny were transferred to OP50-seeded NGM plates. Plates were incubated at 20 °C for 2 days before progeny were assayed for developmental stage.

sRNA sequencing

Each sample of PA14 sRNA was tested for *C. elegans* behaviour before sequencing. The size distribution of sRNA samples was examined on a Bioanalyzer 2100 using RNA 6000 Pico chip (Agilent Technologies). For sRNA sequencing, around 300 ng of sRNA from each sample was first treated with RNA 5′ pyrophosphohydrolase (New England Biolabs) at 37 °C for 30 min, then converted to Illumina sequencing libraries using the PrepX RNA-seq library preparation protocol on the automated Apollo 324 NGS Library Prep System (Takara Bio). In brief, the treated RNA samples were ligated to two different adapters at each end, then reverse-transcribed to cDNA and amplified by PCR using different barcoded primers. The libraries were examined on Bioanalyzer DNA High Sensitivity chips (Agilent) for size distribution, quantified by Qubit fluorometer (Invitrogen), and then pooled at equal molar amount and sequenced on Illumina NovaSeq 6000 S Prime flowcell as single-end 122-nt reads. The pass-filter reads were used for further analysis.

sRNA analysis

Pseudomonas aeruginosa (UCBPP-PA14) sRNA stranded reads were trimmed to remove adapters using Cutadapt (v.1.16.6). Reads were mapped to the CP000438.1 genome using BWA-MEM. For sRNA analysis, count tables were generated using previously annotated intergenic non-coding RNAs (that is, sRNA)[32]. Differential gene expression between the 25-°C plate and the 15-°C plate, and 25-°C plate and liquid conditions, was performed using DESeq2. The principal component analysis plot was generated using DESeq2 on the regularized log-transformed counts.

Strain construction

The *dcr-1* intestinal rescue strain was made by amplifying 1,602 bases upstream of the *vha-6* translational start site, and subsequently using

PCR to fuse the *vha-6* promoter to the *dcr-1* cDNA and 3′ untranslated region (UTR). Purified PCR products were injected at 10 ng/µl with 1 ng/µl *myo-2p::mCherry* into *dcr-1(mg375)* to generate strain CQ636.

E. coli expressing PA14 sRNA strain construction

For cloning the six differentially expressed PA14 sRNA species into *E. coli*, we used previously reported experimentally determined sequences reported[44]. sRNA sequences were amplified from PA14 genomic DNA using the primer pairs described here. The sRNA was cloned into plasmid, pBAD18-Amp[45] which contains an arabinose-inducible promoter upstream of a multiple cloning site. Plasmids were transformed into *E. coli* MG1655. sRNA expression was induced by growing *E. coli* on NGM plates supplemented with 0.1% arabinose. Correct sRNA production was confirmed for each of the six overexpression strains using reverse-transcription PCR. Secondary structure folding predictions for P11 and P11 mutants were performed using Mfold[46]. See Supplemental Table 5 for primers.

PA14 ΔP11 mutant strain construction

The unmarked deletion of the P11 ncRNA was constructed by two-step allelic exchange using plasmid pEXG2[43]. In brief, about 400-bp fragments directly upstream and downstream of the P11 sequence were amplified from genomic DNA using the primer pairs P11-KO-1 and P11-KO-2, and P11-KO-3 and P11-KO-4, respectively. Upstream and downstream fragments were fused together using overlap–extension PCR with primer pair P11-KO-1 and P11-KO-4, and the resulting fragment was cloned into the HindIII site of plasmid pEXG2. The pEXG2 plasmid was integrated into PA14 through conjugation with the donor strain *E. coli* S17. Exconjugants were selected on 30 µg/ml gentamycin, and then the mutants of interest were counterselected on 5% sucrose. Correct deletion was confirmed through PCR using primers P11-seq-5 and P11-seq-5). See Supplemental Table 5 for primers.

E. coli expressing P11 *maco-1* homology mutant

To generate the P11 overexpression construct with mutations disrupting the 17 nt of homology to the *maco-1* gene, fragments to the left and right of the homology site were amplified from the pBAD18-P11 using primer pairs P11-macoI-1and P11-macoI-2, and P11-macoI-3 and P11-macoI-4, respectively, and inserted into the NheI/HindIII-cut pBAD18 plasmid by Gibson assembly. See Supplemental Table 5 for primers.

PA14 ΔP11 survival assay

OP50, PA14, or PA14 ΔP11 were grown in liquid culture and diluted as described in 'Worm preparation for training'. In brief, 200 µl of diluted bacteria was spread to completely cover a 6-cm NGM plate. Plates were incubated for 2 days at 25 °C to allow bacterial growth. Plates were equilibrated to 20 °C before the addition of L4 worms to plates. Assays were performed at 25 °C. Assays were counted every 8 to 10 h until all worms on pathogenic plates died.

Statistical analysis of choice assay data

Populations of worms were raised together under identical conditions and were randomly distributed into treatment conditions. Trained worms were pooled and randomly chosen for choice assays. For all choice assays, each dot represents an individual choice assay plate (about 10–300 worms per plate) with all data shown from at least 3 independent replicates (Supplementary Table 4). Plates were excluded that contained less than 10 total worms per plate. The box extends from the 25th to the 75 percentile, with whiskers from the minimum to the maximum values. Mean differences are shown using Cumming estimation plots[47], with each graphed as a bootstrap sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical bars. All figures in the Article and Supplementary Information represent pooled data from independent experiments. Results from individual experiments are provided in the Supplementary Information. All estimation plots were generated using https://www.estimationstats.com/#/. Additional statistics were generated using Prism 8. Counting of worms on choice assay plates was performed blind with respect to worm genotype and training condition.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

Sequencing data are available from: BioProject under accession number PRJNA553700. Any data related to the study that are not provided in the Article and its Supplementary Information can be obtained upon reasonable request from the corresponding author.

