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Alanine tRNAs translate environment into behavior in *Caenorhabditis elegans*

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Abstract

Caenorhabditis elegans **worms produce and keep imprints of the attractive chemosensory cues to which they are exposed early in life. Imprinting enhances transiently or permanently chemoattraction to early olfactory cues.**

Here we report olfactory imprinting can be transferred from odor-exposed to naive worms via RNA feeding. Biochemical fractionation identified the tRNA^{Ala} (UGC) as the olfactory imprinting molecule. Indeed tRNA^{Ala} (UGC) extracted from odor-exposed woms transfers odor-specific imprints, suggesting odor-stimuli trigger tRNA^{Ala} (UGC) changes. Furthermore mutations in the Elongator complex sub-units 1 or 3 either impair chemo-attractive behavior or stably suppress odor-specific responses upon odor-exposure. Naive wild-type tRNA^{Ala} (UGC) rescue the behavioral phenotypes of Elongator mutant worms. tRNA^{Ala} (UGC) changes triggered by the Elongator complex and the early olfactory environment control both the innate and the experience-modulated *C. elegans* chemoattractive behavior.

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Introduction

Imprinting is an evolutionary conserved form of adaptive plasticity that shapes animal behaviors. It elicits a life-long attachment to external sensory stimuli experienced during an early critical stage of development.

Olfactory imprinting happens in the *C. elegans* worms. A worm exposed to an olfactory cue during a critical period of 12 hours following its hatch, produces life-long imprints that enhance its adult chemosensory response towards this same cue (***Remy and Hobert, 2005***). We have previously shown these imprints are transmitted to the F1 in the absence of the olfactory cue, but not to the F2 generation.

Moreover, if five successive generations experience the same olfactory stimuli during their critical period, imprints are fixed and become stably inherited (***Remy, 2010***). Such trans-generational transmission strongly suggests that odor-imprints produced upon odor-exposure are diffusible messages able to reach worm progeny.

Recent studies show that non-coding RNAs are involved in odor desensitization (***Juang et al., 2013***), and in the trans-generational transfer of responses to starving conditions in *C. elegans* (***Rechavi et al., 2014***). We therefore hypothesized that RNA could convey and transfer the olfactory imprinting information.

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In this study, we show that imprinting is indeed transferred via feeding naive worms on small RNA extracted from odor-exposed animals. We found that such horizontal transmission requires the intestinal double-stranded RNA transporter SID-2.

Using biochemical methods and behavioral screening, we identified the alanine transfer RNAs (tRNA) bearing the 5'-UGC-3' anticodon, or $tRNA^{Ala}$ (UGC), as the unique molecular mediator of olfactory imprinting in *C. elegans*.

Results

We first wanted to test if RNAs could convey olfactory imprints. Olfactory imprinting was induced, as previously described (***Remy and Hobert, 2005***), after exposure to two different olfactory cues, citronellol (CI) or benzaldehyde (BA) (*Figure 1a*, odor-exposure). The efficiency of this induction was tested using the chemotaxis assay described in Methods and ***Figure supplement method***. Total RNA was then extracted from CI-exposed, BA-exposed or water-exposed control worms. These RNAs were fed to naive larvae (***Figure 1a***, RNA-feeding). Once RNA-fed larvae reached the adulthood, they were subjected to the chemotaxis assays. We observed the naive worms fed on RNA from CI or BA-exposed worms migrate significantly faster toward CI or BA, as if they had been themselves odor-exposed (***Figure 1b***).

Olfactory imprinting can therefore be elicited by the exogenous addition of RNA from odor-exposed animals. This observation shows that worms exposed to odors

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during an early critical period produce RNA populations able to transfer an alteration of chemosensory responses.

To investigate if olfactory imprinting via feeding requires uptake and diffusion of RNA, we studied the behavior of worms that do not express one of two *C. elegans* double-stranded RNA specific transporters SID-1 or SID-2 (***Jose et al., 2009***). While SID-2 exclusively localizes to the apical membrane of intestinal cells, SID-1 is more widely expressed through most non-neuronal cells. SID-2 is responsible for the initial binding and internalization of dsRNA from the intestinal lumen (***McEwan et al., 2012***).

We found that both the null *sid-1 (qt2)* and the substitution *sid-2 (qt13)* mutant worms are able to imprint CI after early exposure to the olfactory cue (***Figure supplement 1a***). Furthermore, when fed on RNA from CI-exposed wild-type (wt) worms, the naive *sid-1 (qt2)* worms acquire the CI imprinting behavior (***Figure supplement 1b***). By contrast, worms without a functional SID-2 do not acquire a CI imprint via RNA feeding (***Figure supplement 1b***). Imprinting via RNA feeding thus requires the intestine-specific double-stranded RNA transporter SID-2.

To identify which RNAs are able to transfer odor imprints, we started by separating the large from the small RNAs. We studied the transfer of BA imprinting by feeding naive worms either the “large RNAs fraction” or the “small RNAs fraction”. We observed that only RNAs smaller than 200 nucleotides (nt) are able to trigger

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imprinting (***Figure 2a***). Migration on 3.5% agarose gels separates small RNAs into five fractions (A to E bands, insert on ***Figure 2b***). After RNA-feeding, we observed that the BA imprint was exclusively transmitted to naive worms by the "D" small RNA fraction (***Figure 2b***). Based on co-migration with a double-stranded RNA ladder (L), the "D" RNA population migrates with an apparent mean size of 45 nt on this non-denaturing electrophoresis condition.

Further fractionation on denaturing 7 M urea-15 % polyacrylamide gel (***Figure 3a)*** revealed that the RNA populations "D" is composed of several RNA species we named fraction 1 to fraction 8. We noticed fractions 2 to 7 represent a typical profile of tRNAs (***Figure 3a, left panel***). We therefore performed Northern-blot analyses using two specific *C. elegans* $tRNA^{Gly}$ (UCC) and $tRNA^{Leu}$ (AGG) probes, in order to confirm that the fractions 2 to 7 indeed contain the *C. elegans* tRNA population (***Figure 3a, right panel***).

Each of the 1 to 8 fractions from CI-exposed and from Naive unexposed worms were cut and eluted from the gels. The capacity to transfer a CI imprint was then assayed via feeding naive worms either on the whole "D"-RNA (mixed fractions 1 to 8), on the whole tRNAs population (mixed fractions 2 to 7), or on each fraction, separately (***Figure 3b***). The two 1 to 8 and 2 to 7 mixes extracted from CI-exposed (CI Mix 1-8 and CI Mix 2-7) transferred a CI-specific imprint to naive worms, while the corresponding NA mixes (NA Mix 1-8 and NA Mix 2-7) did not (***Figure 3b***).

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After detailed fraction by fraction analyses, we have found that only the tRNA molecules that co-purify with fraction 5 and 6 significantly enhance migration to CI in naive worms (***Figure 3c***). We however observed a negative effect on CI chemoattraction after feeding fraction 4. Same results were obtained for the transfer of BA imprinting (not shown).

In order to determine the minimal CI-tRNA quantity required to induce such odor-specific behavioral change, CI-tRNA amounts fed to naive were reduced. We found that as low as one femtogram of tRNA, which corresponds to about $2.4x10^4$ tRNA molecules, still efficiently transferred the CI-imprinting behavior to naive worms ***(Figure supplemental 3a).***

This high efficiency suggests that an amplification step might be necessary for tRNA-mediated imprinting. RNA-dependent RNA polymerases (RdRP) are enzymes potentially able to trigger such amplifications (***Duchaine et al., 2006***). In order to assess if *C. elegans* RdRPs are involved in olfactory imprinting, we have studied the establishment of imprinting in worms displaying mutations in the two RdRPs *rrf-1* and *rrf-3* genes. The *rrf-1 (ok589)* and *rrf-1 (pk1417)* mutant worms display a wt N2 olfactory imprinting behavior after odor-exposure. By contrast, the *rrf-3 (pk1426)* mutants are unable to achieve olfactory imprinting. This result suggests an amplification step via the putative RRF-3 RdRP activity is required for the establishment and the transfer of olfactory imprints ***(Figure supplemental 3b).***

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The tRNAs able to transfer the olfactory imprinting behavior to naive might also be able to recapitulate the inheritance pattern of odor-triggered imprinting (***Remy, 2010***). To assess this question, we fed naive N2 worms through seven successive generations on tRNAs fractions able to transmit a CI imprint (CI-tRNAs Mix 2-7 and CI-tRNAs fraction 5). As schematically outlined (***Figure 4a***), we compared the CI responses of the seven tRNAs fed generations and of their naive progeny grown without addition of tRNAs up till the fourth generation. We found that a CI imprint is passed to the first but not to the second naive generation issued from one to five generations of CI-tRNA fed animals. However, CI imprinting is maintained up till the fourth naive generation issued from worms fed on CI-tRNA at least for six successive generations (***Figure 4b***). As negative control, five and six worm generations were also fed on fraction 4 tRNAs which slightly decrease CI response (***Figure 3c***).

In order to assess the stability of inheritance after the 6th tRNA fed generation, we grew more generations without adding tRNAs to worm food.

We found tRNA-triggered imprinting is stably inherited at least until the 19th generation grown without tRNAs in the progeny (***Figure 4c***). Multigenerational feeding on a specific tRNA fraction extracted from odor-exposed worms therefore elicit the same long-term stable behavioral changes as multigenerational odor-exposure.

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We next asked which tRNA molecules could be present in the tRNA-imprinting fractions 5 and 6.

596 functional tRNA genes have been predicted in the *C. elegans* genome (***Duret, 2000; Chan and Lowe, 2009***). To be fully active, tRNAs need to be heavily modified post-transcriptionally (***Agris, 2015; Duechler et al., 2016***). Indeed, the high abundance of tRNA post-transcriptional modifications interfere with reverse-transcription and cDNA synthesis, making tRNA sequencing very challenging. In order to obviate these limitations, alternative methods have been proposed in the recent literature (***Pang et al., 2014; Zheng et al., 2015; Cozen et al., 2015***), however none of them is readily available. Instead of a sequencing strategy, we used biochemical approaches to identify the imprinting tRNAs. We combined streptavidin microbeads purification with Northern blot analysis. This approach revealed the $tRNA^{Ala}$ (UGC) is the unique imprinting tRNA molecule (***Figure 5a***).

For streptavidin microbeads purification, we designed 37 nucleotide-long biotinylated tDNA probes corresponding to the 3' halves of tRNAs, based on the Genomic tRNA database (GtRNAdb) information (described in Methods).

These probes cover the whole set of isoacceptor tRNAs (with anticodons that translate the same aminoacid) and isodecoder tRNAs (with the same anticodon, but a different body of sequence) *C. elegans* genes. For Northern analysis, we designed shorter isoacceptor-specific tDNA probes (described in Methods).

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We screened first for the presence of the imprinting tRNAs using pooled tRNAs eluted from isotype-specific probes. We reasoned that if a tRNAs population purified from a pool of probes do transfer imprinting, this population should contain the imprinting tRNAs. This screen revealed that tRNAs eluted from any pool containing the Alanine tRNAs probes transferred imprinting.

In the example shown in ***Figure 5b (upper panel)***, CI-exposed fraction 5 was subjected to purification by pooled microbeads bound to the six isotype-specific probes for $tRNA^{Glu}$, $tRNA^{Lys}$, $tRNA^{Cys}$, $tRNA^{Gln}$, $tRNA^{Ala}$ and $tRNA^{Leu}$.

The tRNAs eluted from this pool transfer a CI imprint to naive (not shown). We then proceeded with isotype-specific purifications and found that only the tRNAs eluted from $tRNA^{Ala}$ specific probes are able to imprint naive worms.

$tRNA^{Ala}$ specific probes include the 3'-tRNA probes for the three different $tRNA^{Ala}$ isoacceptors used by *C. elegans*, $tRNA^{Ala}$ (AGC), $tRNA^{Ala}$ (CGC) and $tRNA^{Ala}$ (UGC).

To further discriminate between these Alanine tRNAs isoacceptors, we used isoacceptor-specific probes. tRNAs bound to the two probes corresponding to, respectively, the 5' and the 3' halves of the $tRNA^{Ala}$ (UGC) transfer imprinting to naive worms. By contrast, tRNAs from microbeads bearing the $tRNA^{Ala}$ (AGC) or the $tRNA^{Ala}$ (CGC) probes, do not transfer imprinting (***Figure 5b, lower panel)***.

The whole set of tRNAs from naive (NA), CI-exposed (CI) and BA-exposed (BA)

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has been gel-fractionated into 6 fractions (2 to 7). We showed imprinting is only transferred by the tRNAs from fractions 5 and 6 ***(Figure 3c)***. Several types of tRNA molecules are expected to co-migrate in each fraction, including in the imprinting fractions. To identify which tRNAs are present in each fraction, we performed Northern blot analysis. We show here the analysis of naive (N3 to N6), CI-exposed (C3 to C6) and BA-exposed (B4 to B7) fractions using probes for most tRNA isotypes probes used in ***Figure 5b, upper panel***.

Northern blots clearly indicate that while $tRNA^{Leu}$ co-migrate with fractions 3, $tRNA^{Lys}$ migrates mostly in fractions 6, but are also present in fractions 5, and $tRNA^{Gln}$ co-migrate with $tRNA^{Glu}$ mostly in fraction 7, but are also present in fractions 6. The only tRNAs found to be mostly in fractions 5, while also present in fractions 6 are the $tRNAs^{Ala}$ (***Figure 5c***). Northern blot analysis demonstrates the presence of $tRNA^{Ala}$ in the imprinting fractions 5 and 6, which is consistent with the microbeads purification results.

Strikingly, a unique codon-specific tRNA molecule seems to trigger chemo-attraction increase toward two different odorant molecules. To further prove this tRNA does transfer odor-specific imprints, we fed naive on $tRNA^{Ala}$ (UGC) puirified from naive, from CI-exposed and from BA-exposed fractions 5. As shown in ***Figure 5d,*** naive $tRNA^{Ala}$ (UGC) does not alter CI or BA chemo-attraction, while $tRNA^{Ala}$ (UGC) from odor-exposed animals enhance a specific chemo-attraction.

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We hypothesized the $tRNA^{Ala}$ (UGC) might be chemically modified according to the specific odor worms were experienced.

The bases of tRNAs are extensively modified (***Machnicka et al., 2012***). Amongst the most studied tRNA chemical modifications are those affecting Uridine at the wobble position, the first base of the anticodon. These modifications are considered of critical functional importance in codon-anticodon recognition as they might improve tRNA aminoacylation kinetics and prevent translational frame-shifts (***Larsen et al., 2015; Nedialkova and Leidel, 2015; Deng et al., 2015***). Wobble uridine modifications are thought to be modulated by the activity of Elongator (ELP) (***Karlsborn et al., 2014***) a dodecamer complex made of two copies of six different ELP-1 to ELP-6 subunits (***Glatt et al., 2012, Dauden et al., 2017***). Two studies reported behavioral phenotypes in *C. elegans* bearing mutations on the genes encoding the Elongator subunits 1 (*elpc-1*) and 3 (*elpc-3*) (***Chen et al., 2009; Solinger et al., 2010***). As $tRNA^{Ala}$ (UGC) contains a U at the wobble position, we therefore asked if Elongator mutations could also affect the *C. elegans* imprinting behavior. We compared the chemo-attractive responses of the *elpc-1 (ng10), elpc-3 (ng15)* and *elpc-3 (tm3120)* mutant worms to the chemo-attractive responses of wt N2 worms (***Figure 6a***). We assessed chemo-attraction to two odor dilutions (CI and BA 1/300 and 1/200) at two different adult ages (3 and 4 days old). The *elpc-3 (tm3120)* mutants display significantly reduced chemo-attractive responses to all odor dilutions at both tested ages, compared to N2.

