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Endogenous Nuclear RNAi Mediates Behavioral Adaptation to Odor

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SUMMARY

Most eukaryotic cells express small regulatory RNAs. The purpose of one class, the somatic endogenous siRNAs (endo-siRNAs), remains unclear. Here, we show that the endo-siRNA pathway promotes odor adaptation in *C. elegans* AWC olfactory neurons. In adaptation, the nuclear Argonaute NRDE-3, which acts in AWC, is loaded with siRNAs targeting *odr-1*, a gene whose downregulation is required for adaptation. Concomitant with increased *odr-1* siRNA in AWC, we observe increased binding of the HP1 homolog HPL-2 at the *odr-1* locus in AWC and reduced *odr-1* mRNA in adapted animals. Phosphorylation of HPL-2, an in vitro substrate of the EGL-4 kinase that promotes adaption, is necessary and sufficient for behavioral adaptation. Thus, environmental stimulation amplifies an endo-siRNA negative feedback loop to dynamically repress cognate gene expression and shape behavior. This class of siRNA may act broadly as a rheostat allowing prolonged stimulation to dampen gene expression and promote cellular memory formation.

INTRODUCTION

RNA interference (RNAi) has been exploited as a powerful experimental tool in both somatic and germ cells for over a decade (Fire et al., 1998), and organisms ranging in complexity from yeast to humans produce a range of endogenous small RNAs of 20–30 nucleotides in length. Although it is apparent that almost all cells of an organism are actively engaged in some form of endogenous RNAi, its role, particularly in somatic cells, remains unclear (reviewed in Ketting, 2011; Ghildiyal and Zamore, 2009).

Endogenous small RNAs are grouped into three classes according to their biosynthetic origin and the proteins they bind: piwi-RNAs (piRNAs), micro RNAs (miRNAs), and endogenous small interfering RNAs (endo-siRNAs). piRNAs and miRNAs are encoded by genes, whereas in *C. elegans*, endo- siRNAs are produced by RNA-dependent RNA polymerases that use thousands of cellular messenger RNAs (mRNAs) as templates to produce antisense small RNAs (Ghildiyal and Zamore, 2009; Ketting, 2011; Gent et al., 2010; Gu et al., 2009). Small RNAs have been linked to synaptic function and memory formation in mammals (McNeill and Van Vactor, 2012). For instance, the microRNA miR134 was shown to repress context-dependent fear learning and long-term potentiation in mice (Gao et al., 2010), and a piRNA has been shown to promote long-term synaptic facilitation of cultured *Aplysia* sensory neurons (Rajasethupathy et al., 2012). However, the extent to which small RNAs couple environmental stimuli to synaptic plasticity and the mechanism by which small RNAs regulate experience-induced behavioral changes remain a mystery.

Prolonged odor exposure induces a form of behavioral plasticity termed adaptation. *C. elegans* is innately attracted to food-related odors, but the attraction is diminished if starvation accompanies the odor. The resulting odor-adapted state lasts until the animal is fed (Colbert and Bargmann, 1997; Lee et al., 2010). Odor sensation (Bargmann et al., 1993) and adaptation (L'Etoile et al., 2002) occur within the olfactory sensory neuron that is referred to as AWC. Whereas odor sensation requires the guanylyl cyclase (GC) ODR-1, odor adaptation requires downregulation of ODR-1 (L'Etoile and Bargmann, 2000). Decreased intracellular cGMP, in part, drives the cGMP-dependent protein kinase EGL-4 into the AWC nucleus (O'Halloran et al., 2012). Once in the nucleus, EGL-4 is both necessary and sufficient to induce long-lasting odor adaptation (Lee et al., 2010). The mechanism by which nuclear EGL-4 triggers long-lasting odor adaptation is not known.

Small RNAs can regulate gene expression in both the cytoplasm and nucleus. For instance, miRNAs and siRNAs act as guides to target mRNAs for repression in the cytoplasm (reviewed in Ketting, 2011; Ghildiyal and Zamore, 2009). piRNAs and siRNAs can enter nuclei to trigger cotranscriptional gene silencing (nuclear RNAi) (Guang et al., 2008; Le Thomas et al.,

2013). During nuclear RNAi in *C. elegans*, the Argonaute (Ago) NRDE-3 shuttles siRNAs into the nucleus, where it binds nascent transcripts that exhibit sequence complementarity to NRDE-3-associated siRNAs (Guang et al., 2008; Guang et al., 2010). NRDE-3 recruits the conserved nuclear protein NRDE-2 and two nematode-specific proteins, NRDE-1 and perhaps NRDE-4, to RNAi-targeted nascent transcripts to inhibit RNA polymerase II (RNAP II) elongation (Guang et al., 2010; Burkhart et al., 2011). In addition, genes targeted for silencing by the nuclear RNAi pathway accumulate the repressive chromatin mark, H3K9me3 (Guang et al., 2010; Burton et al., 2011). In the *C. elegans* germline, piRNAs and siRNAs trigger nuclear RNAi at thousands of genomic loci (Claycomb et al., 2009; Gu et al., 2009; Ashe et al., 2012; Lee et al., 2012; Shirayama et al., 2012), and the silencing effects can endure for more than five generations (Vastenhouw et al., 2006; Buckley et al., 2012). When nuclear RNAi is disabled, *C. elegans* germlines lose their immortal character (Buckley et al., 2012).

In this paper, we examine the role of RNAi in neurons. Four lines of evidence indicate that, in the AWC olfactory sensory neurons of adult-behaving *C. elegans*, endogenous RNAi promotes odor adaptation by repressing the *odr-1* gene. First, we show that the nuclear RNAi Ago NRDE-3 is required in the AWC neuron to promote adaptation. Second, NRDE-3 coimmunoprecipitates (coIPs) *odr-1*-directed endo-siRNAs, and in adapted animals, we find increased levels of NRDE-3-bound *odr-1* siRNA. Third, odor exposure diminishes the levels of *odr-1* mRNA. Fourth, in odor adaptation, HPL-2, a heterochromatin-binding protein, is loaded onto the *odr-1* locus. Additionally, we find that phosphorylation of HPL-2 at sites that are in vitro targets of the odor-responsive kinase EGL-4 is both necessary and sufficient to promote odor adaptation in the AWC neurons of an intact animal. Our work indicates a mechanism by which environmentally relevant experiences may regulate gene expression, thereby shaping behavior in a specific and dynamic fashion.

RESULTS

The Nuclear RNAi Argonaute NRDE-3 Is Required in the AWC Sensory Neuron for Odor Adaptation

C. elegans is innately attracted to the odor, butanone. Attraction is assessed by the chemotaxis assay shown in Figure 1A, which allows quantification of the behavior in the form of a chemotaxis index (CI) (Bargmann et al., 1993). Naive wild-type animals exhibit a high CI to butanone, which decreases after 80 min of butanone exposure in the absence of food (Colbert and Bargmann, 1995). This experience-dependent decrease in CI is termed long-term olfactory adaptation. If the adapted CI is greater than one half of the naive CI, a strain is considered adaptation defective.

To investigate the role of small RNAs in long-term olfactory adaptation, we examined butanone adaptation in strains defective for major pathways producing RNAi in the soma, including the microRNA, exogenous RNA (exo-RNAi), and endogenous RNAi pathways. Animals lacking Dicer (DCR-1) were defective for adaptation (Figure 1B). Dicer, an RNAase III, processes double-stranded (dsRNA) into small noncoding RNAs (Grishok et al., 2001; Knight and Bass, 2001; Duchaine et al., 2006) that feed into the microRNA, exo-, and endo-siRNA interference pathways (Grishok et al., 2001; Knight and Bass, 2001; Grishok et al., 2005). These data suggest that Dicer-mediated processing of dsRNA is required for adaptation. By contrast, the adapted CI of strains bearing mutations in the pri-miRNA-processing RNase III enzyme Drosha, DRSH-1 (Denli et al., 2004), the miRNA-binding Ago, ALG-2 (Vasquez-Rifo et al., 2012), or the exo-RNAi pathway Ago, RDE-1 (Tabara et al., 1999), were not significantly different from the CI of wild-type controls (Figures 1B and S2 available online). These data suggest that, if Dicer-mediated dsRNA processing is required for butanone adaptation, microRNAs or the exoRNAi pathway are unlikely to mediate this process.

