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Transgenerational Inheritance of an Acquired Small RNA-Based Antiviral Response in *C. elegans*

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SUMMARY

Induced expression of the Flock House virus in the soma of *C. elegans* results in the RNAi-dependent production of virus-derived, small-interfering RNAs (viRNAs), which in turn silence the viral genome. We show here that the viRNA-mediated viral silencing effect is transmitted in a non-Mendelian manner to many ensuing generations. We show that the viral silencing agents, viRNAs, are transgenerationally transmitted in a template-independent manner and work in *trans* to silence viral genomes present in animals that are deficient in producing their own viRNAs. These results provide evidence for the transgenerational inheritance of an acquired trait, induced by the exposure of animals to a specific, biologically relevant physiological challenge. The ability to inherit such extragenic information may provide adaptive benefits to an animal.

INTRODUCTION

The inheritance of acquired traits is a topic of long-standing interest and controversy. Whereas some of the classic Lamarckian ideas have been dismissed (Weismann, 1889), more recent studies suggest that certain traits acquired by an animal during its lifetime may be transmitted to next generations. For instance, in rats, diet-induced obesity was shown to transfer from parent to offspring (Ng et al., 2010), and maternal care influenced multiple aspects of neurobiology and behavior of several ensuing generations (Champagne, 2008). Nevertheless, the controversy over the inheritance of acquired traits remains, as the genetic and mechanistic basis for these observations has remained largely unclear.

Resistance to viruses and other genomic parasites is a trait of crucial importance for the survival of any organism, and consequently, modes of viral resistance can be expected to be under strong evolutionary pressure. One commonly employed defense strategy against viruses and other genomic parasites utilizes the process of RNA interference (RNAi), a gene-silencing process that has been well characterized in *C. elegans* (Fire et al., 1998). An RNAi response can also be triggered by the exogenous application of heterologous double-stranded RNA (dsRNA) that targets any gene of choice. Intriguingly, gene-silencing effects evoked by exogenously added dsRNA can be observed not only in the treated animals, but—in a subset of cases tested—also in the progeny of the treated worms (Alcazar et al., 2008; Fire et al., 1998; Grishok et al., 2000; Vastenhouw et al., 2006). However, whether this transmission involves transgenerationally transmitted RNAs or modifications of DNA or chromatin has not been resolved (Alcazar et al., 2008; Fire et al., 1998; Grishok et al., 2000; Vastenhouw et al., 2006). Moreover, to date no biological function has been attributed to the transmission of RNAi-mediated gene-silencing effects, and biological contexts in which this transmission would be important have not been described.

C. elegans possesses an extraordinary ability to ward off viruses. To date, no viruses have been found to hijack its genome. Nevertheless, a wild *C. elegans* strain has recently been discovered to be infected with a single-strand RNA virus closely related to nodaviruses (Félix et al., 2011). This infected *C. elegans* strain was found to be deficient in mounting an effective RNAi response. In contrast, laboratory strains with an intact RNAi response, such as N2, could not be efficiently infected with this RNA virus (Félix et al., 2011). These observations indicate that the RNAi pathway attacks dsRNA intermediates that single-strand RNA viruses like the natural nodaviruses generate during replication (Figure 1A). Indeed, artificially generated viral infection models have previously shown that *C. elegans* can fight viruses by processing the viral dsRNA trigger into virus-derived, small-interfering RNAs (viRNAs) to guide specific viral immunity by Argonaute-dependent RNAi (Lu et al., 2005; Schott et al., 2005; Wilkins et al., 2005). The ability to respond to specific viruses by the production of targeted viRNAs is an acquired trait, serving as an effective defense mechanism against viruses.

We describe here how we have tested whether this acquired trait is transmitted transgenerationally. We show that an episode of viral expression is memorized in the form of small viRNA molecules that are transmitted through many ensuing generations in the absence of the genetic template and even in the absence of a functional small RNA-generating machinery. These inherited viRNA molecules are capable of protecting ensuing generations from the virus by silencing the expression of the viral genome. We therefore provide here evidence for transgenerational transmittance of extrachromosomal information and suggest a biologically relevant context in which such extrachromosomal

