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Starvation-Induced Transgenerational Inheritance of Small RNAs in *C. elegans*

Oded Rechavi^{1,2,*}, Leah Hour-Ze'evi¹, Sarit Anava¹, Wee Siong Sho Goh³, Sze Yen Kerk², Gregory J. Hannon³, and Oliver Hobert²

¹Department of Neurobiology, Wise Faculty of Life Sciences and Sagol School of Neuroscience, Tel Aviv University, Tel Aviv 69978, Israel

²Columbia University Medical Center, Department of Biochemistry and Molecular Biophysics, Howard Hughes Medical Institute, New York, NY 10032, USA

³Watson School of Biological Sciences, Cold Spring Harbor Laboratory, Cold Spring Harbor, Howard Hughes Medical Institute, New York 11724, USA

SUMMARY

Evidence from animal studies and human famines suggests that starvation may affect the health of the progeny of famished individuals. However, it is not clear whether starvation affects only immediate offspring or has lasting effects; it is also unclear how such epigenetic information is inherited. Small RNA-induced gene silencing can persist over several generations via transgenerationally inherited small RNA molecules in *C. elegans*, but all known transgenerational silencing responses are directed against foreign DNA introduced into the organism. We found that starvation-induced developmental arrest, a natural and drastic environmental change, leads to the generation of small RNAs that are inherited through at least three consecutive generations. These small, endogenous, transgenerationally transmitted RNAs target genes with roles in nutrition. We defined genes that are essential for this multigenerational effect. Moreover, we show that the F3 offspring of starved animals show an increased lifespan, corroborating the notion of a transgenerational memory of past conditions.

INTRODUCTION

The environment's ability to influence inherited traits has been a subject of heated debate for over a century (Koonin and Wolf, 2009). Although the underlying mechanism has been elusive, multiple environmentally induced transgenerational epigenetic effects have been described (Jablonka and Lamb, 2008). In particular, evidence collected from diverse animal species, and broad epidemiologic surveys of different famines in the Netherlands (1944–1945) (Painter et al., 2008), China (1959–1961) (Song et al., 2009), and Russia (1941–1944) (Stanner et al., 1997) have demonstrated that susceptibility to disease is correlated with malnourished ancestors. In particular, it has been shown that there is an increased capacity to transmit epigenetic information about food availability during development before the

prepubertal peak in growth speed (Bygren et al., 2001; Kaati et al., 2007). In all the above-mentioned studies the effects were restricted to the immediate offspring, leaving the possibility open that rather than being transgenerationally inherited, the effects were directly exerted onto the germ cells of the exposed animals (Heard and Martienssen, 2014). Thus, a truly epigenetic effect that could transmit the somatic response to satiety or famine to the generations beyond the immediate next generation remains to be found.

The model organism *Caenorhabditis elegans* has been successfully used for the study of transgenerational epigenetic effects (Greer et al., 2011; Rechavi et al., 2011), in part because of its short generation time, *C. elegans* is particularly suited for studying transgenerational inheritance of dietary history, since worms are often faced with scarcity of nutrients in the wild. In fact, a dedicated genetic program allows worms to reversibly arrest postembryonic development in the first larval stage (L1) in the absence of food (Baugh, 2013). Similar to the situation in humans, it was shown that the *C. elegans* F1 progeny is affected by the maternal diet (Harvey and Orbidans, 2011); again, a genetic effect that extends beyond one generation was not demonstrated to act in response to nutrient availability in *C. elegans*. Nevertheless, it was shown that an association between odor and food (olfactory imprinting), which can only be encoded during the L1 stage (Remy and Hobert, 2005), is memorized for multiple generations (Remy, 2010).

L1 starvation leads to a particularly drastic change in gene expression. The expression of 27% of protein-coding genes is altered, more than the number of genes which are differentially expressed throughout the entire course of larval development (Maxwell et al., 2012). Survival during and recovery from L1 arrest is in part regulated by RNA interference (RNAi), and also depends on microRNA repression of insulin pathway genes (Zhang et al., 2011). Global regulation of small RNA levels during L1 starvation, could be achieved, for example, by the long noncoding RNA *ncs-1*, which was shown to be induced during L1 starvation, and to in vitro “block” DICER, reducing its capacity to process other small RNAs (Hellwig and Bass, 2008).

C. elegans possesses a dedicated RNAi inheritance mechanism that could, in theory, allow memorization of dietary history-dependent small RNA response for multiple generations. Double-strand RNA (dsRNA) spreads systemically and transmits from the soma to the germline in *C. elegans* (Fire et al., 1998). Experimental silencing of certain genes by administration of dsRNA has been demonstrated to persist for more than 80 generations (Vastenhouw et al., 2006). The same inheritance mechanism that acts in response to artificial dsRNA was later shown to also play a role in antiviral and transposon immunity (Rechavi, 2013; Rechavi et al., 2011; Sterken et al., 2014). While the different biogenesis mechanisms are not yet fully understood, endogenous small RNAs (endo-siRNAs) align to thousands of genes across the genome (Grishok, 2013). Recently a number of groups proposed that endo-siRNAs survey all germline-expressed genes, to silence invading elements and license the expression of autogenous sequences (Ashe et al., 2012; Buckley et al., 2012; Shirayama et al., 2012). Specifically, it was suggested that two argonaute proteins, HRDE-1 (*heritable RNAi deficient* or WAGO-9) and CSR-1 (*chromosome-segregation and RNAi deficient*), carry small RNAs that compete over binding to cognate germline-expressed target mRNAs, and the result of this antagonism determines whether a gene

would be transcriptionally silenced (HRDE-1 targets) or licensed (CSR-1 targets) for expression in the germline (Seth et al., 2013). The inherited regulation of germline-expressed genes persists across multiple generations by the action of small RNAs, which are amplified by RNA-dependent RNA Polymerases (RdRPs) (Gu et al., 2012).

Importantly, while it is now clear that RNAi inheritance mechanisms are induced by different exogenous genetic elements, it has not been demonstrated that an environmental change is capable of eliciting a heritable response that involves endogenous small RNAs and endogenous small RNA targets. In all the previous studies, the transgenerational silencing responses were directed against foreign DNA (transposons, viruses, transgenes) (Ashe et al., 2012; Buckley et al., 2012; Lee et al., 2012; Rechavi et al., 2011; Shirayama et al., 2012). Therefore, it is still unknown whether an endogenous, biologically relevant response, which is induced under natural circumstances, could elicit a small RNA response that would be passed on to ensuing generations.

In this paper, we describe a set of small RNAs that are induced by starvation. We show that these starvation-induced small RNAs are transmitted transgenerationally, providing a mean for starved worms to control the expression of relevant genes in consecutive generations.

RESULTS

We first sequenced small RNAs from young adult worms that were either continuously fed or severely starved for 6 days as L1s, and from their F3 progeny (see scheme in Figure 1A). We used a protocol that enables cloning of “secondary” endo-small RNAs (See Extended Experimental Procedures). These are 22 nt long transcripts that possess a 5' guanosine, which are amplified by RdRPs and constitute the most abundant type of endogenous small RNAs (Gu et al., 2009; Pak and Fire, 2007).

We examined the small RNA pools of wild-type (N2), *rde-4* (RNAi Deficient, a DICER interactor), and *hrde-1* worms using principal components analysis (PCA). PCA reduces multidimensional data into two dimensions, so that the relative distance between samples could be determined. The PCA revealed that the three biological replicates cluster together, while the experimental conditions and the different mutants are clearly separated (see processing steps and PCA in Figure 1A, and Figure S1 available online; additional details are provided in the Extended Experimental Procedures).