MUT-7, a putative 3′ to 5′ exonuclease, is required for accumulation of endogenous 22 nucleotide siRNAs that bind the WAGO clade of Agos (Yigit et al., 2006; Lee et al., 2006; Gu et al., 2009) and accumulation of 26 nucleotide siRNAs (Zhang et al., 2011), as well as transposon and transgene silencing, exogenous RNAi, and proper chromosome segregation (Ketting et al., 1999; Tabara et al., 1999; Dernburg et al., 2000; Tops et al., 2005). MUT-7 is also required for nuclear accumulation of NRDE-3 (Guang et al., 2008). HPL-2 is one of two *C. elegans* homologs of Heterochromatin Protein 1 (HP1) (Couteau et al., 2002). HPL-2 is involved in multiple cellular events, including gene regulation and DNA replication and repair (Couteau et al., 2002; Coustham et al., 2006; Black and Whetstine, 2011), as well as transgene silencing and piRNA-mediated gene silencing in the gonad (Grishok et al., 2005; Burkhart et al., 2011; Ashe et al., 2012; Buckley et al., 2012; Shirayama et al., 2012). Strains that lacked MUT-7 or HPL-2 were defective for butanone adaptation (Figure 1B). These results suggest that heterochromatin and possibly small RNAs promote odor adaptation downstream of Dicer.

Using *mut-7* and *hpl-2* promoter fusions to drive expression of GFP-tagged MUT-7 or HPL-2, respectively, we observed GFP expression in many cells, including both AWCs (Figure 1C). To determine whether MUT-7 and HPL-2 act in the AWC neurons, the site of odor sensation and adaptation, we asked whether cell-specific expression of MUT-7 and HPL-2 could rescue the odor adaptation defect of each corresponding mutant strain. Expressing MUT-7 or HPL-2 solely within the AWC neurons (from the AWC-specific *ceh-36*^{prom3} promoter [Etchberger et al., 2007]) of the respective mutant strain rescued its adaptation defects (Figure 1D). These data indicate that MUT-7 and HPL-2 act within AWC neurons to promote odor adaptation.

These factors could be required at the time of odor exposure or developmentally. To distinguish between these possibilities, we used the heat shock promoter p*hsp-16.2* (Stringham et al., 1992) to express each factor in the adult immediately prior to odor exposure. Heat-shock-driven expression restored adaptation to the *mut-7* and *hpl-2* strains (Figure 1E). Consistent with a requirement in the adult, neither morphology nor cell fate of the AWC was altered by loss of HPL-2 or MUT-7 (Figure S1B and Table S1). Together, these results indicate that the adaptation defects of *mut-7*- and *hpl-2*-deficient animals are not due to developmental defects.

To address whether MUT-7 and HPL-2 act in the same molecular pathway, we created *mut-7;hpl-2* and control

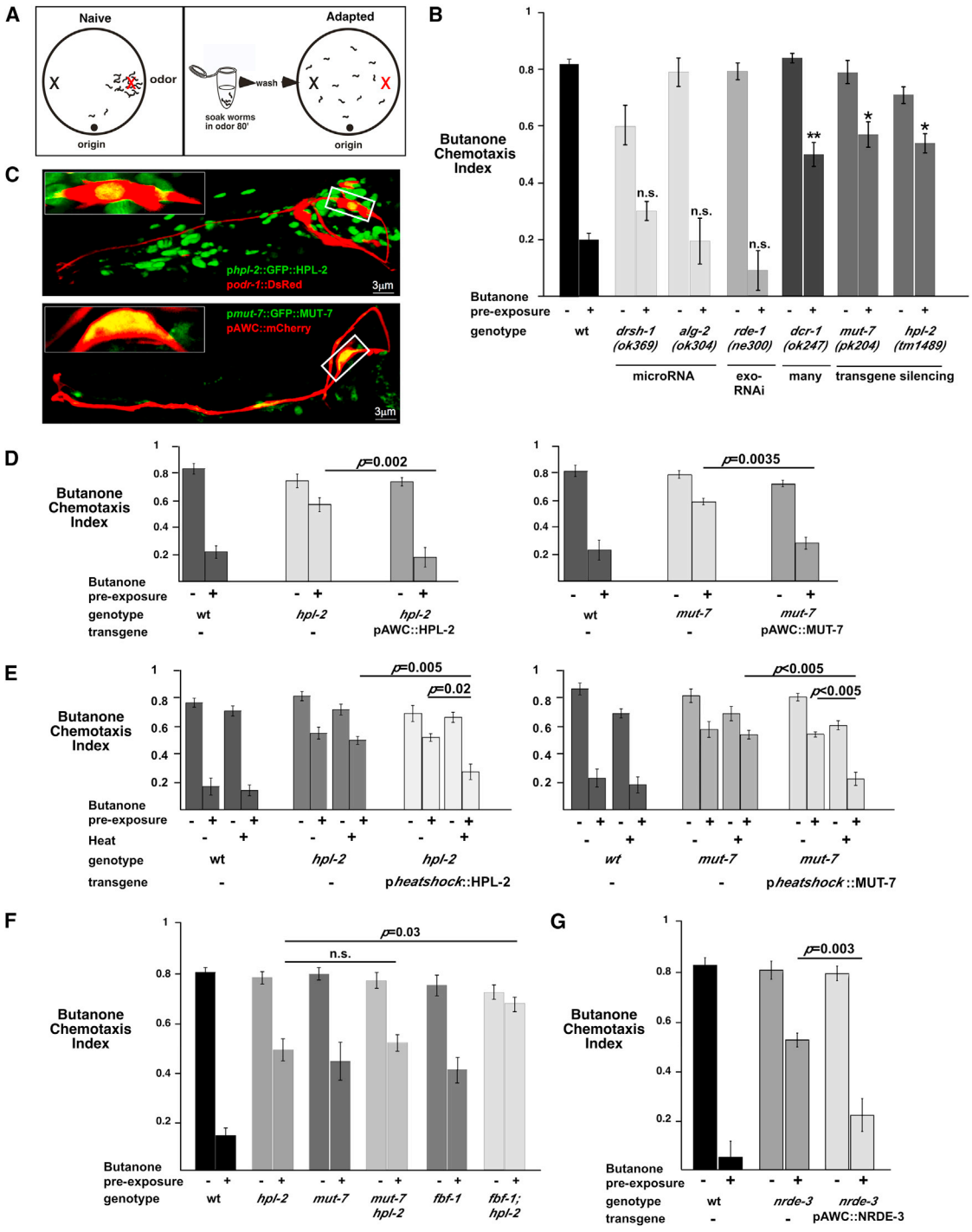

Figure 1. HPL-2 and MUT-7 Act at the Time of Odor Exposure in the AWC Neurons to Promote Adaptation to Butanone

(A) Olfactory adaptation paradigm. Animals exposed to buffer alone (naive) or butanone (adapted) for 80 min are placed at the "origin" of an agar-lined 10 cm Petri dish. Butanone is placed at the red and ethanol at the black "X." Sodium azide (to paralyze the worms) was also placed at each "X." Animals roam plates 2 hr before counting. The CI is calculated by subtracting the number of animals at the diluent from the number at the odor and dividing this by the number of animals that left the origin.

(B) Initial screen of mutant strains defective for siRNA pathways. Bars represent mean CIs of strains of the indicated genotype that had either been incubated with buffer (−) or buffer-diluted butanone (+) for 80 min. Bars for wild-type represent the mean CI of pooled controls for all the strains. All error bars are SEM. The side-by-side comparisons of each strain with wild-type controls are shown in Figure S1A. ** = $p < 0.005$, * = $p < 0.05$, and "n.s." = $p > 0.05$. Unless otherwise noted, all tests were two-tailed Student's t test, and all assays were performed on separate days with >100 animals per assay. *drsh-1*, *alg-2*, *dcr-1*: n = 4; *rde-1* mut-7: n = 6; *hpl-2*: n = 5.

