The EMBO Journal (2012) 31, 3422–3430 | © 2012 European Molecular Biology Organization | All Rights Reserved 0261-4189/12
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THE EMBO JOURNAL

Extremely stable Piwi-induced gene silencing in *Caenorhabditis elegans*

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In recent years, the Piwi pathway has been shown to regulate the silencing of mobile genetic elements. However, we know little about how Piwi pathways impose silencing and even less about *trans*-generational stability of Piwi-induced silencing. We demonstrate that the *Caenorhabditis elegans* Piwi protein PRG-1 can initiate an extremely stable form of gene silencing on a transgenic, single-copy target. This type of silencing is faithfully maintained over tens of generations in the absence of a functional Piwi pathway. Interestingly, RNAi can also trigger permanent gene silencing of a single-copy transgene and the phenomenon will be collectively referred to as RNA-induced epigenetic silencing (RNAe). RNAe can act *in trans* and is dependent on endogenous RNAi factors. The involvement of factors known to act in nuclear RNAi and the fact that RNAe is accompanied by repressive chromatin marks indicate that RNAe includes a transcriptional silencing component. Our results demonstrate that, at least in *C. elegans*, the Piwi pathway can impose a state of gene silencing that borders on 'permanently silent'. Such a property may be more widely conserved among Piwi pathways in different animals.

The EMBO Journal (2012) **31,** 3422–3430. doi:10.1038/emboj.2012.213; Published online 31 July 2012
Subject Categories: RNA
Keywords: chromatin; elegans; piRNA; Piwi; RNAi

Introduction

Organisms need to protect themselves from the invasion of exogenous nucleic acids such as transposons and retroviruses. A widely conserved defence mechanism is based on small RNA molecules that recognize the invading species and trigger a silencing response on that sequence (Malone and Hannon, 2009). Multiple small RNA-based mechanisms have been identified, each capable of recognizing and silencing potentially harmful sequences through unique mechanisms (Ghildiyal and Zamore, 2009). One of these mechanisms is known as the Piwi pathway, named after the Argonaute-family protein Piwi that was first identified in *Drosophila* (Cox *et al*, 1998). Piwi proteins directly bind small RNA cofactors that are better known as piRNAs (Aravin *et al*, 2006; Girard *et al*, 2006; Watanabe *et al*, 2006). Piwi–piRNA complexes have been studied in diverse organisms and in each case a role in the silencing of transposable elements could be demonstrated (Saito and Siomi, 2010; Ketting, 2011).

The *Caenorhabditis elegans* Piwi proteins are named PRG-1 and PRG-2, where PRG-1 is responsible for most, if not all, silencing activities (Batista *et al*, 2008; Das *et al*, 2008). Initially, only a few *C. elegans* piRNAs (also known as 21U RNAs) have been described to target a transposable element although many different piRNAs are produced and many transposons escape 21U-mediated recognition (Batista *et al*, 2008; Das *et al*, 2008). However, more recent studies show that imperfect base-pairing between *C. elegans* piRNAs (also known as 21U RNAs) and target RNAs actually impose gene-regulatory effects on many more targets (Bagijn *et al*, 2012; Lee *et al*, 2012). Bagijn *et al* (2012) also revealed that PRG-1–21U complexes trigger a downstream endogenous RNAi pathway that is mediated by the Argonaute protein WAGO-9 and other endogenous RNAi factors such as MUT-7 and RDE-3. This response is accompanied by the production of a class of small RNA molecules known as 22G RNAs. Interestingly, this is quite similar to how other *C. elegans* Argonaute proteins like ERGO-1, ALG-3/4 and RDE-1 trigger secondary RNAi pathways (Yigit *et al*, 2006; Gent *et al*, 2009, 2010; Conine *et al*, 2010; Vasale *et al*, 2010).

A major question for Piwi pathway biology in general remains how Piwi-targets are silenced. In mice, a link has been suggested between Piwi-pathway activity and DNA methylation (Aravin and Bourc'his, 2008; Aravin *et al*, 2008; Kuramochi-Miyagawa *et al*, 2008), suggesting that in mice the Piwi pathway is set-up to trigger the establishment of a heritable state of gene silencing. However, a causative link between Piwi-pathway activity and DNA methylation has been hard to demonstrate. Besides mammals, in flies it has also been suggested that piRNAs may trigger chromatin-related effects on their targets (Klenov *et al*, 2007; Yin and Lin, 2007; Riddle and Elgin, 2008; Wang and Elgin, 2011). Still, the interactions between the Piwi-pathway and chromatin are unclear at best, especially when target silencing is considered. We now show that the *C. elegans* Piwi (PRG-1) pathway can trigger the establishment of an extremely stable state of gene silencing. Once established, the silencing becomes independent of the initiating Piwi pathway itself. The maintenance of silencing requires previously characterized nuclear RNAi pathway components (Guang *et al*, 2008, 2010; Burkhart *et al*, 2011; Burton *et al*, 2011) and the Argonaute protein WAGO-9. Furthermore, the chromatin at the silenced locus is characterized by repressive histone marks. The silenced state is much more stable than previously reported heritable RNAi (Grishok *et al*,

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Received: 21 June 2012; accepted: 12 July 2012; published online: 31 July 2012

2000; Vastenhouw *et al*, 2006; Alcazar *et al*, 2008; Burton *et al*, 2011), as it is inherited faithfully to 100% of the progeny and can last for tens of generations. Reactivation of the silenced state reveals a striking maternal effect, suggesting that during germline transmission, the silenced state is re-established through nuclear RNAi. In summary, we describe a tractable system that sheds new light on how Piwi proteins can trigger heritable gene silencing effects.