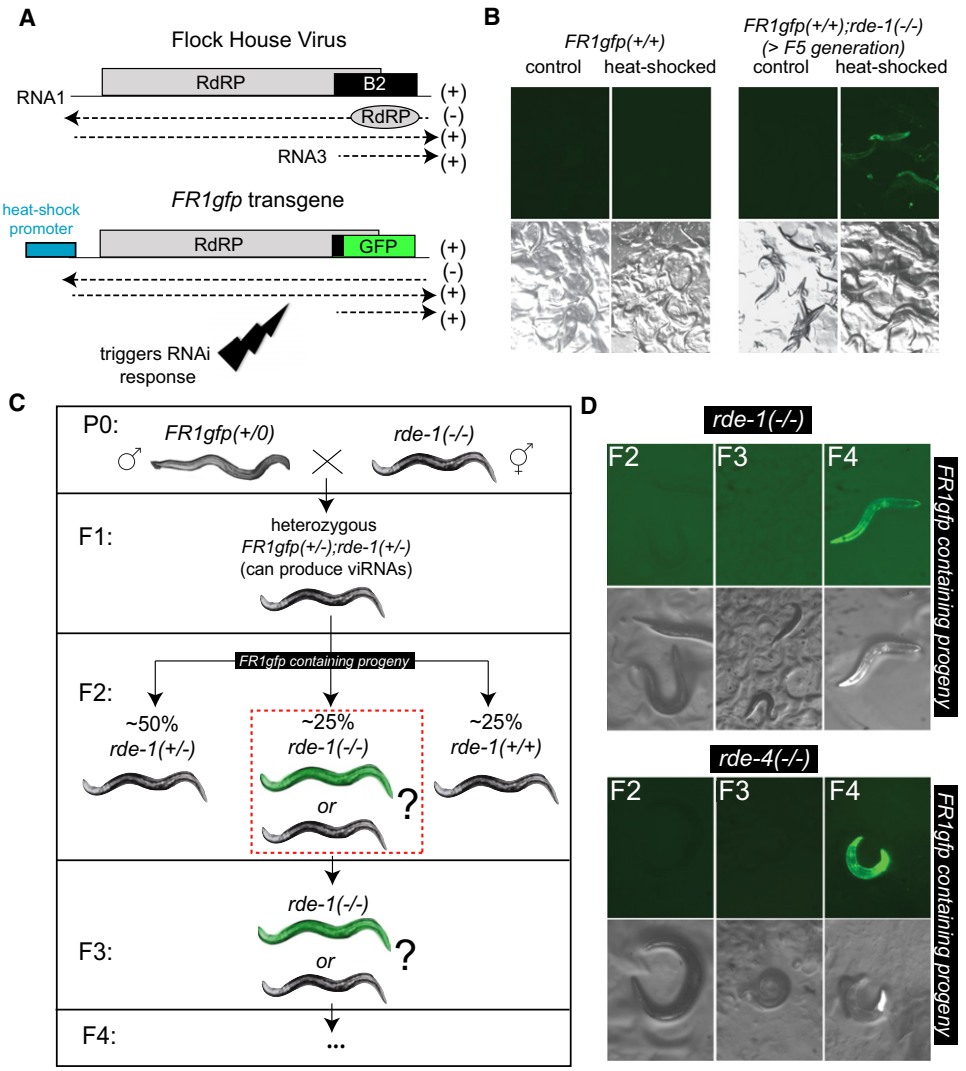

Figure 1. Inheritance of an Antiviral RNAi Response

(A) Schematic presentation of the FHV genome and the *FR1gfp* transgene used in this study (Lu et al., 2009). The RNA2 transcript that produces the capsid is not shown.

(B) Replication of FHV was monitored by recording the expression of GFP in *rde-1* and *rde-4* mutant worms containing the *FR1gfp* transgene 48 hr after heat-shock induction of viral replication. The animals were RNAi deficient for more than five generations.

(C) A scheme depicting the cross that tests the requirement for *rde-1* and *rde-4* in generating virus-silencing viRNAs. Animals shown in the F2 and later generations were all selected to contain the *FR1gfp* transgene as assessed by the dominant coinjection marker *rol-6*. *rde-1(−/−)* animals were scored in the F2 generation for whether they express or do not express GFP after heat-shock induction. As *rde-1(−/−)* animals (or *rde-4* mutant animals) are not capable of initiating an RNAi response, either the *FR1gfp; rde-1(−/−)* F2 generation is not able to protect itself against viral propagation (hence heat-shocked animals would be GFP(+) as indicated schematically with a green animal) or the F2 generation inherits antiviral viRNAs that were produced by earlier generations and can therefore protect itself against the virus (hence heat-shocked animals would be GFP(−) as indicated schematically with a dark animal). As shown in (D), the latter is the case.

(D) DIC and GFP images of heat-shocked *rde-1*(−/−) and *rde-4*(−/−) worms that have been homozygous mutant for *rde-1* or *rde-4* for several generations (as indicated in C) and contain *FR1gfp* as assessed by the *rol-6* transgene marker. The *rde-1* and *rde-4* genotypes were assessed through a linked *unc* marker.

information provides a benefit of potential evolutionary relevance to an organism.

RESULTS

A Reporter-Based System to Visualize RNAi-Mediated Viral Silencing in *C. elegans*

To test the hypothesis of transgenerational inheritance of an acquired trait in a well-controlled setting, we utilized a previously established model to monitor viral propagation, namely, a transgenic worm that supports autonomous replication of the Flock House virus (FHV) (Lu et al., 2005, 2009). FHV is a plus-strand RNA nodavirus that has broad host specificity and is very similar to the virus that was recently identified in a wild *C. elegans* strain (Félix et al., 2011). To bypass the initial steps of infection (cuticle penetration and cell entry), worms were engineered to carry a chromosomally integrated DNA, called *FR1gfp,* corresponding to the RNA1 gene and parts of the RNA3 gene of FHV. The RNA1

Table 1. Viral Silencing in RNAi-Deficient Mutants

Genotype	Generation	GFP/Virus(+) Animals after Heat Shock[c]	Total Number of Animals Examined
Wild-type	any	0%	>100
rde-1 (ne300)	*rde-1(−/−)* P0[a]	100%	50
	rde-1(+/−) F1 cross-progeny[b]	0%	250 (5 experiments)
	rde-1(−/−) F2	0%	250 (5 experiments)
	rde-1(−/−) F3	0%	250 (5 experiments)
	rde-1(−/−) F4	0.71%	882 (5 experiments)
	rde-1(−/−) F5	10.45%	908 (5 experiments)
rde-4 (ne299)	*rde-4(−/−)* P0*	4.9%	102
	rde-4(+/−) F1 cross-progeny[b]	0%	250 (5 experiments)
	rde-4(−/−) F2	0%	250 (5 experiments)
	rde-4(−/−) F3	0%	267 (5 experiments)
	rde-4(−/−) F4	3.4%	441 (5 experiments)
	rde-4(−/−) F5	3.6%	307 (5 experiments)

All animals contain the *FR1gfp* array in the background. Animals were scored in a binary manner as either producing (GFP/virus(+)) or not producing (GFP/virus(−)) any GFP signal. Production of GFP is usually observed in many tissue types. See Figure S1 for further information.

[a] Animals were homozygous for *rde-1* or *rde-4* for at least ten generations.

[b] Cross of wild type with *rde-1(−/−)* or *rde-4(−/−)*, respectively.