The most dramatic changes between the experimental conditions were found to arise from small RNAs that align antisense to gene-coding regions. Past work has shown that endo-siRNAs generally align in the antisense orientation and almost exclusively to exons (Grishok, 2013). This typical pattern was clearly apparent in the differentially expressed small RNAs (Figure 1B). We thus analyzed gene-targeting small RNAs that were differentially expressed between the experimental conditions, and considered a gene as a “putative target” of small RNAs-mediated regulation if multiple small RNAs align to it in

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and six tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2014.06.020>.

the antisense orientation. For short, we dubbed clusters of small RNAs that align in the antisense orientation to particular genes, as “STGs” (small RNAs targeting a given gene, see Figure 1B).

To examine whether there were changes in the small RNA pools between the P0 fed animals and the animals that were starved as L1s (hereby, P0 “starved”), we compared the samples using the DESeq2 method (FDR < 0.1) and distinguished between 22Gs and 26Gs sequences, which are the two different species of endo-siRNAs (Gent et al., 2010; Vasale et al., 2010). In the P0 adults, the 22G STGs (secondary endo-siRNAs) exhibited the most dramatic changes following L1 starvation (578 upregulated, 597 downregulated, see Table S1). However, a statistically significant differential expression in 26G STGs (primary endo-siRNAs) was also observed (20 upregulated, 38 downregulated, see Figures 2A and S2). Although the exact mechanism is not yet clear, it has been shown that this primary small RNA species can induce the biogenesis of secondary 22Gs small RNAs in *cis* or in *trans* (Gent et al., 2010; Han et al., 2009; Montgomery et al., 2012; Vasale et al., 2010). This process probably entails primary small RNA-mediated guiding of RdRPs to the mature mRNAs target, and it is hypothesized that the amplified 22Gs can be sequentially loaded onto either HRDE-1 or CSR-1 (Seth et al., 2013). It is currently very difficult to reliably predict whether primary small RNAs would initiate secondary small RNA production because primary small RNAs were shown to also trigger amplification using very permissive and imperfect base pairing (Ashe et al., 2012; Montgomery et al., 2012; Shirayama et al., 2012). Nevertheless, even when allowing only high degree of complementarity (see Extended Experimental Procedures), 31 putative targets of secondary differentially expressed 22G STGs were also targeted by primary differentially expressed 26G small RNAs (8 upregulated and 23 downregulated) following L1 starvation (Figure 2A). We noticed that 12 out of the 23 STGs that were downregulated in both the 22G and the 26G analysis (Figure 2A) were also found in a previous study that identified a small number (48) of genes that are targeted by ERGO-1-bound and 3'-modified 26G small RNAs (Vasale et al., 2010). These ERGO-1 pathway 26G STGs, that we find to be downregulated upon L1 starvation, were subsequently shown to be stabilized by HENN-1-mediated 3' methylation (Billi et al., 2012; Kamminga et al., 2012; Montgomery et al., 2012).

22G RNAs are the most abundant differentially expressed small RNAs, and since these RNA molecules are amplifiable, and are known to be carried by HRDE-1 and CSR-1, the argonaute proteins that transmit inherited small RNAs across generations (Rechavi, 2013), we hypothesized that this RNA species can be inherited following L1 starvation, and thus focused our analysis on it.

The putative target genes of the differentially expressed 22G STGs were enriched for a number of biological functions (GORilla analysis, Figures 2B and Figure S3), and most significantly for nutrient reservoir activity (FDR < 8.61×10^{-4}). Regulation of nutrient reservoir activity could in theory affect the worm's ability to handle starvation-induced stress. Some of these genes (37/1,175, 1.5-fold enrichment, p value < 0.011) were previously found to be regulated by small RNAs following recovery from dauer arrest, an alternative developmental stage, during which worms are food deprived (Hall et al., 2013).

To test the potential of the differentially expressed STGs to regulate the levels of their cognate target genes, we sequenced mRNAs from the same samples. It is currently not clear to what extent, or depending on which conditions, endo-siRNAs affect their targets. We observed a downregulation in the mRNA levels of 88 genes that were each predicted to be a target of a specific 22G STG, that was upregulated in young adults that experienced starvation as L1s (Figure 2C and Table S3). The observed reduction in mRNA levels is consistent with the canonical mechanism of small RNA-mediated gene silencing. Upregulation of a small number of mRNAs (14), which were similarly predicted to be targets of the upregulated STGs, was also observed. Upregulation of mRNA that are targeted by small RNAs could be the result of indirect interactions, or may be a reflection of the recently described “RNA licensing” or “RNA activation” (RNAa) mechanism (Seth et al., 2013; Wedeles et al., 2013).

To examine whether the small RNA response could be transmitted across generations, we sequenced small RNAs from the fed F3 progeny of the worms that were starved as L1s (hereby referred to as “F3 of starved worms”). Surprisingly, although the “F3 of starved worms” were fed for three generations, clustering of the STGs that were differentially expressed following starvation in the P0 generation revealed that the F3 worms resemble the “starved” parents more than the “fed” parents (Figure S5). We examined which small RNAs were responsible for this effect and found that 26.3% (152/578) of the 22G STGs that were upregulated in their ancestors following L1 starvation were also upregulated in a statistically significant manner in the F3 progeny in comparison to the fed ancestors (hypergeometric test, p value $< 1.399 \times 10^{-71}$, for a typical gene see Figure 3A). In addition to inherited upregulation of STGs, we found that 52% (311/597) of the STGs that were depleted following starvation were depleted also in the F3 progeny (p value $< 1.256 \times 10^{-292}$, Table S2). Thus, the small RNA pools of the F3 worms reflect the changes that occurred following starvation in the P0 generation. As mentioned above, in the P0 generations 31/1,175 differentially expressed STGs which are targeted by secondary 22Gs are also targeted by differentially expressed primary 26G small RNAs STGs, which are predicted by the current model to trigger their biogenesis. We found that levels of 91% of the 22Gs that overlap with 26G STGs were transgenerationally downregulated in the F3 samples (p value $< 5.629 \times 10^{-29}$, fold-enrichment = 26.1, Figure 3B, Tables S2 and S5).

It was previously hypothesized that germline transcription of the mRNA target provides a template on which heritable small RNAs can amplify (Buckley et al., 2012; Burton et al., 2011). We thus asked whether the putative targets of the heritable STGs are enriched for germline expression. We found that 21.7% of the putative targets of the upregulated inherited 22G STGs were germline expressed (based on data from (Reinke et al., 2004), fold-enrichment = 1.5, p value < 0.012 , Figure 3B). This enrichment suggests that inherited small RNAs may indeed preferentially target germline-expressed genes. On the other hand, only 8% of the putative targets of downregulated inherited STGs are germline-expressed genes (fold-depletion = 1.85, p value $< 1.13 \times 10^{-4}$).