Results

Piwi-induced stable gene silencing

Recent work has described a transgenic system in which a specific 21U RNA, named 21UR1, targets the silencing of a single-copy GFP–HIS-58 expressing transgene (Bagijn *et al*, 2012). This transgene will be referred to as '21Usensor' throughout this manuscript. Subsequently, we described (Kamminga *et al*, 2012) that mutations in HENN-1, an enzyme involved in the methylation of 21U RNAs, can re-activate this sensor, despite the fact that 21U RNA levels remain virtually unchanged. Interestingly, significant 21U RNA-mediated repression is still present in *henn-1* mutants, as the observed GFP levels in a *henn-1* mutant background are far below those in a *prg-1* mutant background. Upon culturing of these *henn-1* mutant animals, we noticed that the 21U sensor transgene had a strong tendency to become silenced, a phenomenon that we will refer to as RNA-induced epigenetic silencing (RNAe) throughout this manuscript (Figure 1A). Active transgenes will be marked with '+'. We determined the dynamics of RNAe in a *henn-1* mutant background by scoring the number of animals that lost GFP expression in *henn-1* mutant animals over the course of five generations. This revealed that RNAe is initiated at every generation in a significant number of the progeny (Figure 1B). In all cases tested, when an animal became silenced, 100% of its brood was silenced and remained silent for many generations. We checked whether the 21U sensor has an inherent tendency to become silenced, independent of PRG-1. However, we have never observed spontaneous silencing of an active *21Usensor(+)* in a *prg-1* mutant background, nor did we observe spontaneous silencing of a similar reporter transgene that lacks a 21U RNA-binding site (*non21Usensor*; not shown). Thus, in a *henn-1* mutant background, PRG-1 can initiate an extremely stable form of gene silencing. RNAe can also be initiated in *henn-1* wild-type animals, as we can identify animals carrying the 21U sensor in a *henn-1* wild-type background that fail to activate the 21U sensor transgene upon RNAi against *prg-1* (Figure 1C). Interestingly, we have observed that dsRNA-induced silencing of a *21Usensor(+)* transgene can also initiate a form of silencing that resembles RNAe (not shown). We have not observed any spontaneous reactivation of either Piwi-induced or RNAi-induced silenced *21Usensor(RNAe)* transgenes during day-to-day culturing and manipulation for a period of more than 6 months. We conclude that RNAe is much more stable than any previously described RNAi-related heritable silencing effects in *C. elegans* (Grishok *et al*, 2000; Vastenhouw *et al*, 2006; Alcazar *et al*, 2008; Burton *et al*, 2011), although we note that antiviral responses in *C. elegans* have been reported to be similarly stable (Rechavi *et al*, 2011). In all further studies described in this manuscript we made use of PRG-1-initiated RNAe.

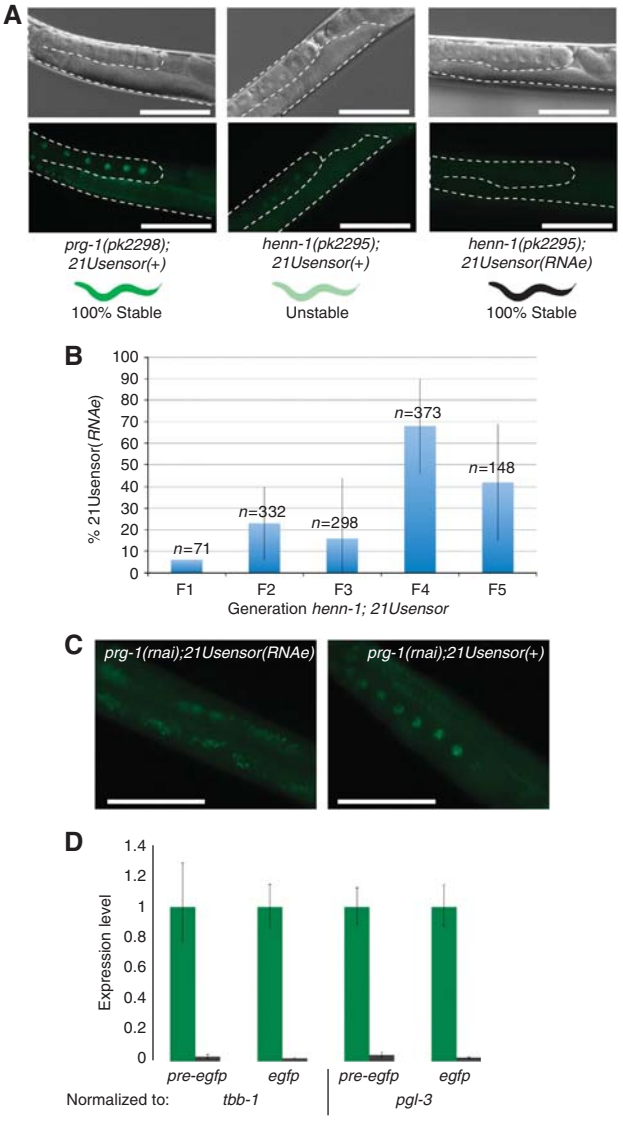

Figure 1 Piwi-initiated silencing of a single-copy transgene. (**A**) Images of animals with the indicated genotypes. The gonads are outlined with a dashed line. GFP expression from the *21Usensor* is nuclear and chromosome bound due to the HIS-58 fusion. '100% stable' indicates no observed spontaneous changes in GFP expression during months of culturing. Scale bars are 100 μm. (**B**) Quantification of spontaneous *21Usensor* silencing in a *henn-1* mutant background. *Y*-axis shows percentage of silenced animals in the broods of up to 10 individuals. *X*-axis displays the generation following the isolation of a single GFP-positive *henn-1;21Usensor* founder animal. Error bars reflect standard deviation of between 5 and 10 replicates. The F1 was scored on only one plate. (**C**) Animals wild-type for *henn-1* but carrying the *21Usensor* were treated with RNAi against *prg-1*. Ten activated the sensor (right panel), 26 did not (left panel). Further culturing showed that the transgene is indeed stably silenced in animals that failed to activate (not shown). Note that these numbers not necessarily reflect the initiation rate of silencing (RNAe) but rather may reflect the fraction of RNAe animals present in the strain at the time of the experiment. Scale bars are 100 μm. (**D**) q-RT–PCR on mature mRNA and pre-mRNA isolated from *21Usensor(RNAe)* (black bars) and *21Usensor(+)* (green bars; set to 1) animals, both in a *prg-1* mutant background. The qPCR was normalized to the two different reference genes indicated. Error bars reflect standard error based on three replicates.