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By contrast, the *elpc-1 (ng10)* and *elpc-3 (ng15)* mutant worms show a wt chemoattraction (***Figure 6a***). We therefore assessed olfactory imprinting in *elpc-1 (ng10)* and *elpc-3 (ng15)* worms and found that, as opposed to wt, an early CI-exposure significantly decreases adult CI responses, compared to naive (***Figure 6b***).

These results indicate the 355 bp deletion in the Elongator sub-unit 3 gene in *elpc-3 (tm3120)* mutants impairs the implementation of chemo-attractive responses. By contrast, the *elpc-3* R327C substitution in *elpc-3 (ng15)* mutants or the 2050 bp deletion of *elpc-1* in *elpc-1 (ng10)* mutants allows a wt development of chemoattraction.

However, the two *elpc-1 (ng10)* and *elpc-3 (ng15)* mutations impair olfactory imprinting. In theses mutants, the outcome of early odor-exposures on adult chemosensory responses is to decrease odor-specific chemo-attraction, as opposed to wt. Moreover, we found this decrease stably inherited over generations, at least until the 7th unexposed generation (***Figure 6c***).

In the wt worms, odor-exposure or feeding on $tRNA^{Ala}$ (UGC) from odor-exposed, trigger the same behavioral change: both trigger a specific enhancement of chemoattraction ***(Figure 5)***. To further demonstrate odors and $tRNA^{Ala}$ (UGC) from odor-exposed indeed convey the same behavioral effects, we fed the *elpc-1 (ng10)* and *elpc-3 (ng15)* mutant worms on $tRNA^{Ala}$ (UGC). We found that $tRNA^{Ala}$ (UGC) - but not $tRNA^{Ala}$ (AGC) or $tRNA^{Ala}$ (CGC) - from odor-exposed decrease chemo-

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attraction in these mutants, as does odor-exposure (***Figure 6d***).

Depending on Elongator complex activities, exposure to odors or to $tRNA^{Ala}$ (UGC) from odor-exposed wild-type can stably alter *C. elegans* odor-specific responses in two opposite ways.

Our results suggest ELPC play a role in the odor-triggered chemical modifications of $tRNA^{Ala}$ (UGC). To further address this question, we purified $tRNA^{Ala}$ (UGC) from naive and CI-exposed N2 wt, *elpc-1 (ng10),* and *elpc-3 (ng15)* worms. The behavioral effects of these $tRNA^{Ala}$ (UGC) were assessed via feeding naive N2, naive *elpc-1 (ng10)* and naive *elpc-3 (ng15)*.

We observed that $tRNA^{Ala}$ (UGC) purified from CI-exposed wt, *elpc-1 (ng10)* or *elpc-3 (ng15)* increased CI chemo-attraction of wt N2 worms (***Figure 7***, strain tested: N2).

The same tRNA molecule by contrast decreased CI chemo-attraction of the *elpc-1 (ng10)* and the *elpc-3 (ng15)* mutant worms (***Figure 7***, strains tested: N2, *elpc-1 (ng10)* and *elpc-3 (ng15)*).

The same pattern of responses was obtained after BA exposure, meaning $tRNA^{Ala}$ (UGC) from BA-exposed wt, *elpc-1 (ng10)* and *elpc-3 (ng15)* increased BA chemo-attraction in wt while they decreased BA chemo-attraction in *elpc-1 (ng10)* and *elpc-3 (ng15)* (data not shown). **Figure 7** data indicate $tRNA^{Ala}$ (UGC) from odor-exposed wt and from odor-exposed *elpc* mutants transfer the same behavioral

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changes to naive animals. It seems thus that the ELPC is not involved in the odor-triggered modifications of $tRNA^{Ala}$ (UGC). Rather, ELPC might be involved downstream odor-modified $tRNA^{Ala}$ (UGC) to control olfactory imprinting.

It has been shown that the yeast Elongator complex interacts with tRNAs (***Glatt et al., 2012, Dauden et al. 2017***).

Our results suggest a $tRNA^{Ala}$ (UGC) chemical form supports the development of worms chemo-attractive responses. This form would be made by the wt worms, by the *elpc-1 (ng10)* and by the *elpc-3 (ng15)* worms, but not by the *elpc-3 (tm3120)* worms. Upon odor-exposure, the same odor-modified forms of $tRNA^{Ala}$ (UGC) might be produced by wt, by *elpc-1 (ng10)* and by *elpc-3 (ng15)*. The Elongator activity defects in the two later mutants however does not allow olfactory imprinting: instead, odors and odor-modified forms of $tRNA^{Ala}$ (UGC) stably suppress odor-specific responses in these mutant worms.

We therefore hypothesize that the Elongator complex could be both involved in the synthesis of the $tRNA^{Ala}$ (UGC) wt form, and downstream the odor-modified $tRNA^{Ala}$ (UGC) forms to trigger imprinting.

To further address these questions, we have proceeded to rescue the Elongator mutants phenotypes by feeding on $tRNA^{Ala}$ (UGC) from wt naive N2 worms.

We found that feeding *elpc-3 (tm3120)* mutants on $tRNA^{Ala}$ (UGC) extracted from the whole tRNA population (N2 naive tRNAs) or from the tRNA fraction 5 of naive (N5

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fraction) fully restored a wild-type chemoattractive response to CI and to BA in these mutants ***(Figure 8a)***. We next asked if the negative olfactory imprinting phenotype we have seen in the *elpc-1 (ng10)* and *elpc-3 (ng15)* mutants can be also rescued by the same form of $tRNA^{Ala}$ (UGC). We observed that the presence of wt naive $tRNA^{Ala}$ (UGC) during odor-exposure indeed rescued CI chemo-attraction in CI-exposed *elpc-1 (ng10)* mutants ***(Figure 8b)*** and rescued BA chemo-attraction in BA-exposed *elpc-3 (ng15)* mutants ***(Figure 8c)***.

We performed rescue experiments using the $tRNA^{Ala}$ (UGC) form produced by the naive *elpc-3 (tm3120)* mutant worms. We found that this form is unable to oppose the reppressive effect of CI-exposure on *elpc-1 (ng10)* CI chemoattraction (***Figure Supplement 8a***). Moreover, when provided during the development of N2 wt animals, it inhibit the development of CI and BA chemo-attraction. We observed the same inhibiting effects on wt N2 chemoattraction after feeding on $tRNA^{Ala}$ (UGC) extracted from CI-exposed or from BA-exposed *elpc-3 (tm3120)* mutants (***Figure Supplement 8b).*** The odor-exposed *elpc-3 (tm3120)* mutants are unable to produce the odor-modified forms of $tRNA^{Ala}$ (UGC) with imprinting transfer activity. These imprinting forms might thus be made from the naive wt form and would not be made from the naive *elpc-3 (tm3120)* mutant form.

Our data suggest worms olfactory neurons produce odor-specific chemically modified $tRNA^{Ala}$ (UGC) upon attractive odorants stimulations. The *C. elegans* genome

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encodes around 1300 functional seven transmembrane receptors, presumably coupled to G-proteins. These receptors, when expressed in chemosensory neurons, are thought to support odor specificity of the chemoattractive responses *(**Hart et al., 2010**)*.

In *C. elegans*, only two pairs of chemosensory neurons, AWA and AWC, are required for chemotaxis to volatile attractants (***Bargmann, 1993***). The major signal transduction pathway utilised by the AWC sensory neurons appears to be through the DAF-11/ODR-1 guanylyl cyclases and the TAX-2/TAX-4 cGMP-gated channels (***Bargmann, 2006***). ODR-1 is required downstream all AWC-expressed odorant receptors and the *odr-1 (n1936)* mutants are defective for all AWC-mediated chemosensory responses.

We hypothesised worms defective for the transduction of odor signals into behaviors would not be able to couple odor stimuli to modification the $tRNA^{Ala}$ (UGC) molecules. In order to test this hypothesis we first analysed the chemotaxis behavior in *odr-1 (n1936)* mutants. These worms display an impaired chemotaxis to the two attractive odorants benzaldehyde (BA) and beta-citronellol (CI) at the dilutions used in this study ***(Figure 9a)***, suggesting BA and CI olfactory receptors are expressed by the same AWC chemosensory neurons. We then compared the behavioral activity of the $tRNA^{Ala}$ (UGC) extracted from CI-exposed wt worms to the activity of the $tRNA^{Ala}$ (UGC) extracted from CI-exposed *odr-1 (n1936)*. We found $tRNA^{Ala}$ (UGC) produced by CI-stimulated *odr-1 (n1936)* mutants are unable to transfer a CI imprint

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to naive animals: they do not enhance CI attraction in wt and do not decrease CI attraction in *elpc* mutants (***Figure 9b***). Same results were obtained after BA-exposure (not shown). This data suggest the AWC-specific guanylate-cyclase ODR-1 might be required for odor-triggered modifications leading to the imprinting forms of $tRNA^{Ala}$ (UGC).

Based on the present data, we hypothesize the model shown in ***Figure 10***. Attractive odorants bind putative specific membrane G-protein coupled chemoreceptors expressed by the olfactory receptor neurons AWC. Odor to receptor interaction activates the AWC-specific olfactory transduction pathway that includes a trimeric G protein and the guanylate-cyclase ODR-1 which controls the levels of cyclic-GMP (cGMP) and the cGMP-gated ion channel TAX-2/TAX-4.

Naive unexposed wt N2 worms produce the Naive form of $tRNA^{Ala}$ (UGC) required for the development of the wt chemo-attractive behavior. The Elongator sub-unit 3 might be required to produce the Naive form of $tRNA^{Ala}$ (UGC).

Each odor might trigger specific chemical modifications of the naive form of $tRNA^{Ala}$ (UGC). These alterations require a functional olfactory transduction pathway. Transient or stable enhancement of odor-specific responses resulting from imprinting are equally triggered by early odor-exposure or by feeding odor-modified $tRNA^{Ala}$ (UGC). The *elpc-1 (ng10)* and *elpc-3 (ng15)* elongator mutant worms produce the naive form of $tRNA^{Ala}$ (UGC). They implement chemoattraction as wt, and

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synthesize odor-specific forms of $tRNA^{Ala}$ (UGC). They are however unable to imprint, suggesting imprinting also require Elongator complex functions. In these imprinting-deficient mutants, odor-exposure and feeding odor-modified $tRNA^{Ala}$ (UGC) result in stably inherited suppression of odor-specific responses.

Discussion

Our study relies on the ability some RNA molecules have to enter the *C. elegans* body via feeding, using specific RNA transporters present at the lumen of intestinal cells. We show here olfactory imprinting through RNA feeding requires the SID-2 intestinal protein. It has been demonstrated that SID-2 selectively allows the transfer of large (at least 25 bp) double-stranded RNAs, while it does not mediate the uptake of short double-stranded RNAs, single-stranded RNAs or of the 60 nt long pri-miRNAs (***McEwan et al., 2012***). The inherited behavioral changes we describe here are clearly elicited only by mature tRNAs. Indeed, RNA fractions of lower sizes are unable to transfer imprinting: these fractions include all the known small non-coding RNA (miRNA, piRNA and siRNA) as well as putative tRNA fragments. We cannot exclude however that imprinting requires the processing of ingested mature tRNAs into fragments (***Megel et al., 2015***).

Further fractionation of tRNAs from odor-exposed worms indicated identical tRNA fractions are able to transfer both benzaldehyde or citronellol imprints to naive unexposed animals. Furthermore, the use of tRNA-specific probes in Northern blots

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and affinity purification analyses revealed that odor-specific imprinting is exclusively transferred by the tRNA molecule which translates the 3'-ACG-5' codon into Alanine.

We found this $tRNA^{Ala}$ (UGC) controls *C. elegans* chemoattractive responses together with the Elongator complex. Although not yet demonstrated at the molecular level, our findings suggest the chemical structure of $tRNA^{Ala}$ (UGC) can be modified by the Elongator complex and by odor-stimuli. The behavior of the *elpc-3 (tm3120)* mutants suggest Elongator would be responsible for producing the wt naive $tRNA^{Ala}$ (UGC) structure required for the developpement of chemoattraction.

Odor-exposure would alter the $tRNA^{Ala}$ (UGC) chemical structure in an odor-specific way. However, the behavioral issues of such odor-specific modifications might be elongator-dependent. Odor-modified $tRNA^{Ala}$ (UGC) enhances chemoattraction in wild-type, while it decreases chemoattraction in *elpc-1 (ng10)* and *elpc-3 (ng15)* mutants. Both behavioral changes can be fixed in the progeny, indicating the involvement of epigenetic mechanisms leading to stable inheritance.

More investigations are necessary to describe the mechanisms by which odors and Elongator would modify the chemical structure of this tRNA molecule and how these modifications could stably alter odor-specific responses.

In *C. elegans*, as in mammalian species, olfactory transduction takes place in olfactory receptor neurons cilia. Benzaldehyde and citronellol would bind to yet

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unknown seven transmembrane G-protein coupled olfactory receptors (OR) present in the chemosensory neurons AWC cilia membranes. We showed odor-induced $tRNA^{Ala}$ (UGC) modifications may occur dowstream the ODR-1 guanylate-cyclase in the odor-transduction pathway. In AWC, different G-alpha proteins, ODR-3, GPA-2, GPA-3 and GPA-13 (***Lans et al., 2004***) transduce odor signals to the guanylate cyclases ODR-1/DAF-11, regulating cGMP levels which in turn control the opening of the cyclic-nucleotide gated ion channel TAX-2/TAX-4 (***Bargmann, 2006***). Worms with the *tax-2 (p671)* and *tax-4 (p678)* mutations affecting respectively the beta and alpha subunit of the TAX-2/TAX-4 channel, do not produce CI-imprinting tRNAs upon CI-exposure (data not shown), suggesting odor-induced tRNA changes may require a functional cGMP-gated channel. How tRNA modifications could be coupled to sensory signaling remains to be discovered. However, the odor-specificity of odor-induced and of tRNA-triggered imprinting supports the idea of a spatial insulation of odor signaling in AWC cilia, as already suggested (***L'Etoile and Bargmann, 2000***).

It would be of interest to see whether other AWC-sensed attractive odorants also trigger the production of odor-specific forms of $tRNA^{Ala}$ (UGC) able to mediate odor-specific behavioral changes.

Up or down-regulation of odor-specific responses could involve the differential expression of specific olfactory receptors (OR). The genome of *C. elegans* encodes more than 1300 GPCRs, many of which are believed to be chemoreceptors

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(***Robertson and Thomas, 2006***). The nematode respond to many volatil odorants but has only three pairs of olfactory neurons, suggesting each neuron would express a large number of chemoreceptors. The way each worm's odor-responsive neuron expresses specific sets of OR, and represses the expression of all others, has never been investigated.

In mammalian species, each olfactory neuron express only one allele of a member of the OR gene family; the precise mechanisms by which such monogenic monoallelic receptor choice is achieved is not fully undestood. These mechanisms involve nuclear compartimentalization, repression/derepression through epigenetic marks made by the chromatin-modifying enzyme Lysine demethylase 1 (LSD-1) and stabilization of functionally expressed OR by the odor-signaling pathway (reviewed in ***Degl'Innocenti A and D'Errico A, 2017***). Whether tRNAs participate to OR choice in mammalian olfactory neurons has not been investigated. Conversely, worms might use identical epigenetic and activity-dependent mechanisms to drive OR choice and expression in chemo-sensory neurons.