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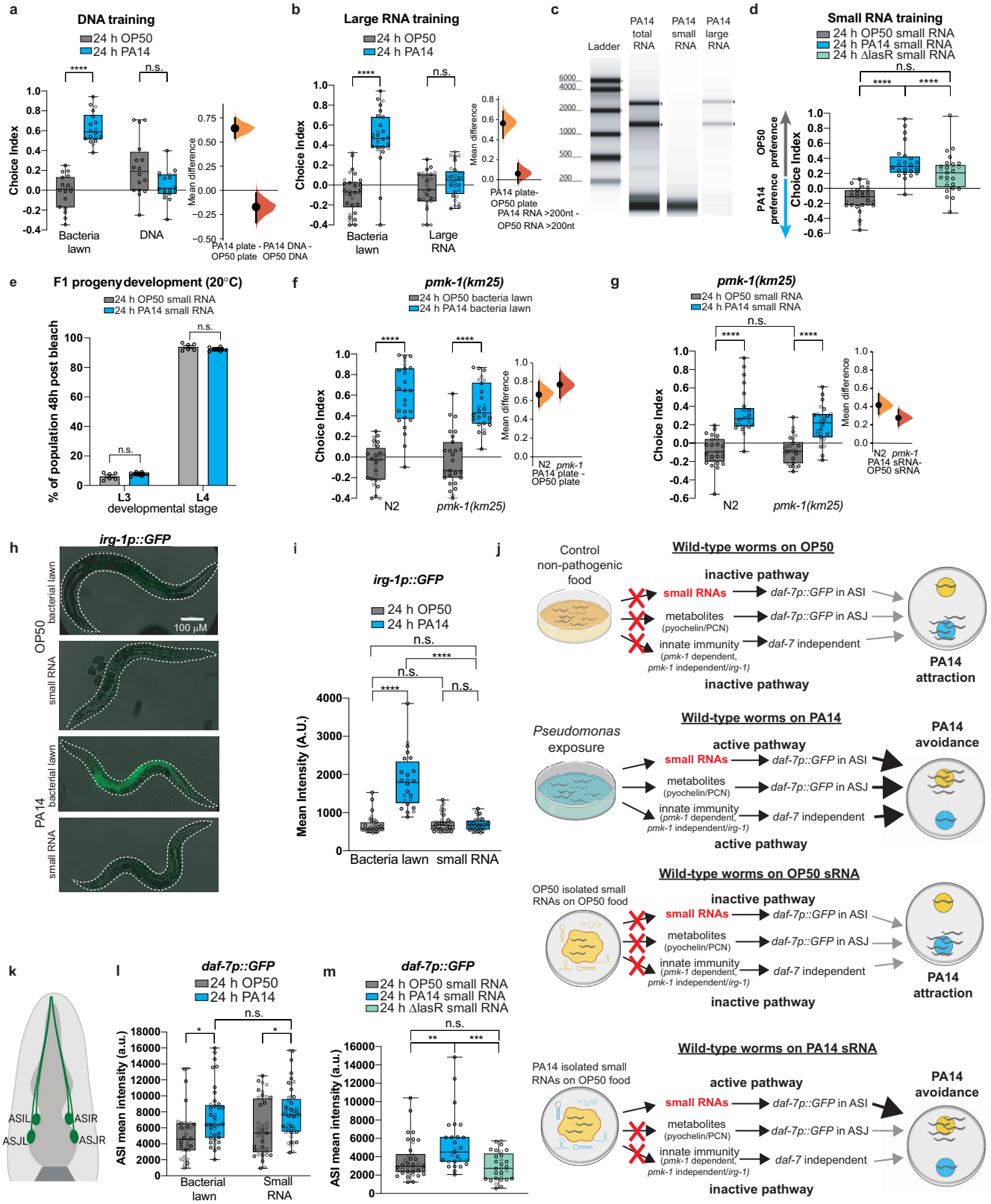
Author contributions R.K., R.S.M., G.D.V., Z.G. and C.T.M. designed experiments. R.K. and R.S.M. performed experiments and analysed data. G.D.V. constructed P11 *E. coli* and PA14 mutant strains. L.R.P. and R.K. analysed small-RNA-seq data. R.K., R.S.M. and C.T.M. wrote the manuscript.

Competing interests The authors declare no competing interests.