[c] Numbers are averaged over several experiments. Individual experiments and animals are shown in Figure S1.

of all known nodaviruses encodes the viral RNA-dependent RNA polymerase (RdRP) (Félix et al., 2011). The subgenomic RNA3 transcript encodes the B2 protein, a viral suppressor of RNAi essential for FHV propagation in *D. melanogaster* (Li et al., 2002). B2 was largely replaced in *FR1gfp* by *gfp* as previously described (Figure 1A) (Lu et al., 2009). Transcription of the viral RNA in the *FR1gfp* transgene is initially triggered by a heat-inducible promoter (Figure 1A) (Lu et al., 2009), and ensuing viral replication (including the replication of the subgenomic *gfp*) is then carried out autonomously by the RdRP (Lu et al., 2009). Generally, RdRP-catalyzed replication of FHV progresses at a vigorous rate, reaching levels as high as those of ribosomal RNAs (rRNAs) (Ball et al., 1994; Johnson and Ball, 1997). Transgenic worms that were heat-induced to initiate the expression of the virus show robust silencing of the B2-deficient FHV mutant virus because the RNAi pathway generates viRNAs that target viral RNA for degradation (Figure 1B) (Lu et al., 2005, 2009). Worms mutant in individual components of the RNAi pathway do, however, display robust virus production as monitored by strong GFP expression throughout the animal (Figure 1B; Table 1), as previously reported (Lu et al., 2009).

Transgenerational Inheritance of the Antiviral Response

To test whether *C. elegans* can remember an episode of viral propagation and pass this memory to its progeny, perhaps in the form of viRNAs, we performed a set of genetic crosses (Figure 1C). Specifically, we generated animals that are heterozygous for two different RNAi-defective mutants, *rde-1* or *rde-4* (both coding for RNA-binding proteins required for the initiation of an RNAi response [Aoki et al., 2007; Parrish and Fire, 2001; Steiner et al., 2009]), and that contain the heat-inducible *FR1gfp* viral transgene in the genetic background (see Experimental Procedures for how the genotypes were determined and how virus expression was induced). Due to the presence of one functional copy of *rde-1* or *rde-4,* these heterozygous animals display a robust antiviral silencing response and therefore display no GFP fluorescence upon heat induction of virus expression (Table 1; Figure 1C). Throughout this paper, we call this phenotype "GFP/virus(−)." Self-fertilization of these heterozygous animals results in the generation of *rde-1* or *rde-4* homozygous animals. Such homozygous mutant progeny are expected to be unable to silence the virus and therefore should be GFP/virus(+) upon heat-shock induction of viral expression. However, we find that homozygous mutant offspring of *rde-1* and *rde-4* heterozygotes show robust viral silencing upon heat-shock induction, as evidenced by animals being GFP/virus(−) (Figures 1C and 1D; Table 1). This observation cannot be simply explained by maternal deposition of wild-type *rde-1* or *rde-4* activity from the heterozygous parents because it has been explicitly demonstrated that neither gene activity is maternally transmitted (Blanchard et al., 2011). Moreover, the silencing effect persists through several generations of homozygous *rde-1* or *rde-4* mutants. Only in the F4 generation of *rde-1(−/−)* and *rde-4(−/−)* worms did a small percentage of the animals start to express the virus, as measured by GFP fluorescence (Table 1; Figure S1 available online). Among the progeny of F4 GFP/virus(+) worms, we found an increasing number of worms that failed to silence the virus and were thus GFP/virus(+) (Table 1; Figure S1). This inherited silencing effect can eventually "wear off," as evidenced by continued isolation and propagation of GFP/virus(+) worms that then produced progenies that were 100% GFP/virus(+) upon heat-shock induction (Figure S1).

Crossing GFP/virus(+), homozygous *rde-1(−/−)* mutant worms, in which the inherited antiviral silencing effect has worn off, with RNAi-competent wild-type males produces cross-progeny in which the viral genome is again silenced (Figure 2A). This demonstrates that the restitution of the RNAi machinery, achieved through provision of a functional copy of *rde-1*, re-establishes the antiviral response. When *rde-1(−/−)* males, rather than wild-type males, were crossed to the same GFP/virus(+) > F5 generation *rde-1* homozygous mutant hermaphrodites, the heat-shocked cross-progeny remained GFP/virus(+) because they still lacked the ability to mount an RNAi response (Figure 2A). This control experiment also demonstrates that unpairing of the transgenic DNA during meiosis is insufficient by itself to trigger silencing, as has been observed in Neurospora ("meiotic silencing by unpaired DNA") (Shiu et al., 2001).

It was previously shown that cells infected with FHV produce highly abundant RNA transcripts, as abundant as rRNAs (Ball et al., 1994; Johnson and Ball, 1997). Our heat-shock induction of viral transcripts should therefore not produce nonphysiological levels of RNAs. We nevertheless tested whether much lower-level induction of viral transcripts can induce an inherited