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Table 1. Olfactory Adaptation Requires a Nuclear RNAi Pathway

Gene (Allele)	Gene Function	Butanone Adaptation[a]
dcr-1(ok247)[b]	RNase III nuclease	defective
rde-4(ne337)	double-stranded RNA binding	defective
drsh-1(ok369)	RNase III nuclease	partially chemotaxis defective
rrf-1(pk1417)	RNA-dependent RNA polymerase	wild-type
rrf-2(ok210)	RNA-dependent RNA polymerase	wild-type
rrf-3(pk1426)	RNA-dependent RNA polymerase	partially defective
drh-1(tm1329)	RNA helicase (RIG-I)	wild-type
drh-2(ok951)	RNA helicase (RIG-I)	defective
drh-3(ne4253)	RNA helicase	chemotaxis defective
rde-3(ne3364)	β-nucleotidyl transferase	defective
mut-7(pk204)	3′-5′ exonuclease	defective
rde-1(ne300)	exo-RNAi Argonaute	wild-type
ergo-1(gg98)	Argonaute	wild-type
alg-2(ok304)	microRNA Argonaute	wild-type
quintuple	5 Argonautes	wild-type
MAGO12	12 Argonautes	defective
nrde-3(gg66)	nuclear RNAi Argonaute	defective
nrde-2(gg91)	NRDE-3 binding nuclear factor	defective
nrde-1(gg88)	NRDE-2/3-chromatin associated	defective
hpl-2(tm1489)	histone H3 lysine 9 trimethyl binding (HP1)	defective

[a]Behavioral assays are shown in Figure S1A.
[b]Heterozygous animals are marked with hT2::GFP(I,III).

fbf-1;hpl-2 double-mutant animals. We found that the ability of *hpl-2* or *mut-7* single-mutant animals to adapt to odors was similar to the ability of *mut-7;hpl-2* double-mutant animals (Figure 1F), but the adaptation defects of *hpl-2* were enhanced in the *fbf-1;hpl-2* double-mutant strain. These data indicate that MUT-7 and HPL-2 likely act in the same pathway within AWC to promote odor adaptation at the time of odor exposure.

To probe the involvement of nuclear RNAi in adaptation, we examined the nuclear Ago, NRDE-3. NRDE-3 interacts with a subset of endo-22GRNAs and shuttles them into the nucleus, where they direct cotranscriptional gene silencing (Guang et al., 2008). NRDE-3 is expressed in the AWC neurons (B.-T.J. and N.D.L., unpublished data), and the NRDE-3 null (*nrde-3(gg66)*) was unable to adapt to butanone (Figure 1G). These adaptation defects were rescued by expressing NRDE-3 solely in the AWC neuron (Figures 1G and S1C), demonstrating that the nuclear RNAi Argonaute NRDE-3 acts in AWC to promote odor adaptation.

To better characterize the nuclear RNAi pathway, we surveyed adaptation in siRNA-defective strains that were deemed chemotaxis proficient (Table 1 and Figure S1A). In *C. elegans*, RNAi can be broken down into three steps: trigger processing, amplification, and silencing (reviewed in Pak et al., 2012). We found that trigger processing factors, Dicer and its partner RDE-4 (Tabara et al., 2002; Duchaine et al., 2006), are required for adaptation. The siRNA-amplifying RNA-dependent RNA polymerase (RdRP), RRF-3 (Simmer et al., 2002), was partially required as *rrf-3(pk1426)* animals failed to adapt in five out of eight trials. The silencing factor NRDE-3, along with its nuclear complex of NRDE-2 (Guang et al., 2010) and NRDE-1 (Burkhart et al., 2011), were each required. These results suggest that adaptation requires trigger processing, possibly RdRP amplification, and nuclear Ago-mediated silencing.

Biochemical and genetic analyses have implicated additional factors in RNAi. Of the many factors shown to associate with Dicer, DRH-2 (a Dicer-related DExH-box helicase [Tabara et al., 2002]) and RDE-3 (a β-nucleotidyl transferase) (Duchaine et al., 2006) were required for adaptation. Taken as a whole, our genetic analysis indicates that the nuclear RNAi pathway is likely to act in the AWC neuron to promote odor adaptation downstream of DCR-1/RDE-4-mediated small RNA production.

odr-1 mRNA Decreases in Odor-Adapted Animals

To identify a target for siRNA in adaptation, we used quantitative real-time PCR to probe endo-22GRNAs that map to AWC-expressed genes (see Supplemental Information). We found that the *odr-1*-derived 22GRNAs, odr-1.6 and odr-1.7, as well as the *unc-40*-derived 22GRNA, unc-40.2, gave the most robust signals. *odr-1* encodes a GC whose downregulation is required for odor adaptation (L'Etoile and Bargmann, 2000), and *unc-40* encodes an axon guidance and synaptogenesis factor

(C) HPL-2 and MUT-7 are expressed in AWCs. Fluorescent confocal images of wild-type animals expressing the putative *hpl-2* (top) or *mut-7* (bottom) promoters driving GFP-tagged versions of each protein. AWC is marked with *ceh-36*^{prom3} promoter driving mCherry. Anterior is at the left for both images. Figure S1B is associated with this panel.
(D) Expression of HPL-2 or MUT-7 in AWC rescued the adaptation defects of each mutant. HPL-2 (left graph, third pair of bars) or GFP-tagged MUT-7 (right graph, third pair of bars) was expressed in AWC from p*ceh-36*^{prom3} in *hpl-2(tm1489)* or *mut-7(pk204)*, respectively. **p = 0.002, *p = 0.0035, and n > 5 for each.
(E) Expression of HPL-2 or MUT-7 at the time of odor exposure rescued adaptation defects. *hpl-2(tm1489)* (left) or *mut-7(pk204)* (right) transgenic for the respective cDNA under the control of the heat shock promoter (p*hsp16-2)* were heated (+) 1 hr before odor exposure. Heat-treated animals' exposed CI's were significantly different from either before heating (p = 0.02, *hpl-2*; p < 0.005, *mut-7*) or from nontransgenic animals that had been heated (p = 0.005, *hpl-2*; p < 0.005, *mut-7*), n > 5 for each.
(F) HPL-2 and MUT-7 act in the same genetic pathway for adaptation. Mean naive (−) and exposed (+) CIs of animals of the indicted genotype. The adaptation defects of the *fbf-1(ok91)* strain are due to loss of the translational control pathway (Kaye et al., 2009) that acts in parallel with *hpl-2*.
(G) Expression of NRDE-3 in AWC rescued the adaptation defects of the *nrde-3* mutant strain. Mean CI of naive (−) and exposed (+) wild-type, *ndre-3(gg66)*, and NRDE-3 expressed in AWC (p*ceh-36*^{prom3}) of the *nrde-3(gg66)* mutant strain. Figure S1D is associated with this figure.
Error bars for each panel are SEM.