Examination of the list of 463 potential targets (see Table S6) of the inherited STGs using GOrilla revealed functional enrichments that resembled the enrichments that were detected in the P0 generation, and most notably where again enriched for nutrient reservoir activity

(Figure 3C). Interestingly, differentially expressed heritable STGs align to the entire family of the vitellogenins (6/6, 38.7-fold enrichment, p value $< 2.950 \times 10^{-10}$), worm yolk proteins that provision the egg, and are encoded in different locations in the genome (DePina et al., 2011). Biogenesis of the secondary 22G STGs that target the vitellogenins could be initiated by dsRNA precursors. On the complementary strands of all the vitellogenins, there are genes which are transcribed in the opposite direction, suggesting that cotranscription of these genes and the vitellogenins could lead to the formation of dsRNA (Figure S4). 20 F box proteins (5-fold enrichment, p value $< 5.057 \times 10^{-9}$) were also found to be targeted by heritable STGs. While the function of these proteins in the starvation response of *C. elegans* is unknown, in plants homologous proteins monitor protein turnover following stress (Meloche and Roux, 2012).

To examine whether inherited small RNAs could regulate these enriched genetic pathways across multiple generations, we analyzed the mRNA expression pattern of the genes that were targeted by inherited STGs using transcriptome sequencing. The expression levels of 96/463 genes that are targeted by differentially expressed heritable small RNAs were changed between the F3 progeny of starved worms and the fed P0s (Table S4). We could not detect any distinct pattern of up or downregulation when we plotted the inherited small RNAs against their cognate mRNAs. However, separate examination of the mRNA targets of the heritable small RNAs that were previously shown to bind HRDE-1 or CSR-1 (Buckley et al., 2012; Claycomb et al., 2009), revealed opposite trends of regulation. We observed a downregulation of 65.3% (17/26) of the mRNAs that were targeted by HRDE-1-bound small RNAs, and upregulation of 68.7% (11/16) of the mRNAs that were targeted by CSR-1-bound small RNAs. Interestingly, a strong correlation ($R^2 = 0.907$, p value < 0.00026) was detected between the levels of inherited CSR-1 STGs and germline expressed, but not somatically expressed, cognate mRNA targets (Figure 4A).

Previous studies described that levels of DICER-dependent small RNAs (both endo-siRNAs and microRNAs) are altered by L1 starvation (Hellwig and Bass, 2008; Reinke et al., 2004; Zhang et al., 2011), and therefore in theory the levels of these small RNAs, if inherited, could be affected in future nonstarved generations. The dsRNA-binding protein RDE-4 is a specific component in the DICER endo-siRNA pathway (Lee et al., 2006). Conveniently, while RDE-4 was shown to play a role in response to different stresses (Mansidor et al., 2011), multiple generations of *rde-4* mutants can be followed (unlike DICER mutants, which are sterile), since these mutants are surprisingly fertile and lack obvious phenotypes under normal growth conditions. Moreover, RDE-4 operates upstream to ERGO-1, and as mentioned above, we found that a large fraction of the differentially expressed 26G STGs were previously found to bind this argonaute (Vasale et al., 2010). We therefore examined which small RNAs are induced by L1 starvation and persist until adulthood in *rde-4* mutants and whether small RNA inheritance following L1-starvation is abrogated in these animals. We found that inheritance of most (93%) of the STGs is dependent on RDE-4. Only 33 out of the 463 (Table S5) STGs that were found to be inherited following L1-starvation in N2 were also found to be inherited following L1-starvation in *rde-4* animals (Figure 4B). In addition to the significant reduction in the inherited small RNAs response, while in N2 expression of 96 out of 463 genes (20%) that were found to be targeted by heritable STGs

were changed, the mRNA levels of only 33 putative targets (7%) were changed in *rde-4* animals. RDE-4 was previously hypothesized to function specifically in response to stress. We observed that more STGs are reduced in *rde-4* animals in comparison to N2 animals when comparing the starved conditions (1,496 genes) or the F3 of starved worms (2,686 genes), relative to the smaller difference which is observed between the fed parents (1,024 genes).

hrde-1 hermaphrodites, but not *csr-1* hermaphrodites, are fertile at nonrestrictive temperature (Buckley et al., 2012; Conine et al., 2013), and so the progeny of starved *hrde-1* mutants could be examined. We found in additional sequencing experiments that 96.8% of the heritable STGs were no longer inherited in *hrde-1* mutants (13/463) (Figure 4B and Table S5), in line with HRDE-1's role in transgenerational shuttling of heritable small RNAs. 25.9% of these inherited STGs were previously shown to physically bind HRDE-1 (3.7-fold enrichment, p value $< 1.139 \times 10^{-40}$) (Buckley et al., 2012). In contrast to the clustering pattern of the STGs that was observed in N2 animals, in *hrde-1* animals the "fed" parents clustered with the "F3 of starved worms," separately from the "starved" parents. These patterns are consistent with the reduced small RNA inheritance in *hrde-1* mutants. In *rde-4* animals, no clear clustering pattern was observed, possibly because of RDE-4's upstream role in biogenesis of small RNAs (Figure S5) (Gent et al., 2010; Vasale et al., 2010).

To assess whether starvation also alters the physiology of the progeny of starved parents, we performed lifespan assays. We compared the lifespan of animals whose great-grandparents had experienced starvation at the first larval stage with animals whose great-grandparents were continuously fed. In three independent experiments, we observed a significant increase in the lifespan of animals whose ancestors had experienced starvation (37% increase, p value < 0.0001 ; 70% increase, p value < 0.0001 ; 22% increase, p value = 0.004, Figure 5 and Figure S6). At this point, we do not know whether inherited RNA molecules are responsible for this lifespan effect, but the lifespan effect independently corroborates the notion that there is a transgenerational memory of dietary history.

DISCUSSION

We discovered that changes in small RNA (22Gs) levels that follow L1-starvation-induced developmental arrest are passed on for at least three generations (Figure 6). While it was previously shown that RNAi could act transgenerationally, in all the previous studies heritable silencing was directed against foreign DNA introduced into the worm. This is the first study that demonstrates that an endogenous response could trigger small RNA inheritance. The targets of these inherited small RNAs include sets of genes involved in nutrition. This epigenetic response is compromised in *rde-4* mutants, and transgenerational transmission of the starvation-induced small RNAs depends on the germ-line-expressed nuclear argonaute HRDE-1.

Some small RNAs in *C. elegans*, for example PIWI-interacting RNAs (piRNAs) and microRNAs, are independent transcriptional units regulated by dedicated promoters (Bagijn et al., 2012; Grishok, 2013). Although the biogenesis mechanism is at the moment poorly

understood, endo-siRNAs in general, and RDE-4-dependent 26G and 22G endo-siRNAs in particular, are not independently transcribed but originate from mRNA transcripts (Billi et al., 2014). 26Gs and 22Gs are antisense sequences that are produced as short independent transcripts by nonprocessive RdRPs (26Gs by RRF-3 and 22Gs by RRF-1 and EGO-1) that use spliced mRNAs as a template. Some, but not all of the amplified 22G directly depend on primary 26Gs that prime their production (Grishok, 2013). Thus, it is possible that the mRNA response, either during or following severe L1 starvation, triggers the starvation-induced changes in endo-siRNAs, simply because endo-siRNAs levels are determined based on the availability of the cognate mRNA template. Alternatively, changes in small RNA production could be, in theory, initiated by starvation-induced expression or modification of particular biogenesis protein factors or by starvation-induced bidirectional transcription that would result in dsRNA formation, which could serve as substrate for small RNA production. Irrespective of the nature of the initiation mechanism, our findings suggests that a transient trigger (L1-starvation) is memorized by the generation of an ensemble of small RNAs which are then available to target mRNAs in ensuing generations in the absence of the original starvation trigger (Figure 6).