We quantified the silencing effect imposed by RNAe through q-RT–PCR on both mature and primary transcripts. This revealed that both mRNA and pre-mRNA levels are

strongly reduced in backgrounds showing RNAe (Figure 1D). These data indicate that RNAe may act at both the transcriptional and post-transcriptional levels.

Genetics of RNAe

Next we looked into the genetics of the RNAe silenced transgene. First, we determined whether the silenced state was dependent on the presence of PRG-1. Interestingly, while PRG-1 is absolutely required to initiate RNAe, it is dispensable during maintenance of RNAe (Supplementary Figure S1; Table I). This reveals that PRG-1 purely acts as an initiator of RNAe. Furthermore, established RNAe silencing is inherited via both the male and the female germline, since animals carrying just one *21Usensor(RNAe)* transgene copy do not express GFP, independent of whether the transgene comes from the father or the mother (not shown).

To probe if RNAe can act *in trans*, we performed crosses in which an active, germline-expressed transgene lacking 21U RNA-binding sites (*non21Usensor(+)*) was put into animals carrying *21Usensor(RNAe)* transgenes. The *non21Usensor* transgene is integrated at the same site in the *C. elegans* genome as the *21Usensor* transgene. First, we crossed *prg-1* mutant males carrying a *21Usensor(RNAe)* transgene with hermaphrodites containing the *non21Usensor(+)* transgene and analysed F2 offspring carrying *non21Usensor* transgenes only. These experiments revealed that, although transmission is not complete, the RNAe-state of the *21Usensor* can be imposed *in trans* (Figure 2A). The original *21Usensor (RNAe)* transgene remained silent during this experiment (not shown). We note that for some unexplained reason, PRG-1 seems to be able to counteract RNAe spreading *in trans*, since we consistently observe that in *prg-1* mutant backgrounds significantly more animals harbour *non21Usensor (RNAe)* transgenes (Figure 2A). This may indicate that PRG-1 could be involved in setting up anti-silencing responses as well. Nevertheless, this cross illustrates that RNAe can act *in trans*.

To separate the *in trans* effect from potential *de novo* RNAe initiation events in the F1 we repeated the cross, but now in a completely *prg-1* mutant background, analysing GFP expression in the F1 (Figure 2B). This confirmed that an RNAe silenced transgene can act *in trans*, but only via the female germline. A *21Usensor(RNAe)* transgene brought in via the sperm is not able to efficiently induce silencing upon a *non21Usensor(+)* transgene (Figure 2B). This illustrates that the female germline carries a dominant factor that sets the expression status of the transgene. Such a factor may involve a diffusible agent and/or direct allelic interactions. To examine this further, we performed an experiment in which we introduced a *non21Usensor(+)* transgene into an oocyte that has just lost a *21Usensor(RNAe)* transgene. In this scenario, no allelic interactions between the two transgenes are possible. Still, such oocytes are capable to impose silencing upon the active transgene brought in via the sperm (Figure 2C). Again, sperm does not contain such a dominant activity (Figure 2C). These data indicate that the *in trans* activity of RNAe includes a diffusible, cytoplasmic agent in the female germline.

Table I Genetic requirements for RNAe

Genotype	Germline GFP
prg-1(pk2298)	−
mut-7(pk204)	++[a]
mut-7(pk204); prg-1(pk2298)	++[a]
wago-9(tm1200)	+/−[a]
wago-9(tm1200); prg-1(pk2298)	+[a]
wago-10(tm1332)	−
wago-11(tm1127)	−
C04F12.1(tm1637)	−
wago-10(tm1332); wago-11 (tm1127)	−
wago-11(tm1127); C04F12.1(tm1637)	−
nrde-1(gg088)	+[b]
nrde-1(gg088); prg-1(pk2298)	++[b]
nrde-2(gg091); prg-1(pk2298)	++[a]
nrde-3(gg066)	−
smg-2(e2008)	−
smg-5(r860)	−

[a]First generation homozygous mutant animals did not show GFP expression.
[b]First generation homozygous mutant animals was not analysed.

RNAe-associated small RNAs

Next, we tried to detect small RNAs derived from the *(non)21Usensor(RNAe)* transgenes in different genetic backgrounds. As published (Bagijn *et al*, 2012), we could easily detect 22G RNAs upon 21U RNA-induced silencing of the *21Usensor(+)* transgene (Figure 3A). Similar small RNAs were also observed, although at reduced levels, in strains carrying transgenes silenced through RNAe and placed in a *prg-1* mutant background. Since 22G RNAs that are directly triggered through PRG-1 are absent in these backgrounds, these results demonstrate that a 22G-like population can be maintained independently of Piwi-pathway activity. To analyse these small RNAs in further detail we cloned small RNAs from strains carrying a *21Usensor(RNAe)* or *21Usensor(+)* transgene. This confirmed that GFP-derived 22G-like RNAs are present specifically in the RNAe displaying background (Figure 3B; Supplementary Tables S1 and S2). Like previously reported 22G-RNAs, the majority of GFP-matching small RNA reads have a G-nucleotide at their 5′ end (Figure 3C) and show a size preference of 22 nucleotides (Figure 3D). We note that the cloned 22G-like RNAs are located quite far upstream from the 21UR1 recognition site that initiates the silencing, and seem to be further upstream compared with the 22G RNA sequences that were described by Bagijn *et al* (2012). This may reflect spreading of 22Gs to more distant, upstream regions during establishment or maintenance of RNAe, compared with 22G RNA production that is triggered directly by PRG-1.