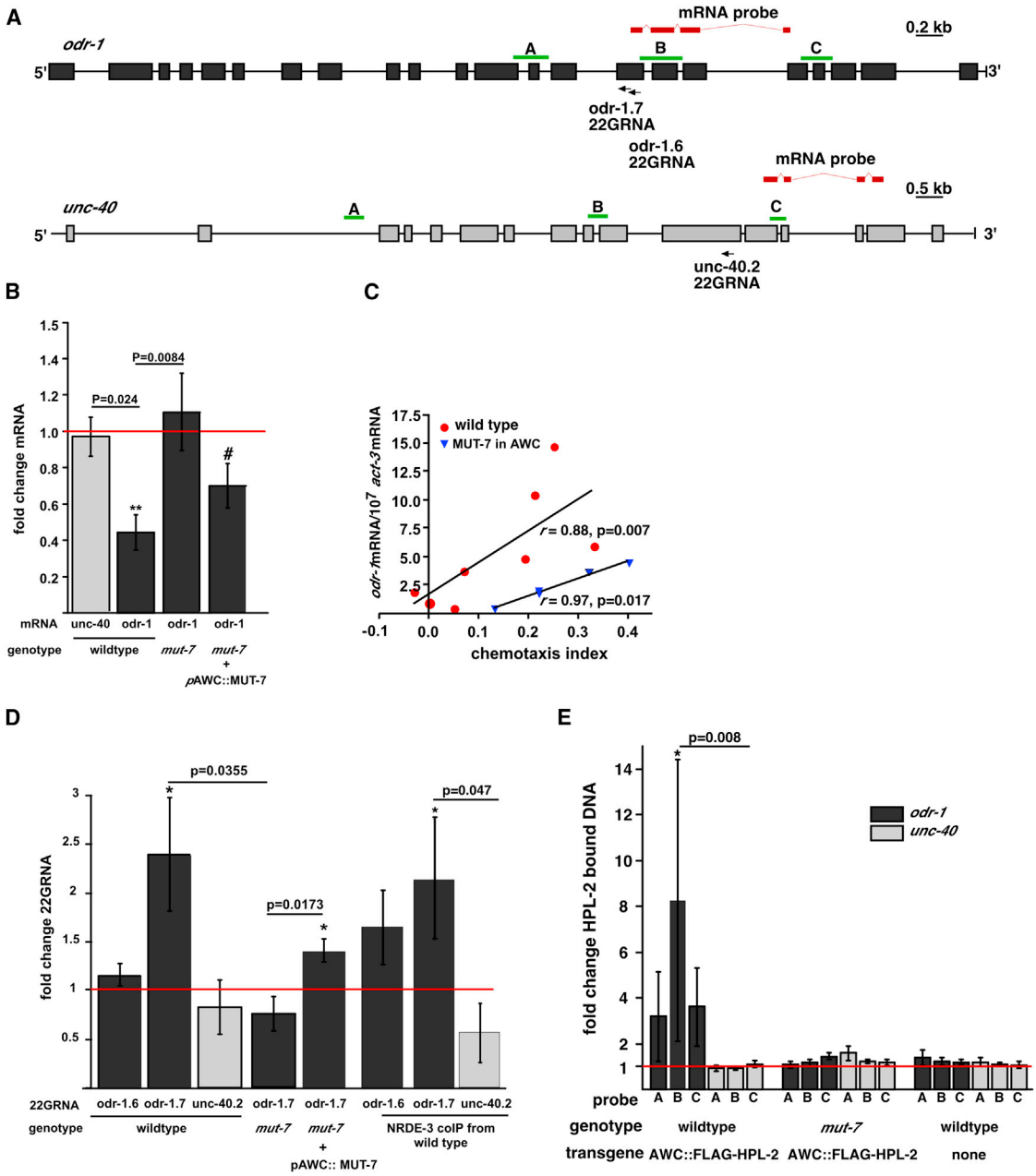

Figure 2. Prolonged Odor Stimulation Dynamically Regulates *odr-1*-Derived 22G RNAs, Association of HPL-2 with the *odr-1* Locus, and Levels of *odr-1* mRNA

(A) Diagram of the *odr-1* and *unc-40* genes. The *odr-1* and *unc-40* 22GRNAs examined are indicated with arrows below the gene. The PCR amplicons for ChIP-qPCR are in green. PCR amplicons for mRNA analysis are in red.

(B) Prolonged odor exposure decreases *odr-1* mRNA levels. Bars represent the mean fold change in *unc-40* (gray) or *odr-1* (black) mRNA level as a function of odor exposure (adapted/naive). RNA from animals of the indicated genotype was normalized to *act-3* mRNA. The red line indicates "no change," and the significance of the difference between a sample and "no change" was assessed using a two-tailed Wilcox signed rank test. ** indicates that median of sample and "no change" are different; $p < 0.005$. # indicates a difference of $p = 0.034$ (nonparametric, pair-wise comparison) in medians between the naive and adapted values of the mRNA. p values displayed are from two-tailed Mann-Whitney test of medians. Chemotaxis behavior for each population and the individual data points for each pair are shown in Figure S2A. Error bars represent SEM, and $n > 3$.

(C) Chemotaxis behavior correlates with the level of *odr-1* mRNA in butanone-adapted animals. The butanone CI of odor-exposed animals was compared with their *odr-1* mRNA level (mRNA levels normalized to *act-3* mRNA). Red circles indicate wild-types, and blue triangles indicate *mut-7(pk204)* animals expressing MUT-7 solely within the AWC neurons. r is Pearson's correlation coefficient, and p is from a two-tailed Student's t test (wild-type, $n = 8$; AWC MUT-7 rescue, $n = 5$).

(D) Prolonged odor exposure increases NRDE-3-bound *odr-1* 22GRNA levels. The first five bars represent mean fold change in total 22GRNAs normalized to odor-insensitive sn2343 RNA in adapted versus naive animals of the indicated genotype. Error bars represent SEM. Red line indicates no change. * = $p < 0.03$,

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(Hedgecock et al., 1990; Colon-Ramos et al., 2007). The gene structure, along with the amplicons derived from mRNA, 22GRNA, and genomic DNA, are indicated in Figure 2A.

Nuclear RNAi silences gene expression, leading to lower levels of target mRNA. To determine whether *odr-1* message levels are decreased in odor-adapted populations, we performed quantitative real-time PCR on RNA collected from the same samples that showed behavioral adaptation to butanone (Figure S2A). We found that *odr-1* mRNA decreased by approximately one half in odor-adapted as compared to naive populations (Figure 2B, second bar). By comparison, *unc-40* mRNA levels were unchanged (Figure 2B, first bar). In *mut-7(pk204)* animals, *odr-1* mRNA levels were not odor responsive (Figure 2B, third bar, Figure S2A for individual assays and behavior), but expression of MUT-7 solely within AWC partially restored odor responsiveness (Figure 2B, fourth bar). Thus, in odor-adapted populations, the *odr-1* mRNA decreases, and these changes depend on odor exposure as well as functional MUT-7.

To understand whether the modest decrease in *odr-1* mRNA (Figure 2B) has a behavioral consequence, we asked whether the level of *odr-1* mRNA correlates with the CI of odor-adapted populations. We found that the levels of *odr-1* mRNA correlated strongly with odor attractiveness (Figure 2C). The correlation between CI and *odr-1* mRNA was even stronger in the *mut-7(pk204)* strains that expressed MUT-7 solely in the AWC neuron (Figure 2C). This indicates that the decreases we observe in *odr-1* mRNA in AWC could be responsible for the stably diminished odor attractiveness that is the hallmark of long-term adaptation.

In the analysis described above, we examined mRNA from whole worms, but two lines of evidence indicate that this drop in mRNA occurs within the AWC neurons: loss of *odr-1* leads to the adapted phenotype, and this is rescued by expression of ODR-1 in the AWC neurons (L'Etoile and Bargmann, 2000), and overexpression of ODR-1 in AWC alone blocks adaptation (L'Etoile and Bargmann, 2000). Taken together, the data implicate downregulation of the *odr-1* gene in AWC in butanone adaptation.

odr-1-Directed 22GRNA Increases in the AWC Sensory Neuron of Adapted Animals

To determine whether there is evidence for the endo-RNAi pathway acting in adaptation, we used quantitative real-time PCR to compare the levels of *odr-1* and *unc-40* 22GRNA species in naive and butanone-adapted populations. We found that expression of the *odr-1* 22GRNA odr-1.7 increased by more than 2-fold in adapted animals compared to naive controls (Figure 2D, second bar, and Figure S2B). The levels of a less abundant 22GRNA, odr-1.6, and unc-40.2, however, did not change significantly (red line indicates a ratio of 1:1 for adapted to naive levels) (Figure 2D, first and third bars, and Figure S2B). Thus, a 22GRNA (odr-1.7) complementary to the *odr-1* gene increases in animals adapted to odor.