Alanine tRNAs have distinctive features that could have important functional consequences. Human 5'-tiRNA^{Ala} fragments, produced after cleavage of mature tRNA^{Ala} within the anticodon loop by angiogenin, inhibit translation via the displacement of the m7GTP cap structures from cap-binding translation initiation complexes (***Lyons et al., 2017***). An angiogenin-like enzymatic activity leading to the

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formation of 5'-tiRNA^{Ala} fragments, has however not been yet described in *C. elegans*. In all species, tRNA^{Ala} display streches of consecutive guanine residues at their 5' ends. This conserved motif is believed to favor the formation of intermolecular G-quadruplex structures (***Lyons et al., 2017***). The existence of G-quadruplex structures in worm's tRNA^{Ala} might be of importance regarding the epigenetic control of chemo-attractive behavior. Indeed the chromatin-remodelling Lysine Demethylase-1 (LSD-1), the main epigenetic regulator of olfactory receptor choice in mouse olfactory neurons, interacts with RNA G-quadruplexes (***Lyons et al., 2013***).

C. elegans produces three Alanine tRNA with the respective anticodons 5'-AGC-3', 5'-CGC-3', and 5'-UGC-3'. The chemoattractive behavior is controlled only by the later tRNA^{Ala} with an Uridine at the first 5' anticodon base, the wobble position.

The presence of un-modified U or A at the wobble position allows non-canonical Watson-Crick base pairing, increasing the number of mRNA codons recognized by a single tRNA molecule. Modified nucleosides in the anticodon region, especially at the wobble position, have been suggested to be important for restriction of codon-anticodon interactions (***Gustillo et al., 2008***). The Lysine, Glutamine and Glutamic acid tRNAs isolated from *elpc-3* (*tm3120*) mutants lack the mcm5s2U and ncm5U nucleosides which instead contain s2U (***Chen et al., 2009***). Elongator sub-units 1 and 3 are required for the addition of the mcm5 and ncm5 side chains to wobble uridine residues in these three tRNAs. How *C. elegans* Alanine tRNAs Uridines are modified

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is unknown. Besides at the wobble position, Uridines are however also present at positions 31, 32 and 37 in the anticodon loop of $tRNA^{Ala}$ (UGC).

We showed the BA and CI chemoattraction defect of *elpc-3* (*tm3120*) mutants can be fully rescued via feeding on the wild-type naive form of $tRNA^{Ala}$ (UGC). *elpc-3* (*tm3120*) is a 355 bp deletion in *elpc-3* which supresses both the ELPC-3 C-terminal Lysine Acetyl-Transferase (KAT) and SAM domains. SAM domain proteins have been indeed involved in the activity of several tRNA-modifying enzymes as the methyl-thiotransferases YqeV and MiaB or the 4-demethylwyosine synthase TyW1 (***Hori, 2014***). However, nothing is known about the putative tRNA-modifying activities of ELPC-3 SAM domain. The R327C substitution in *elpc-3 (ng15)* mutants and, surprisingly, the absence of a ELPC-1 sub-unit in the 2050 bp deleted *elpc-1 (ng10)* mutants, do not affect worm chemotaxis development. These two later mutations however also stably suppress odor-specific chemo-attractive responses, but only after odor stimulation or after odor-modified $tRNA^{Ala}$ (UGC) exposure.

The six sub-units Elongator complex is highly conserved from plants to human. It has been involved in a great variety of nuclear and cytoplasmic cellular functions, including transcription elongation, chromatin remodeling, exocytosis, zygotic paternal DNA demethylation (***Okada et al., 2010***), and neuronal development (***Chen et al., 2009; Solinger et al., 2010; Creppe et al., 2009***).

The functions of most tRNA post-transcriptional modifications remain to be

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discovered (***Torres et al., 2014; Schimmel, 2018***). tRNAs and tRNAs genes not only control translational (***Kirchner and Ignatova, 2015; Avihu et al., 2013***) but also transcriptional fine-tuning (***Woolnough et al., 2015; Pratt-Hyatt et al., 2009; Kirkland and Kamakaka, 2015***). Importantly for epigenetic inheritance, tRNAs also control the plasticity of genome architecture via the dynamic positioning of nucleosomes and the modulation of chromatin domains boundaries (***Raab et al., 2012; McFarlane and Whitehall, 2009; Talbert and Henikoff, 2009***). This work highlights the role of a tRNA and the tRNA modifying Elongator complex at the interface between environmental inputs and stably inherited behavioral changes. It set the stage of elucidating how environmental information, here olfactory cues, could regulate the secondary and tertiary tRNA structures, which will in turn control biological functions.

We hope this research is paving the way for more investigation on how the huge combinatorial potential of (epi)transcriptomes may organize the interplay between environments and (epi)genomes.

The combined versatility of tRNA molecules and diversity of Elongator activities may have a great potential to produce and stably maintain non-genetic adaptations to the diverse environmental challenges biological organisms face during their life-time (***Bohacek and Mansuy, 2015; Jablonka and Lamb, 2015***).

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Material and Methods

Strains and culture conditions

We used the wild-type *C. elegans* reference strain N2, unless otherwise stated. Worms with the *sid-1 (qt2), rrf-1 (ok589), rrf-1 (pk1417)* and *rrf-3 (pk1426)* mutations were obtained from the *Caenorhabditis* Genetics Center (CGC). The *sid-2 (qt13)* mutant was obtained from Craig Hunter. The elongator mutants *elpc-1 (ng10)* or *ikap-1 (ng10), elpc-3 (ng15)* and *elpc-3 (tm3120)* were from Jachen A. Solinger. Worms were cultured on E. coli OP50 at 20°C using standard protocols.

Odor-exposure for olfactory imprinting

Benzaldehyde and β-citronellol (Sigma-Aldrich) were diluted to the 1/300 in water. Odor-exposures were done by suspending a 4 μl drop of these dilutions on the lids of worm culture dishes during the critical plasticity period corresponding to the first 12 hours of post-hatch development (***Remy JJ and Hobert O, 2005***).

Chemotaxis assay

The chemotaxis assay used in this study is schematically outlined in the Supplement Figure Method. It is based on the population chemotaxis assay originally described by C. I. Bargmann (***Bargmann CI, 2006***). Several modifications were made in the procedure and in chemotaxis index calculation. Changes aimed at more accurately

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compare the chemo-attraction of worm populations to moderately attractive odorants (the 1/300 dilution of benzaldehyde or citronellol used here), using 20 adult worms per condition.

40 ml of low-salt (1mM Ca^{++}, 1mM Mg^{++}, 5mM KPO4) agar (20 g/l) were poured in 12x12 cm square plates. Assay plates were allowed to dry at room temperature for at least three days before use. Worms were individually transferred from culture dishes to the middle of the square assay plates. To establish a homogeneous odor gradient, so that all worms were submitted to the same olfactory stimulus, 3 drops of odor-dilution (4 μl each) were suspended on the lids at one side, each placed at a distance of 3 cm from the others. At the opposite side of the lids were placed 3x 4 μl of water. 6 drops of 4 μl of 1M NaN3 were added at both sides of the agar plate to immobilize animals that reached edges.

At time 0, 20 worms were placed on the middle line of the squared plate, every worm being at a distance of 6 cm from odor sources. Assays performed on squared plates allowed the indexation of all worm positions between the starting line (time 0, position 0 cm) and the odor source (position + 6 cm), usually four times at 10, 20, 30 and 40 minutes from time 0. The mean value of all indexed positions (in cm from the starting line) of each of the 20 worms represented the Mean Migration Index (MMI). Each experiment shown in the paper was performed at least 4 times. MMI (Means +/-

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S.E.) values were compared using unpaired data with unequal variance Student t-tests performed with the KaleidaGraph program.

Assays were always performed so as to compare synchronized worm populations. In Figure 7, results are expressed as Mean Imprinting Indices, which is a subtraction of two MMI. For instance, MMI of CI-tRNAs fed population *minus* MMI of naive-tRNAs fed population.

RNAs and tRNAs fractionation and purification

The large and small RNAs (< 200 nt) were separated using the NORGEN RNA Purification Kit (Norgen Biotek Corp). Small RNAs were further size-fractionated on 3.5% low-melting agarose (Nusieve GTG) gels. RNA was quantified with the Nanodrop 2000 (ThermoScientific). To purify *C. elegans* transfer RNAs, small RNA fractions (under 150 nt in size) were prepared as described (***Maréchal-Drouard L et al., 1995***). In brief, RNA was incubated in a 2 M lithium chloride solution over-night at 4°C. After a centrifugation at 16000 g for 30 min at 4°C, the supernatants containing the tRNAs were recovered. Transfer RNAs were thereafter precipitated with 0.1 volume of 1 M sodium acetate pH 4.5 solution and 2.5 volumes of ethanol over-night at -20°C. Pelleted tRNAs were dissolved in water and loaded on 15 % polyacrylamide with 7 M Urea and 1 X TBE gels. After Ethidium bromide staining, gel slices were cut from the gels. The tRNAs were eluted over-night at room

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temperature in a solution composed of 0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1 mM EDTA and 0.1% SDS. After phenol extraction, tRNAs were ethanol precipitated and finally recovered in 10 µl water.

Specific tRNA molecules isolation

To purify tRNA molecules, we used the µMACS Streptavidin MicroBeads Kit (Miltenyi Biotec). We synthesized 37 nt long 3'-biotinylated DNA probes compementary to the 3' half of the *C. elegans* tRNAs. Oligonucleotide sequences (listed in the Supplementary material) were deduced from the *C. elegans* GtRNAdb data. The sources of RNA from which specific tRNAs were isolated were either the whole small RNA populations or the gel-eluted tRNA fractions showed in Figure 3. Procedure was as indicated by the µMACS Kit, except that the annealing buffer was made of 10 mM Tris HCl pH 7.5, 5 mM MgCl2. tRNA elutions from the microbeads were in 200 µl TE. For each feeding and behavioral assay, we used 10µl eluate per worm culture dish, as described in the RNA-feeding section below.

Northern blots analyses

RNAs fractionated on a denaturing 7M Urea/15 % polyacrylamide gel were transferred onto Hybond-N+ membrane (Amersham Pharmacia Biotech). Membranes were then hybridized with ^{32}P labeled probes specific to *C. elegans* tRNAs:

tRNA^{Ala}(AGC) : 5'- CTACCACTGAGTTATACCCCC - 3'

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tRNA^{Ala}(CGC) : 5' - TACCCCTGAGCTATACCCCC - 3'

tRNA^{Ala}(UGC) : 5' - TATGGGGAATCGAACCCCA - 3'

tRNA^{Leu}(AAG) : 5'- TGGTGAAGAGAGTGGGATTCGAAC - 3'

tRNA^{Gly}(UCC) : 5'- TGGTGCGTTCGGGGGGAATCGAAC - 3'

tRNA^{Lys}(UUU) : 5'- ACCAACTGAGCTAAGGAGGC -3'

tRNA^{Glu}(UUC) : 5'- AACCACTAGACCACATGGGA -3'

tRNA^{Gln}(UUG) : 5'- AACCGCTACACCATGGAACC - 3'

RNA-feeding

NGM agar plates were loaded with 40 μl of OP50 culture, which, after drying, formed approximately a 100 mm^2 spot. Volumes of 10 μl RNA to be assayed were deposited per *E. Coli* spot and shortly dried. Synchronised naive embryos were spawned on the RNA-loaded plates, and worms grown at 20°C until adulthood. For imprinting inheritance after multi-generational tRNA feeding, N2 worms were grown from the spawn embryo to the adult laying stage on 1 ng CI-tRNA-loaded cultures plates (F1). Part of the next generation was grown on a new CI-tRNA-loaded culture plates - the second tRNA-fed generation F2 -, another on a regular plate without CI-tRNA - the first naive generation F1+1. F3 is the progeny of F2 grown on tRNA

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while F2+1 is the progeny of F2 grown without tRNA. The CI chemo-response of the naive generations from the seven successive tRNA fed generations (F1 to F7) was determined as described.

Supplementary material

Biotinylated oligonucleotides used for *C. elegans* tRNAs microbeads purification:

1) Ala AGC trna1 V : 5' TGGAGGTTTGGGGAATTGAACCCCAGCCCTCTCCCAT 3'

2) Ala CGC trna 140 V: 5' TGGAGGCACGGGGGATTGAACCCCGGACTTCCCGCAT 3'

3) Ala TGC trna 264 X : 5' TGGAGGTATGGGGAATCGAACCCCAGACTTCTCGCAT 3'

4) Ala TGC trna 264 X : 5' ATGCAAAGCCAGCGCTCTACCCCTGAGCTATACCCCC 3'

5) Arg ACG trna250 X : 5' CGACCACGGCAGGATTCGAACCTACAATCTTCTGC 3'

6) Arg CCG trna 39 II : 5' AGCTCGCGGAGGGACTTGAACCCCCATTCCCGGTTCC 3'

7) Arg CCT trna2 IV : 5' CGACCGAGGCAGGACTCGAACCTGCTGTCTTCGGTTT 3'

8) Asn GTT trn 46 X : 5' CGCTCCCGGTGGGCTCGAATCACCTTTCGGTTAA 3'

9) Asp GTC trna 15 VI : 5' CTCCCCGGCCGGGAATTGAACCCGGGTCTCGCATGTG 3'

10) Cys GCA trna 75 V: 3' CTAGCTCTCCAGGGACCAAGTTGAGGCCCACGGGGGA 5'

11) GlnCTG trna 87 X : 3' CTTAGGACGCTGGGCTCAAGTTTAGAGCCACCCTGGA 5'

12) Gln TTG trna38 X : 3' CTTAGGACGCTGGGCTCAAGTTTAGAGCCACCTTGGA 5'

13) Glu CTC trna49 X : 3' GTGGGTATTCCGGCCCCAAGCTAAGGGGCGTTGCTTT 5'

14) Glu TTC trna 55 V : 3' GTGGGTGCGCCGGGCCCAAGCTAAGGGCCGTACCCTT 5'

15) Gly CCC trna57 I : 3' ACCATTGTCTCGCGCCCAAGCTTAGGGCAGGTGGCGT 5'

16) Gly TCC trna 68 X : 3' GTTCGTAAGCTGCCCCCAAGCTAAGGGGAGCTTGCGT 5'

17) His GTG trna 24 V : 3' CCGGCACCGCTGCGACCAAGCTAAGGTCGTCGTCCGT 5'

18) Ile AAT trna 17 X : 3' TTCGGGTTCCAGCGTCCAAGCTGGGGACGACCGCCGT 5'

19) Ile TAT trna 19 I : 3' CAATTTGGTCAGCGCCCAAGCTTAGGGCGGGCCCCGT 5'

20) Leu AAG trna9 II : 3' GGGAAGCCCCCGCACCCAAGCTTAGGGAGAGAGAAGT 5'

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21) LeuCAA trna206 X : 3'AGCATACCCACGCACCCAAGCTTAGGGTGAAGCACGT 5'

22) Leu CAG trna2 I : 3'AGAGGCCTCCCGCGTCCAAGCTTAGGACGCCTGCCGT 5'

23) LeuTAA trna 85 X: 3' GGGAGGCCCCCGCACCCAAGCTTAGGGTGAGAGTAGT 5'

24) LysCTT trna 109X: 3' ATTAGACCAACAGCGCCAAAGCTCGGGGCGTAACCCA 5'

25) Lys TTT trna 69 III : 3' TTAGAATTCCAGTCCCCAAGCTCAGGGGATCCACCGA 5'

26) Met CAT trna 144 X: 3' TTGGGTCTCCAGCCACCTAGCTTTGGTGAGCGACGAT 5'

27) Met CAT trna 168 V: 3' TTAGACTTCCAGCACTCAAGCTCGGAGTGGCCCTCGT 5'

28) Phe GAA trna 28 I : 3' TTTAGCAATCCAGTGGTCAAGCTAGGACCAAGCCCGT 5'

29) Pro AGG trna50 X : 3' CACGTTCTCTAGGGCCCAAGCTAGGGGCCAAGCTGGG 5'

30) ProCGG trna 82 IV: 3' CACGCTCTCCAGGGCCCAAGCTAAGGGCCAAGCCGGG 5'

31) ProTGG trna 50 II : 3' CACGCTCTCCAGGGACCAAGTTAGGGGCCAAGCCGGG 5'

32) Ser AGA trna 7 II : 3' CCGAGACGGGCGCATCCAAGCTTAGGACGACTGACGC 5'

33) Ser CGA trna 50 I : 3' CCGAGACGGGCGCATCCAAGCTTAGGACGACTGACGC 5'

34) SerGCT trna 104 X: 3' CCCAAAGGGGCGCACTCAAGCTTAGAGTAGAACTAGC 5'

35) Thr AGT trna 69 II : 3' TTTGTCTTCCAGCGACCAAGCTAAGGTCGTACTCCGT 5'

36) Thr CGT trna 160 V : 3' TTTGTCTTCCAGCTGCCAAGTTAGGGCAGACCCCCGT 5'

37) Thr TGT trna 78 II : 3' TAGTTATCCAGGCCCCAAGCTGGGGAGCATTCCCAGT 5'

38) Trp CCA trna 41 X : 3' CTAGCTTTCCATCCCGCAAGCTAGGCGAGTCACCAGT 5'

39) Tyr GTA trna 92 X : 3' TAGGAATCCAGTGACCAAGCTTAGGCCAAGCTGCCT 5'

40) Val AAC trna 37 IV : 3' TGTGTCTTCCAGCCACCAAGCTCGGGCGGGCTCTAGT 5'

41) ValCAC trna 151 V : 3' TGCGTCTTCCAGCGGCCAAGCTTGGGCCGGTCCTGGA 5'

42) Val TAC trna 101 X : 3' TGCGTCTTCTAGCGGCCAAGCTTGGGCCGATCCTGGA 5'

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Figure Legends

Supplement Figure Methods.