Additional information

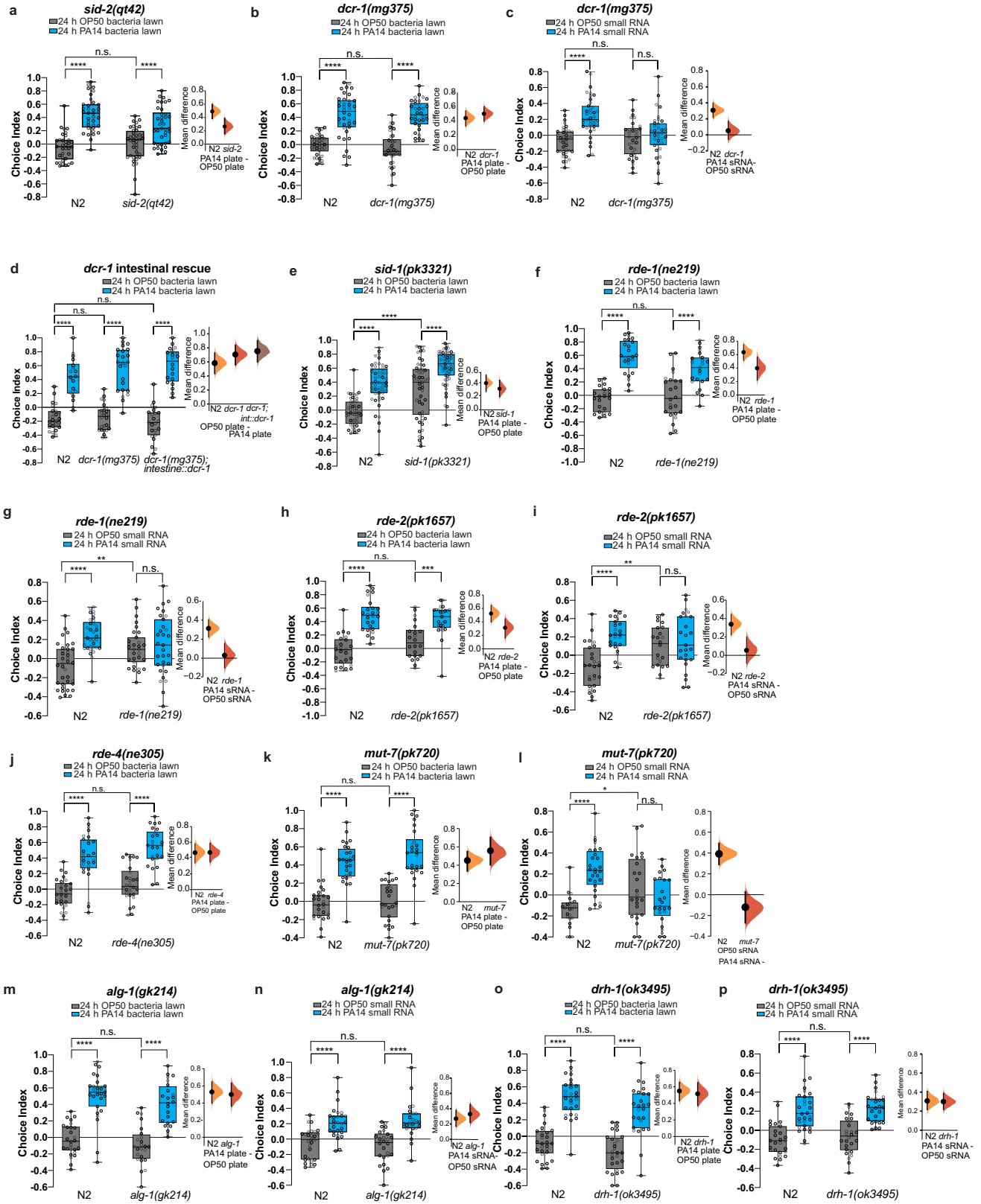
Supplementary information is available for this paper at https://doi.org/10.1038/s41586-020-2699-5.

Correspondence and requests for materials should be addressed to C.T.M.



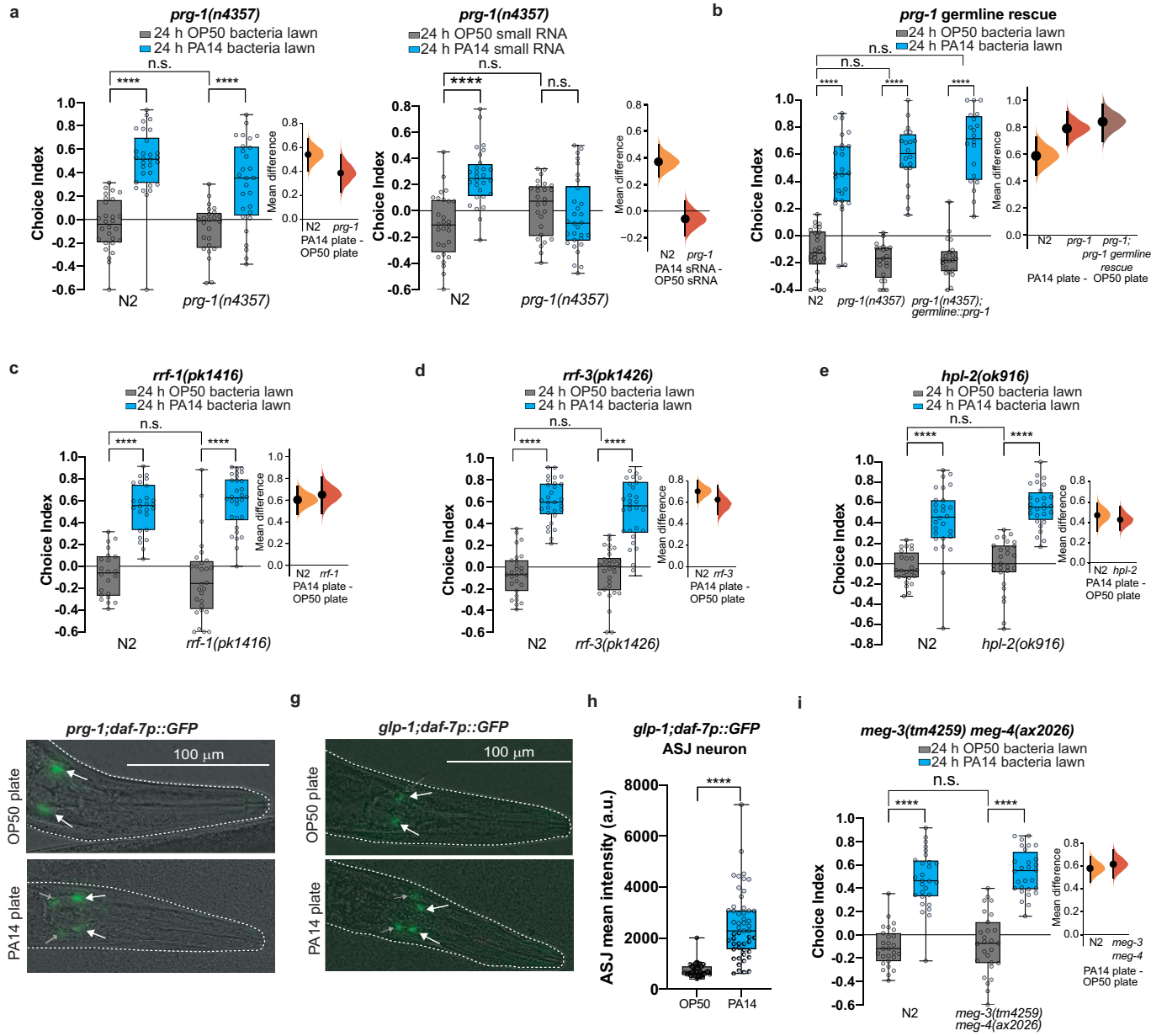
Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | PA14 sRNAs induce maternal PA14 avoidance and increased *daf-7* expression in the ASI neurons. a, Worms exposed to a PA14 bacterial lawn for 24 h learn to avoid PA14 in a choice assay, but PA14 DNA exposure alone does not induce maternal avoidance of PA14. **b**, Training with large RNA (>200 nt) isolated from bacterial lawns of PA14 is not sufficient for maternal PA14 avoidance. **c**, Bioanalyzer results of isolated PA14 total RNA and fractionated small and large RNAs. RNA levels were normalized for worm training. **d**, *ΔlasR* sRNA exposure does not induce PA14 learned avoidance. **e**, Development of progeny of PA14 sRNA-trained mothers was not delayed compared to progeny of OP50-trained mothers. $n = 6$ plates per condition with 23–142 progeny per plate, mean ± s.e.m. **f**, *pmk-1(km25)* worms learn to avoid PA14 when exposed to PA14 bacteria lawns. **g**, *pmk-1(km25)* is not required for PA14 sRNA-induced pathogenic learning. **h**, *irg-1p::gfp* expression is induced by PA14 bacterial lawn exposure, but not by PA14 sRNAs alone. **i**, GFP intensity from **h** was quantified, $n = 33$, 24, 43 and 35 worms, left to right. **j**, Model of PA14 bacteria lawn and sRNA learning. **k**, Location of ASI and ASJ neurons. **l**, The 24-h exposure of worms to a PA14 lawn or PA14 sRNA increases *daf-7p::gfp* expression in the ASI neurons. Two-way ANOVA, Tukey's multiple comparison test. $n = 35$, 41, 41 and 49 neurons, left to right. **m**, *ΔlasR* sRNA exposure does not induce changes in *daf-7p::gfp* ASI expression. One-way ANOVA, Tukey's multiple comparison test. $n = 38$, 27 and 29 neurons, left to right. Biological replicates: 2 (**a**), 3(**b**–**d**, **f**–**i**, **m**), 6 (**l**). For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) with data shown from all replicates. For box plots, centre line is the median, box extends from the 25th to the 75th percentile; whiskers denote minimum–maximum values. One-way (**d**, **m**) and Two-way ANOVA (**a**, **b**, **e**–**g**, **i**, **l**), Tukey's multiple comparison test. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P < 0.0001$, n.s., not significant. Mean differences are shown using Cumming estimation plots[47], with each graphed as a bootstrap sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical bars. See Supplementary Table 4 for exact sample sizes (n) and P values.

