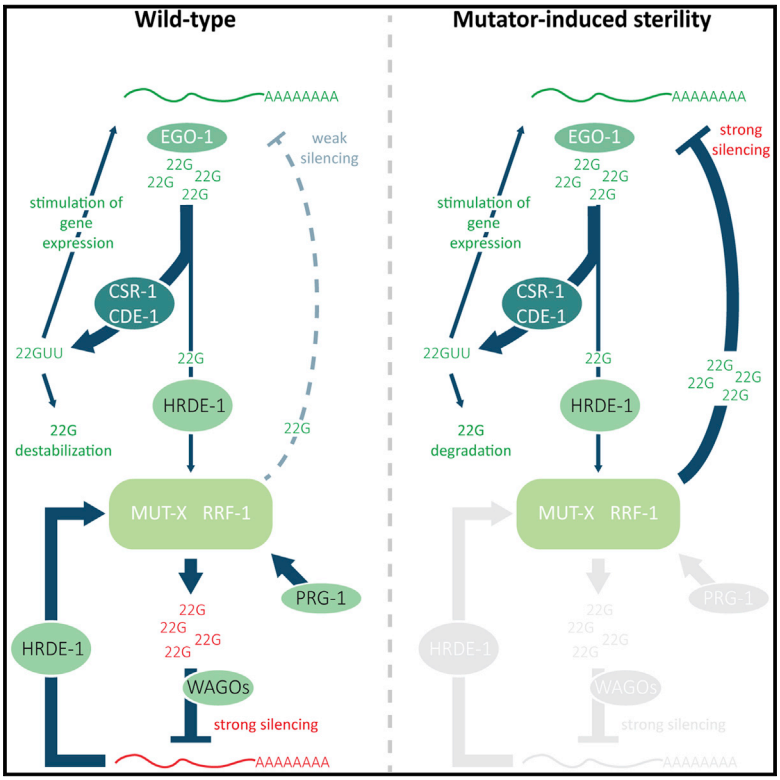
Short Article

Developmental Cell

Maternal piRNAs Are Essential for Germline Development following De Novo Establishment of Endo-siRNAs in *Caenorhabditis elegans*

Graphical Abstract



Highlights

- Maternal 21U RNAs are required to start de novo transposon silencing in *C. elegans*
- PRG-1, HRDE-1, and WAGO-1/WAGO-2/WAGO-3 coordinate silencing of Tc1 transposition
- Mutators drive silencing of germline genes in absence of parental 21U and 22G RNA
- CDE-1-mediated 22G RNA turnover prevents silencing of germline genes by HRDE-1

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In Brief

de Albuquerque et al. demonstrate that in *C. elegans*, transgenerational memory of piRNAs and endogenous siRNAs is required for proper germ cell development. Without this memory, the silencing machinery cannot properly silence transposons and, in addition, starts to act on genes that should be expressed.

de Albuquerque et al., 2015, Developmental Cell *34*, 448–456
August 24, 2015
http://dx.doi.org/10.1016/j.devcel.2015.07.010

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Maternal piRNAs Are Essential for Germline Development following De Novo Establishment of Endo-siRNAs in *Caenorhabditis elegans*

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http://dx.doi.org/10.1016/j.devcel.2015.07.010

SUMMARY

The Piwi-piRNA pathway represents a germline-specific transposon-defense system. *C. elegans* Piwi, *prg-1*, is a non-essential gene and triggers a secondary RNAi response that depends on mutator genes, endo-siRNAs (22G-RNAs), and the 22G-RNA-binding Argonaute protein HRDE-1. Interestingly, silencing of PRG-1 targets can become PRG-1 independent. This state, known as RNAe, is heritable and depends on mutator genes and HRDE-1. We studied how the transgenerational memory of RNAe and the piRNA pathway interact. We find that maternally provided PRG-1 is required for de novo establishment of 22G-RNA populations, especially those targeting transposons. Strikingly, attempts to re-establish 22G-RNAs in absence of both PRG-1 and RNAe memory result in severe germline proliferation defects. This is accompanied by a disturbed balance between gene-activating and -repressing 22G-RNA pathways. We propose a model in which CSR-1 prevents the loading of HRDE-1 and in which both PRG-1 and HRDE-1 help to keep mutator activity focused on the proper targets.