Schematic outline of the chemotaxis assay.

Chemotaxis assays were performed on square plates in order to index the worms' positions between the starting line (time 0, position 0 cm) and the odor source (position + 6 cm) or water source (position - 6 cm). 3 ml of odor solution or water are added to the lids of the plates. 20 synchronised worms are placed at the starting line at time 0. Worms start their migration and their position is carefully noted, usually at four time-points, at 10, 20, 30 and 40 minutes past time 0. Worms that migrate up the odor gradients are positively indexed (from 0 to 6). Worms that migrate down the odor gradients towards water are negatively indexed (from 0 to – 6). The mean value of all indexed positions at all noted times (in cm from the starting line) of each of the 20 worms involved in the assay is the Mean Migration Index (MMI).

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Figure 1. RNA extracted from odor-exposed worms transfer olfactory imprinting to naive unexposed worms via feeding.

(a) Schematic depiction of worms odor-exposure and RNA-feeding. Upper part: a drop of 4 µl of citronellol (CI) or benzaldehyde (BA) or water (for control unexposed) is placed on the lid of each culture plate. Worms produce long-lasting odor-specific imprints when exposed to odors for 12 hours post-hatch at 20°C, the critical period for olfactory imprinting (*Remy and Hobert, 2005*). Worms exposed to odors during this period are collected at the adult stage. RNA is extracted from each collected population. Bottom part: a 10 µl drop of purified RNA is placed on worm food (*E. coli* 0P50) to be ingested by naive larvae.

(b) Chemotaxis assays performed on naive adults fed on different RNA populations. Worms fed on RNA from CI-exposed (CI-RNA, blue column) and worms fed on RNA from BA-exposed (BA-RNA, red column) migrate faster toward a CI source and a BA source, respectively, compared to worms fed on RNA from naive animals (Naive-RNA, white columns). Mean Migration Index (MMI) was determined as described in Supplemental Material (experimental repeats > 4, ***p-value<0.001).

Figure Supplement 1.

(a) Wild-type N2 larvae and larvae with mutations inactivating the *sid-1* or *sid-2* genes were odor-exposed to CI 1/300 or not exposed, as control naive. The Mean

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Migration Indices of naive and of CI-exposed wt N2, *sid-1 (qt2)* and *sid-2 (qt13)* worms in CI 1/300 gradients are shown. (experimental repeats > 4, ***p-value<0.001).

(b) *sid-1 (qt2)* and *sid-2 (qt13)* mutants were fed on the indicated amounts (from 0 to 100 ng) of total RNA extracted from CI-exposed wt N2 animals. Mean Migration Indices to CI 300 were determined at the adult stage (experimental repeats > 4, ***p-value<0.001).

Figure 2. A specific population of small RNA transfers olfactory imprinting.

(a) RNA from BA-exposed and unexposed naive was fractionated into two populations: the larger than 200 nucleotides fraction (Large-RNA) and the smaller than 200 nucleotides fraction (Small-RNA). Worms fed on 10 ng of Large BA-RNA migrate in a BA gradient as worms fed on 10 ng of Large Naive-RNA. By contrast, worms fed on 10 ng of Small BA-RNA display enhanced BA responses, compared to worms fed on 10 ng of Small Naive-RNA or to worms fed on 10 ng of Large BA-RNA (experimental repeats > 4, ***p-value < 0.001). (b) 3.5% low-melting agarose gels fractionate BA-RNA and Naive-RNA into five A to E discrete bands (left side of insert). Naive worms were fed on each of the five gel-purified NA-RNA and BA-RNA populations. Compared BA 1/300 MMI shows that only the BA-RNA band D is

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able to transfer a BA-imprint to naive (experimental repeats > 4, ***p-value < 0.001). (L), NEB double stranded RNA ladder.

Figure 3. Olfactory imprinting is triggered by transfer RNAs (tRNAs).

(a) Left part: Total small RNA from naive (NA) and from CI-exposed (CI) were fractionated on a 7M Urea 15% polyacrylamide gel and stained with ethidium bromide (EtBr). Band 1 is the 5S rRNA, bands 2 to 7 are tRNAs, while band 8 is made of unknown RNAs. The L well contains the NEB single stranded RNA ladder. Right part: Northern blot analyses were performed on NA and CI RNA blots with two radiolabeled probes specific to the *C. elegans* $tRNA^{Leu}$(AAG) and $tRNA^{Gly}$(UCC). (b) After elution from the polyacrylamide gels, fractions 1 to 8 or fractions 2 to 7 from naive (NA Mix 1-8 and NA Mix 2-7) and from CI-exposed (CI Mix 1-8 and CI Mix 2-7) were pooled. Naive worms were fed on each pool and assayed for CI response. Both of the pooled CI mixes trigger a faster migration (MMI) in CI gradients than the corresponding NA mixes (experimental repeats > 4, **p=0.006; ***p<0.001). (c) Naive N2 were fed on each of the 1 to 8 individual fractions from naive and from CI-exposed. Only feeding on the tRNA fractions 5 and 6 from CI-exposed enhances migration in CI gradients (MMI), compared to all other tRNA fractions (experimental repeats > 4, ***p<0.001).

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Figure Supplement 3.

(a) Femtogram (fg, 10^{-15} g) amount of tRNA elicits an olfactory imprint. Naive worms were grown with the indicated amounts of the CI-tRNA pool (fractions 2-7) in food. Worms fed on CI-tRNA quantities above 1 fg migrate faster (MMI mean of 2.26 ± 0.28) than worms fed on CI-tRNA quantities below 1 fg (MMI mean of 0.58 ± 0.3). (b) Worm larvae with the indicated mutations inactivating the *rrf-1* or *rrf-3* genes were odor-exposed to CI 1/300 or water as control naive. The behavior of CI-exposed worms was compared to the behavior of unexposed naive worms. Both *rrf-1* alleles displayed a wild-type imprinting behavior, whereas the *rrf-3* mutant is unable to produce an olfactory imprint (experimental repeats ≥ 4, **p-value < 0.01).

Figure 4. CI-imprinting can be fixed and stably inherited after feeding six generations on tRNAs extracted from CI-exposed worms.

(a) CI-imprinting segregation in the progeny of multigenerational CI-tRNAs fed worm populations (schematic representation). Worm populations were fed 1 ng of pooled CI-tRNA fractions 2-7, of CI-tRNA fraction 5 for one (F1) to seven (F7) successive generations. Four naive generations grown without addition of tRNAs were obtained from each of the seven (F1 to F7) CI-tRNAs fed populations. As negative control, we performed mutigenerational feeding with the CI-tRNA fraction 4 that do not transfer CI-imprinting. Fractions 2-7 and fraction 5 CI-tRNAs fed worms

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passed a CI-imprint to the first naive generation. Imprinting is definitely lost at the second naive generation issued from one to five CI-tRNAs fed generations; it is stably maintained in worms issued from six and seven CI-tRNAs 2-7 and 5 fed generations. (b) Behavior of the fourth naive generations issued from worms fed on CI-tRNAs pool 2 to 7 or fraction 5 for one to seven generations (F1+4 to F7+4) was compared to behavior of Naive-tRNA fed worms. The F6+4 and F7+4 worms migrate significantly faster than Naive-RNA fed animals in CI gradients ($n > 4$, ***p-value < 0.001).

(c) Behavior of the 19th generation of naive worms issued from ancestors fed for six successive generations on the non-imprinting CI-tRNAs fraction 4 as negative control (CI tRNA 4), on the imprinting CI-tRNAs pool 2 to 7 (CI Mix 2-7), or on the imprinting CI-tRNAs fraction 5 (CI tRNA 5) (***p-value < 0.001).

Figure 5.

tRNA^{Ala} (UGC) transfers BA and CI olfactory imprinting.

(a) Schematized strategy used to identify tRNA^{Ala} (UGC) as the imprinting tRNA. We designed isotype and isoacceptor-specific DNA probes corresponding to the 3' halves of all *C. elegans* tRNAs. Large 37 nt probes were 3'-biotinylated and used for tRNA purification on streptavidin microbeads, as described in the methods. tRNAs from the imprinting tRNA fractions 5 were purified using pools of isotype-specific

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probes. tRNA eluted from each pool was assayed to test the imprinting transfer to naive. tRNA-specific probes were radiolabeled for Northern blot analysis, as described in the methods. (b1) Fraction 5 CI-tRNAs were hybridized to the isotype-specific 3'-biotinylated DNA probes for the 3' halves of $tRNA^{Glu}$, $tRNA^{Lys}$, $tRNA^{Cys}$, $tRNA^{Gln}$, $tRNA^{Ala}$ or $tRNA^{Leu}$ bound on streptavidine-microbeads. Naive worms were fed on tRNAs eluted from the microbeads and tested for migration in CI 1/300 gradients (***p-value < 0.001). (b2) Fraction 5 CI-tRNAs were eluted from microbeads bound to the three Alanine codon-specific (3'-AGC, 3'-CGC, 3'-UGC and 5'-UGC) probes. Naive worms were fed on tRNAs eluted from the microbeads and tested for migration in CI 1/300 gradients (***p-value < 0.001). (c) Northern blot analysis on tRNA fractions from Naive (N3 to N6), on tRNA fractions from CI-exposed (C3 to C6) and on tRNA fractions from BA-exposed (B4 to B7) N2 worms. Hybridizations were performed with radiolabeled oligonucleotides specific for $tRNA^{Lys}$ (UUU), $tRNA^{Gln}$ (UUG), $tRNA^{Glu}$ (UUC), $tRNA^{Leu}$ (AAG), and $tRNA^{Ala}$ (AGC, CGC, UGC). (d) Fractions 5 tRNAs extracted from BA-exposed and from CI-exposed were eluted from microbeads bound to the Alanine 3'-UGC specific probe. Naive wt worms fed on these tRNAs were assayed for BA and for CI chemotaxis. Control naive are unfed wt N2 worms (***p-value < 0.001).

Figure 6. The *C. elegans* chemo-attractive behavior requires the elongator complex (ELPC).

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(a) Mean Migration Indices (MMI) to CI 1/300, CI 1/200, BA 1/300 and BA 1/200 were determined for 3 and 4 days old wt N2, elongator sub-unit 3 (*elpc-3*), and elongator sub-unit 1 (*elpc-1*) mutant worms (experimental repeats ≥ 4, **p-value < 0.01, ***p-value < 0.001).

(b) Elongator mutant *elpc-1 (ng10)* and *elpc-3 (ng15)* larvae were exposed or not (naive controls) to CI 1/300. CI 1/300 MMI was determined as described on 4 days old worms (experimental repeats ≥ 4, **p-value < 0.01).

(c) Elongator mutant *elpc-3 (ng15)* larvae were exposed or not (naive controls) to CI 1/300 or to BA 1/300. Worms from all following generations were not odor-exposed. Chemo-attractive responses to CI 1/300 and to BA 1/600 of the 7th unexposed generations were determined as described (experimental repeats ≥ 4, ***p-value < 0.001).

(d) Elongator mutant *elpc-1 (ng10)* and *elpc-3 (ng15)* larvae were grown in the presence of $tRNA^{Ala}$ (AGC), $tRNA^{Ala}$ (CGC) or $tRNA^{Ala}$ (UGC) purified from the gel-extracted tRNA fraction 5 of BA-exposed wt worms. BA 1/600 MMI was determined as described on 4 days old worms (experimental repeats ≥ 4, **p-value < 0.01, ***p-value < 0.001).

Figure 7. Odor-exposed *elpc-1 (ng10)* and *elpc-3 (ng15)* elongator mutants produce $tRNA^{Ala}$ (UGC) with wild-type imprinting activities.

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Wild-type (wt) N2, *elpc-1 (ng10)* and *elpc-3 (ng15)* larvae were or not early exposed to CI 1/300, as described. $tRNA^{Ala}$ (UGC) was purified from adult worms using the complementary DNA probe bound to microbeads, as described. Naive wt, naive *elpc-1 (ng10)* and naive *elpc-3 (ng15)* worms were fed on these $tRNA^{Ala}$ (UGC). Their Mean Imprinting Index (MII) to CI 1/300 was determined at the adult stage, as described. Results are expressed as Mean Imprinting Index (MII). MII represents the difference between mean migration index (MMI) after feeding on $tRNA^{Ala}$ (UGC) from CI-exposed MMI after feeding on $tRNA^{Ala}$ (UGC) from naive. A positive MII indicates $tRNA^{Ala}$ (UGC) from CI-exposed increased worm migration to CI, compared to $tRNA^{Ala}$ (UGC) from naive. A negative MII results by contrast indicates $tRNA^{Ala}$ (UGC) from CI-exposed decreased worm migration to CI compared to naive $tRNA^{Ala}$ (UGC).

Figure 8. Wild-type naive $tRNA^{Ala}$ (UGC) rescues the behavioral phenotypes of elongator mutants.

(a) *elpc-3 (tm3120)* mutant worms were grown during their whole development in the presence or in the absence of $tRNA^{Ala}$ (UGC) either purified from whole tRNA population of naive wt worms (N2 naive tRNAs) or from gel-extracted fraction 5 of naive tRNAs (N5 fraction). MMI to CI 1/300 and to BA 1/600 was determined at the adult stage as described (***p-value < 0.001).

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(b) *elpc-1 (ng10)* mutant worms were unexposed (Naive), CI-exposed (CI exp) or CI-exposed in the presence of $tRNA^{Ala}$ (UGC) purified from naive wt worms (CI exp + $tRNA^{Ala}$ (UGC). CI 1/300 MMI was determined at the adult stage as described (***p-value < 0.001).

(c) *elpc-3 (ng15)* mutant worms were unexposed (Naive), BA-exposed (BA exp) or BA-exposed in the presence of $tRNA^{Ala}$ (UGC) purified from naive wt worms (BA exp + $tRNA^{Ala}$ (UGC). BA 1/600 MMI was determined at the adult stage as described (***p-value < 0.001).

Figure supplement 8. The $tRNA^{Ala}$ (UGC) extracted from naive *elpc-3 (tm3120)* mutants inhibit chemo-attraction and do not rescue imprinting.