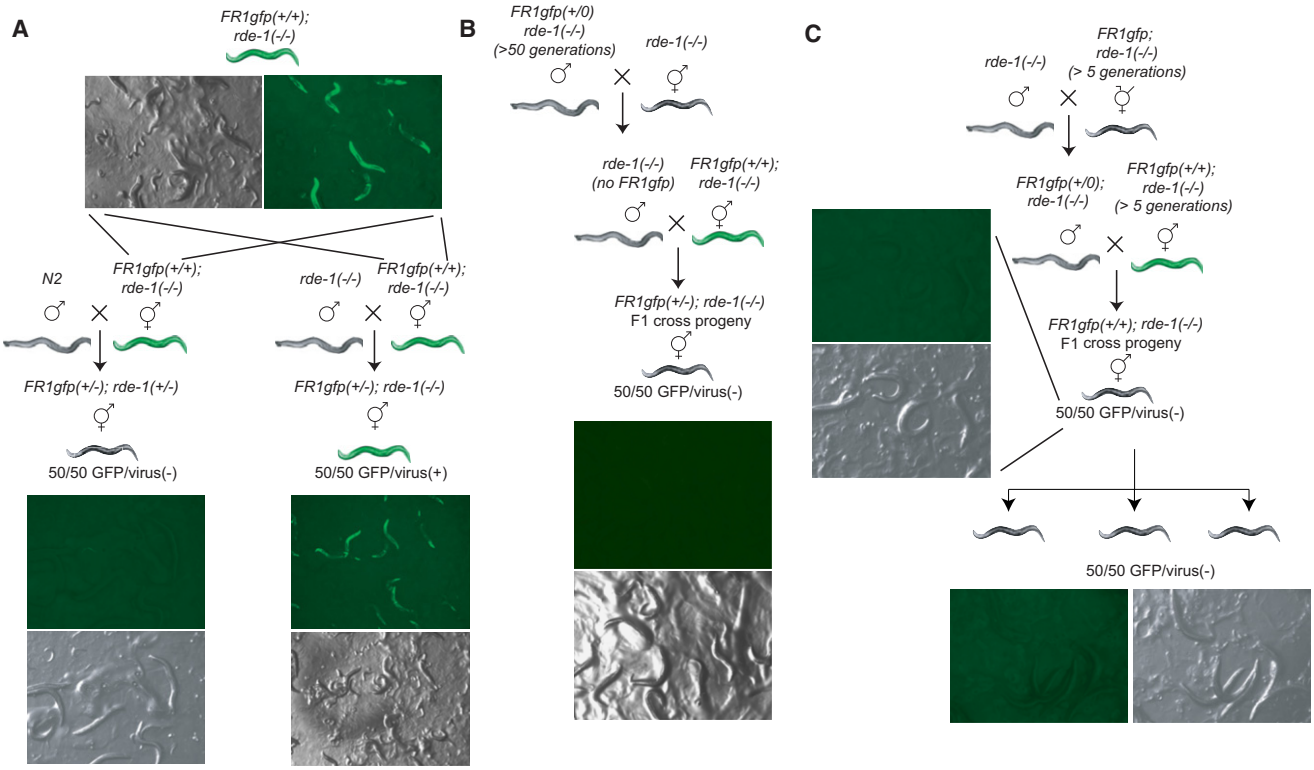

Figure 2. Genetic Analysis of the Transgenerational Inheritance of an Antiviral Response

Animals of all the genotypes schematically shown in this figure were tested for whether they express GFP after heat shocking adult animals (see Experimental Procedures), as a measure to assess viral silencing; dark animals do not express GFP after heat shock, green animals do. Numbers are shown for the most relevant genotypes. The X-linked *FR1gfp* transgene is present only when specifically indicated.

(A) Reconstitution of the RNAi machinery resilences viral production.

(B) The antiviral RNA silencing can pass through the sperm and is independent of the presence of the viral template. Because the *FR1gfp* array is on the X chromosome, F1 males originating from the cross of male *FR1gfp(+/0)* (indicating hemizygosity); *rde-1(−/−)* with *rde-1(−/−)* hermaphrodites will not contain the array.

(C) Long-term-silenced worms contain a nonchromosomally encoded dominant spreading signal. Crossed animals carry the same genotype in regard to the transgene and *rde* locus, yet one strain is long-term silenced, whereas the other has lost its ability to silence. The long-term-silenced strain is able to silence the nonsilent strain in *trans*. Viral silencing was always assessed by heat-shock treatment to induce the *gfp*-tagged viral transcript. If the *FR1gfp* transgene was not present in a strain, it is not shown in the genotype.

response. To this end we made use of the inherent, slight leakiness of the heat-shock promoter at 15°C and simply maintained the strain containing *FR1gfp* at 15°C. We find that the *rde-1(−/−)* F2 progeny of the cross between wild-type males and *rde-1(−/−)* animals is able to silence viral propagation even if viral replication in the F1 *rde-1(+/−)* cross-progeny was kept at 15°C (50/50 animals are GFP/virus(−)). These results indicate that even very low levels of viral product are sufficient to trigger an antiviral response.

In addition to examining the impact of the small RNA biogenesis genes *rde-1* and *rde-4* on viral silencing, we also checked other components of the RNAi pathway (*mut-2, mut-7, mut-14, mut-16, rde-2, ergo-1, csr-1,* and *C04F12.1*). We find that the activity of these genes is not required for virus silencing as their elimination did not result in GFP/viral expression (>50 animals tested for each gene), possibly due to the documented redundancy of the RNAi silencing machinery in the worm, which contains 27 known Argonautes (Yigit et al., 2006).

Transgenerational Transmission of the Antiviral Response Is Template Independent

We next tested whether the antiviral RNA agent can be passed to ensuing generations independently of the viral template. To this end, we used the genetic strategy shown in Figure 2B. We crossed *rde-1* mutant males that carry the X-linked *FR1gfp* transgene and display silencing because of the inherited RNA agent (and therefore are GFP/virus(−)) to *rde-1(−/−)* hermaphrodites that never encountered the viral transgene. Cross-progeny males from this cross do not carry the X-linked *FR1gfp* due to its X linkage. These cross-progeny *rde-1(−/−); FR1gfp(−/0)* males were crossed with GFP/virus(+), F5 generation *rde-1(−/−); FR1gfp(+/+)* hermaphrodites. We find that the *rde-1(−/−)* progeny of this cross have their viral GFP signal eliminated (Figure 2B). This experiment demonstrates that the antiviral agent can be transmitted in the absence of its template. As a side note, the experiment also shows that the antiviral agent can be transmitted through sperm, consistent with previous

experiments that tested the transmission of silencing effects against exogenously provided dsRNA (Alcazar et al., 2008; Grishok et al., 2000).