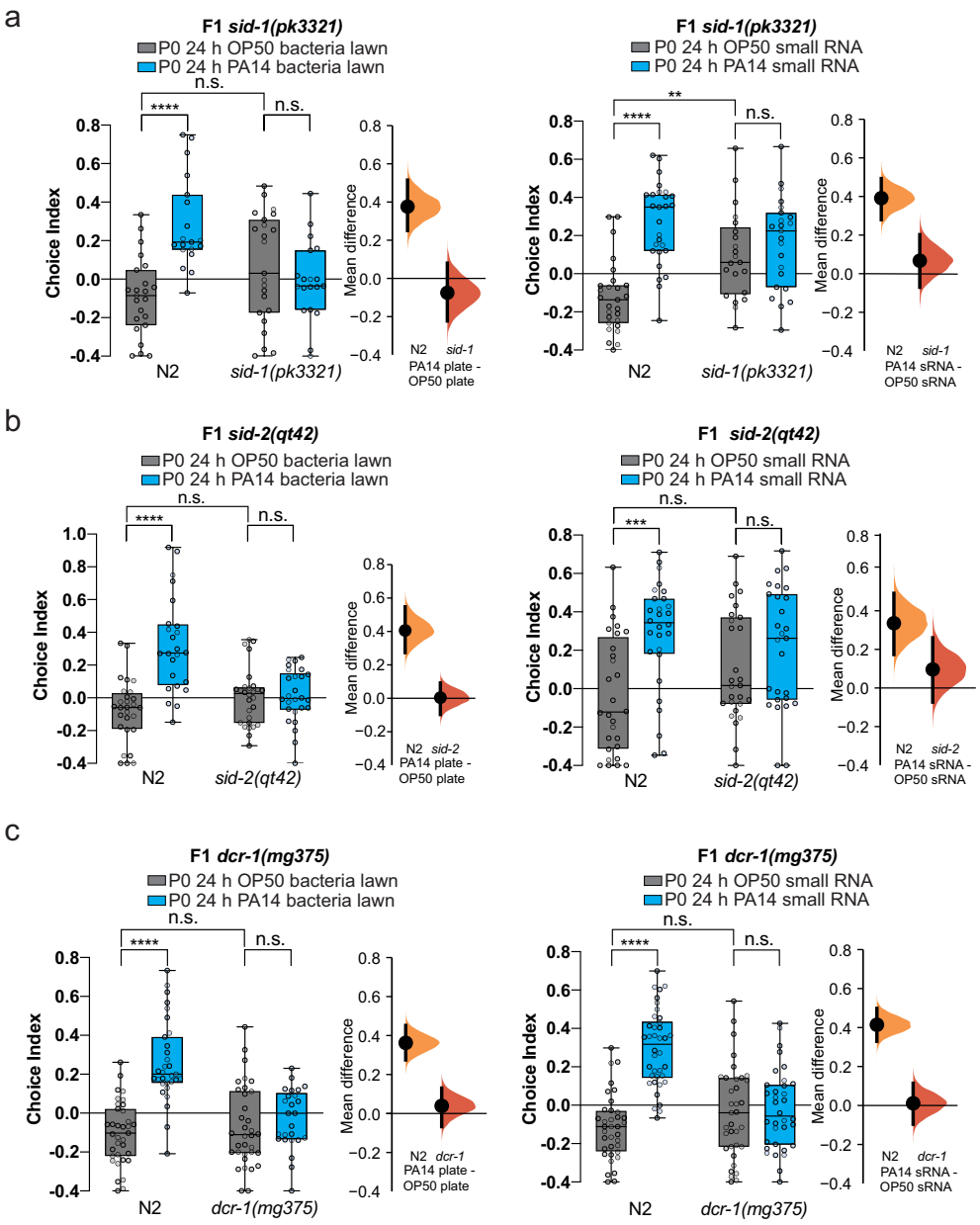


Extended Data Fig 2 | See next page for caption.