INTRODUCTION

The Piwi-piRNA pathway is an RNAi-related mechanism that is essential for germ cell development in most organisms (Ghildiyal and Zamore, 2009; Ketting, 2011; Malone and Hannon, 2009). Loss of this pathway results in strong upregulation of transposon activity, apoptosis, and blocks at various stages of meiosis. In contrast, the *C. elegans* Piwi pathway has been shown to be not acutely required in germ cells (Batista et al., 2008; Cox et al., 1998; Das et al., 2008; Wang and Reinke, 2008). Also the impact of PRG-1 on transposon silencing is very limited (Das et al., 2008). Intriguingly, *prg-1* mutant animals have a so-called mortal germline (Mrt) phenotype (Simon et al., 2014), meaning that the germline deteriorates over generations.

PRG-1 uses Piwi-interacting RNAs (piRNAs; in *C. elegans* named 21U RNAs) to identify targets, on which it triggers the production of endogenous short-interfering RNAs (endo-siRNAs; 22G RNAs) (Bagijn et al., 2012; Lee et al., 2012). This occurs in a process that depends on an RNA-dependent RNA polymerase (RdRP) and mutator proteins (Zhang et al., 2011), in so-called mutator foci (Phillips et al., 2012) that flank bigger peri-nuclear aggregates (P granules). Animals lacking mutator activity display defects in RNAi and activation of various transposable elements (Ketting et al., 1999; Tabara et al., 1999). Different Argonaute proteins, including WAGO-1 (Gu et al., 2009), PPW-1 (Simon et al., 2014), and HRDE-1 (Ashe et al., 2012; Buckley et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012), act as recipients for the 22G-RNA output of mutators. Interestingly, whereas *hrde-1* mutants also display a Mrt phenotype (Buckley et al., 2012), mutator mutants do not (Simon et al., 2014). However, mutator genes do affect germline mortality because they are required for the suppression of the Mrt phenotype of *prg-1* by *daf-2* (Simon et al., 2014).

As mentioned, PRG-1 can silence target genes through the involvement of mutator genes. Interestingly, such PRG-1-initiated silencing can become PRG-1 independent (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). This state, referred to as RNAe, can be faithfully inherited across many generations and depends on mutators, the nuclear 22G-RNA-binding Argonaute protein HRDE-1, and chromatin factors (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). In this light it is interesting to note that transposon activation is much stronger in mutator mutants than in *prg-1* mutants, as one of the most active transposons in *C. elegans*, Tc1, is still mostly inactive in *prg-1* mutants (Das et al., 2008). Possibly, transposon silencing depends for a large extent on the PRG-1-independent, but mutator-dependent RNAe-related silencing memory.

In parallel to a memory that transmits silencing, *C. elegans* gametes also transmit information on genes that are active (Conine et al., 2013; Seth et al., 2013; Wedeles et al., 2013). This requires the Argonaute proteins ALG-3, ALG-4, and CSR-1. In fact, CSR-1 can reactivate genes that have been silenced through PRG-1 and mutator activity (Seth et al., 2013). The molecular mechanisms behind this activation are currently not clear. These may involve chromatin-related effects (Claycomb et al., 2009; Wedeles et al., 2013) but could also relate to 22G-RNA turnover, since we previously described an enzyme named CDE-1 that is required for CSR-1-bound 22G-RNA turnover through non-templated uridylation of CSR-1-bound 22G RNAs (van Wolfswinkel et al., 2009). Whatever its mechanism of action, the CSR-1