These measurements of 22GRNAs reflect levels throughout the animal, including the germline (Gu et al., 2009). To determine whether odr-1.7 22GRNA is regulated by odor specifically in AWC, we analyzed 22GRNA from animals that expressed MUT-7 only in AWC (Figure 1D). Though total odr-1.7 22GRNA levels were insensitive to odor exposure in *mut-7*-defective animals, expression of MUT-7 in AWC restored odor responsiveness to this species of 22GRNA (Figure 2D, fourth and fifth bars, and Figure S2B). Thus, the levels of odr-1.7 22GRNA are increased by odor exposure when a factor required for 22GRNA accumulation (Gu et al., 2009) is expressed solely within the AWC neuron.

odr-1 siRNAs Are Loaded onto NRDE-3 in Adaptation

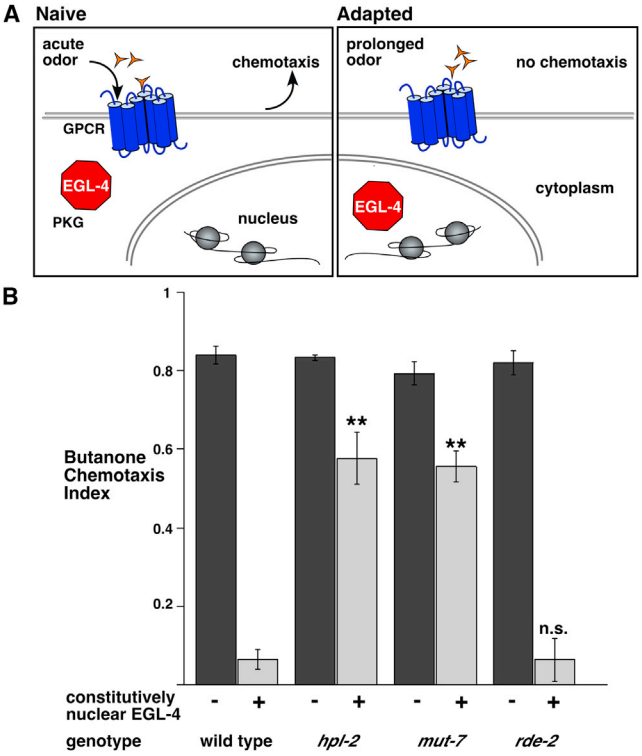
To better understand how the nuclear RNAi pathway might function in odor adaptation, we asked whether odr-1.7 or unc-40.2 22GRNAs associate with NRDE-3. We probed this association by IPing 3XFLAG-tagged NRDE-3 (see Figure S2C for behavior). We found odr-1.6, 1.7 and unc-40.2 coimmunoprecipitated with NRDE-3. The level of odr-1.7 22GRNA in association with NRDE-3 was increased significantly in adapted animals (Figure 2D, seventh bar). By contrast, levels of coimmunoprecipitated odr-1.6 or unc-40.2 22GRNA were not changed in the same animals, indicating that NRDE-3 specifically binds more odr-1.7 22GRNA in adapted animals. This finding supports a model in which ODR-1 mRNA is reduced by NRDE-3/odr-1.7 22GRNA, mediating downregulation of the *odr-1* gene.

HPL-2 Associates with the *odr-1* Locus in Odor-Adapted AWC Neurons

One biochemical readout of siRNA/NRDE-3-directed silencing is increased heterochromatin deposition at the targeted locus (Burkhart et al., 2011; Guang et al., 2010; Gu et al., 2012). To understand whether odr-1.7/NRDE-3 might target the *odr-1* locus in the odor-adapted AWC neuron, we expressed 3XFLAG-tagged heterochromatin associated factor, HPL-2, from the *odr-3* promoter (which drives expression in AWCs and four other neurons; see Figure S2D for behavior). When we performed chromatin immunoprecipitation (ChIP) of HPL-2 followed by qPCR on naive and behaviorally adapted populations, we found that ChIP of the *odr-1* locus was increased in adapted AWC neurons (Figure 2E). The greatest increase in HPL-2-associated

Wilcoxon signed-rank test for median values versus no change. p values displayed are the comparison of medians using an unpaired two sample Mann Whitney nonparametric t test, n > 3. Figure S2B is associated with this panel. The last three bars represent mean fold change in p*nrde-3*::NRDE-3 coIPed 22GRNA (n = 6) normalized to the odor-insensitive X-cluster. Error bars represent SEM. * = p < 0.04, Wilcoxon signed rank test for median values versus no change. Displayed p values are from a pairwise, one-tailed t test, p = 0.0469 of medians. Figure S2C shows the behavior.

(E) Prolonged odor exposure specifically increases HPL-2 binding to the *odr-1* locus in a MUT-7-dependent fashion. The mean ratio of 3XFLAG-HPL-2 expressed in AWC (p*odr-3*) coimmunoprecipitated *odr-1* (dark bars) or *unc-40* (light bars) in adapted versus naive animals is shown above the genotype of each population. Error bars represent SEM. Also indicated is the PCR-amplified, coIPed region of each locus corresponding to "A," "B," and "C" in (A). Coimmunoprecipitated DNA from each locus was normalized to input. This was then normalized to the ratio of IPed *act-3* to input. *act-3* levels were odor insensitive. *p = 0.031, one-tailed Wilcoxon signed-rank test comparing median values to no change (the red line). The median value of *odr-1* B was compared to *unc-40* B; p = 0.0079 using a two-tailed Mann Whitney test. n = 5. The final set of bars represents background from nontransgenic animals. Figure S2D shows the behavior.



C

genotype	% population with nuclear EGL-4 in AWC	
	naive	butanone exposed
wild type	12 (n=68)	99 (n=99)
hpl-2	12 (n=86)	89 (n=114)
mut-7	5 (n=88)	89 (n=93)

Figure 3. HPL-2 and MUT-7 Act Downstream of Nuclear EGL-4

(A) Current model for long-term olfactory adaptation of the AWC neuron. Acute stimulation of AWC localized G-protein-coupled receptors (GPCR) by odor (left) causes animals to chemotax toward the odor. After prolonged odor exposure (right), the cGMP-dependent protein kinase (PKG) EGL-4 translocates to the nucleus to cause animals to ignore the odor for prolonged periods of time.

(B) Once in the nucleus, EGL-4 requires HPL-2 and MUT-7 to promote adaptation. The chemotaxis index of the indicated strains that express constitutively nuclear EGL-4 from a transgene (+) were compared to their siblings that did not carry this transgene (−). *rde-2* is a control, adaptation-proficient strain (Figure S1A). Importantly, all animals were naive to butanone. $n > 3$ with >100 animals analyzed per assay. $^{**}p < 0.0001$, two-tailed Student's t test. Bars represent the mean CIs, and the error bars represent SEM.

(C) HPL-2 and MUT-7 are not required for odor-induced nuclear entry of EGL-4. GFP-tagged EGL-4 was expressed in either wild-type, *hpl-2(tm1489)*, or *mut-7(pk204)* strains. Animals were exposed to buffer alone (naive) or butanone for 80 min before imaging. The percentage of the population that showed nuclear EGL-4 in one AWC neuron was determined.

ChIP (8-fold higher in adapted than in naive) was located just downstream of the region encoding odr-1.7. Further, the odor-dependent increase was not seen at the *unc-40* locus. As a specificity control for the 22GRNA pathway, we performed ChIP from *mut-7* loss-of-function animals, which show no increase in odr-1.7 22GRNA levels in response to odor and likewise show no increase in *odr-1* ChIP (Figure 2E). These results show that *odr-1* is a target for increased HPL-2 association in the odor-adapted AWC. Though this is not the only interpretation, these results are most consistent with nuclear RNAi targeting this locus.

HPL-2 Is a Direct Phosphorylation Target of the Odor-Responsive Kinase, EGL-4

How might an environmental signal such as odor intersect with the endogenous nuclear RNAi pathway to mediate adaptation? Prolonged odor stimulation causes nuclear accumulation of the cGMP-dependent protein kinase EGL-4 (Figure 3A) (O'Halloran et al., 2009; Lee et al., 2010), and nuclear EGL-4 is both necessary and sufficient to induce long-term odor adaptation. Indeed, expression of constitutively nuclear EGL-4 (NLS-EGL-4) in AWC decreased chemotaxis toward inherently attractive odors even in naive animals (Figure 3B) (Lee et al., 2010; O'Halloran et al., 2009). MUT-7 or HPL-2 could thus act by promoting nuclear accumulation of EGL-4. However, we found that nuclear accumulation of EGL-4 was not altered in *mut-7* or *hpl-2* mutant strains (Figure 3C). Three lines of evidence led us to hypothesize instead that EGL-4 promotes adaptation by phosphorylating and activating MUT-7 and HPL-2. First, we found that constitutively nuclear EGL-4 required both HPL-2 and MUT-7 to induce adaptation in naive animals (Figure 3B). Second, predicted EGL-4 phosphorylation sites within MUT-7 and HPL-2 (Figure 4A) are required for adaptation (Figures 4B, 4C, and S3). Third, expression of phospho-defective MUT-7 in wild-type animals caused adaptation defects, suggesting that MUT-7 phosphorylation is required for this behavioral change (Figure S3D).