Long-Term Silencing Is Transmitted in a Non-Mendelian Manner and Requires the RNA-Dependent RNA Polymerase *rrf-1*

One notable feature that became apparent throughout handling *FR1gfp; rde-1(−/−)* and *FR1gfp; rde-4(−/−)* animals over the course of many months was that in many animals the GFP/virus(+) signal never reappeared after the initial construction of those strains. This indicates that in addition to the "fading" (~3 generations) mode of silencing that we described above, a second and more stable mode of inherited silencing can also take place. Such a long-term mode of inherited silencing was also previously observed in response to exogenously added dsRNA (Alcazar et al., 2008; Vastenhouw et al., 2006). Based on genetic loss-of-function analysis, it has been proposed that this long-term silencing effect requires specific chromatin-modifying factors (Vastenhouw et al., 2006). Similarly, it has previously been reported that transgenes can become silenced over many generations (Kelly et al., 1997) (a scenario unlikely to apply here because the *rol-6* injection marker that is present on the *FR1gfp*-containing array is still expressed in these animals). A number of chromatin factors have been identified whose knockdown affects either exogenous RNAi-mediated, long-term silencing (*hda-4, K03D10.3, isw-1, mrg-1*) or transgene silencing (*mes-2, mes-3, mes-4, mes-6, mys-1, M03C11.3, zfp-1, rba-1, cin-4, gfl-1)* or both (*hda-4, K03D10.3, isw-1, mrg-1*) (Kim et al., 2005; Vastenhouw et al., 2006; Wang et al., 2005). We tested all of these factors in the same manner as they were tested in earlier studies and found that the silencing of heat-shock-induced *FR1gfp* expression is not affected upon knockdown of any of these factors (>50 animals tested for each gene).

To further test the transgene silencing issue, but also to examine whether long-term-silenced worms carry a trait that segregates in a Mendelian manner (as would be expected from a silenced transgenic array or from a mutation in some other, secondary locus), we conducted a genetic experiment schematically shown in Figure 2C. We crossed long-term-silenced (i.e., GFP/virus(−)), *FR1gfp*-containing animals that were more than F5 generation homozygous for *rde-1(−/−)* with >F5 generation *rde-1(−/−); FR1gfp* animals that had lost their silencing ability after a few generations and were therefore GFP/virus(+) upon viral induction. Because the RNAi machinery is not reinstated in the cross-progeny (all animals used are *rde-1(−/−)*), a hypothetical genomically encoded locus that suppresses GFP/virus production should segregate in a Mendelian manner in the ensuing generations; similarly, if the GFP/virus(−) worms simply had their transgene silenced, three-quarters of the progeny should be GFP/virus(+). If, in contrast, the silencing agents are diffusible *trans*-acting factors (as would be expected from viRNAs), then all F2 progeny should be silenced. We observed that all the F2 progeny had the virally produced GFP signal eliminated (Figure 2C). This experiment indicates that inherited silencing in *rde-1(−/−); FR1gfp* worms, in which the virus is still silenced after >F5 generation, is achieved by a spreading and DNA-independent element.

It is conceivable that long-term silencing would be maintained by constant amplification of the original RNAi response (Alcazar et al., 2008; Groenenboom et al., 2005). Even though the endogenous RdRP *rrf-1* has been shown in the past to amplify viRNAs (Parameswaran et al., 2010; Schott et al., 2005), we find that *rrf-1* is not required for the initial transgenerational viral silencing that is observed in *rde-1* or *rde-4* homozygous mutant animals that were derived from RNAi-competent parents (Figure 3A). Intriguingly, however, *rrf-1* dependence is observed in animals that have been long-term silenced in the absence of *rde-1* (Figure 3B). We draw this conclusion from the reinstated ability of the virus to be expressed (i.e., a GFP/virus(+) phenotype) in the *rrf-1(−/−)* homozygous F3 progeny of a cross between stably silenced GFP/virus(−) >F5 generation *rde-1(−/−); FR1gfp* worms and *rde-1(−/−); rrf-1(−/−)* double mutants (Figure 3B).

Physical Detection of Inherited viRNA Molecules

Next, we used small RNA isolation and ensuing deep sequencing to try to detect viRNAs transgenerationally transmitted from RNAi-competent parents. Although both *rde-1* and *rde-4* mutants are required for initiation of a de novo (but not inherited) RNAi response, we chose to sequence viRNAs from *rde-4* mutant animals rather than from *rde-1* mutant animals because of the different roles that the two proteins play in the RNAi pathway. *rde-4* mutants are depleted of siRNAs because the RDE-4 protein acts upstream in the dsRNA processing pathway by binding long dsRNA and guiding it toward dicing (Aoki et al., 2007). *rde-1*, on the other hand, is not defective in primary small RNA synthesis (or primary viRNA synthesis) (Wu et al., 2010) because its protein activity is required only for the separation of the dsRNA duplex but not for the accumulation of short dsRNAs (Aoki et al., 2007; Parrish and Fire, 2001; Steiner et al., 2009). We chose to use a cloning protocol that enriches for rare *rde-4*-dependent primary small RNAs (Parameswaran et al., 2010) because *rde-4* (as well as *rde-1)* animals are not defective in secondary siRNA production (Blanchard et al., 2011) and thus can theoretically continue to amplify secondary siRNAs de novo if even a single "trigger" siRNA is inherited (Groenenboom et al., 2005). Detected primary siRNAs, however, are guaranteed to be derived from the original RNAi-competent parents because *rde-4* mutant animals cannot produce them (Alcazar et al., 2008; Groenenboom et al., 2005).

Thus, we prepared and sequenced small RNA libraries from four different types of animals: (1) *FR1gfp*-transgenic worms that can mount an RNAi response and should contain viRNAs (positive control); (2) *rde-4(−/−)* mutants (negative control as no viRNAs are expected to be produced); (3) *FR1gfp; rde-4(−/−)* worms that are two generations away from their *rde-4(+/−)* grandparents, i.e., worms that can themselves not produce viRNAs but may have inherited viRNAs from their grandparents; and (4) the F3 progeny of wild-type animals that contained the *FR1gfp* transgene (and therefore produced viRNAs) but have lost this transgene through outcrossing with nontransgenic wild-type worms. This tests whether the silencing reagent can exist without its template.