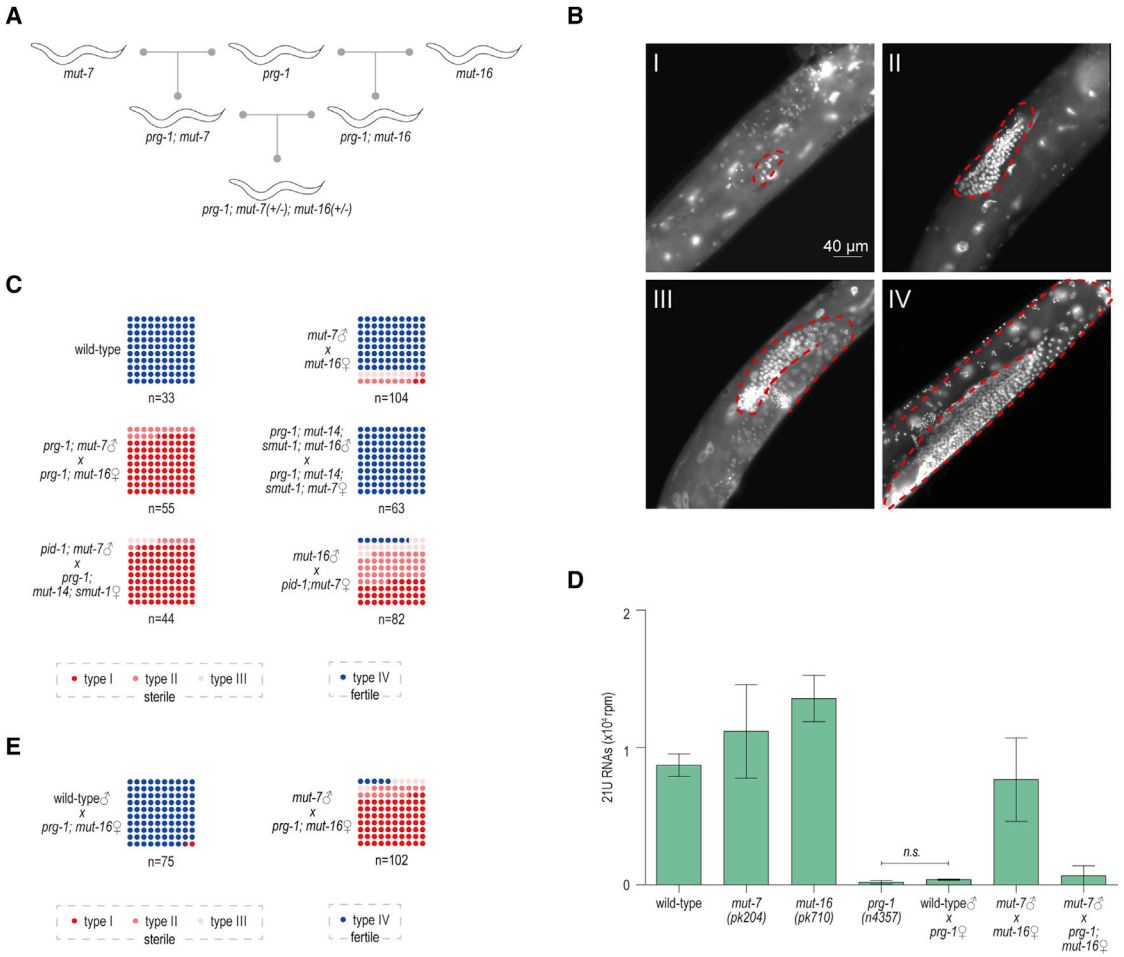

Figure 1. Mutator Genes Induce Sterility in Absence of Maternal 21U RNAs

(A) Schematic depicting the crosses performed to erase RNAe memory from *prg-1* mutant animals.