MUT-7 and HPL-2 might be direct targets of the EGL-4 kinase; thus, we asked whether NLS-EGL-4 phosphorylates these factors in vitro. We were unable to purify full-length MUT-7, so we focused on HPL-2. We found that *C. elegans* expressed immunopurified NLS-EGL-4 phosphorylated recombinant HPL-2 and that the level of ^{32}P incorporation diminished when the predicted PKG phosphorylation sites within HPL-2 were mutated (Figures 4D and S3G). We therefore conclude that these sites are direct targets of EGL-4 in vitro. Thus, it is likely that HPL-2, a nuclear protein, is directly phosphorylated by EGL-4 once it enters the AWC nucleus.

Phosphorylation of HPL-2 at EGL-4 Target Sites Is Both Necessary and Sufficient to Promote Odor Adaptation

If nuclear EGL-4 promotes odor adaptation by phosphorylating HPL-2 or MUT-7, then mimicking phosphorylation at consensus sites is predicted to promote adaptation in naive animals. To test this, we replaced the serines and threonines at each predicted EGL-4 phosphorylation site in MUT-7 and each in vitro verified site in HPL-2 with the phosphomimetic, glutamic acid (Mansour et al., 1994). Expression of the phosphomimetic form of MUT-7 in wild-type worms had no effect on chemotaxis. Because only ~50% of known functions of phosphorylated residues can be mimicked by glutamic acid substitutions (Maciejewski et al., 1995), we can make no conclusions about MUT-7 phosphorylation. However, expressing the phosphomimetic form of HPL-2 in wild-type animals substantially reduced naive attraction to butanone, whereas expression of the wild-type HPL-2 had no effect (Figure 4E). Thus, mimicking phosphorylation of HPL-2 at EGL-4 target residues is sufficient to

promote behavior that resembles the adapted state. When each site was analyzed individually, we found that HPL-2(S155E), which lies in the chromo shadow domain (CSD), had the greatest effect (Figure S3E).

HPL-2 (all S/T to E) could act as a gain-of-function mutation that engages the adaptation machinery in the absence of odor, or it could nonspecifically diminish AWC function. To distinguish between these possibilities, we expressed HPL-2 (all S/T to E) in mutants that lack the downstream adaptation-promoting factor, OSM-9 (Colbert and Bargmann, 1995). These animals were able to chemotax significantly better to butanone than the parental strain (Figure 4F). Thus HPL-2(all S/T to E) promotes adaptation upstream of OSM-9. We conclude that phosphorylation of HPL-2 at EGL-4 target sites is sufficient to promote adaptation even in the absence of odor exposure. Importantly, EGL-4 is the only PKG in *C. elegans* that is required for odor adaptation (Figure S3H). Thus, it is likely that odor acts via EGL-4 to activate HPL-2.

To understand whether the siRNA pathway was required for HPL-2(all S/T to E) to induce adaptation, we asked whether *mut-7* was required for this gain-of-function phenotype. Loss-of-function MUT-7 (*mut-7(pk204)*) suppressed the ectopic adaptation seen in naive animals expressing HPL-2(all S/T to E) (Figure 4F). Thus, phosphorylation of HPL-2 is both necessary and sufficient for adaptation, but it requires fully functional MUT-7. This is consistent with the ChIP studies in Figure 2E that show that accumulation of HPL-2 at the *odr-1* locus of adapted worms requires functional MUT-7. The observation that HPL-2(allS/T to E) promotes adaptation in the naive animal—and yet loss of MUT-7 blocks this adaptation—indicates that, in the naive animal, there is sufficient MUT-7-dependent RNAi to engage the adaptation process.

DISCUSSION

An emerging paradigm is that small noncoding RNAs provide memory of nonself gene expression (Shirayama et al., 2012); this work extends the role of siRNAs to encoding memory of the environment and experience. We provided evidence that, in the olfactory sensory neurons (AWCs) of adult-behaving *C. elegans*, endogenous RNAi promotes odor adaptation by repressing the *odr-1* gene (Figure 5). Our data show that, in response to prolonged odor exposure, *odr-1*-directed 22GRNAs increase, and this increase is most likely to occur in the AWC neuron (Figure 2D). We demonstrated that these 22GRNAs are loaded on to the nuclear Ago, NRDE-3 (Figure 2D), that acts in AWC (Figure 1G). NRDE-3 may shuttle the *odr-1* 22GRNA into the AWC nucleus, and we have direct evidence that the HP1 homolog, HPL-2, is loaded on to the *odr-1* gene in response to odor (Figure 2E). We provide in vitro evidence that HPL-2 can be phosphorylated by nuclear EGL-4 (Figure 4). Mimicking phosphorylation of HPL-2 is sufficient to evoke adaptation behavior. Phosphorylation of HPL-2 would repress the *odr-1* gene and ultimately lead to the reduced levels of *odr-1* mRNA seen in adapted animals (Figure 2B). This reduction in *odr-1* mRNA correlates strongly with behavior (Figure 2C). One gap in this model is that we do not know whether NRDE-3 or *odr-1* 22GRNA bind the *odr-1* locus. An alternate explanation is that *odr-1* is repressed by a factor that is itself negatively regulated by a second factor that is repressed by NRDE-3 and the RNAi pathway. In this scheme, the repressive factor that binds to the *odr-1* regulatory regions would set up repressive chromatin marks that center at the same part of the *odr-1* gene that encodes the *odr-1* 22GRNA bound by NRDE-3. However, the proposed model is more parsimonious and consistent with the data than the alternative model and leads to the exciting hypothesis that RNAi may act broadly as a biological rheostat to allow stimulation to dampen gene expression and may promote cells to alter their responses as a function of previous stimulation.

Specificity of Odor Adaptation within AWC Neurons

Butanone adaptation does not affect attraction to benzaldehyde or isoamyl alcohol (Colbert and Bargmann, 1995), so how would downregulation of ODR-1, a GC required for all AWC responses (L'Etoile and Bargmann, 2000), specifically adapt the butanone response? The other odors are sensed by both left and right AWCs, and butanone is sensed by only one AWC (Wes and Bargmann, 2001). Indeed, prolonged butanone exposure results in nuclear EGL-4 in only one AWC (Lee et al., 2010). Thus, reducing the levels of ODR-1 in the butanone responsive neuron should not affect chemotaxis mediated by the other AWC. Furthermore, each odor requires different factors for adaptation (Colbert and Bargmann, 1995), and thus, each response may have unique sensitivity to the level of ODR-1.