Although we observed an enrichment for heritable targeting of germline genes, in accordance with previous studies on gene targets of HRDE-1-bound 22Gs (Buckley et al., 2012), many genes that were found to be targeted by heritable small RNAs were not previously found to be expressed in the germline. We observed that a large percentage of the genes (52%) which are targeted by both 26G and 22G following starvation were previously annotated as ERGO-1 targets, and it was shown that although ERGO-1-bound small RNAs are produced during oogenesis, they mostly regulate zygotic genes that are not expressed in the germline (Han et al., 2009; Reinke et al., 2004). It is not clear whether endogenous small RNAs, similarly to exogenous ones (Fire et al., 1998), move systemically in *C. elegans*; if this is the case, it is also possible that certain small RNAs were transmitted from the soma to the germline.

It is not known which features in a given mRNA transcript render it suitable for serving as a template for the production of 26G or 22G small RNAs. Presumably secondary structures (perhaps simply dsRNA), induce the recruitment of small RNA biogenesis factors (Billi et al., 2014). Hence, in theory, for each starvation-regulated gene, there could be a different biogenesis pathway that produces the specific starvation-provoked small RNAs. We found that genes that are involved in nutrition, and most notably all the vitellogenin genes, are under heritable small RNA control. Since on the complementary strands of all the vitellogenin genes there are genes that are transcribed in the opposite direction, we hypothesize that cotranscription and ensuing formation of dsRNA are the trigger for small RNA formation in this case (Figure S4). Regulation of the levels of yolk proteins that provide resources to the egg (DePina et al., 2011) by cotranscription-induced small RNA production upon starvation, could be a method to monitor the amount of food that is allocated to the next generations. Similarly, heritable small RNAs were found to target multiple genes that are involved in fat regulation, protein turnover, stress resistance, longevity, and reproduction, all with the potential of affecting the survival of the progeny (Table S6).

Since heritable small RNAs are hypothesized to act as memory of “self” and “nonself” genes (Rechavi, 2013), it was previously suggested the endo-siRNAs “buffer” the genome against new or foreign genes that might be deleterious (Fischer et al., 2011; Vasale et al., 2010). In addition to upregulation of inherited endo-siRNAs, we observed, upon stress, an inherited downregulation of many endo-siRNAs. It is possible that releasing silenced genes from endo-siRNA-mediated repression is a bet-hedging strategy, which could benefit the progeny if some of these “dangerous” and otherwise silent genes prove useful in overcoming stress such as food deprivation.

In parallel to the characterization of the inherited small RNA response, we also observed that F3 progeny that were derived from L1-starved parents live longer. The putative gene targets of the transgenerationally inherited small RNAs were enriched for functions that are related to aging. The inherited increased longevity could be directly dependent on gene regulation that is enforced by the inherited small RNAs (for example, the *kin-29*, *gcy-23*, *polg-1*, *coq-3*, and *aco-1* genes, were shown to affect lifespan and are targeted by heritable small RNAs), or, since different small RNA species compete over common limiting resources (for example, binding to shared proteins), it could indirectly stem from a tilt in the balance within the small RNA pool, or by a heritable reduction in the productivity of the germline. Future studies will reveal if L1-starvation-induced and inherited small RNA gene regulation is related to the observed heritable increased longevity, and whether these transgenerational effects prepare the progeny for similar famines, or are adaptive in other ways.

Over the course of evolution worms and humans specialized in different regulatory epigenetic processes. For example, *C. elegans* have an expanded argonaute family (Yigit et al., 2006), and RdRP-mediated small RNAs amplification (Sijen et al., 2001; Smardon et al., 2000), but no DNA methylation (Simpson et al., 1986). Thus, until the proper experiments are performed, it cannot be assumed that the mechanism for memorizing starvations that we describe here is the mean by which mammals achieve the same end. Nevertheless, it was recently found in mice that traumatic depression leaves a trace of microRNAs in sperm (Gapp et al., 2014). Therefore, it is of great importance to study whether noncoding RNAs play a role in the epigenetic effects that were observed following starvation in humans as well.

EXPERIMENTAL PROCEDURES

Small RNA Library Preparation and Sequencing

All the experiments were done three times (independent biological triplicates). Worms were lysed using the TRIzol reagent (Life Technologies), repetitive freezing, thawing, and vortexing. The total RNA samples were treated with tobacco acid pyrophosphatase (Epicenter), to ensure 5' monophosphate-independent capturing of small RNAs. Libraries were prepared using the TruSeq Small RNA protocol (illumina) according to the manufacturer's protocol. The resulting cDNAs were separated on a 4% agarose E-Gel (Invitrogen, Life Technologies), and the 140–160 nt length species were selected. cDNA was purified using the MinElute Gel Extraction kit (QIAGEN). Libraries were sequenced using an Illumina HighSeq2500 instrument.

mRNA Library Preparation and Sequencing

Total RNA was isolated using the TRIzol reagent (Life Technologies), and libraries were prepared using the Low Sample (LS) protocol (Illumina) according to the manufacturer's protocol. Libraries were sequenced using an Illumina HighSeq2500 instrument.

Worm Strains and L1 Starvation Protocol

Standard culture techniques were used to maintain the nematodes on NGM plates seeded with OP50 bacteria. Strains employed in this work were as follows: The wild-type strain (Bristol N2), WM35 *rde-4(ne299) unc-69(e587)*, and YY538: *hrde-1(tm1200)*. The nematodes were kept fed for at least five generations before the beginning of each experiment. Synchronization of the worms at the L1 stage was achieved using "egg prepping." Half of the eggs were transferred to NGM plates with OP50 bacterial lawn ("fed" condition) and the other half to NGM plates without food ("starved" worms). Worms that hatched on plates without food were kept starved as L1s for 6 days. The starved worms were then collected using M9 buffer and transferred to NGM plates containing food. All worms ("fed" and "starved") were collected for sequencing as young adults 2 days after they reached the L4 stage.

Small RNA-seq Analysis

For details see the Extended Experimental Procedures. In brief, the adaptor sequences were removed using CutAdapt. Clipped reads were mapped to WormBase 220 version of the genome (WS220) using Bowtie2 (v. 2.1.0). Reads which aligned antisense to genes were counted using htseq-count and Ensembl-based gff file (version 66). We used DESeq2, an R package, to determine differential expression of STGs, and considered an STG to be differentially expressed if its FDR value was less than 0.1. Hierarchical clustering was done using the pvclust package.

RNA-seq Analysis

Reads were mapped to WormBase 220 version of the genome (WS220) using Bowtie (v. 2.1.0). Reads were counted using htseq-count and Ensembl-based gff file (version 66) and normalized for RPM (reads per million). We considered a gene to be differentially expressed if its fold-changed was greater than 1.5 and it had total reads of more than 10 RPM per comparison.