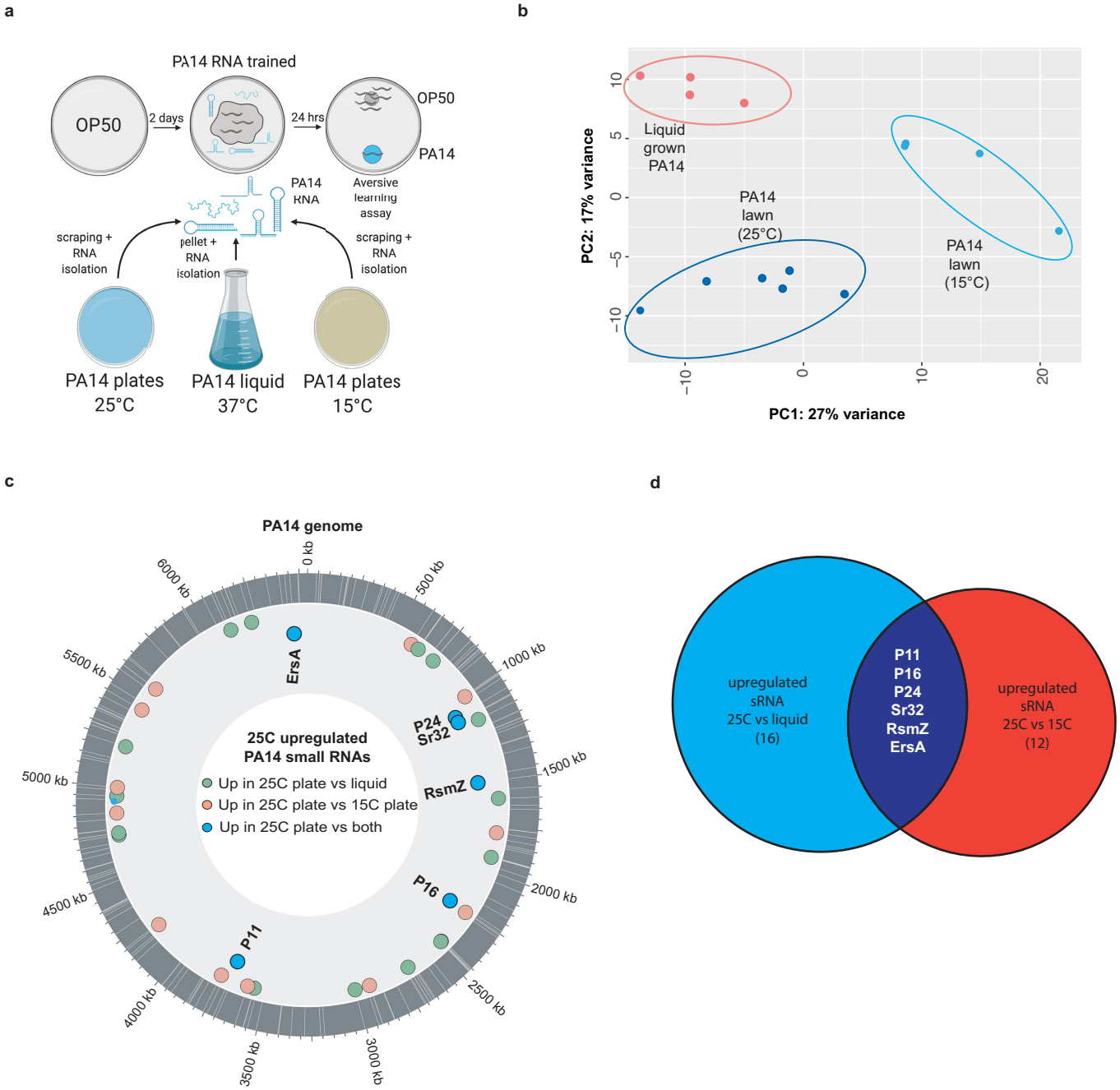
Extended Data Fig 2 | sRNA and bacterial lawn training of *C. elegans* RNAi pathway mutants. a, Wild-type and *sid-2(qt42)* worms were trained on OP50 or PA14 bacterial lawns for 24 h and tested for learned PA14 avoidance. **b**, **c**, Wild-type and *dcr-1(mg375)* worms were trained on OP50 or PA14 lawns (**b**) or OP50 or PA14 sRNA (**c**). **d**, Wild-type, *dcr-1(mg375)* and *dcr-1(mg375);vha-6p::dcr-1* worms were trained on OP50 or PA14 bacterial lawns for 24 h and tested for learned PA14 avoidance. Training and choice assays for *dcr-1(mg375)* and *dcr-1(mg375); vha-6p::dcr-1* worms were performed on the same plates. For choice assays, transgenic worms expressing the rescue construct and pharyngeal mCherry were counted using a fluorescence dissecting microscope. Non-transgenic, non-fluorescent *dcr-1(mg375)* sibling worms from the same plates were also counted and the results are shown. **e**, Wild-type and *sid-1(pk3321)* worms were trained on OP50 or PA14 bacterial lawns for 24 h and tested for learned PA14 avoidance. *sid-1* mutants have a constitutively high naive avoidance, but are able to learn avoidance after training on a PA14 lawn, but not after training on sRNAs. **f**–**j**, Wild-type, *rde-1(ne219)* (**f**, **g**), *rde-2(pk1657)* (**h**, **i**) or *rde-4(ne305)* (**j**) worms were trained on OP50 or PA14 bacterial lawns (**f**, **h**, **j**) or sRNA (**g**, **i**) for 24 h and tested for learned PA14 avoidance. *rde-1*, *rde-2* and *rde-4* mutants are able to learn avoidance following bacteria lawn training, but do not additionally avoid PA14 following sRNA training. **k**, **l**, Wild-type and *mut-7(pk720)* worms were trained on OP50 or PA14 lawns (**k**) or sRNA (**l**) for 24 h and tested for learned PA14 avoidance. These mutants (in **e**–**l**) have high naive preference after training on sRNAs only. **m**–**p**, Wild-type and *alg-1(gk214)* (**m**, **n**) or *drh-1(ok3495)* (**o**, **p**) worms were trained on OP50 or PA14 bacterial lawns (**m**, **o**) or sRNA (**n**, **p**) for 24 h and tested for learned PA14 avoidance. Biological replicates: 3 (**d**, **f**, **h**–**p**), 4 (**a**–**c**, **g**), 6 (**e**). For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) with data shown from all replicates. For box plots, centre line is the median, box extends from the 25th to the 75th percentile; whiskers denote minimum–maximum values. Two-way ANOVA (**a**–**p**), Tukey's multiple comparison test. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P < 0.0001$, n.s., not significant. Mean differences are shown using Cumming estimation plots[47], with each graphed as a bootstrap sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical bars. See Supplementary Table 4 for exact sample sizes (n) and P values.