(a) *elpc-1 (ng10)* mutant worms were unexposed (Naive), CI-exposed or CI-exposed in the presence of $tRNA^{Ala}$ (UGC) extracted from naive wt worms (CI exp + $tRNA^{Ala}$ (UGC) wt naive) or in the presence of $tRNA^{Ala}$ (UGC) purified from naive *elpc-3 (tm3120)* mutant worms (CI-exp + $tRNA^{Ala}$ (UGC) *elpc-3 (tm3120)* naive). CI 1/300 MMI was determined at the adult stage as described (***p-value < 0.001). (b) Wt N2 worms were fed on $tRNA^{Ala}$ (UGC) extracted from naive wt as control, from naive *elpc-3 (tm3120)* mutants and from CI-exposed or BA-exposed *elpc-3 (tm3120)* mutants. CI 1/300 and BA 1/600 MMI were determined at the adult stage as described (*p-value < 0.05, ***p-value < 0.001).

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Figure 9. The olfactory transduction *odr-1 (n1936)* mutants do not produce odor-imprinting tRNA^{Ala} (UGC).

(a) Chemotaxis to CI 1/600, CI 1/300, BA 1/600 and BA 1/300 were determined for 4 days old wt N2 and for *odr-1 (n1936)* mutant worms. Mean Migration Indices for each odor dilution were determined as described (experimental repeats ≥ 4, **p-value < 0.01).

(b) tRNA^{Ala} (UGC) was purified from naive unexposed and CI 1/300-exposed wt N2 worms, from naive unexposed and CI 1/300-exposed *odr-1 (n1936)* mutant worms. Naive wt, *elpc-1 (ng10)*, and *elpc-3 (ng15)* elongator mutant worms were fed on these tRNA^{Ala} (UGC). Mean Migration Index to CI 1/300 was determined at the adult stage as described (***p-value < 0.001).

Figure 10. Summarized hypothesis

Attractive odorants bind putative specific membrane G-protein coupled chemoreceptors expressed by olfactory receptor neurons AWC. The Naive form of tRNA^{Ala} (UGC) is required for the development of *C. elegans* chemo-attractive behavior. Each odor might specifically chemical modify the Naive tRNA ^{Ala} (UGC). These modifications require a functional olfactory transduction pathway. Transient or stable enhancement of odor-specific responses resulting from imprinting are equally triggered by early odor-exposure or by feeding odor-modified tRNA^{Ala} (UGC).

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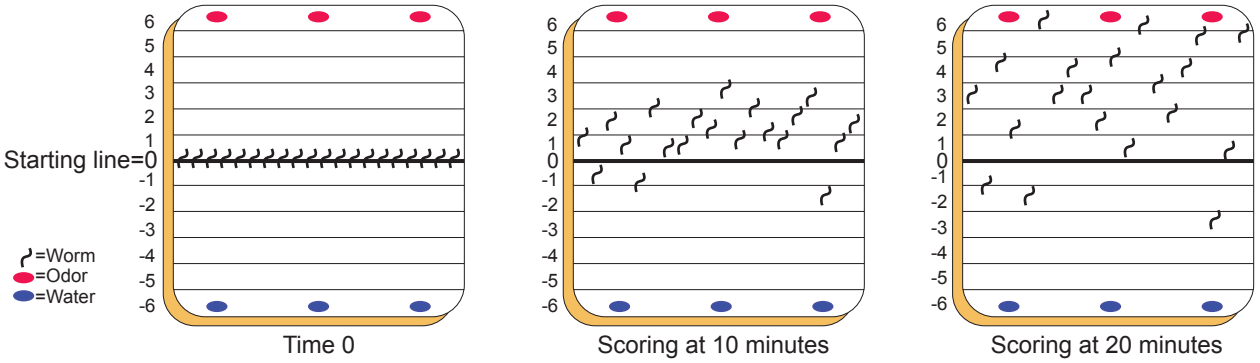
Supplement Figure Method



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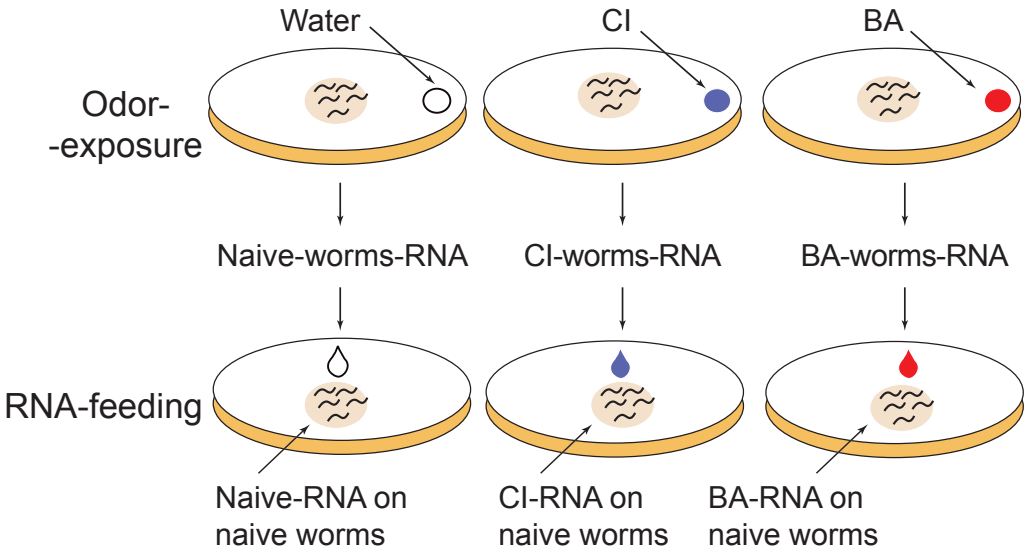
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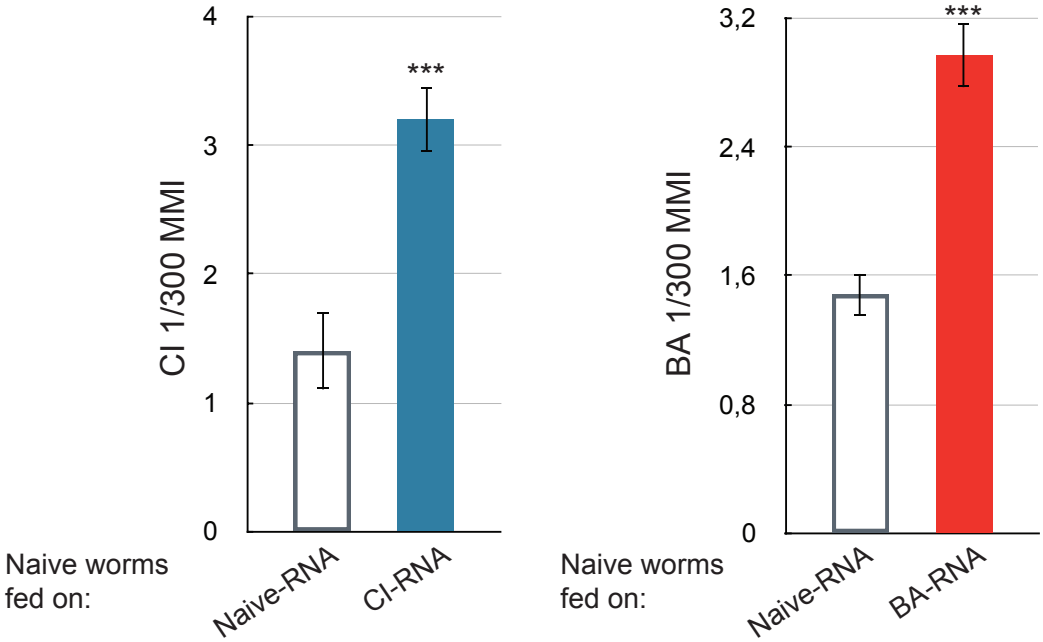
b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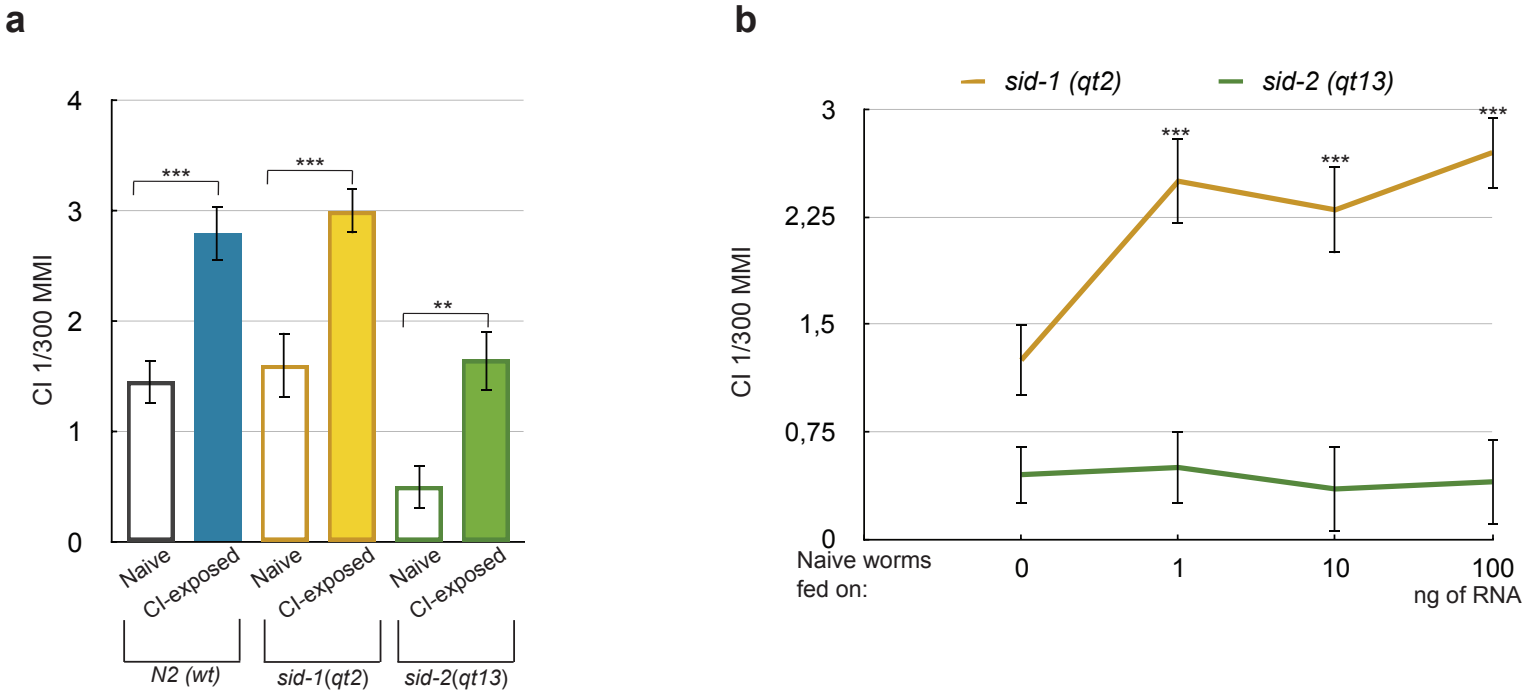
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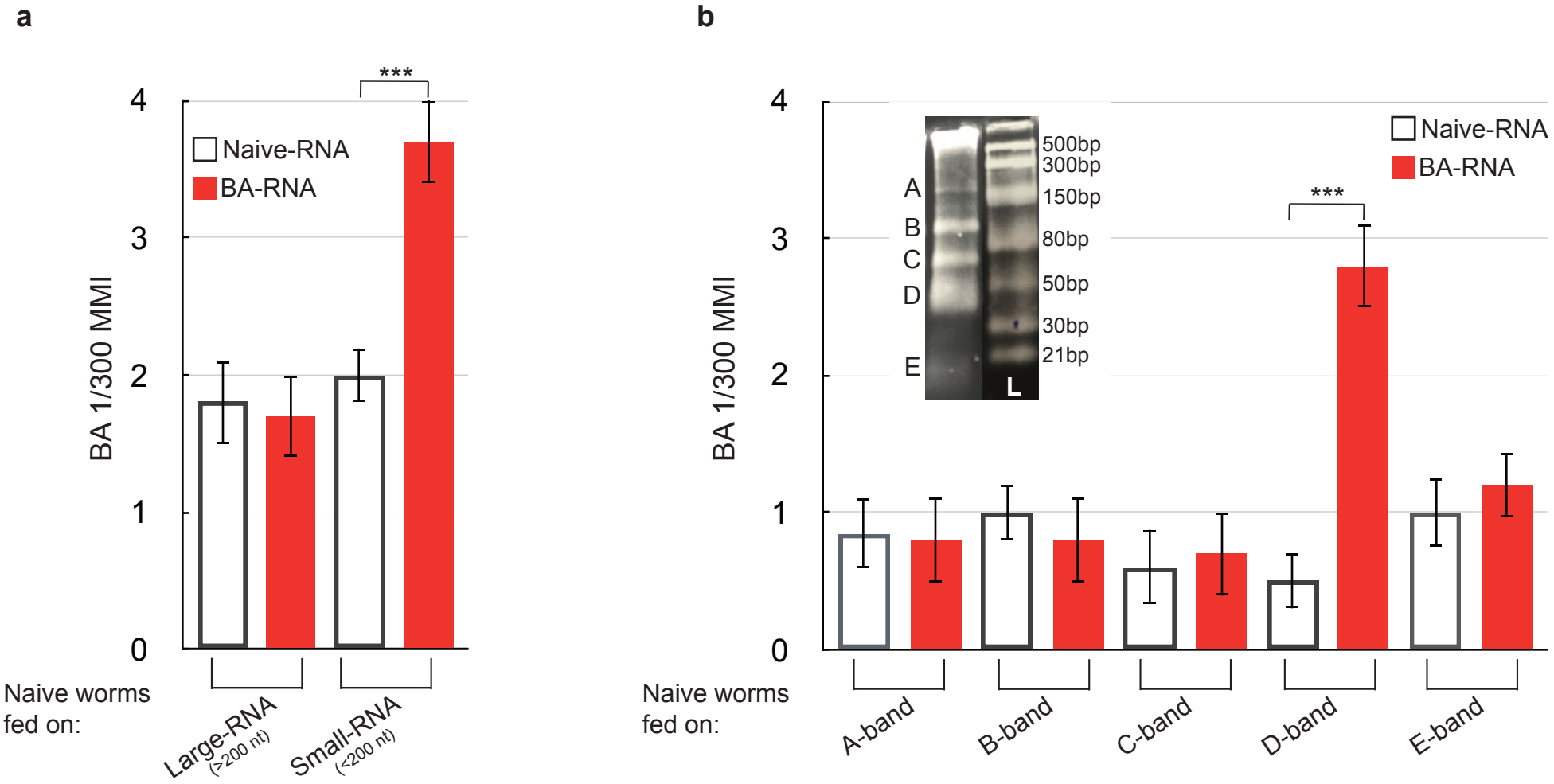
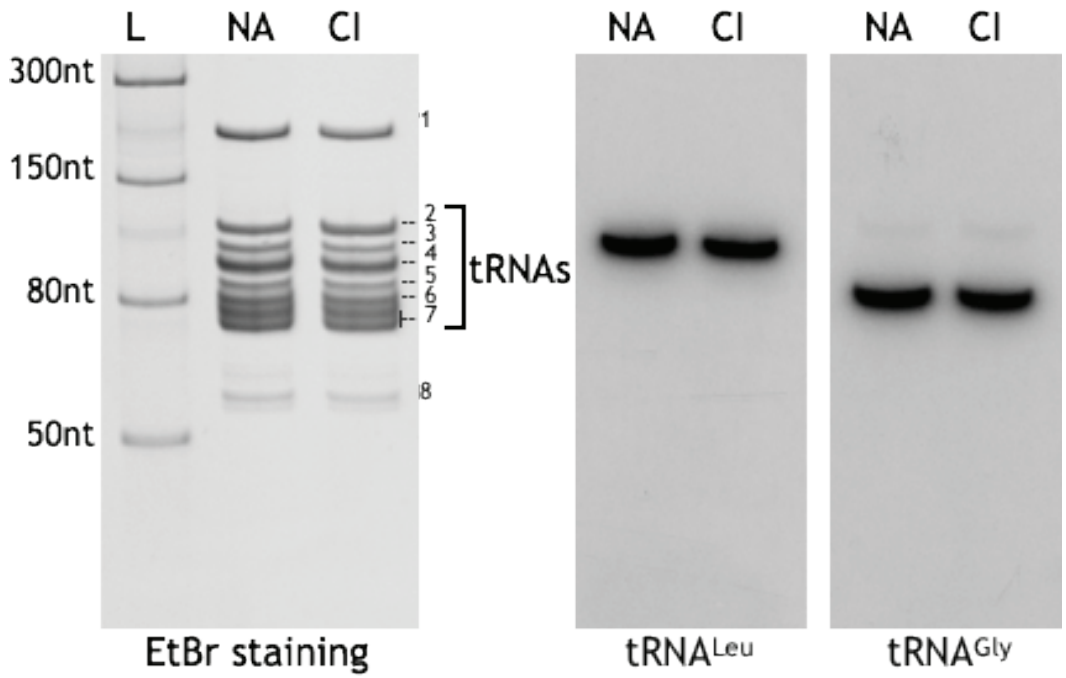
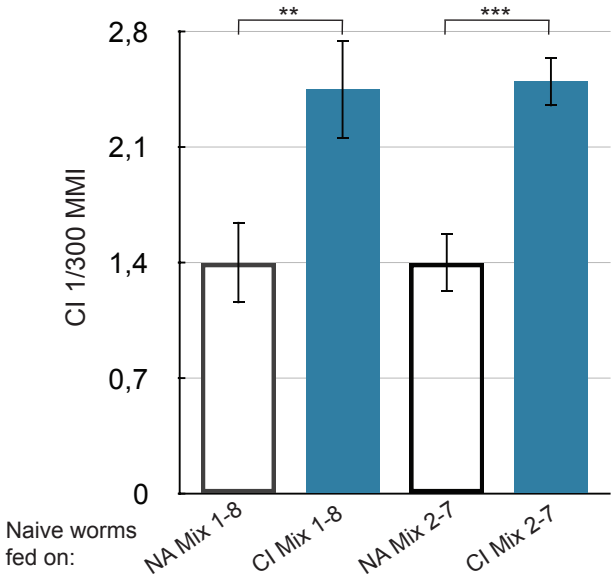
100
ng of RNA