Genetic requirements of RNAe

We proceeded to identify genes that are required for RNAe. First, we checked the involvement of MUT-7, a well-known endogenous RNAi factor that recently has been shown to be required for PRG-1-mediated silencing (Bagijn *et al*, 2012). We found that RNAe also depends on MUT-7 (Table I; Supplementary Figure S2). Interestingly, *mut-7* behaves as a maternal effect gene in these crosses: loss of MUT-7 only reactivates the sensor in the second generation of homozygosity. This result indicates that RNAe may be established in the germline of the parents. It also indicates that MUT-7 is not required for the maintenance of established RNAe within an individual. Two other genes previously implicated in the biogenesis of some 22G RNAs are *smg-2* and *smg-5* (Gu *et al*, 2009). Mutation of neither of these genes restored

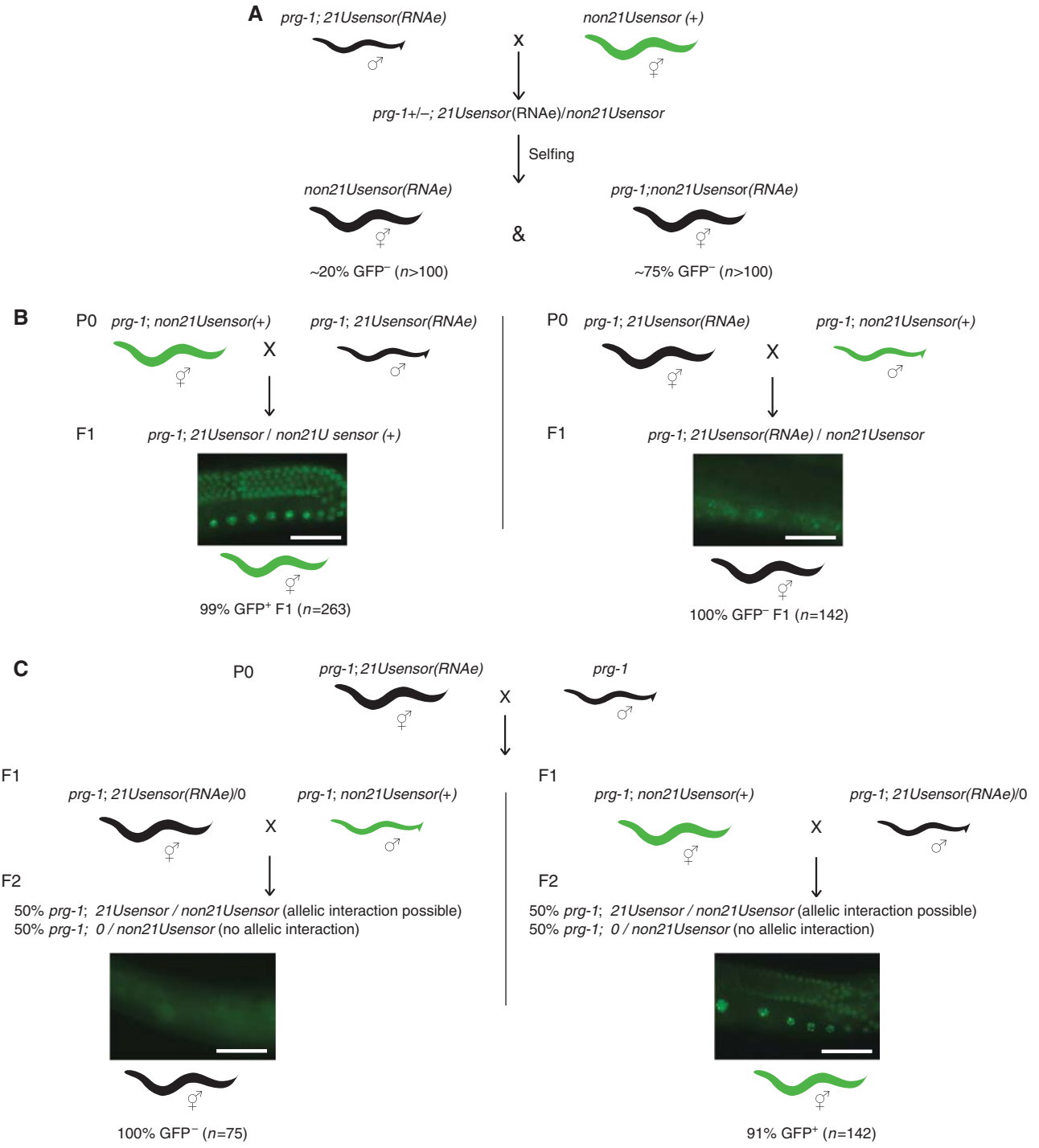

Figure 2 Genetics of RNAe. (**A**) Crossing scheme illustrating the *trans*-acting capabilities of an RNAe silenced transgene. (**B**) Crossing scheme illustrating *in trans* silencing activities of a *21Usensor(RNAe)* transgene in the female, but not in the male germline. Scale bars are 100 μm. (**C**) Crossing scheme displaying *in trans* silencing activity in oocytes and sperm that have lost the *21Usensor(RNAe)* transgene. In the F2 two possible genotypes can be found, but a (nearly) homogeneous GFP expression status for was observed. Scale bars are 100 μm.

GFP expression of the RNAe silenced transgene (Table I). The RNA-dependent RNA polymerase producing the RNAe-related 22G RNAs still has to be identified.

Next we set out to identify Argonaute proteins involved in RNAe. We first tested *wago-9*, also known as *hrde-1* (Buckley *et al*, 2012), since it was recently shown to function in the Piwi pathway (Bagijn *et al*, 2012). Disruption of WAGO-9 function reactivated the RNAe-silenced transgene, again only in animals coming from a *wago-9* homozygous mutant hermaphrodite (Table I; Supplementary Figure S2). We note, however, that the GFP levels in *wago-9* animals are notably lower than those observed in other mutant backgrounds, including *mut-7*, suggesting redundancy. Based on protein sequence, WAGO-9 is related WAGO-10, WAGO-11 and NRDE-3. We crossed mutant alleles of *wago-10*, *wago-11* and *nrde-3* into RNAe backgrounds but detected no reactivation of