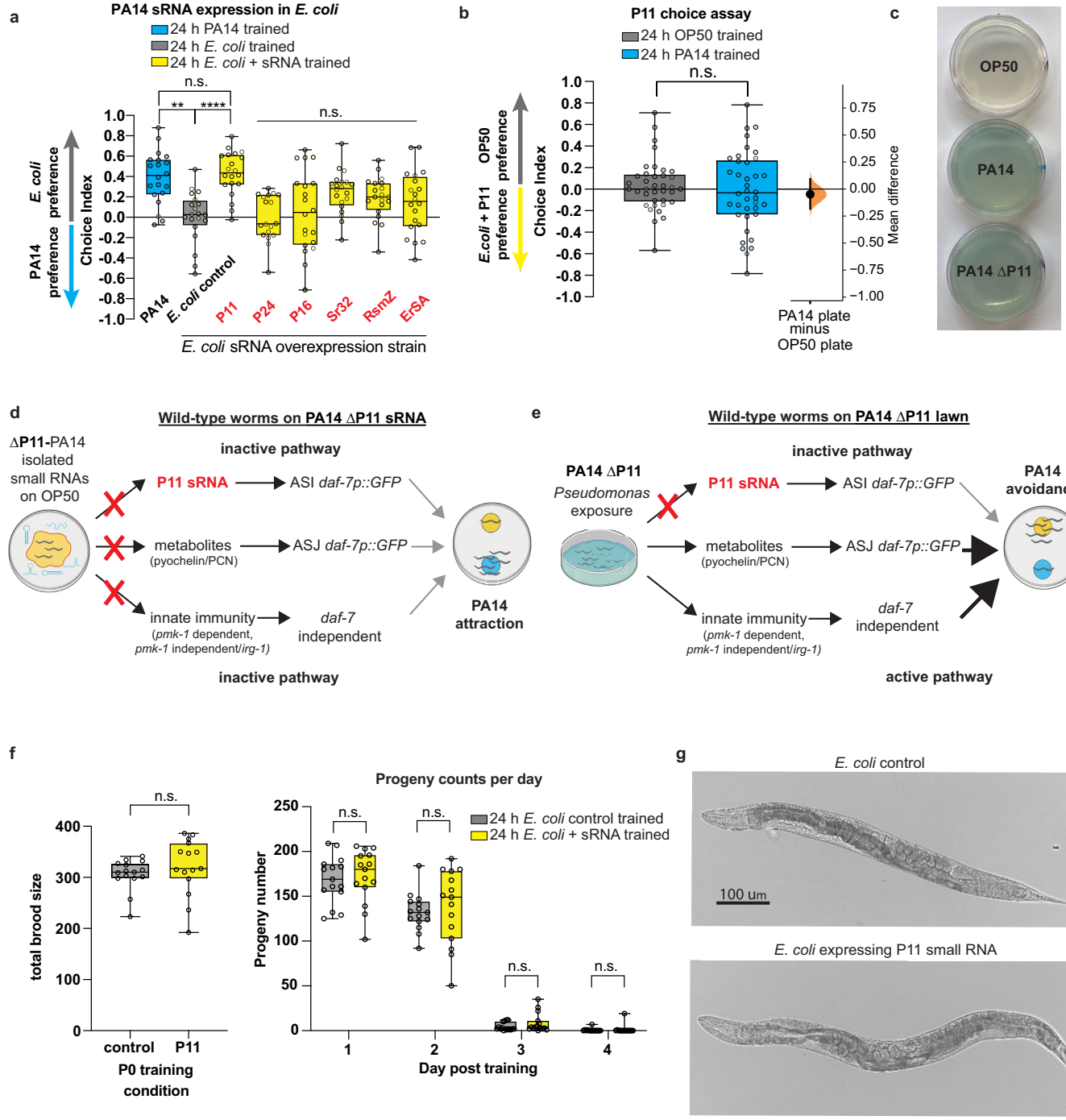
Extended Data Fig. 3 | sRNA and bacterial lawn training of *C. elegans* sRNA pathway and germline mutants. **a**–**e**, Wild-type and *prg-1(n4357)* (**a**), *prg-1* germline rescue (**b**), *rrf-1(pk1417)* (**c**), *rrf-3(pk1426)* (**d**), or *hpl-2(ok916)* worms (**e**) were trained on OP50 or PA14 bacterial lawns or sRNA (**a**, right) for 24 h and tested for learned PA14 avoidance. **f**, **g**, Representative images of OP50- or PA14-trained *prg-1;daf-7::gfp* (**f**) or *glp-1;daf-7::gfp* (**g**) mothers. ASI neuron = white arrow, ASJ neuron = grey arrow. **h**, Germline-less *glp-1* mutants induce *daf-7p::gfp* expression in the ASJ neuron after PA14 lawn exposure. $n = 42$ (OP50), 46 (PA14) neurons, two-tailed Student's *t*-test. **i**, Wild-type and *meg-3(tm4259) meg-4(ax2026)* worms were trained on OP50 or PA14 bacterial lawns for 24 h and tested for learned PA14 avoidance. Biological replicates: 3 (**a**–**i**). For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) with data shown from all replicates. For box plots, centre line is the median, box extends from the 25th to the 75th percentile; whiskers denote minimum–maximum values. Two-way ANOVA (**a**–**e**, **i**), Tukey's multiple comparison test. ****$P < 0.0001$, n.s., not significant. Mean differences are shown using Cumming estimation plots[47], with each graphed as a bootstrap sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical bars. See Supplementary Table 4 for exact sample sizes (n) and P values.