a
b
BA 1/300 MMI
Naive-RNA
BA-RNA
***
Naive worms fed on:
Large-RNA (>200 nt)
Small-RNA (<200 nt)
A
B
C
D
E
L
500bp
300bp
150bp
80bp
50bp
30bp
21bp
A-band
B-band
C-band
D-band
E-band

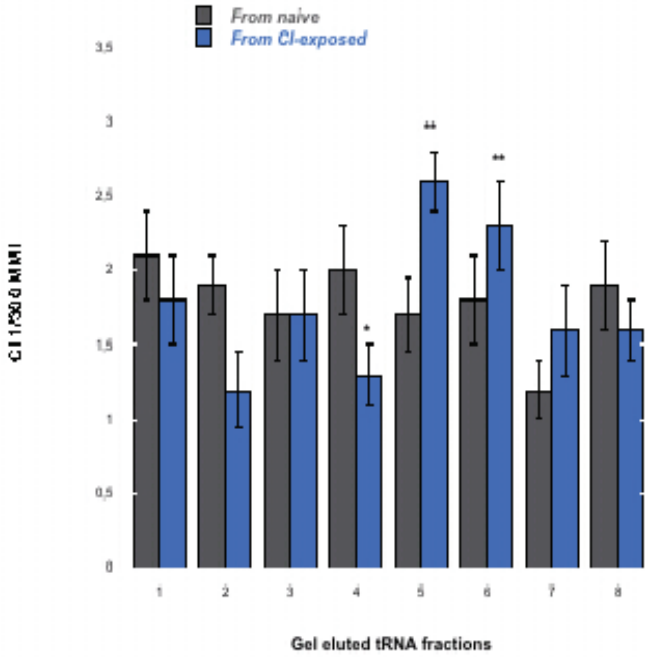
a


L
NA
CI
NA
CI
NA
CI
300nt
150nt
80nt
50nt
1
2
3
4
5
6
7
8
tRNAs
EtBr staining
tRNA^Leu
tRNA^Gly

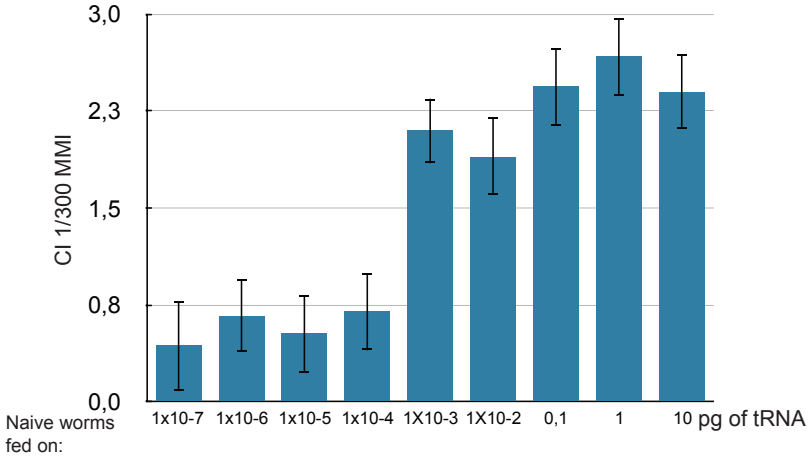
b


**
***
2,8
2,1
1,4
0,7
0
CI 1/300 MMI
Naive worms fed on:
NA Mix 1-8
CI Mix 1-8
NA Mix 2-7
CI Mix 2-7

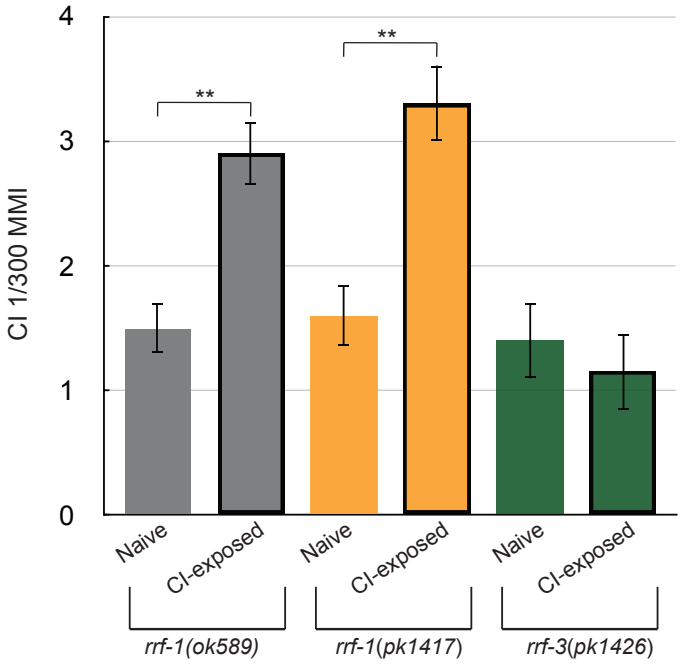
c


From naive
From CI-exposed
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
Gel eluted tRNA fractions

a


3,0
2,3
1,5
0,8
0,0
CI 1/300 MMI
Naive worms fed on:
1x10-7
1x10-6
1x10-5
1x10-4
1X10-3
1X10-2
0,1
1
10 pg of tRNA

b


4
3
2
1
0
CI 1/300 MMI
**
**
Naive
CI-exposed
Naive
CI-exposed
Naive
CI-exposed
rrf-1(ok589)
rrf-1(pk1417)
rrf-3(pk1426)

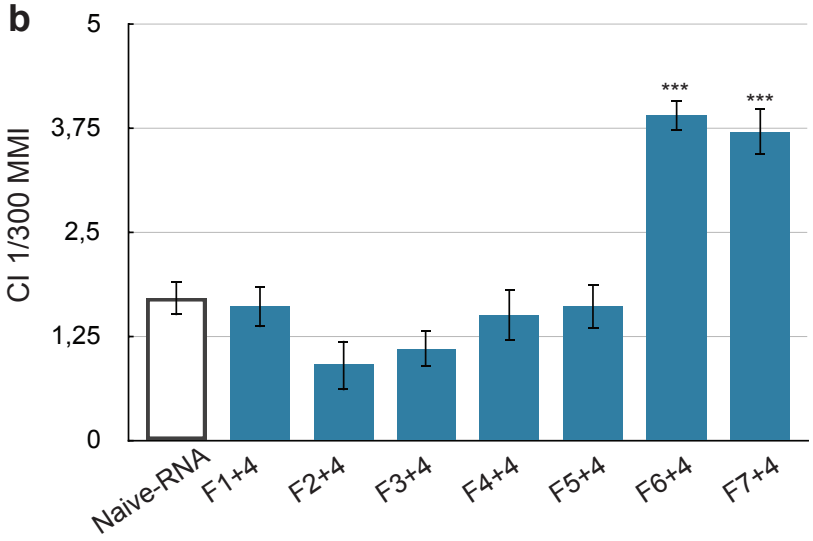
a

0 **0** 0 0 0 F1+4
0 **0** **0** 0 0 0 F2+4
0 **0** **0** **0** 0 0 0 F3+4
0 **0** **0** **0** **0** 0 0 0 F4+4
0 **0** **0** **0** **0** **0** 0 0 0 F5+4
0 **0** **0** **0** **0** **0** **0** 0 0 0 F6+4
0 **0** **0** **0** **0** **0** **0** **0** 0 0 0 F7+4

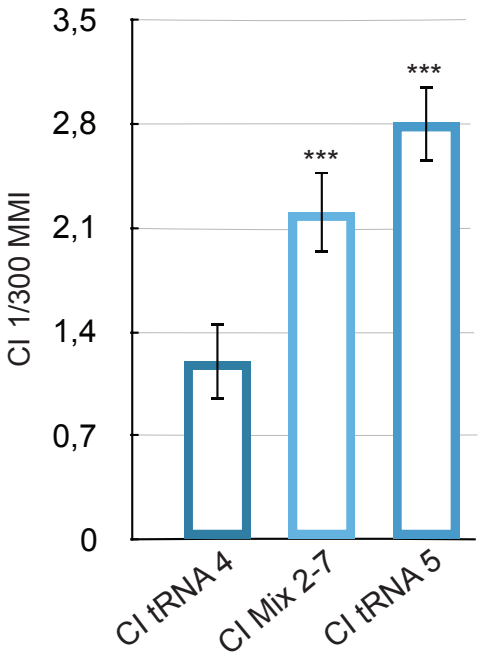
Generations

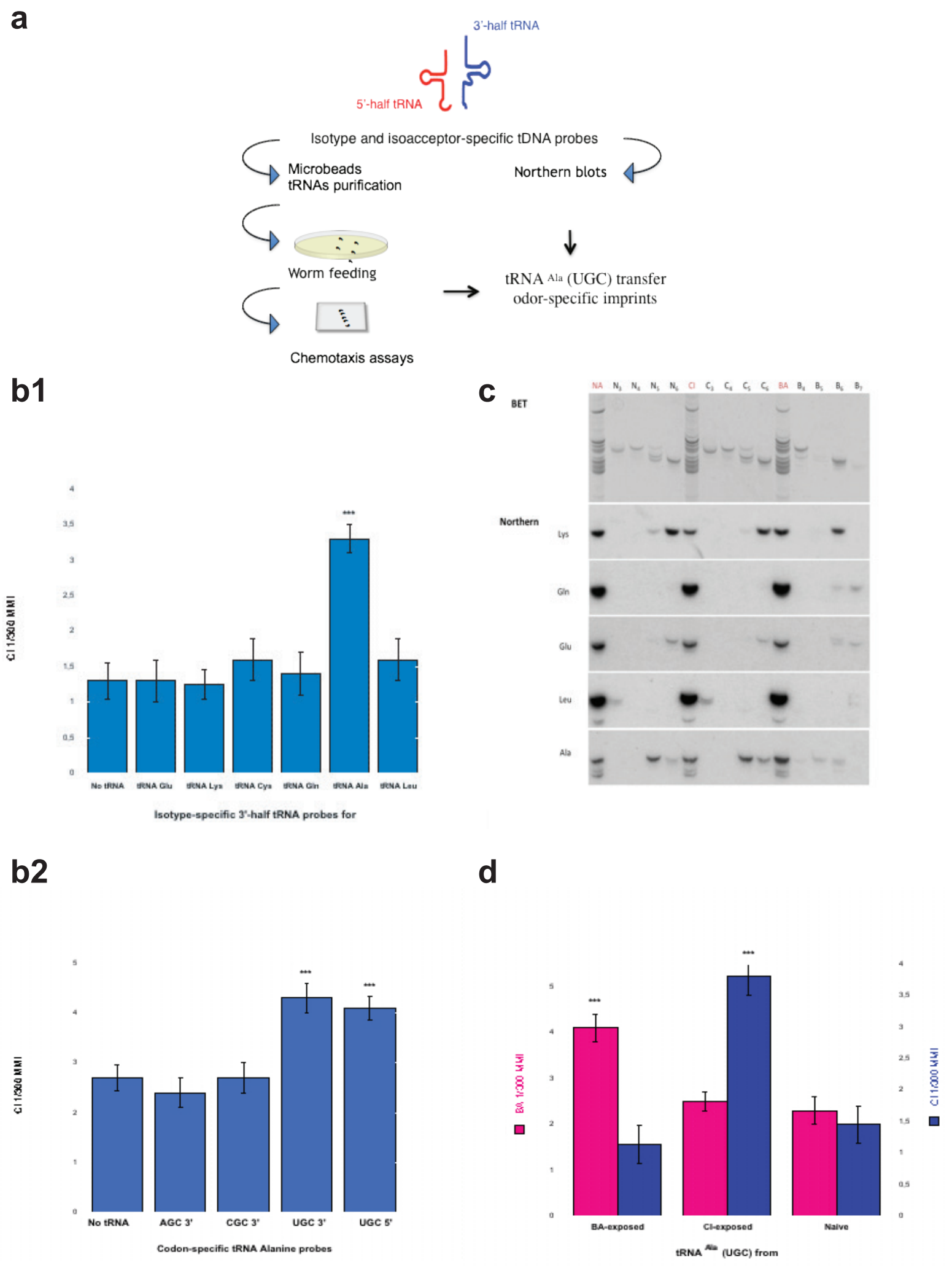
0 = CI-tRNA fed generation, **imprinting** behavior
0 = No-CI-tRNA added, **imprinting** behavior
0 = No-CI-tRNA added, naive behavior