The Nuclear RNAi Pathway Acts with HP1 in Odor Adaptation

We found that the nuclear Argonaute NRDE-3 is required in AWC for odor adaptation, and it binds *odr-1* siRNA in an odor-dependent fashion. Prior work showed that NRDE-3 acts in the nucleus along with NRDE-2, NRDE-1, and NRDE-4 to establish H3K9me3 marks on the target locus, thereby silencing transcription (Burkhart et al., 2011; Burton et al., 2011; Gu et al., 2012; Guang et al., 2010). This connection between endo-siRNA, H3K9me3 marks, and gene silencing was originally reported in *S. pombe*, in which silencing involves deposition of H3K9me3 marks directed by siRNAs produced from pericentromeric repeat regions and the mating type locus (Aygün and Grewal, 2010). In *pombe*, these siRNAs induce a transcriptional silencing complex (RITS) that localizes chromatin to specific nascent transcripts. A feed-forward silencing loop is established as chromodomain proteins, including the HP1 homolog, and methyltransferases are nucleated by RITS complexes and in turn recruit more methyltransferases. Concurrently, RNA-dependent RNA polymerase complexes (RDRCs) are recruited, thus increasing siRNA production (Hayashi et al., 2012; Rougemaille et al., 2012; Yamanaka et al., 2013). A direct link between chromatin, RNAi, and RITS was demonstrated when the CSD of *pombe* HP1 was shown to interact with several members of the RNAi and RITS machinery via the HP1-binding protein, Ers1 (Rougemaille et al., 2012). Because Ers1 interacted specifically with the CSD of yeast HP1, and we show that in *C. elegans*, phosphorylation of this domain is sufficient to induce adaptation, we speculate that the *C. elegans* HPL-2 CSD likewise nucleates RNAi factors on genes such as *odr-1* whose silencing promotes adaptation. Indeed, because loss of *mut-7* suppressed the gain-of-function HPL-2(S155E), MUT-7 may either act along with or

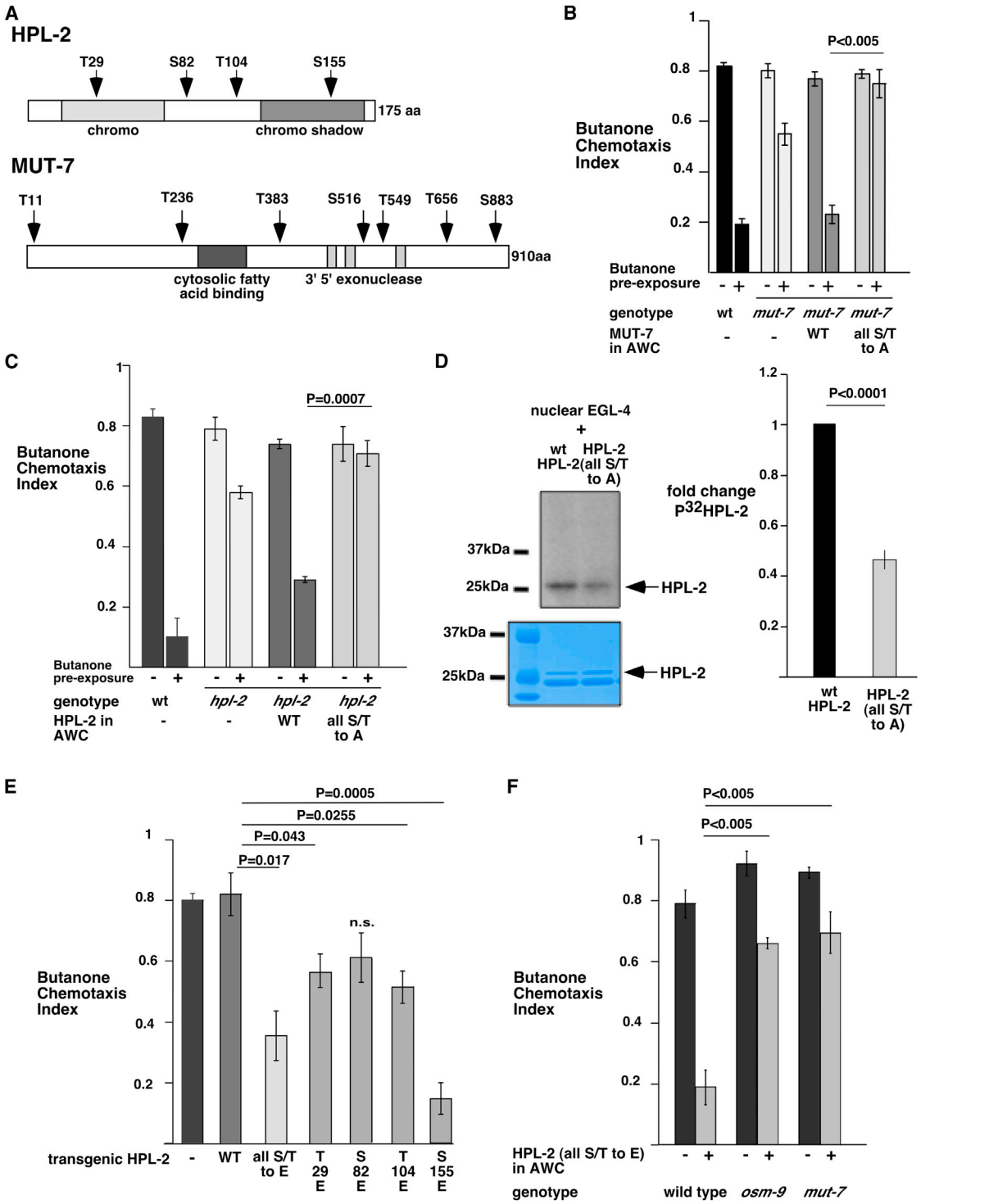


Figure 4. Phosphorylation of HPL-2 and MUT-7 at Predicted PKG Sites Is Required for Adaptation

(A) Schematic of HPL-2 and MUT-7. (Top) HPL-2 contains an N-terminal chromodomain, a C-terminal chromo shadow domain, and four predicted PKG phosphorylation sites. (Bottom) MUT-7 contains two predicted functional domains—cytosolic fatty acid binding domain and 3′ to 5′ exonuclease—and seven predicted PKG phosphorylation sites.

(B) Phosphorylation of predicted PKG target sites in MUT-7 is required for adaptation. Mean CIs of wild-type or *mut-7(pk204)* strains expressing the indicated form of MUT-7 in AWC. Figure S3D shows individual lines. n = 3 and p value is from a two-tailed Student's t test. The lines rescued the sterility defects of *mut-7(pk204)* (Figure S3B).

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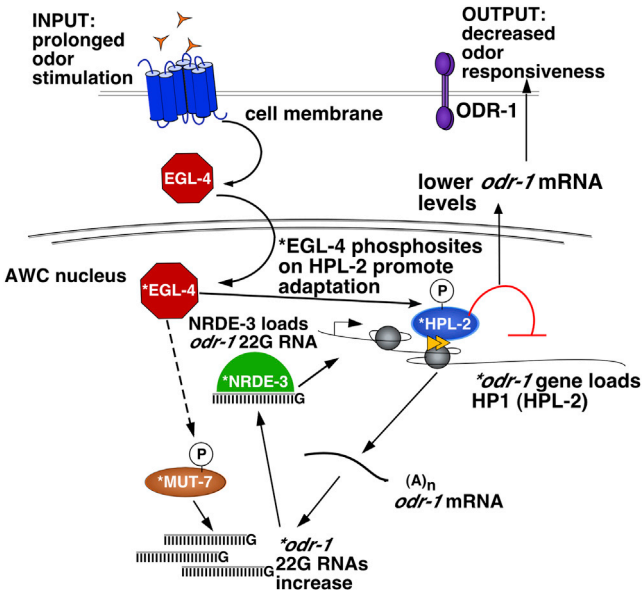

Figure 5. Prolonged Stimulation Induces Long-Term Olfactory Adaptation in the AWC Neurons via an siRNA and Chromatin-Remodeling-Dependent Process

Model for how prolonged butanone stimulation may lead to long-lasting olfactory adaptation in the AWC neuron. Asterisks indicate processes and factors shown to act in AWC. Odor exposure stimulates a seven transmembrane GPCR at the cell membrane and causes EGL-4 to enter the nucleus where it phosphorylates HPL-2 (solid arrow) and may also phosphorylate MUT-7 (dashed arrow). Phosphorylated HPL-2 promotes adaptation in a 22GRNA dependent process by binding to H3K9me3 (yellow flags). Phosphorylated MUT-7 boosts levels of *odr-1* 22G RNAs. These siRNAs act as guides within the NRDE-3 Ago complex to direct H3K9me3 to *odr-1* gene. Phosphorylated HPL-2 would repress transcription of the *odr-1* gene (red inhibitory bar). Finally, lower levels of *odr-1* mRNA decreases the animal's attraction to butanone.

downstream of activated HPL-2. Thus, our data are consistent with HPL-2 being recruited to siRNA-targeted loci by H3K9me3 marks and perhaps also nucleating an RNAi-based feed-forward loop in an analogous fashion to its role in *S. pombe*.