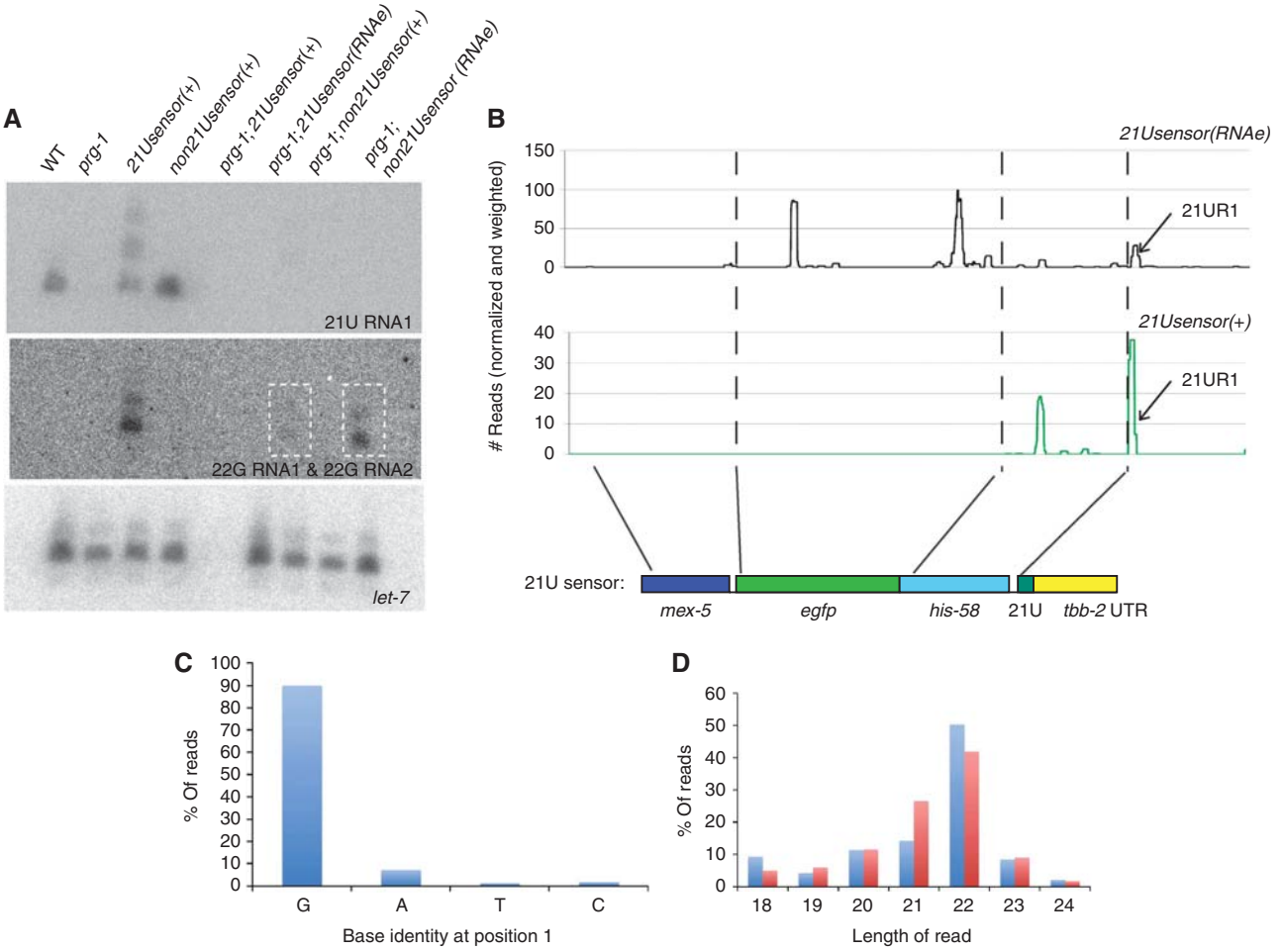

Figure 3 RNAe-associated small RNAs. (**A**) Northern blot, probed for 21UR1 (top panel), 21Usensor-derived 22G RNA (middle panel; probe just upstream of the 21UR1 recognition site), and *let-7* (bottom panel). WT: wild-type animals. (**B**) Cloning frequencies (normalized to miRNAs and weighted to correct for non-unique reads) of small RNA reads from the two indicated genetic backgrounds (both wild-type for *prg-1*). Note that only *egfp* is specific for the transgene. The arrow indicates 21UR1 sequences for which the transgene has an engineered recognition site (dark green box). (**C**) Frequencies of base identities found at the 5′ end of cloned small RNAs that are anti-sense to *egfp*. Virtually no sense-RNA sequences matching to *egfp* were recovered. (**D**) Length distribution of small RNAs anti-sense to *egfp* (blue) and of small RNAs anti-sense to endogenous genes (red).

GFP expression (Table I; Supplementary Figure S2). Thus, the activity mediating the remaining silencing activity in *wago-9* mutants remains to be identified.

Finally, we tested whether *nrde-1* and *nrde-2* are required for maintenance of RNAe. NRDE-1 and NRDE-2 are factors known to interact with nascent RNA and the nuclear Argonaute protein NRDE-3 and play a role in the establishment of repressive chromatin and the inhibition of transcription elongation at target loci following RNAi (Guang *et al*, 2008, 2010; Burkhart *et al*, 2011; Gu *et al*, 2012). Interestingly, disruption of both *nrde-1* and *nrde-2* results in restoration of GFP expression (Table I; Supplementary Figure S2), suggesting that RNAe at least partially acts at the chromatin level.

Interestingly, both *nrde-1* and *wago-9* mutants display enhanced de-silencing activity in *prg-1* mutant backgrounds compared with *prg-1* wild-type backgrounds, while the *prg-1* mutation alone does not reactivate the RNAe sensor at all (Table I; Supplementary Figure S2). Thus, upon disruption of these two genes, PRG-1 re-engages in silencing of the 21U sensor, independent of WAGO-9 and NRDE-1, again suggesting redundancy. This effect is not observed in *mut-7* mutant animals (Table I; Supplementary Figure S2), suggesting that MUT-7 acts at an early step during PRG-1-mediated silencing, while WAGO-9 and NRDE-1 act downstream of a branching point in the pathway.