Lifespan Assays

N2 worms in the late L4 stage growing at 20°C were transferred to fresh NGM plates with OP50 bacterial lawn. The first day of adulthood is day 1 in the survival curves. Animals were scored as "alive," "dead," or "lost" every other day. Animals that failed to display touch-provoked movement were scored as "dead." Animals that died from causes other than aging, such as sticking to the plate walls, were scored as "lost." Animals were transferred to fresh plates every 2 days. Each assay consisted of 8 NGM plates with 15 worms in each plate and every experiment was conducted three times. All lifespan experiments were performed at 20°C. Survival curves were plotted and statistical analyses (log-rank tests) were performed using the SPSS V 15 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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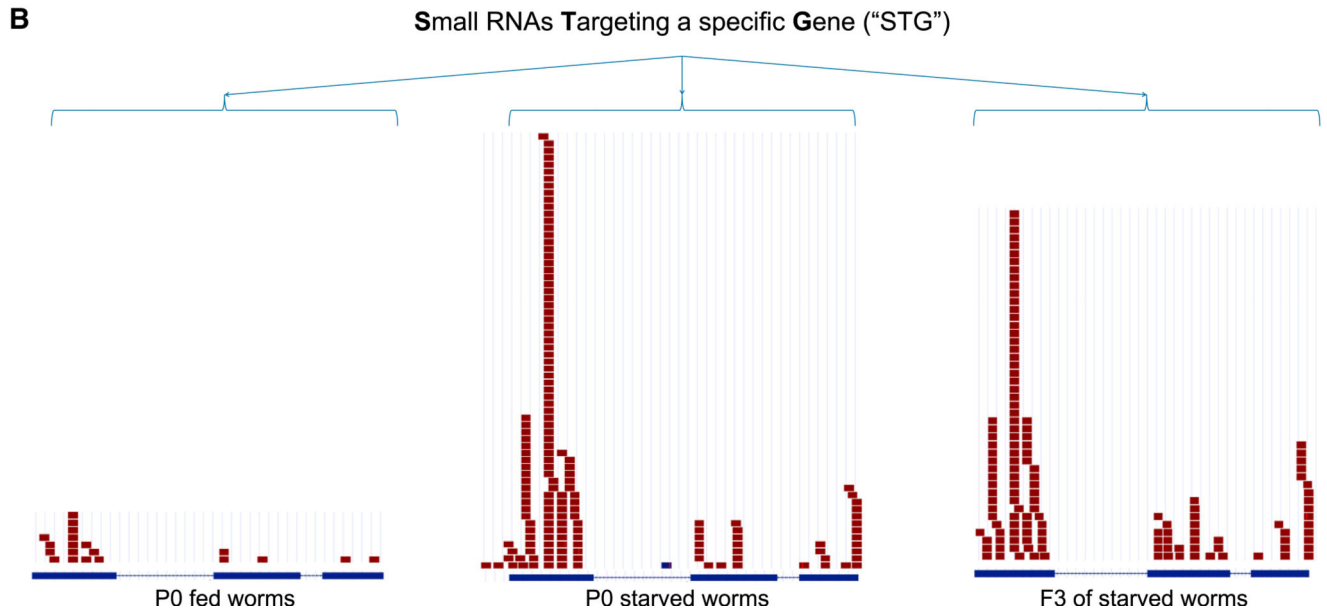
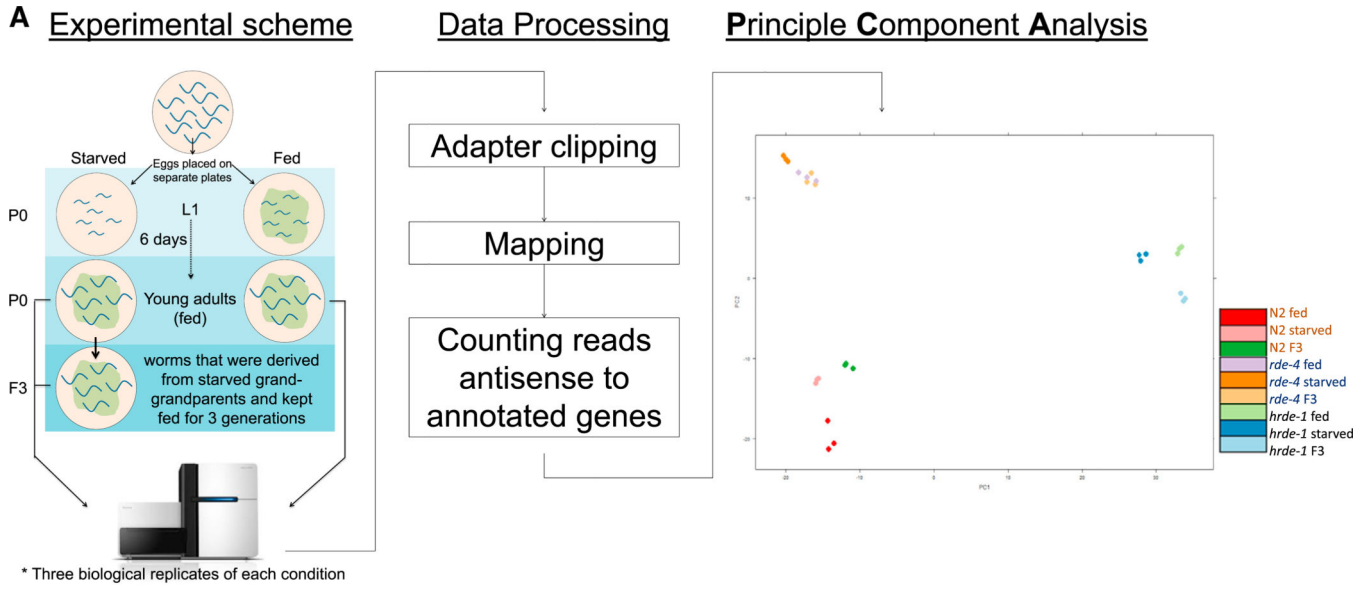


Figure 1. A Protocol for Analyzing the Starvation-Induced Transgenerational Small RNA Response

(A) Aschematic description of the experimental protocol: secondary (RdRP-amplified) small RNAs were cloned and sequenced from three biological replicates of young adult worms that were either continuously fed or severely starved for 6 days as L1s and from their F3 progeny. Following adapters trimming, the reads were mapped to the WS220 version of the *C. elegans* genome. Reads which mapped antisense to annotated gene were counted and then analyzed by principle component analysis to reveal the similarities/differences between the experimental conditions (see Figure S1 for an additional PCA of reads which were mapped to the entire genome).

(B) Visualization in the UCSC browser of the *vhl-1* gene. An STG (small rnas targeting a given gene) is defined as the sum of the small RNAs that are antisense to a certain gene (see Table S1 for the fold-change and p value ranking of all the STGs).