In agreement with the functional assays, we detected different viRNAs complementary to several regions of the viral genome in the positive control (*FR1gfp*), no viRNAs in the negative control

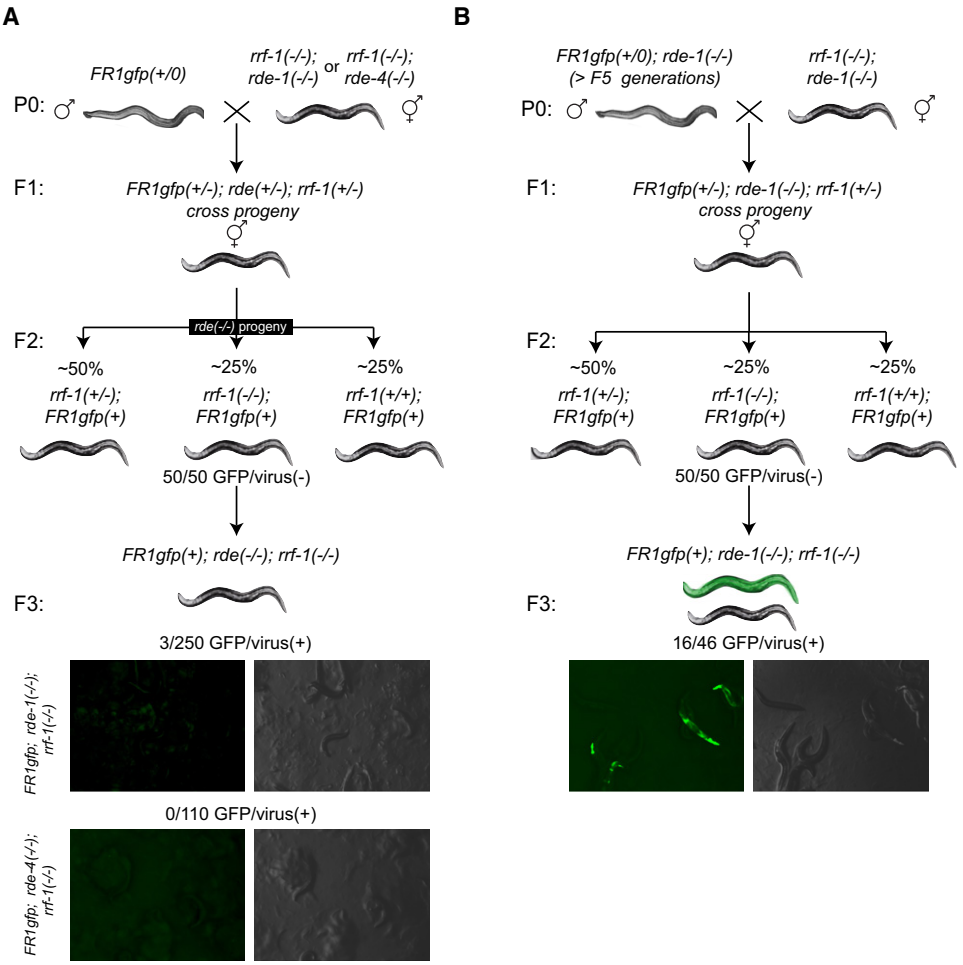

Figure 3. The RdRP Amplification by *rrf-1* Is Required Only for Long-Term Silencing

Animals of all the genotypes schematically shown in this figure were tested for whether they express GFP after heat shocking adult animals (see Experimental Procedures), as a measure to assess viral silencing; dark animals do not express GFP after heat shock, green animals do. Numbers are shown for the most relevant genotypes. *FR1gfp(+)* indicates that animals contain the *FR1gfp* array, but we did not test whether it is contained in the homozygous (*FR1gfp(+/+)*) or heterozygous (*FR1gfp(+/−)*) state. If the *FR1gfp* transgene was not present in a strain, it is not shown in the genotype.

(A) RNAi-competent grandparents initiate an RNAi response that is sufficient for viral silencing for at least two generations in the absence of *rrf-1*.

(B) The virus escapes long-term silencing (GFP/virus(+)) when *rrf-1* is neutralized in otherwise GFP/virus(−) >F5 generation *rde-1(−/−)* animals.

(*rde-4(−/−)*), and a number of viRNAs in the worms that could not generate their own viRNAs and thus inherited these viRNAs from their grandparents (F3 generation *FR1gfp; rde-4(−/−)*) (Table 2; Figure 4). Moreover, we detect viRNAs in the worms in which the *FR1gfp* transgene had been crossed out, confirming that the viRNAs transmit in a template-independent manner. The inherited viRNAs matched the two most abundant types of viRNAs detected in the positive control (Figure 4), and these viRNAs were all of the reverse orientation (negative strand, which typically exists in much lower quantities than the positive strand; Félix et al., 2011); both observations make it highly unlikely that the detected viRNAs merely represent unspecific breakdown products of the viral RNA. In regard to the low number of inherited viRNA reads, it needs to be considered that our protocol enriches specifically for rare primary viRNA species. Moreover, these viRNA species are derived from a response mounted in RNAi-competent grandparents and are therefore possibly diluted over the course of several generations. Taken together, our results support the genetic experiments that argue for the existence of *trans*-acting factors that are transmitted in a non-Mendelian manner to ensuing generations.

DISCUSSION

We have described here a series of genetic experiments that provide support for the existence of non-Mendelian, multigenerational inheritance of extrachromosomal information. This information is transmitted in the form of small RNAs, viRNAs, which are induced by an episode of viral replication and which are propagated through the germline in a non-template-dependent manner. Our results therefore support the Lamarckian concept of the inheritance of an acquired trait.