RNAe induces repressive chromatin

To follow-up on the suggestion that RNAe induces changes at the chromatin level, we compared the chromatin states of the *21Usensor(+)* and *21Usensor(RNAe)* transgenes using ChIP-qPCR. In order to focus our experiments on the maintenance phase of RNAe, we performed these experiments in a *prg-1* mutant background. To control for our ChIP efficiency, we first checked whether transposon Tc1 was enriched in our ChIP-qPCR experiments. This was indeed the case (Supplementary Figure S3). We then probed three distinct regions throughout the transgene. Consistent with the above results, we find H3K9 tri-methylation to be significantly enriched in all tested regions of the *21Usensor(RNAe)* transgene (Figure 4A), while the *21Usensor(+)* transgene did not show this enrichment. Among the tested regions is one upstream promoter fragment, indicating that repressive chromatin formation is not restricted to transcribed areas. H3K9

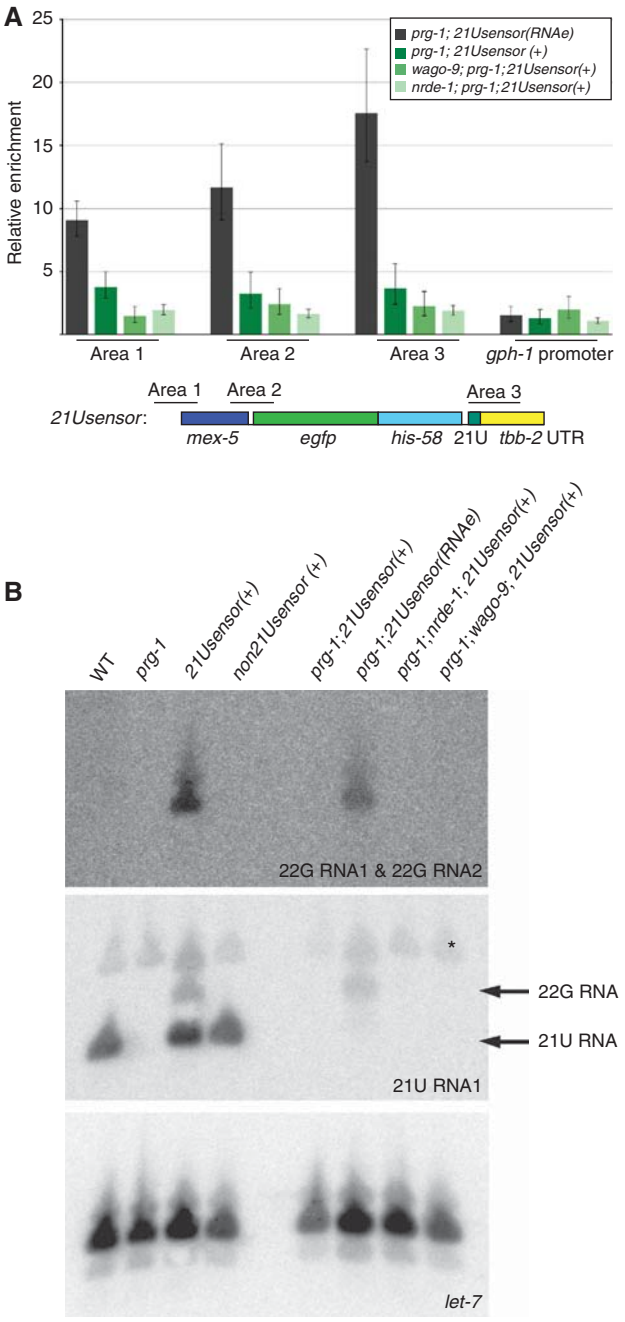

Figure 4 RNAe affects chromatin. (**A**) ChIP-qPCR on animals carrying *21Usensor(+)*, *21Usensor(RNAe)* and *21Usensor(RNAe)* transgenes reactivated by *nrde-1* and *wago-9* mutations. All strains also carried the *prg-1(pk2298)* allele. H3K9 tri-methylation was probed in the indicated regions of the transgene. ChIP-qPCRs were normalized against the *gph-1* promoter. Error bars reflect standard error, based on three replicates. (**B**) Northern blot for 22G and 21U RNAs in genetic backgrounds in which the *21Usensor(RNAe)* transgene has been reactivated. As loading control *let-7* is shown. *: non-specific signal. WT: wild-type. Note that the 21UR1 probe also visualizes 22G RNAs.

tri-methylation was lost upon disruption of *nrde-1* or *wago-9* (Figure 4A), just like RNAe-associated 22G RNAs (Figure 4B).

Discussion

In conclusion, we describe a phenomenon, RNAe, in which the *C. elegans* Piwi pathway can initiate a state of gene silencing that is extremely stable across generations. Similar findings were recently reported by others as well (Ashe *et al*, 2012; Lee *et al*, 2012; Shirayama *et al*, 2012). Although the mechanism behind RNAe has to be further elucidated, we present a model for RNAe based on the data presented in this manuscript (Figure 4B). RNAe taps into a nuclear RNAi pathway that has been suggested to interfere with transcriptional elongation (Guang *et al*, 2010). The transitivity of the silencing effect suggests that the involved factors are not stably bound to chromatin, but can diffuse through the nucleus and/or the cytoplasm. RNAe can be clearly separated into two phases: initiation and maintenance (Figure 5). Initiation can be through different input signals, as both PRG-1 and double stranded RNA can trigger RNAe. This initiation phase is accompanied by the production of 22G RNAs. In case of PRG-1 triggered silencing, these initiating 22G RNAs depend on the presence of PRG-1 and the response appears to be a relatively local event as these 22G RNAs are found close to the 21U recognition site on the targeted mRNA (Bagijn *et al*, 2012).