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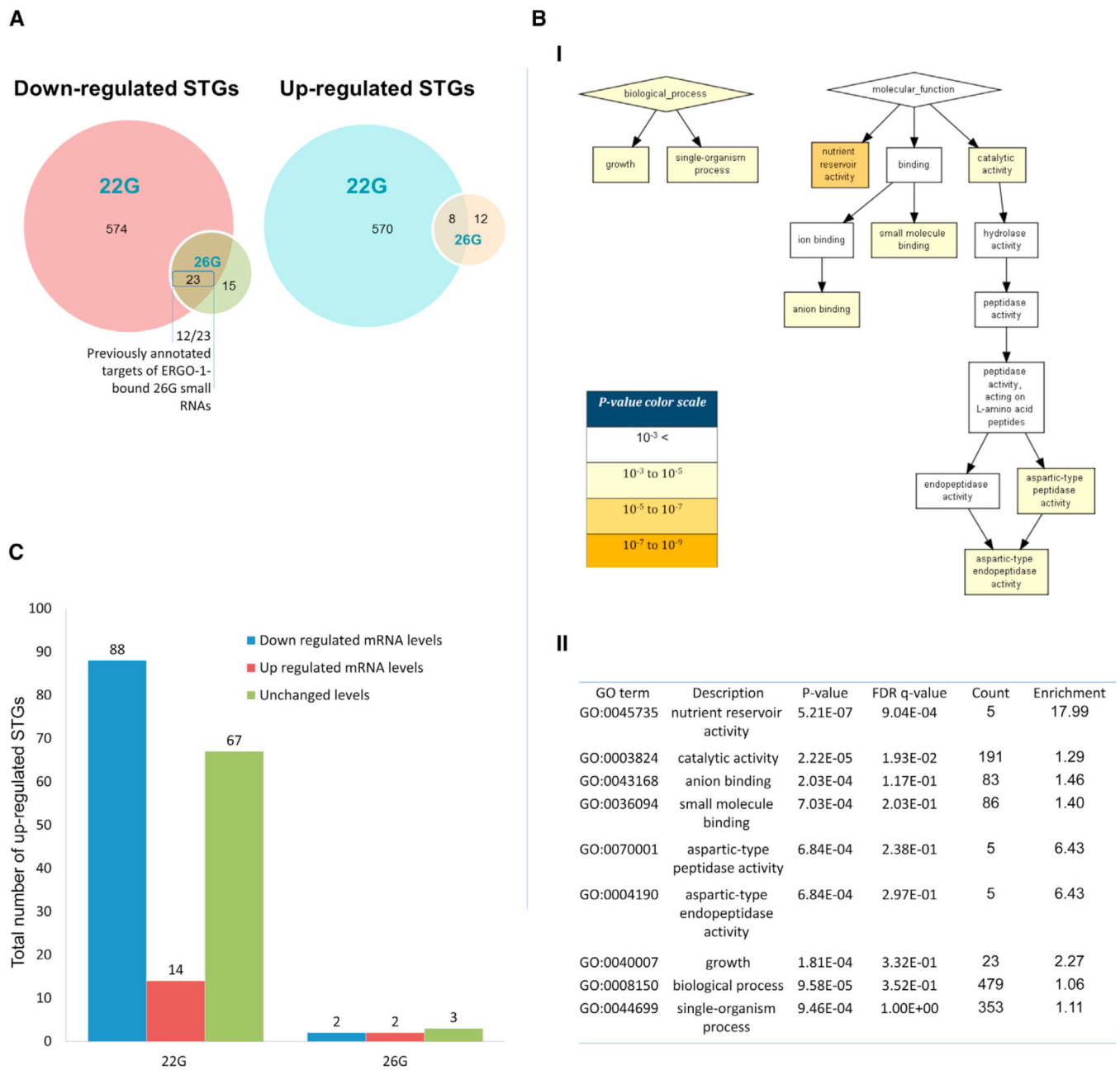


Figure 2. Transcriptome Analysis of P0 Adults that Experienced Starvation at the L1 Stage
 (A) Overlaps of up/downregulated STGs of the different endo-siRNA species (see Figure S2 for an MA Plot visualization of the differentially expressed STGs of each small RNA species).
 (B) A functional annotation of the 22G STGs (secondary small RNAs which are 22 nt long and possess a 5' guanosine) that are differentially expressed following starvation in wild-type animals. Annotation was performed using the GOrilla web-server. (I) Functional enrichment map. (II) A table of the specific GO terms, statistical significances, the number of genes that share each specific GO term, and their relative enrichments. An additional GO

analysis, which also takes into account the fold-change ranking of the differentially expressed STGs is presented in Figure S3.

(C) Changes in the mRNA levels that correspond to changes in STGs. The x axis represents the two species of small RNAs that were examined: 22Gs (secondary small RNAs) and 26Gs (primary small RNAs which are 26 nt long and possess a 5' guanosine). The y axis represents the total number of STGs which were upregulated following L1-starvation, in correspondence to the changes in mRNA levels (see Table S3 for fold-change ranking of the genes).

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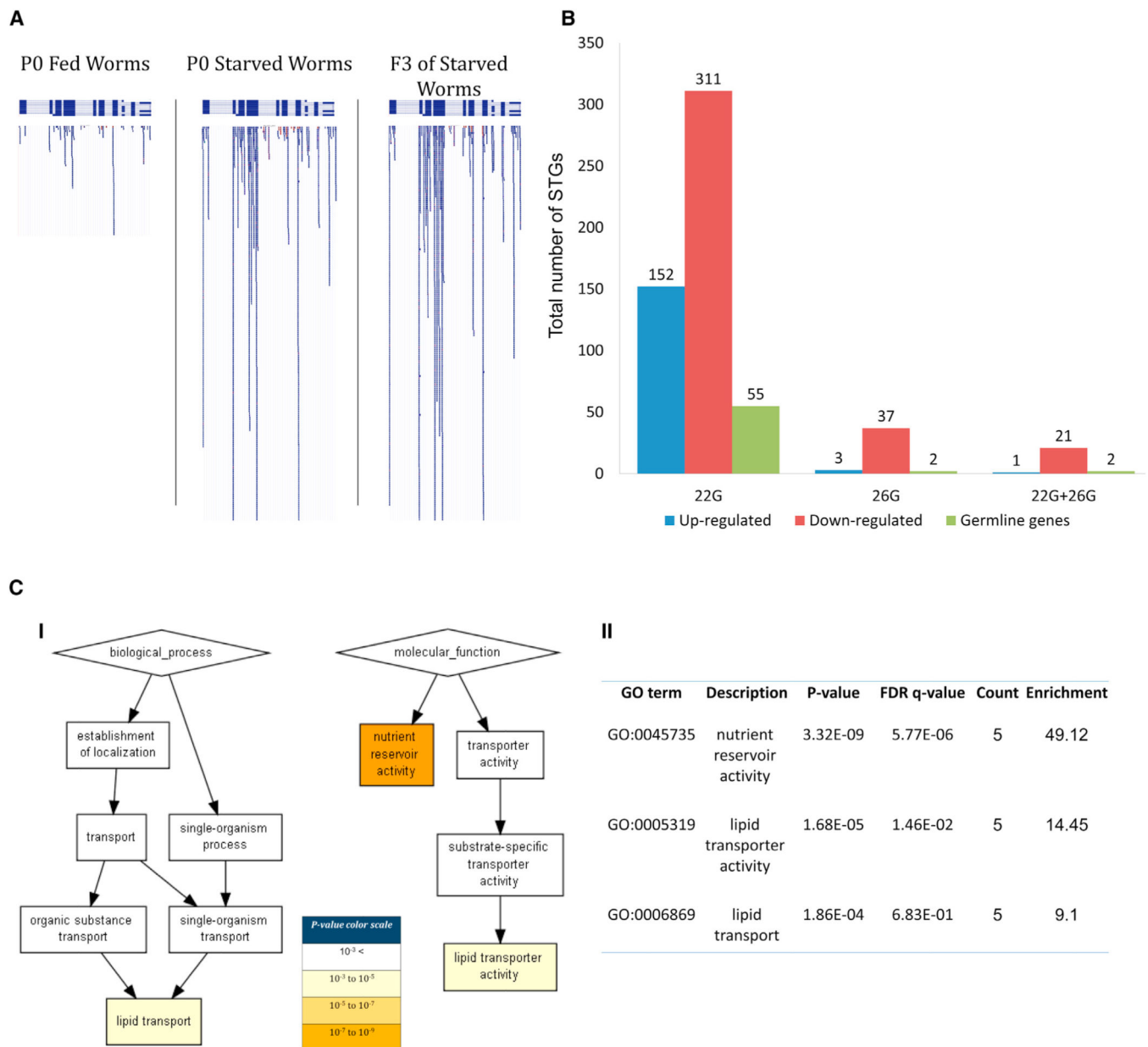


Figure 3. Transgenerational Inheritance of Small RNAs Following L1 Starvation

(A) An example of a gene (*hlh-30*) targeted by heritable 22Gs. See Table S2 for the fold-change and p value ranking of all the inherited STGs.