Table 2. Molecular Identification of Transgenerationally Inherited viRNAs

Genotype	# 20–30 bp Sequences[a]	# viRNAs against FHV[b]	viRNA Reads per Total # Small RNA Reads (× 10^6)
FR1gfp	3,641,082	115	31.6
F3 generation *FR1gfp; rde-4(−/−)*	1,131,284	10	8.8
F3 generation after *FR1gfp* outcross	567,549	2	3.5
rde-4(−/−)	966,052	none	none

[a] Numbers correspond to reads after adaptors and noise have been removed (see Experimental Procedures).
[b] See Experimental Procedures for more information and comments about these viRNA reads and Figure 4 for the mapping of the viRNAs on the viral genome.

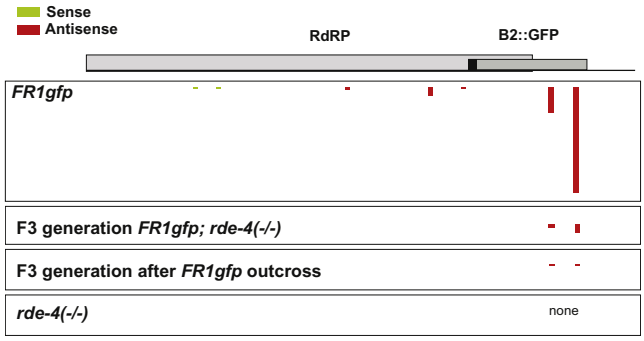

Figure 4. viRNA Match to Specific Epitopes in the Viral Genome
The number and strandedness of 20–30 nt viRNAs are shown with respect to their alignment to the FHV genome. The thickness of lines, which indicate the location of individual reads, is proportional to number of reads. Note that the transmitted viRNA reads match to the main two epitopes of the virus. Clustering of epitopes to the 3′ end of FHV has been noted before (Parameswaran et al., 2010). See Experimental Procedures and Table 2 for more details on the reads.

Our results suggest a physiologically relevant role for these inherited molecules that may lie in transmitting antiviral protection to future generations. We infer this notion from our use of a transgenic system that expresses a viral genome that is from the same family as a natural virus recently discovered in a wild isolate in France (Félix et al., 2011). This transgenic system permitted us to measure viral expression and silencing through a GFP signal rather than relying on the relatively subtle phenotypes induced by the natural virus. Moreover, the use of a well-defined minimal transgenic system in which the viral genome can be unambiguously removed through genetic crosses eliminates potentially confounding issues of viral contamination and also enabled us to show that an antiviral response is transgenerationally segregated in the absence of a template. Lastly, because the *FR1gfp* transgene does not harm the worm (unlike the natural virus), it can be used to study inherited immunity without fear of accidentally selecting for worms that acquired viral resistance through secondary mutations.

Our findings suggest a physiological context for the previously reported transmission of gene-silencing effects elicited by exogenously added dsRNA (Alcazar et al., 2008; Fire et al., 1998; Grishok et al., 2000; Vastenhouw et al., 2006). As these previously described inherited effects show a nonunderstood specificity for only some target genes, it was unpredictable a priori whether an antiviral RNAi response, triggered by perhaps only a few dsRNA molecules, would also be transgenerationally transmitted and be effective in ensuing generations. Moreover, the mechanistic basis for previously reported transmission of gene-silencing effects has been controversial (Alcazar et al., 2008; Fire et al., 1998; Grishok et al., 2000; Vastenhouw et al., 2006). Aside from suggesting biological context, our results also indicate that the inheritance of the viral silencing effect is mediated by inherited small RNA molecules acquired after viral replication.

It is conceivable that other biological functions may be controlled by transgenerationally transmitted, extrachromosomal agents as well. For example, one may speculate that the recently described inheritance of an olfactory memory (Remy, 2010) or the transgenerational inheritance of longevity traits (Greer et al., 2011) could also be the result of inherited small RNA molecules. Intriguingly, our deep sequencing of *rde-4(−/−)* animals not only identified transgenerationally transmitted viRNA molecules but also suggests that several classes of *rde-4*-dependent endo-siRNAs may be inherited (O.R., G.M., and O.H., unpublished data), indicating that the transmission of extrachromosomal information may be a common phenomenon. Such a mode of inheritance may provide adaptive advantages to an animal.

EXPERIMENTAL PROCEDURES

Genetics

Worms were cultured on NGM plates seeded with OP50 bacteria. *FR1gfp* transgenic worms were a kind gift from S.W. Ding (Lu et al., 2009). Animals carrying the *FR1gfp* transgene were followed by monitoring their rolling behavior caused by the *rol-6(d)* transgene marker. In order to follow worms that were homozygous for RNAi-deficiency mutations *((rde-1(ne300)* or *rde-4(ne299))* after crossing to *FR1gfp* animals, we used the WM36 *rde-1(ne300) unc-42(e270)* and WM35 *rde-4(ne299) unc-69(e587)* strains, in which the *rde* mutations are linked to *unc* marker mutations. RNAi-deficient *FR1gfp* worms OH10354 and OH10356 were created by singling Unc Rol worms for >10 generations (OH10354 genotype: *FR1gfp; rde-1(ne300) unc-42(e270)* and OH10356 genotype: *FR1gfp; rde-4 (ne299) unc-69(e587)*). Worms with the OH10435 genotype *FR1gfp; rde-1(ne300) unc-42(e270); rrf-1(ok589)* and the OH10436 genotype *FR1gfp; rde-4 (ne299) unc-69(e587); rrf-1(ok589)* were created using standard genetic crosses.