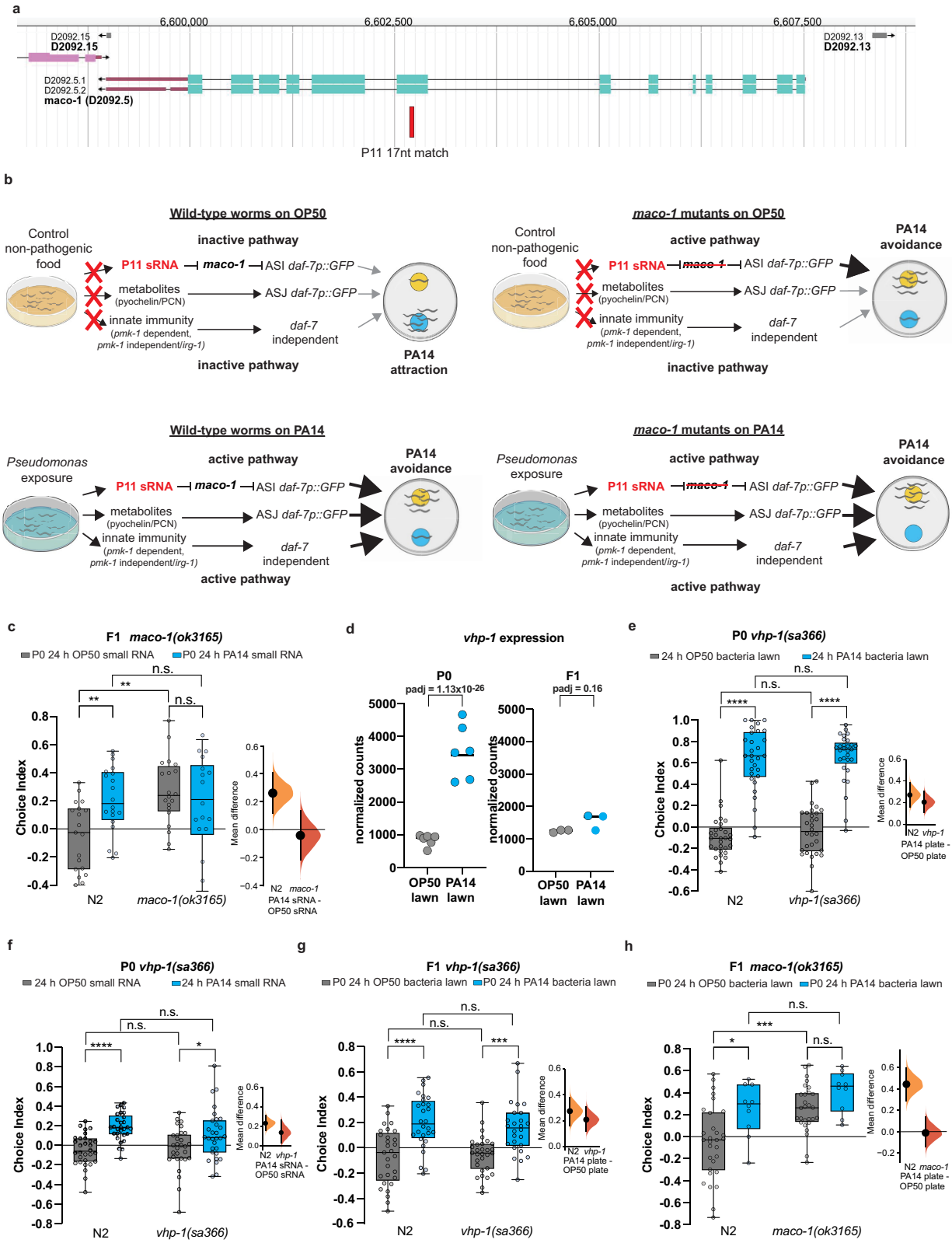
Extended Data Fig. 4 | *sid-1*, *sid-2* and *dcr-1* F_1 progeny of plate and sRNA trained parent worms. a–**c**, Progeny of *sid-1* (**a**), *sid-2* (**b**) and *dcr-1* (**c**) mutants are defective in both transgenerational pathogen avoidance following maternal bacterial lawn (left) and PA14 sRNA training (right). Biological replicates: 3 (**a**–**c**). For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) with data shown from all replicates. For box plots, centre line is the median, box extends from the 25th to the 75th percentile; whiskers denote minimum–maximum values. Two-way ANOVA (**a**–**c**), Tukey's multiple comparison test. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P < 0.0001$, n.s., not significant. Mean differences are shown using Cumming estimation plots[47], with each graphed as a bootstrap sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical bars. See Supplementary Table 4 for exact sample sizes (*n*) and *P* values.