Maintenance of RNAe is also accompanied by 22G RNA biogenesis. Interestingly, these maintenance-related 22G RNAs are distinct from the initiating 22G RNAs, as they are independent of PRG-1 and appear more upstream on the targeted mRNA. Like all 22G RNA studied so far, these small RNAs are most likely made by one of the RNA-dependent RNA polymerases RRF-1 and/or EGO-1. They are dependent on MUT-7 and WAGO-9, the latter being a likely acceptor for these 22G RNAs. Furthermore, the maintenance associated 22G RNAs are lost upon disruption of *nrde-1*, consistent with the previously proposed role for *nrde-1* in inheritance of 22G RNAs following RNAi (Burton *et al*, 2011). It will be interesting to analyse the requirements and the dynamics of these two phases of 22G RNA production in further detail.

Given our finding that RNAe can be maintained within one generation in the absence of MUT-7 or WAGO-9, maintenance of RNAe seems to involve two distinct steps: a MUT-7/WAGO-9-dependent and a MUT-7/WAGO-9-independent step. The MUT-7/WAGO-9-independent phase may represent the heterochromatic nature of the silenced locus. The observed H3K9 tri-methylation of the transgene under RNAe conditions may keep it silent independent of ongoing RNAi. Following the parallels from the RNAi-chromatin pathway in *Schizosaccharomyces pombe*, the MUT-7/WAGO-9-dependent phase may reflect a requirement to re-initiate heterochromatin formation at each germline transmission by a nuclear RNAi pathway. Consistent with this notion, H3K9 tri-methylation and 22G RNAs are lost from RNAe silenced transgenes upon disruption of *wago-9* or *nrde-1*. These data would suggest that WAGO-9, like its sequence-related paralog NRDE-3, is a nuclear Argonaute. Indeed, recently published papers confirm this idea (Ashe *et al*, 2012; Buckley *et al*, 2012; Shirayama *et al*, 2012). Since MUT-7 has also been described to be in the nucleus (Tops *et al*, 2005), we have to consider the possibility that RNAe-related 22G RNA biogenesis may be (partly) nuclear.

The just described mechanisms are characterized by redundancies that are revealed by differences in expression levels upon reactivation of an RNAe affected transgene through different mutations. One source of redundancy may be found in different types of RNAi-like pathways acting in parallel; for example, nuclear and cytoplasmic pathways.

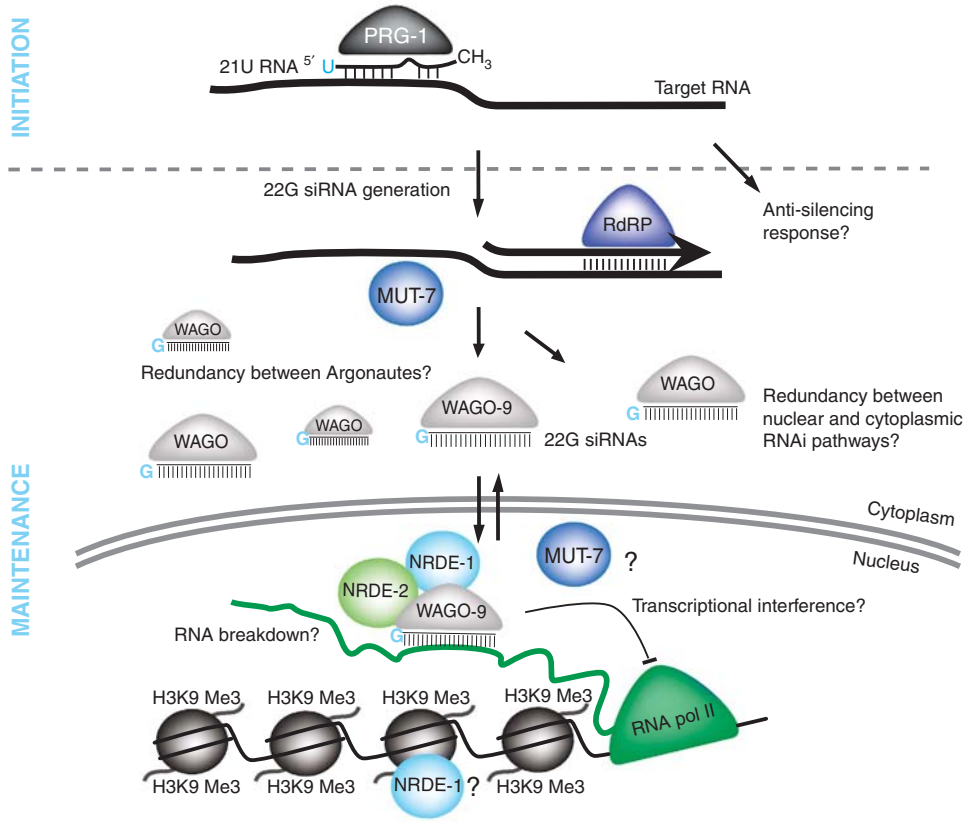

Figure 5 Model for RNAe in *C. elegans*. PRG-1, but also long dsRNA, can induce a form a stably inherited gene silencing, named RNAe. The maintenance of RNAe across generations depends on the exo-ribonuclease MUT-7, on the previously identified nuclear RNAi factors NRDE-1 and NRDE-2 and on the Argonaute WAGO-9. In analogy with published work on NRDE-1/NRDE-2 and the nuclear Argonaute NRDE-3, we hypothesize that NRDE-2 and NRDE-1 bind to WAGO-9, while NRDE-1 may be bound to RNAe chromatin directly as well. The silencing pathway branches downstream of MUT-7, but upstream of WAGO-9. In addition to silencing, our results suggest that PRG-1 may also act to prevent silencing. See main text for further discussion.

A second source of redundancy may be found between Argonaute proteins acting redundantly within similar, or identical pathways. Further experiments are required to resolve these issues.