(B) The total number of inherited STGs of different small RNA species and their overlapping targets.

(C) A functional annotation of STGs that are differentially expressed in an inherited manner following L1-starvation in WT animals. (I) GOrilla functional enrichment map. (II) A table of the specific GO terms, statistical significances, the number of genes that share each specific GO term, and their relative enrichments (see Figure S4 for genomic visualization of the nutrient reservoir activity genes).

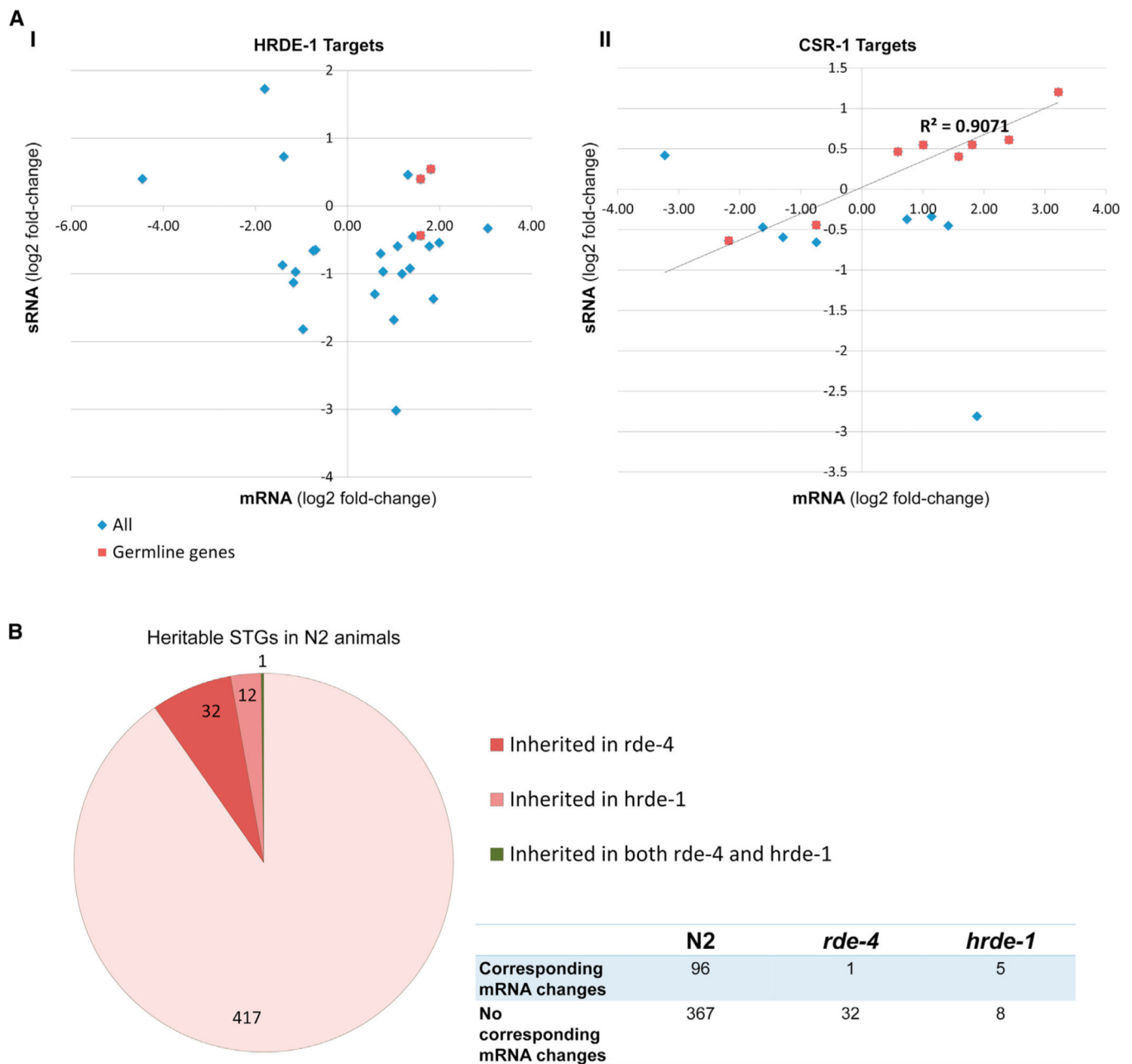


Figure 4. Transgenerationally Inherited Small RNAs Differentially Regulate Their Cognate mRNAs and Are RDE-4 and HRDE-1-Dependent

(A) Plotting of the log₂-fold changes of the inherited STGs, versus the mRNA log₂-fold changes of their cognate targets. Shown separately are (I) HRDE-1 (II) CSR-1 targets (Buckley et al., 2012; Claycomb et al., 2009). Somatic genes are represented by blue dots and germline-expressed genes are represented by red dots. Shown is a linear correlation line ($R^2 = 0.907$) between the inherited STGs that were previously shown to bind CSR-1, and their germline-expressed mRNA targets.

(B) The majority of the inherited STGs are depleted in *rde-4* and *hrde-1* mutants. Shown are the numbers of the small minority of STGs which are nevertheless inherited in the RNAi mutants and their corresponding mRNA changes.