Chromatin Marks in Behavior

HPL-2 loads onto the *odr-1* locus in odor-adapted AWCs. This may reflect deposition of a heterochromatic mark. Such marks have been implicated in both neuronal development, as well as in stimulus-induced changes in behavior. H3K9me3-mediated silencing of all but the active olfactory receptor allows for monoallelic expression of odor receptors in the mammalian nasal epithelium (Magklara et al., 2011). In rodents, behavioral addiction to cocaine has been shown to increase H3K9me2 marks within a key brain reward region (Renthal et al., 2009), and regulation of H3K9 methylation is important for addiction-induced neuroplasticity (Maze et al., 2010). These studies highlight the importance of histone methylation marks in regulating long-term behavioral states and may indicate that recruitment of these marks to specific locations could be a key regulated process. It remains to be seen whether such marks can be directed to genes via the action of endo-siRNAs.

Evidence that mammals have a dicer-dependent class of 22GRNAs is currently lacking (Babiarz et al., 2011). In *S. pombe*, however, siRNA species derived from protein-coding genes were not identified until nuclear exosome deficient cells were used (Yamanaka et al., 2013). Such degradation processes might also conceal endo-siRNAs in higher eukaryotes. Though no RNA-dependent RNA polymerase has yet been identified in mammals, it is possible that other classes of small RNAs such as mitrons (miRNAs processed from introns) play an analogous function in the mammalian brain or that RNA polymerase I, II, or III might be recruited to produce small antisense RNAs (Filipovska and Konarska, 2000; Lehmann et al., 2007; Greco-Stewart et al., 2009). These RNAs could similarly direct deposition of chromatin marks and affect behavior.

Odor Regulates Chromatin Changes

Our work indicates that an environmental signal is likely to act via a kinase to amplify the small RNA-directed process. Kinases have been widely appreciated to effect behavioral responses: mitogen-activated protein kinases, calcium calmodulin-dependent protein kinase II, protein kinase C, and protein kinase A can each contribute to the formation of long-term memory subsequent to repeated training (Dash et al., 2007; Gerstner et al., 2009). Indeed, EGL-4 acts via a histone deacetylase (HDA class I) in the nucleus of uterine epithelium cells to promote egg laying (Hao et al., 2011). Here, we demonstrate that the HP1 homolog, HPL-2, is a direct target of this odor-dependent kinase.

In both yeast and mammals, HP1 phosphorylation has been shown to regulate HP1's repressive activity in response to inter- and intracellular signals. Although many studies highlighted the important role played by modifications of the CD (Shimada and Murakami, 2010), our observations suggest that modifications of the CSD may be equally important. The CSD serves as a

(C) Phosphorylation of HPL-2 at predicted PKG sites is required for adaptation. CIs of animals of the indicated genotype that expressed the indicated form of HPL-2 cDNA in AWC are shown. n > 3 with >100 individuals per assay. p value is from an unpaired Student's t test.

(D) Nuclear EGL-4 phosphorylates HPL-2 in vitro. (Left) 3XFLAG-nuclear EGL-4 kinase was immunopurified from worms (behavior in Figure S3F) and incubated with purified HPL-2 and ^{32}P ATP. The reactions were resolved on a gel and stained with Coomassie blue as loading control (lower) followed by autoradiography (upper). (Right) Quantification of five independent kinase assays. ^{32}P phosphorylated HPL-2 was normalized to Coomassie stained band. Values shown for mutant HPL-2 substrate are shown as fold reduction of phosphorylation relative to HPL2-wild-type, which was set to 1. Error bars represent mean ± SEM ($p < 0.0001$; two-tailed Student's t test, n = 5).

(E) Phosphorylation of HPL-2 at a predicted PKG phosphorylation site in the CSD is sufficient to decrease butanone chemotaxis in naive animals. CIs of wild-type animals expressing the indicated form of HPL-2 in AWC, n > 3. Figure S3E shows CIs of individual lines. All strains expressed similar levels of the indicated transgenes as assessed by GFP intensity. p values are from a two-tailed Student's t test.

(F) Phosphorylation of HPL-2 at the EGL-4 phosphorylated sites is sufficient to promote adaptation in naive animals. CIs of naive wild-type, *osm-9*, or *mut-7* animals either expressing the phosphomemetic HPL-2(S/Tto E) (+) or not (–). In all panels of this figure, the bars represent the mean values, and the error bars represent SEM; n > 5. p value is from an unpaired two-tailed Student's t test.

platform for the assembly of other chromatin (Couteau et al., 2002) and RNAi (Rougemaille et al., 2012) -associated proteins and may therefore represent an attractive target for dynamic regulation of transcriptional states. The CSD is required for HP1 homodimerization and formation of an interaction platform with proteins containing the PxVxL interaction motif (Cowieson et al., 2000; Thiru et al., 2004). Though basal silencing requires phosphorylation of the CSD (Zhao et al., 2001), our data indicate that CSD phosphorylation may also be used for signal responsive silencing in neurons.

Other kinases may act in a similar fashion to EGL-4 in other cells and organisms to allow developmental or environmental signals to enhance small-RNA-dependent gene silencing. By regulating RITS, all siRNA-producing loci could be coordinately silenced at a point in time, and the ensuing chromatin changes would ensure stable silencing. Such widespread silencing by siRNAs may allow experiences to alter expression of whole cohorts of genes in the context of both development and behavior.

EXPERIMENTAL PROCEDURES

Worm Strains

For a complete list of strains used, please see the Supplemental Information. Bristol N2 was the wild-type strain.

Plasmid Construction and Transgenic Strains

Details of plasmid construction can be found in the Supplemental Information.

Behavior

Behavioral assays were conducted on day one adults as described (Colbert and Bargmann, 1995). More details are presented in the Supplemental Information. For heat shock experiments, worms on their original growth plates were exposed to 30°C for 1 hr and then recovered at 20°C for 2 hr prior to behavioral assays.

Kinase Assay with Nuclear EGL-4

To evaluate nuclear NLS FLAG-EGL-4 kinase activity, 100 μg of worm lysate was immunoprecipitated using anti-FLAG M2 magnetic beads (Sigma-Aldrich). Bead-bound immunoprecipitates were washed extensively with kinase buffer. Then kinase assays were performed directly on the beads by adding 1.5 μg of substrate (HPL-2 WT, HPL-2[all S/T-A], or Histone H1), 2 μCi ^{32}P ATP (PerkinElmer), and 25 μM cGMP (Sigma-Aldrich).

Details are in the Supplemental Information.

Isolation of NRDE-3-Associated Small RNA

50–60 plates of adult animals expressing 3XFLAG::GFP::NRDE-3 were collected, and half the population was incubated with SBasal alone, and the other half was incubated with SBasal plus butanone for 80 min. Behavior of ~100 animals from each was assessed. Extracts were made from the remaining animals as described (Guang et al., 2008 and Supplemental Information).

Isolation of HPL-2-Associated DNA

p*odr-3*::3XFLAG::GFP::HPL-2 was integrated into the genome and outcrossed five times. 100 plates of adult animals were harvested, and half were exposed to buffer and half to butanone and buffer. ~100 animals from each were assayed. The remaining animals were processed for ChIP (Gerstein et al., 2010). Only populations that showed an adapted CI of 0.05–0.3 were used. Details of the ChIP are in the Supplemental Information. Quantitation of coimmunoprecipitated DNA is described in the Supplemental Information.

Quantitative Real-Time PCR

For RNA analysis, 5 plates of day one adult animals were collected and treated to the adaptation protocol, and their behavior was assessed. Total RNA was isolated as described in the Supplemental Information. Total RNA from entire worms was used in 22GRNA and mRNA quantitation as described in Supplemental Information.

To quantify HPL-2-associated DNA, ChIP results were analyzed by qPCR using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies).

The primers were specific to the *odr-1*, *unc-40*, or *act-3* loci. The levels of the housekeeping gene *act-3* did not change with odor. Please see Extended Experimental Procedures and Table S2 for details and primers.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.08.006.

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