b

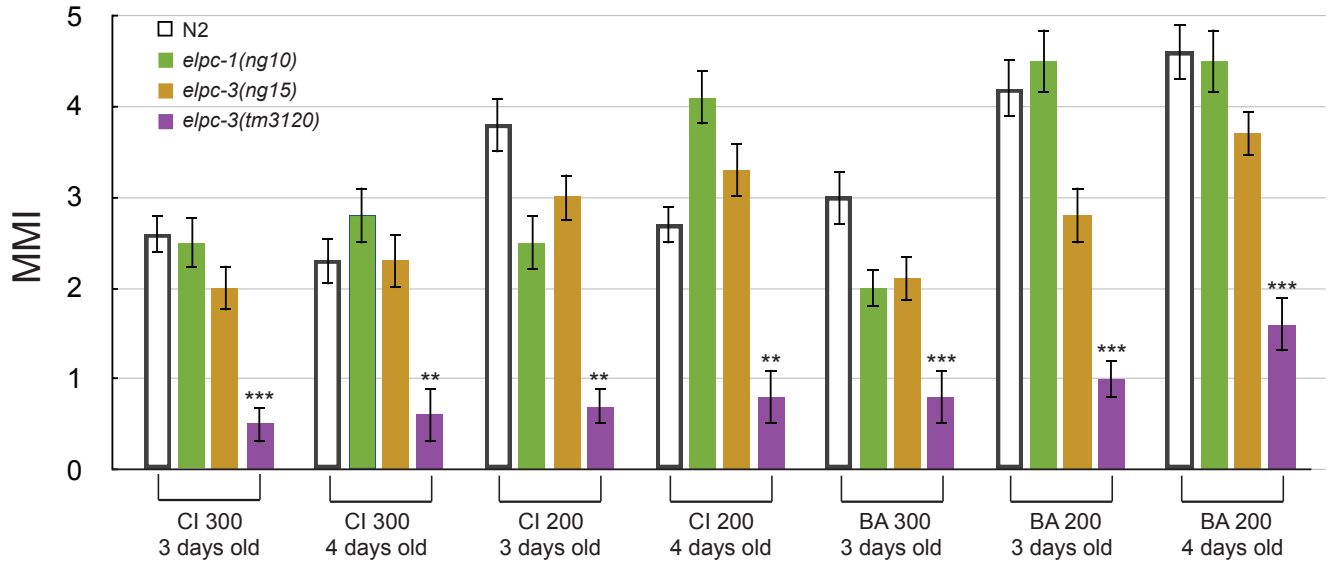


c

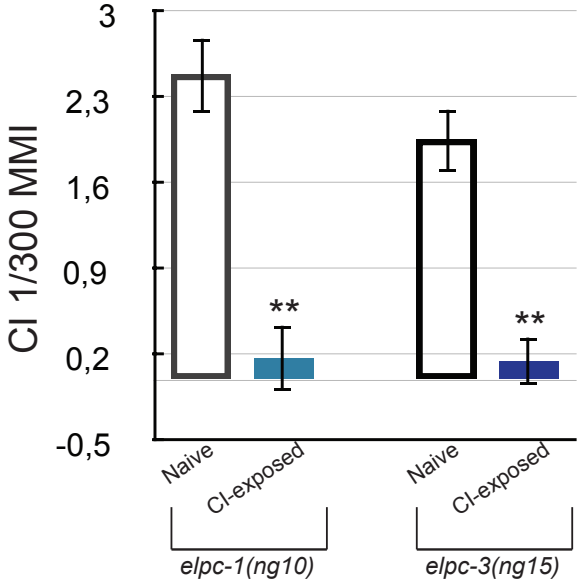



a
3'-half tRNA
5'-half tRNA
Isotype and isoacceptor-specific tDNA probes
Microbeads
tRNAs purification
Northern blots
Worm feeding
Chemotaxis assays
tRNA Ala (UGC) transfer
odor-specific imprints
b1
Isotype-specific 3'-half tRNA probes for
No tRNA
tRNA Glu
tRNA Lys
tRNA Cys
tRNA Gln
tRNA Ala
tRNA Leu
c
BET
Northern
Lys
Gln
Glu
Leu
Ala
b2
Codon-specific tRNA Alanine probes
No tRNA
AGC 3'
CGC 3'
UGC 3'
UGC 5'
d
BA-exposed
Cl-exposed
Naive
tRNA Ala (UGC) from

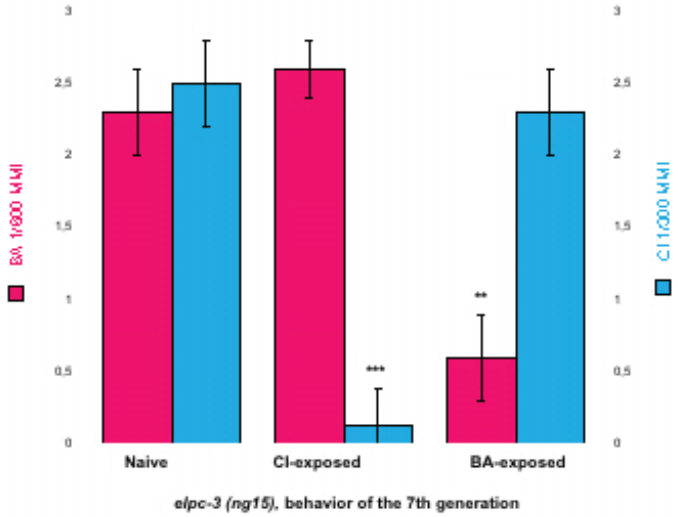
a


N2
elpc-1(ng10)
elpc-3(ng15)
elpc-3(tm3120)
MMI
0
1
2
3
4
5
***
**
**
**
***
***
***
CI 300 3 days old
CI 300 4 days old
CI 200 3 days old
CI 200 4 days old
BA 300 3 days old
BA 200 3 days old
BA 200 4 days old

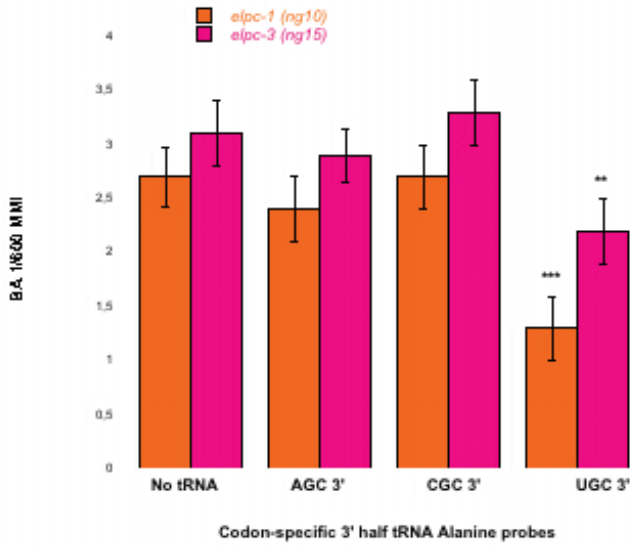
b

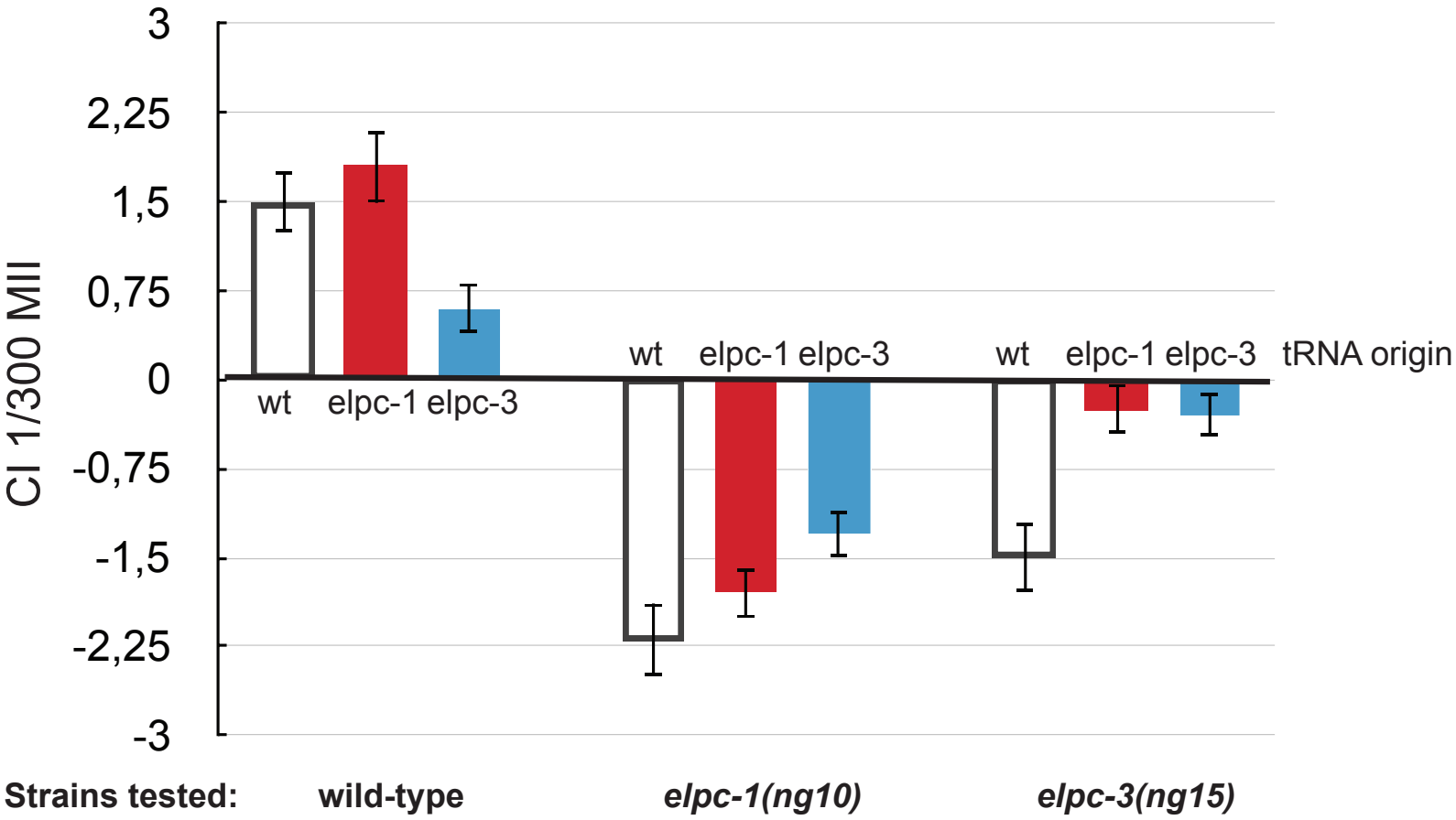

CI 1/300 MMI
-0,5
0,2
0,9
1,6
2,3
3
**
**
Naive
CI-exposed
Naive
CI-exposed
elpc-1(ng10)
elpc-3(ng15)

c

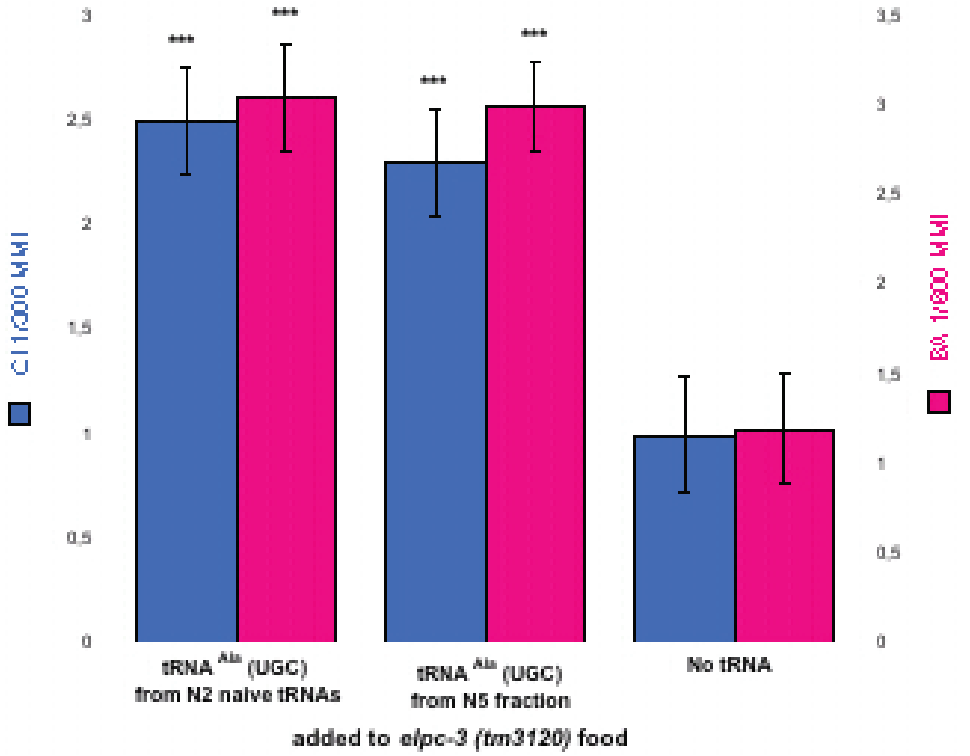

BA 1/600 MMI
CI 1/300 MMI
**
***
Naive
CI-exposed
BA-exposed
elpc-3 (ng15), behavior of the 7th generation

d

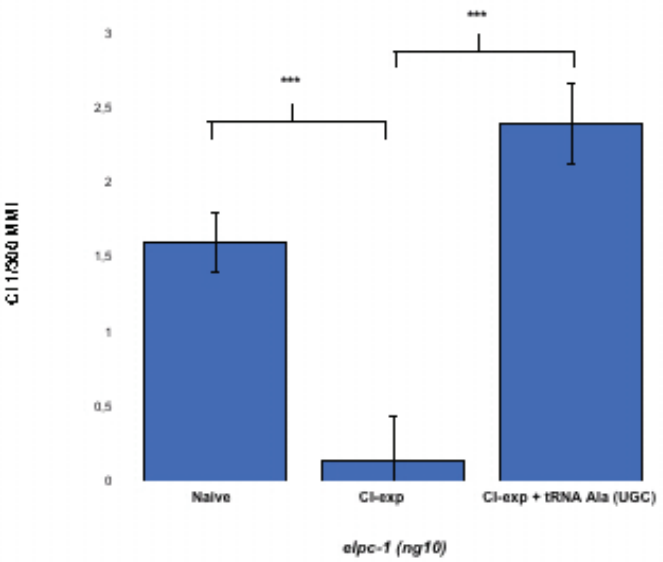

elpc-1 (ng10)
elpc-3 (ng15)
BA 1/600 MMI
***
**
No tRNA
AGC 3'
CGC 3'
UGC 3'
Codon-specific 3' half tRNA Alanine probes


3
2,25
1,5
0,75
0
-0,75
-1,5
-2,25
-3
CI 1/300 MII
wt
elpc-1
elpc-3
wt
elpc-1
elpc-3
wt
elpc-1
elpc-3
tRNA origin
Strains tested:
wild-type
elpc-1(ng10)
elpc-3(ng15)

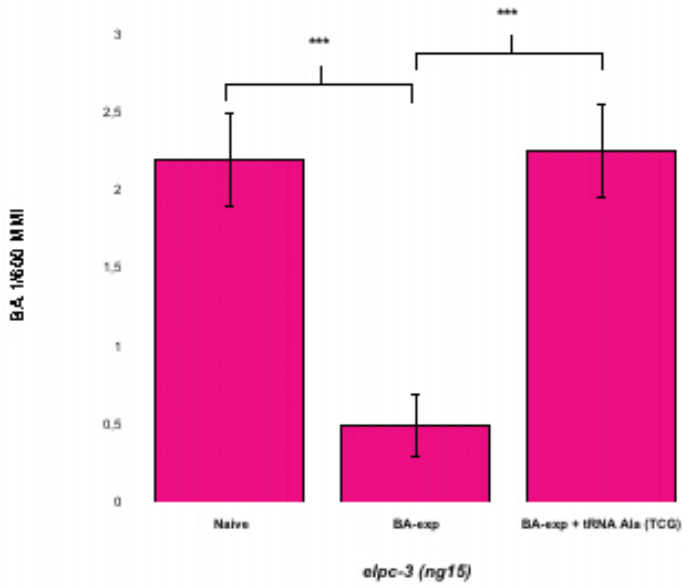
a


CI 1/600 MMI
BA 1/600 MMI
***
tRNA Ala (UGC) from N2 naive tRNAs
tRNA Ala (UGC) from N5 fraction
No tRNA
added to elpc-3 (tm3120) food