To assess the dependency of inherited antiviral protection on chromatin-remodeling factors, we used the MT15795 *isw-1(n3294)* and SS186 *mes-2(bn11)* strains and also preformed RNAi feeding to knock down these and a number of other chromatin modulators (*isw-1, M03C11.3, mrg-1, rba-1, cin-4, mes-2, mes-3, mes-4, mes-6, mys-1, hda-4, gfl-1, zfp-1)*. The RNAi assays were performed using a previously described bacterial feeding protocol (Kamath et al., 2003). Briefly, NGM agar plates containing 6 mM IPTG and 100 μg/ml ampicillin were seeded with bacteria expressing dsRNA. Ten *FR1gfp* L4 hermaphrodites were placed onto these plates and grown at 20°C. The P0 worms and the F1 progeny of these worms were heat shocked as young adults and scored for GFP expression 48 hr after.

Measurement of Viral Silencing

All the animals in which viral expression was measured contained the *FR1gfp* array in the background. Our standard procedure of triggering viral

propagation is to heat shock young adults first for 1 hr at 37°C and then for an additional 3 hr at 33°C. Forty-eight hours later, individual animals were scored in a binary manner as either producing ("GFP/virus(+)") or not producing ("GFP/virus(−)") any GFP signal. Production of GFP is usually observed in many tissue types. If animals with the *FR1gfp* transgene are kept at 15°C, RNAi-deficient animals will not produce a readily detectable GFP signal; however, we notice that even at 15°C there appears to be some small residual *FR1gfp* expression as those animals are able to produce viRNAs that can silence viral replication upon genetic crosses (see text). Such leakiness of heat-shock promoter expression has been noted in many instances in the literature (e.g., Poole et al., 2011).

Molecular and Computational Analysis of Small RNAs

Small RNA libraries were constructed using a protocol that enriches for Dicer products that harbor a single phosphate at the 5′ end of the RNA (Zamore et al., 2000), such as primary siRNAs but unlike RdRP products that harbor triphosphate ends (Parameswaran et al., 2010). This was the protocol of choice because we were interested in identifying viRNAs that are guaranteed to be derived from the original RNAi-competent parents (Alcazar et al., 2008), and *rde-4* animals cannot produce primary siRNAs (Grishok et al., 2000; Parrish and Fire, 2001). *rde-4* animals are, however, not defective in secondary siRNA production (Blanchard et al., 2011) and can therefore continue to amplify secondary siRNAs de novo; such autonomously produced siRNAs will not be distinguishable from inherited ones. The detection of rare primary siRNAs is important, as even a single "trigger" siRNA can induce a full-blown RNAi response that is not proportional to the primary trigger (Groenenboom et al., 2005).

Worms were lysed using the TRIzol reagent, and repetitive freezing, thawing, and vortexing were done as previously described (Lee and Ambros, 2001). The mirVana kit (Ambion) was then used for isolation of <200 nt RNAs following size selection of small RNAs of 18–30 bases by denaturing polyacrylamide gel fractionation. Small RNA libraries were constructed using the Illumina v1.5 Small RNA Cluster Generation Kit and sequenced using the Illumina/Solexa GAIIX platform (Illumina, Inc., San Diego, CA, USA). Sequences were preprocessed to remove flanking adaptor sequences and noise prior to analysis using the Hannon lab FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Reads were then size filtered using PRINSEQ (Schmieder and Edwards, 2011) so that only small RNAs in the range 20–30 nt were considered for alignment. Alignments of viRNAs to the FHV genome were performed using Geneious v5.4.3 (word length = 6) (Drummond et al., 2011). Aligned reads were further examined for similarity to the *C. elegans* genome using BLASTN (Zhang et al., 2000) to rule out any small RNAs that might match both *C. elegans* and FHV genomes. No reads matching both FHV and *C. elegans* genomes were found.

The sequence analysis revealed a strong antisense polarity bias (113/115 viRNA reads in *FR1gfp* and all inherited reads), consistent with the notion that some of the much more abundant secondary viRNAs (that typically exhibit such a bias; Sijen et al., 2007) are nevertheless captured despite the use of a protocol that enriches mostly for primary siRNAs (Parameswaran et al., 2010). This can possibly be due to nonenzymatic or post-extraction loss of 5′ phosphates (Gent et al., 2010). However, a strong 3′ end bias that is typical to primary siRNAs (Sijen et al., 2007) and to (FHV-targeting) primary viRNAs in particular (Parameswaran et al., 2010) was observed in the detected viRNAs, suggesting that the cloning protocol successfully enriched for primary siRNAs. The fact that 98.26% of the viRNA transcripts were of the negative orientation, even though synthesis of sequences with this orientation is very rare during nodavirus replication (Félix et al., 2011), makes it highly unlikely that they represent nonspecific degradation products.

Only reads containing 0–2 mismatches to FHV were considered for analysis. For consideration as a "viral epitope" ("viRNA hot spot"), an epitope had to contain at least one read with 0–1 mismatches, and reads containing 2 mismatches were only counted when they corresponded to an epitope without mismatches. Sixty-five percent of all reads contained 0 mismatches, 24% contained 1 mismatch, and 10% contained 2 mismatches.

We furthermore note that in addition to the perfectly matching inherited viRNAs, we detected other unique viRNAs with mismatches in accordance to the template-derived *FR1gfp* RNA (these sequences were not present in the negative control). The ability to evade treatment by many drugs is granted to RNA viruses by poor proofreading transcription machinery (error every $\sim 10^4$ bp) (Lauring and Andino, 2010). Such viruses exist as swarms of similar variants that are continuously regenerated by their own polymerases, which amplify mutated versions of related sequences (Lauring and Andino, 2010). Even though we cannot exclude simple sequencing errors, it is attractive to speculate that some of the mismatched viRNA reads that were identified in our sequencing data have been produced off such mutated viral copies.

ACCESSION NUMBERS

The sequence data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, http://www.ncbi.nlm.nih.gov/geo, with accession number GSE33334.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at doi:10.1016/j.cell.2011.10.042.

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