Extended Data Fig. 5 | RNA sequencing of sRNAs from PA14. a, sRNA training protocol using RNA isolated from PA14 cultures grown at 25 °C or 15 °C on plates, or in liquid culture. **b**, Principal component analysis of PA14 sRNA sequencing. **c**, Upregulated sRNAs in 25-°C grown PA14 compared to 15 °C-grown and liquid-grown PA14 sRNAs. The outermost grey circle represents the PA14 genome, with previously identified sRNAs that were upregulated (DESeq2, adjusted $P \leq 0.05$) in the 25-°C plate condition relative to 15-°C plate grown PA14 (pink) or liquid-grown PA14 (green) are indicated. Overlapping sRNAs upregulated in the 25-°C plate condition relative to both other conditions are noted (blue dots). sRNAs annotated with white lines. **d**, Venn diagram showing the number of PA14 sRNAs upregulated at 25-°C growth conditions compared to liquid growth (blue circle) or 15-°C growth (red circle). The six shared upregulated sRNAs are shown.



Extended Data Fig. 6 | Identification and testing of the differentially regulated PA14 sRNA. a, P11 expressed in *E. coli* induces PA14 avoidance in a choice assay between PA14 and *E. coli* strain MG1655. Worms were trained on OP50, PA14, *E. coli* strain MG1655 containing empty vector (control), or *E. coli* strain MG1655 expressing PA14 small RNAs (red). Following training, a choice assay was performed between *E. coli* strain MG1655 and PA14. Similar to Fig. 3c, worms trained on PA14 or *E. coli* expressing P11 exhibited PA14 avoidance behaviour. One-way ANOVA with Tukey's multiple comparison test. **b**, PA14 plate-trained worms do not avoid *E. coli* expressing P11 compared to OP50 in a choice assay. Two-tailed Student's *t*-test. **c**, PA14 ΔP11 bacteria grown on NGM plates produce pyocyanin (blue pigment on plates) similar to PA14. **d**, **e**, Model showing induced learning pathways for wild-type worms on isolated PA14 ΔP11 sRNA (**d**) or on PA14 ΔP11 bacteria lawns (**e**). **f**, Exposure to *E. coli* expressing P11 does not affect total brood size (left) (two-tailed Student's *t*-test), or the number of progeny hatched per day (right). Two-way ANOVA with Tukey's multiple comparison test. $n = 15$ worms were analysed per condition. **g**, Worms exposed to control (top) or *E. coli* expressing P11 appear healthy after 24 h of training. Biological replicates: 2 (**a**), 3 (**b**, **c**, **g**). For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) with data shown from all replicates. For box plots, centre line is the median, box extends from the 25th to the 75th percentile; whiskers denote minimum–maximum values. $^{**}P \leq 0.01$, $^{****}P < 0.0001$, n.s., not significant. Mean differences are shown using Cumming estimation plots[47], with each graphed as a bootstrap sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical bars. See Supplementary Table 4 for exact sample sizes (n) and P values.



Extended Data Fig. 7 | See next page for caption.

Extended Data Fig. 7 | P11 region of homology with *maco-1* and behaviour of *vhp-1* mutants. a, Wormbase depiction of the *maco-1* genomic locus and the region of P11 homology (red). **b**, Model depicting induced learning pathways in wild-type or *maco-1* mutant worms as a result of OP50 or PA14 lawn exposure. **c**, F_1 progeny of OP50 or PA14 sRNA-exposed *maco-1* mutant mothers were tested for PA14 avoidance behaviour. **d**, *vhp-1*expression is increased in PA14-exposed mothers, but not in F_1 progeny[3]. Mean; DESeq2 adjusted *P* values are shown. P_0, $n = 6$ replicates per condition; F_1, $n = 4$ replicates per condition. **e**, The *vhp-1(sa336)* mutation is a null allele (early stop codon)[40]. Wild-type and *vhp-1(sa366)* worms were trained on OP50 or PA14 bacterial lawns for 24 h and tested for learned PA14 avoidance (top). **f**, Wild-type and *vhp-1(sa366)* worms were trained on OP50 or PA14 sRNA for 24 h and tested for learned PA14 avoidance. **g**, Progeny of *vhp-1(sa366)* PA14 lawn-trained worms inherit PA14 avoidance behaviour. **h**, F_1 progeny of OP50 or PA14 lawn-exposed *maco-1* mutant mothers were tested for PA14 avoidance behaviour. Biological replicates: 3 (**c**, **e**–**h**). For choice assays, each dot represents an individual choice assay plate (average of 115 worms per plate) with data shown from all replicates. For box plots, centre line is the median, box extends from the 25th to the 75th percentile; whiskers denote minimum–maximum values. Two-way ANOVA (**c**, **e**–**h**), Tukey's multiple comparison test. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P < 0.0001$, n.s., not significant. Mean differences are shown using Cumming estimation plots[47], with each graphed as a bootstrap sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical bars. See Supplementary Table 4 for exact sample sizes (*n*) and *P* values.

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Data exclusions	Any choice assay plates containing fewer than 10 worms were excluded from any condition tested. This was done to improve reliability of the calculated choice indices. Based on this study exclusion criteria were previously established.
Replication	Experiments were repeated independently at least three times.
Randomization	Populations of animals were raised together under identical conditions and were randomly distributed into treatment conditions. Trained animals were pooled and randomly chosen for choice assays.
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Laboratory animals	Caenorhabditis elegans, hermaphrodites, age L4-Day 1 (Wild type N2 Bristol). All mutations used are in the N2 Bristol background.
Wild animals	The study did not involve wild animals.
Field-collected samples	The study did not involve samples collected from the field.
Ethics oversight	No ethical approval was required.

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