(B) DAPI staining showing the range of germline defects in worms whose mutator activity was restored in the absence of PRG-1. Gonads are outlined by the dashed line. The germlines of individual animals were classified into one of four categories: type I—none or very few germline cells; type II—some germ cells, but no apparent differentiation; type III—some germ cells with apparent differentiation; type IV—wild-type germline. Types I–III animals are sterile.

(C and E) Quantification of the observed germline defects in wild-type animals where the mutator pathway was reactivated in different genetic backgrounds. Each dot represents 1%.

(D) Levels of 21U RNA determined by small RNA-seq of total RNA from L1 larvae of the indicated genotype. Error bars represent SD between biological duplicates.

See also Figure S1.

pathway is extremely important since loss of its activity leads to embryonic lethality and sterility (Claycomb et al., 2009; Qiao et al., 1995; Rocheleau et al., 2008; Yigit et al., 2006).

We set out to test the idea that transposons are kept silenced through RNAe-related memory and that PRG-1 is required specifically for the initiation of transposon silencing and not for maintenance. To do this we first erased the mutator-mediated silencing memory from *prg-1* mutant animals, and then re-activated this memory system. Indeed, we find that transposon-targeting 22G RNAs require PRG-1 to re-establish, and we demonstrate that PRG-1 and HRDE-1 synergistically act to silence Tc1 transposition. In addition, our experiments reveal an acute requirement for PRG-1 for proper germ cell development. We propose that this defect is related to mis-targeted mutator complexes that start to act, through HRDE-1 and WAGO-1/WAGO-2/WAGO-3, on transcripts that are normally protected from silencing through CSR-1.

RESULTS

Mutator-Induced Sterility in *prg-1* Mutants

To erase RNAe memory from *prg-1* mutants, in the presence of intact mutator activity, we created two strains (*prg-1;mut-7* and *prg-1;mut-16*). Both strains lack 21U RNAs and RNAe-related memory. These lines exhibit strong transposon mobilization (see below) and are fertile. Cross offspring of these two lines will remain *prg-1* defective but will have mutator activity, allowing one to address whether *prg-1* is required to initiate transposon silencing (Figure 1A). Unexpectedly, however, the offspring of these crosses are completely sterile (Figures 1B,

1C, and S1A–S1C). We first checked the generality of this phenotype by combining *prg-1* mutation with other mutator alleles. For this we used *mut-14* and *smut-1*, two redundant RNA helicases (Phillips et al., 2014). Animals lacking both *mut-14* and *smut-1* were shown to be RNAi defective, and we show here that *mut-14;smut-1* double mutants cannot maintain RNAe (Figure S1D). When *prg-1;smut-1;mut-14* mutant hermaphrodites are crossed with *mut-7* animals that lack PRG-1 activity, again a strong sterility phenotype develops (Figure 1C). If the sterility really comes from the re-establishment of mutator activity, the above crosses should yield fertile offspring if a third mutator protein is kept inactive. Indeed, *prg-1* mutant animals in which *mut-7* and *mut-16* are complemented while *mut-14* and *smut-1* are kept homozygous mutant are fertile (Figure 1C). Consistent with the fact that PRG-1 acts through 21U RNAs, re-establishment of mutator activity in absence of PID-1, a factor required for 21U-RNA biogenesis (de Albuquerque et al., 2014), results in the same phenotype (Figure 1C).

Overall, the germ cell count in these animals is strongly reduced, and no or only limited numbers of mature germ cells are present. Both gonad arms tend to contain similar numbers of germ cells (Figure S1E). The germ cells that are still present in these animals express variable levels of the germ cell marker PGL-1 (Figure S1F) (Kawasaki et al., 1998). We did not detect expression of a somatic, neuronal gene *unc-119* in the remaining germ cells (data not shown), indicating that these germ cells are not subject to gross germ cell-to-soma transformation. Interestingly, the precise gonadal phenotype varies strongly among individuals (Figure 1B). Collectively, our data show that the *prg-1* pathway is required for normal germ cell development upon de novo establishment of mutator activity. The variable nature of the developmental defect would be consistent with rather stochastic molecular defects underlying the sterility phenotype.