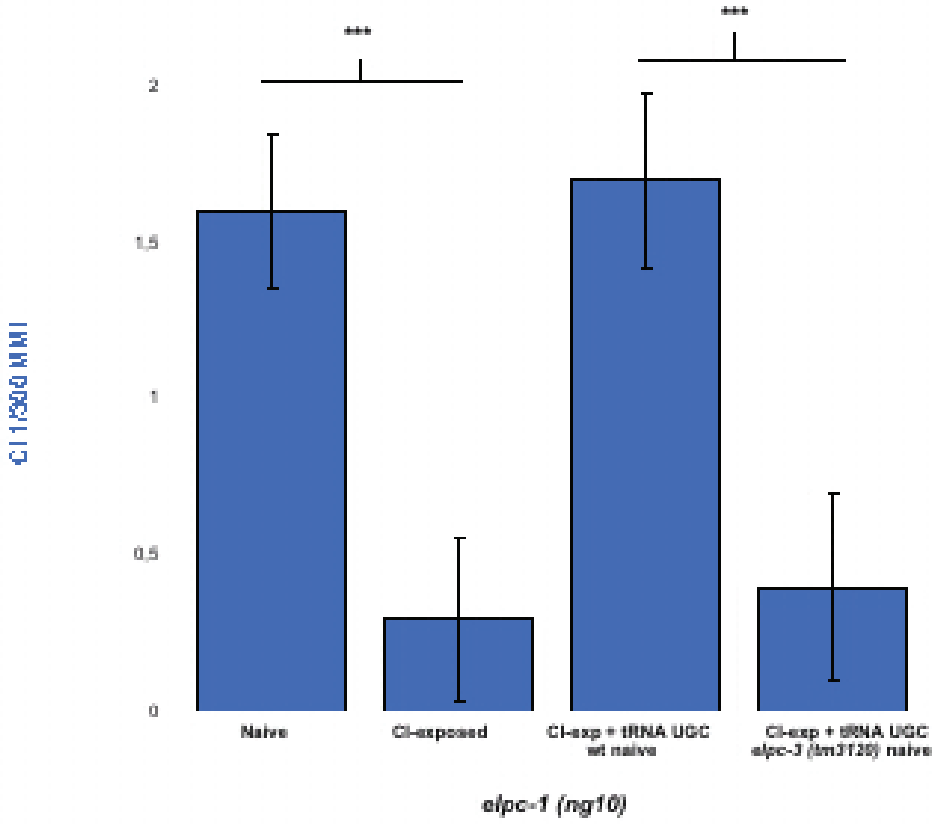
b


CI 1/600 MMI
***
Naive
CI-exp
CI-exp + tRNA Ala (UGC)
elpc-1 (ng10)

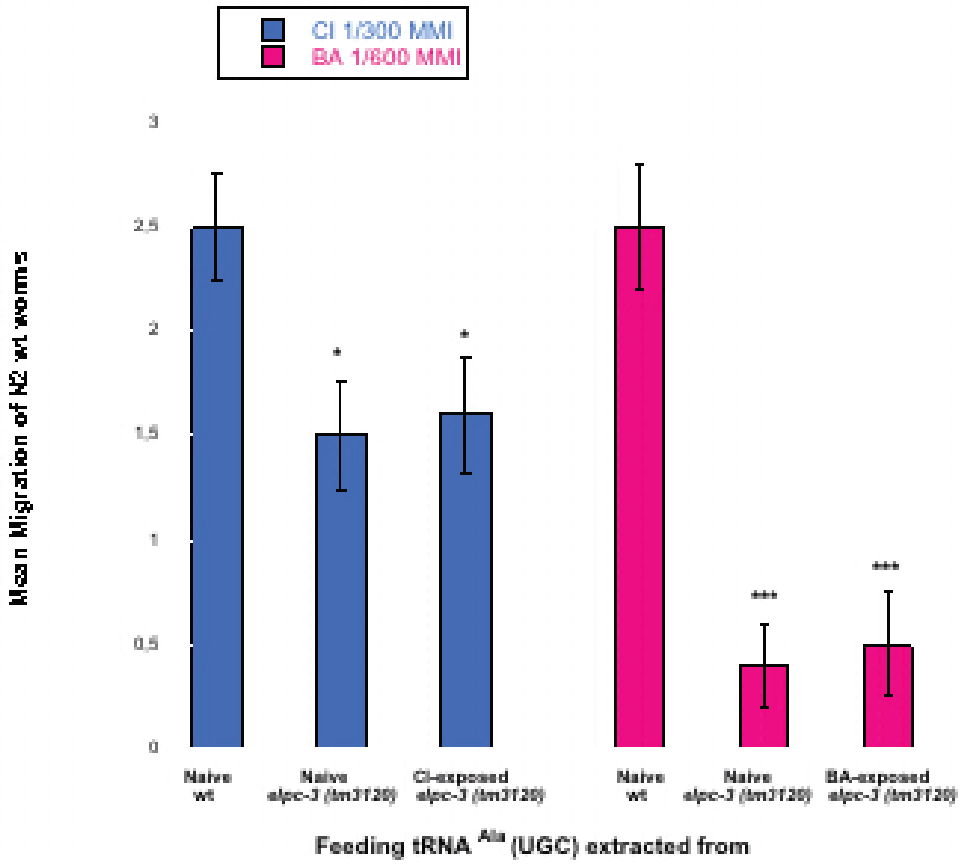
c


BA 1/600 MMI
***
Naive
BA-exp
BA-exp + tRNA Ala (TCG)
elpc-3 (ng15)

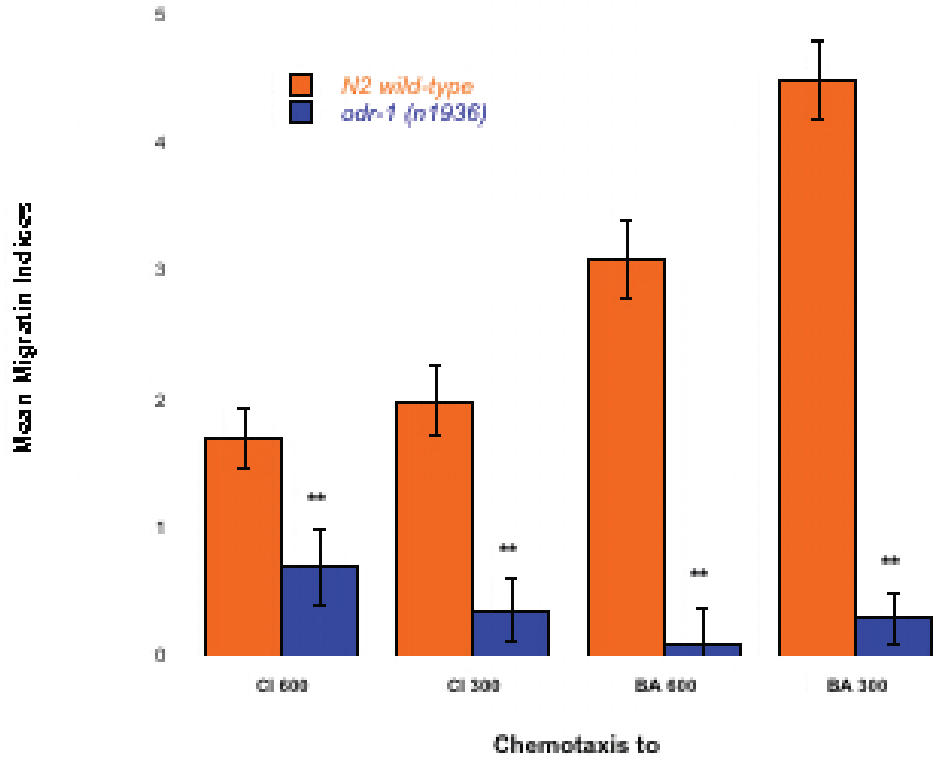
a


***
***
2
1,5
1
0,5
0
Naive
CI-exposed
CI-exp + tRNA UGC wt naive
CI-exp + tRNA UGC alpc-3 (tm3120) naive
alpc-1 (ng10)

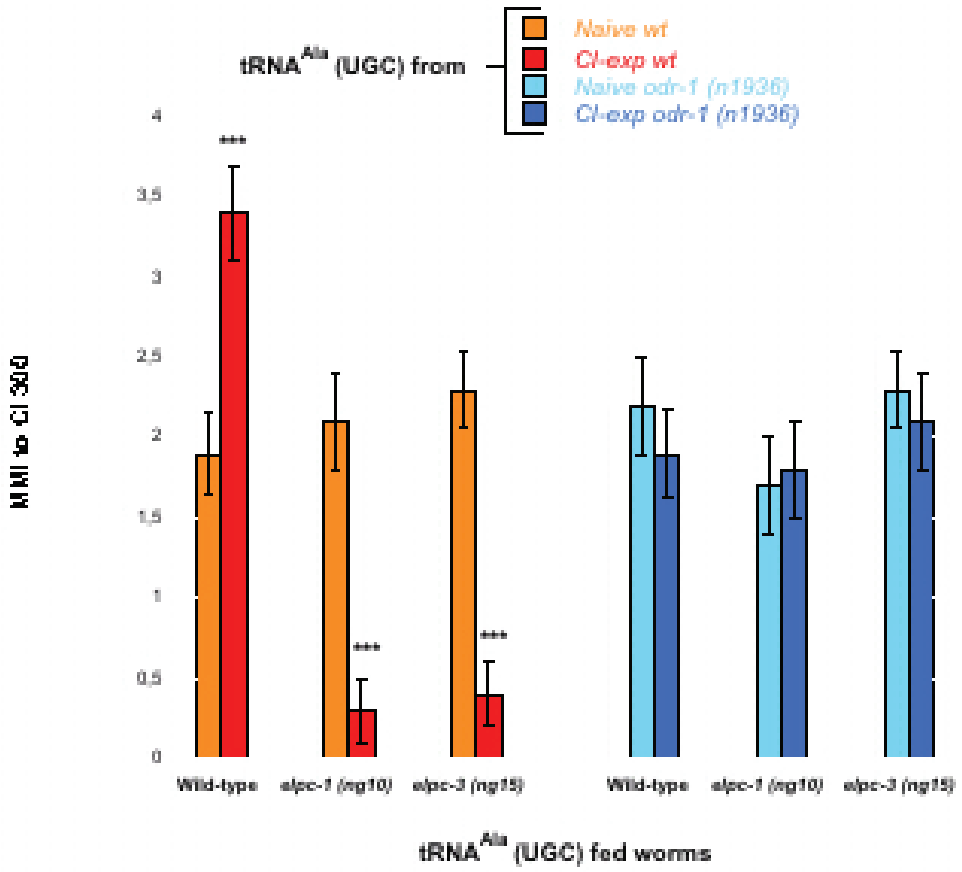
b

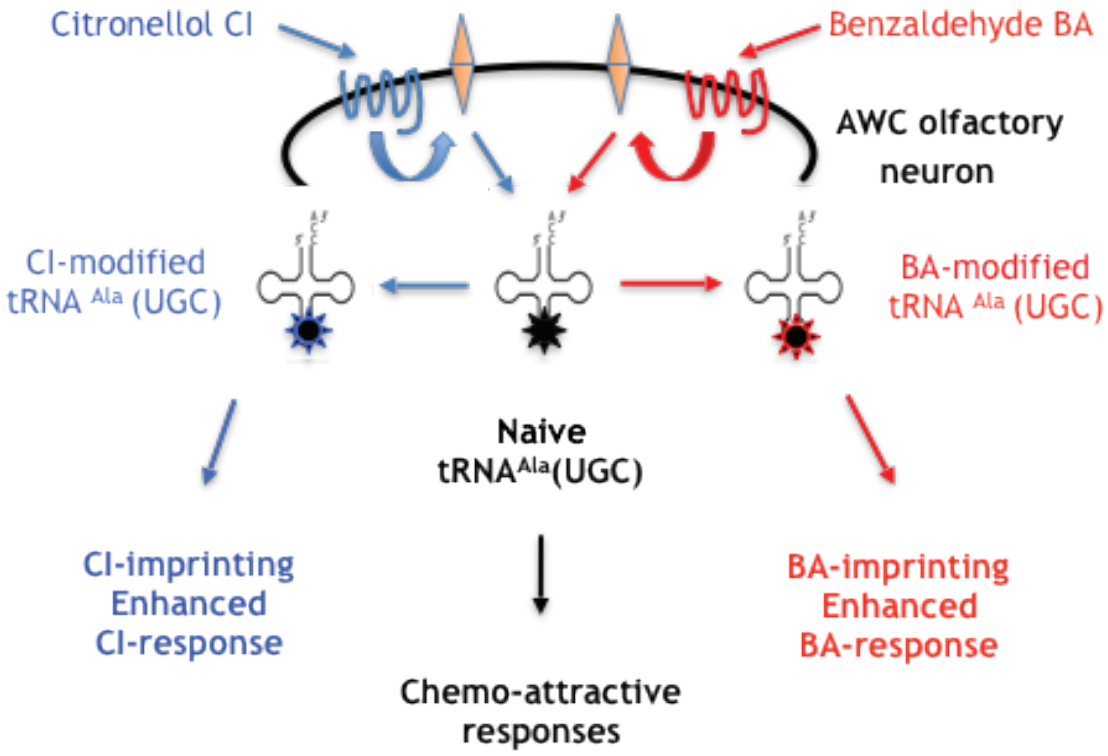

CI 1/300 MMI
BA 1/600 MMI
Mean Migration of N2 wt worms
3
2,5
2
1,5
1
0,5
0
*
*
***
***
Naive wt
Naive alpc-3 (tm3120)
CI-exposed alpc-3 (tm3120)
Naive wt
Naive alpc-3 (tm3120)
BA-exposed alpc-3 (tm3120)
Feeding tRNA Ala (UGC) extracted from

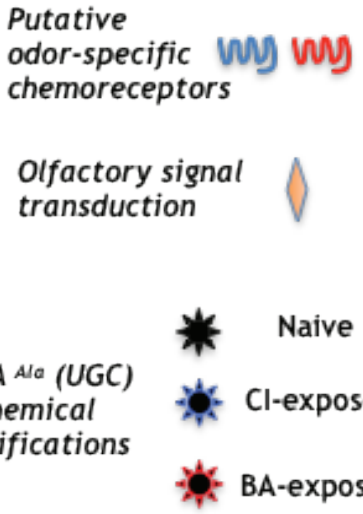
a


N2 wild-type
odr-1 (n1936)
Mean Migration Indices
0
1
2
3
4
5
**
**
**
**
CI 600
CI 300
BA 600
BA 300
Chemotaxis to

b


tRNA^Ala (UGC) from
Naive wt
CI-exp wt
Naive odr-1 (n1936)
CI-exp odr-1 (n1936)
MMI to CI 300
0
0,5
1
1,5
2
2,5
3
3,5
4
***
***
***
Wild-type
alpc-1 (ng10)
alpc-3 (ng15)
Wild-type
alpc-1 (ng10)
alpc-3 (ng15)
tRNA^Ala (UGC) fed worms


Citronellol CI
Benzaldehyde BA
AWC olfactory neuron
CI-modified tRNA Ala (UGC)
BA-modified tRNA Ala (UGC)
Naive tRNAAla(UGC)
CI-imprinting Enhanced CI-response
BA-imprinting Enhanced BA-response
Chemo-attractive responses


Putative odor-specific chemoreceptors
Olfactory signal transduction
tRNA Ala (UGC) chemical modifications
Naive
CI-exposed
BA-exposed