Our findings indicate that PRG-1 may also be involved in generating an anti-silencing response, since the *in trans* effect of RNAe seems to be more effective in *prg-1* mutants. Furthermore, we detected a dominant activity in the female germline that is capable of preventing RNAe establishment on an active transgene. In relation to these findings, it is interesting to note that we find 22G RNA derived from the endogenous part of the 21U sensor transgene (*his-58*), and that these apparently do not trigger RNAe. Moreover, these *his-58* 22G RNAs are reduced when RNAe has been triggered, while the GFP 22G RNAs increase in abundance. We speculate that the *his-58* derived small RNA may reflect an anti-silencing pathway that is also guided by small RNA molecules, potentially triggered by PRG-1 as well. Such a model, in which repressing and activating activities compete has recently also been suggested by others (Ashe *et al*, 2012; Shirayama *et al*, 2012).

Finally, we note that RNAe is reminiscent of stable gene silencing effects known as paramutation (Erhard and Hollick, 2011; Pilu, 2011). These effects were originally described in maize but were later detected in mammals as well. It will be interesting to further decipher RNAe and learn what is responsible for the extreme stability across generations and whether other Piwi pathways can initiate paramutation-like effects as well. In this light, the recently reported piRNA-mediated *trans*-generational effects in *Drosophila* (Grentzinger *et al*, 2012) may be of particular interest.

Materials and methods

Worm culture

C. elegans was grown on OP50 bacteria according to standard laboratory conditions. The alleles used in this study are the following: *prg-1(pk2298)*, *henn-1(pk2295)*, *wago-9(tm1200)*, *wago-10(tm1332)*, *wago-11(tm1127)*, *C04F12.1(tm1637)*, *nrde-1(gg088)*, *nrde-2(gg091)*, *nrde-3(gg066)*, *mut-7(pk204)*, *smg-2(e2008)*, *smg-5(r860)*.

RNAi

RNAi was performed as described (Kamath *et al*, 2003), using bacterial strains expressing dsRNA for the indicated target genes.

Transgenics

The transgene alleles were previously described in Bagijn *et al* (2012). 21Usensor: mjIS144. non21Uensor: mjIS145.

Microscopy

GFP fluorescence was scored on a Leica DM6000 microscope and on a Zeiss M2Bio microscope. Images were taken on the DM6000 with fixed exposure times and illumination.

Northern blotting analysis

Total RNA was isolated using RNA lysis buffer and Trizol. Subsequently, small RNA was isolated with the Mirvana kit. Northern blotting was done as described previously (Kamminga *et al*, 2010). In all, 20 μg of small RNA was loaded on a 12% polyacrylamide gel and blotted according to standard procedures. Probe sequences:

21U-R1: GCACGGTTAACGTACGTACCA
let-7: AACTATACAACCTACTACCTCA.
22G-1: AAAGTGGTCAAGCACGGTTAAC
22G-2: AGTAAACCCAGCTTTCTTGTAC

22G-1 and 22G-2 probes were mixed before hybridization in Ambion hybridization buffer (ULTRAhyb-Oligo). Blots were exposed to phosphor-imager screens that were scanned on a BAS-2500 imager.

ChIP qPCR

ChIP was performed as described before (Mukhopadhyay *et al*, 2008). Antibodies: H3K9me3 (Abcam, Cat# ab8898). The qPCR was performed in triplicate and revealed almost identical results in two biological duplicates.

Primers:

TC1 F1	aaccgttaagcatggaggtg
TC1 R1	cacacgacgacgttgaaacc
gph-1_promoter F3	gcgcaagtttctgctgtttt
gph-1_promoter R3	cggaagattcacaagaagcaa
21Usensor mex_1 F1	gaccatgattacgccaagcta
21Usensor mex1 R1	TTTAATTCGGTGCGCCTTTA
21Usensor mex2 F1	ACTTTCCCCAAAATCCTGCT
21Usensor mex2 R1	CCTTCACCCTCTCCACTGAC
21Usensor egfp F1	GTCAGTGGAGAGGGTGAAGG
21Usensor egfp R1	TCGAGAAGCATTGAACACCA
21Usensor his58 F2	ACCGCTGTCCGTTTGATTCT
21Usensor his58 R2	GAAGAAGGGAATGCTTGAAAGG

q-RT–PCR

Primers used for qPCR:

RT_egfp_intron_F	CATATTTAAATTTTCAGGTGCTGAAGTCAAG
RT_egfp_intron_R	GTTGTGTCTAATTTTGAAGTTCTGAAAATTT AAATCAG
RT_tbb-1_F	GAGGCCAACAATGGCAAATACGTTCC
RT_tbb-1_R	CCACCTCCAAGAGAGTGTGTGAGC
RT_pgl-3_F1	CCCACTGCTCCCTCAAAGCG
RT_pgl-3_R1	CAGTCCTTGGGCGAACTTTTTGAAG
RT_egfp_F	CTACCTGTTCCATGGCCAAC
RT_egfp_R	AGTTAACTTTGATTCCATTCTT

Deep sequencing

Libraries for deep sequencing were prepared as described in and sequenced on an Illumina platform (Kamminga *et al*, 2012). RNA samples were not treated to remove 5′-tri-phosphate groups, hence 22G cloning frequencies are relatively low. Bioinformatic analysis was essentially performed as previously described (Kamminga *et al*, 2012), using custom scripts to map reads to transgenic DNA. Small RNA sequences have been deposited at GEO, accession number GSE39226.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

The research here described was funded by the following grants to RFK: an ERC Starting Grant from the Ideas Programme of the European Union Seventh Framework Programme (202819), the European Union Sixth Framework Programme Integrated Project SIROCCO (LSHG-CT-2006-037900) and two grants from NWO (ECHO 700.57.006 and Vici 724.011.001). MVA was supported by an Erasmus grant and is a student of the Masters in Evolutionary and Developmental Biology, University of Lisbon, Portugal. We thank Bruno Albuquerque for assistance in qPCR design and Josien van Wolfswinkel for discussions. Some of the strains used in this study were provided by the NRBP, Japan.

Author contributions: ML, PvB and LJTK designed, performed and analysed the experiments. MVA and EFR performed and analysed the experiments. EB performed bioinformatics analysis. RFK designed the study, analysed data and wrote the paper with input from all the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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