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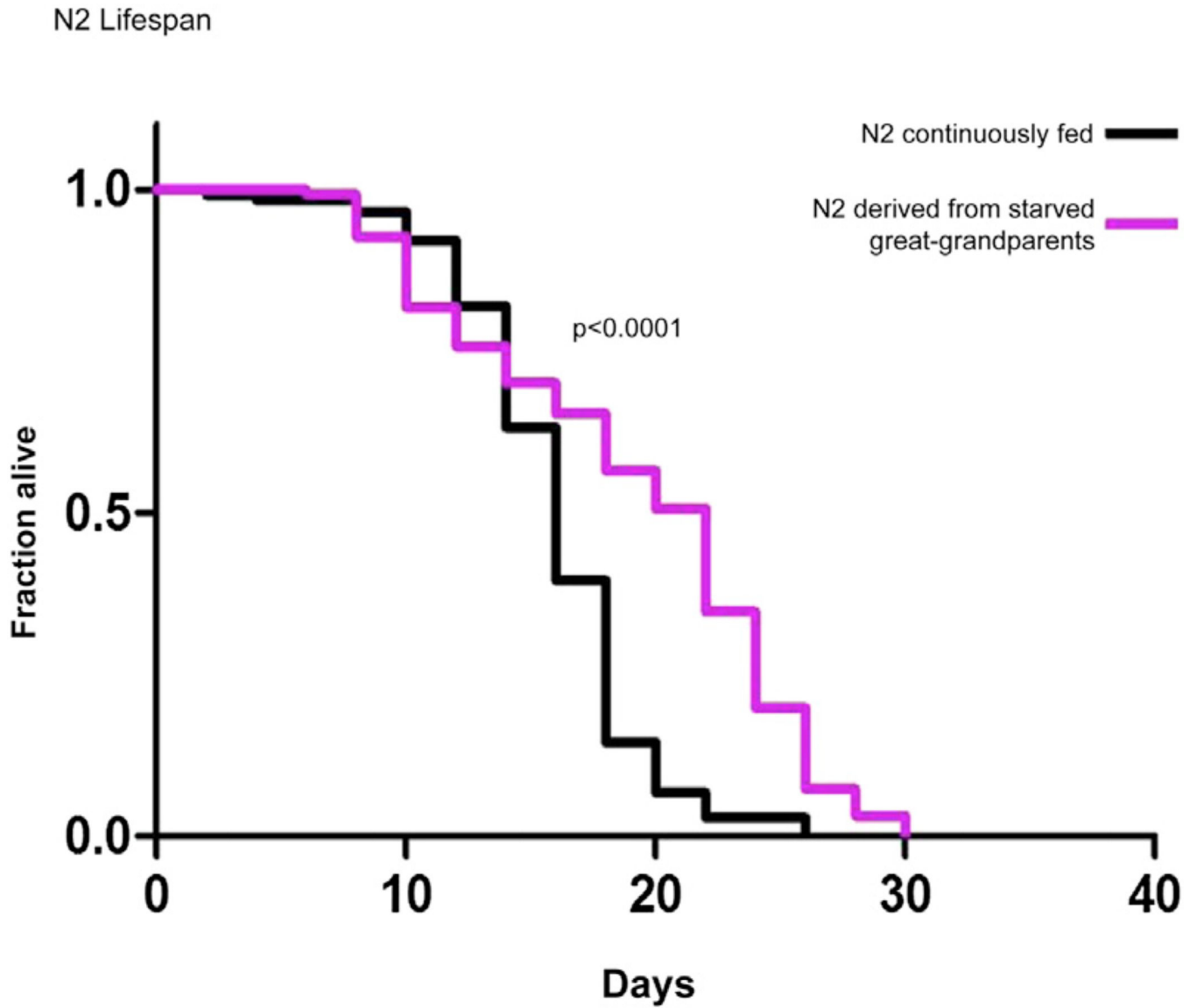


Figure 5. Animals with a Starvation History Show an Extended Lifespan

A comparison of the lifespan of N2 worms whose great-grandparents had experienced starvation at the first larval stage with animals whose great-grandparents were continuously fed. The x axis shows lifespan in days of adulthood. The y axis shows the fraction of worms alive. N2 derived from starved great-grandparents show extended lifespan in comparison to N2 that were continuously fed over generations (live 37% longer, log-rank, $p < 0.0001$). Each of the lifespan experiments was performed three times (data from two additional experiments are shown in Figure S6).

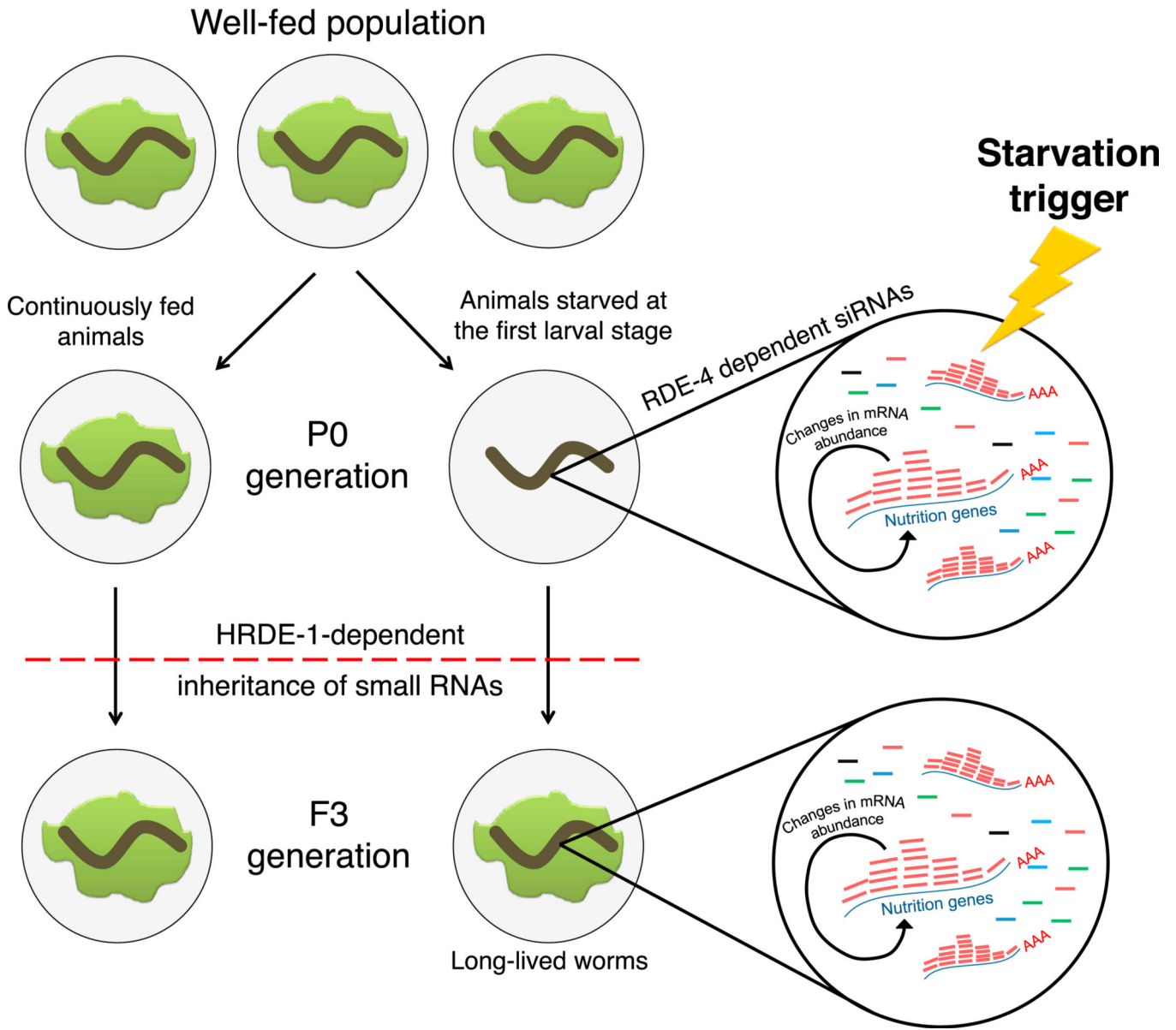


Figure 6. A Model for L1 Starvation-Induced Transgenerational Inheritance
 Food (bacterial lawn) is shown in dark green. Starvation-induced transcripts, which are transcribed either during or after worms experience L1 starvation, enable the production or depletion of specific RDE-4-dependent small RNAs, which regulate the levels of their mRNA targets (involved in nutrition). The production of the heritable small RNAs might be initiated in the germline, or, if endogenous small RNAs act systemically, it could originate also in somatic tissues. The changes in small RNAs persist in the absence of the trigger (starvation) to the next generations. The germline-expressed argonaute, HRDE-1, carries the heritable 22G small RNAs in the germline.