Parental Effects of Mutator-Induced Sterility

PRG-1-mediated silencing has a strong maternal component (de Albuquerque et al., 2014). Consistent with this, we find that *prg-1*/+ L1-stage offspring from *prg-1* mutant hermaphrodites have as few 21U RNAs as straight *prg-1* mutant L1 larvae (Figure 1D), showing that the vast majority of 21U RNAs detected in wild-type L1 larvae are of maternal origin. We then tested whether maternal or paternal PRG-1 could rescue the sterility and found that loss of maternal PRG-1 is sufficient to trigger sterility upon re-establishment of mutator activity (Figure 2B, right panel). Loss of only maternal (Figures 1C and 1E) or paternal (data not shown) mutator activity does not result in sterility. We conclude that PRG-1 affects fertility mainly through the maternal lineage, whereas mutator activity acts both maternally as well as paternally.

PRG-1 and HRDE-1 Cooperate to Silence Tc1 Transposition

We next analyzed small RNAs isolated from animals displaying mutator-induced sterility. In order to reduce potential secondary effects stemming from the developing germ cell phenotype, we focused our sequencing efforts on L1 and L2 larvae. We used homozygous wild-type and corresponding mutant strains as controls as well as offspring from a cross between two mutator mutants that have wild-type PRG-1 activity. For all samples, two or three biological replicates were processed, and progeny from crosses were hand-picked to make sure cross-progeny were analyzed. Finally, we included random barcodes in the small RNA libraries for the identification of unique ligation events.

We first looked at the abundance of transposon-targeting 22G RNAs. As expected, these 22G RNAs are largely missing in mutator mutants, and their levels stay stable in wild-type and in *prg-1* mutants (Figure 2A). This shows that the majority of these 22G RNAs are inherited in a PRG-1-independent manner, consistent with ongoing transposon silencing in *prg-1* mutants. When mutator activity is de novo established, transposon-targeting 22G RNAs start to build up in L2 stage, in a process that depends on PRG-1 (Figures 2A and 2B). This strongly suggests that PRG-1 is required to initiate transposon silencing in *C. elegans*, whereas it is not required for the 22G-RNA-mediated memory of it.

To obtain more direct evidence of this idea, we determined the germline reversion frequency of the *unc-22::Tc1(st136)* allele. We confirmed that in *prg-1* mutants this allele reverts at a very low (Das et al., 2008) but reproducible frequency of $\sim 10^{-5}$ (Figure 2C). In contrast, we could not detect reproducible reversion events in *hrde-1* mutants. Strikingly, in *prg-1;hrde-1* double mutants we observe a 100-fold increase in Tc1 excision frequencies, comparable with those in *mut-16* mutants (Figure 2C). Loss of PRG-1 does not further increase Tc1 activity in *mut-16* mutants (Figure 2C), consistent with the idea that PRG-1-mediated silencing is fully dependent on mutator activity (Bagijn et al., 2012). Since PRG-1 seems to act primarily via secondary Argonaute proteins, and Tc1 silencing is still intact in *hrde-1* mutants, it is likely that additional Argonaute proteins participate in Tc1 silencing. Indeed, we find that *wago-1; wago-2; wago-3* triple-mutant animals display activation of Tc1 at levels similar to those observed in *prg-1;hrde-1* double mutants or *mut-16* single mutants (Figure 2C). These data suggest that both HRDE-1 and PRG-1 act through WAGO-1/WAGO-2/WAGO-3 to silence Tc1.

Inappropriate Gene Silencing in Animals with Mutator-Induced Sterility

Since transposon activation per se does not result in sterility in *C. elegans*, the question remains: What causes sterility upon reactivation of mutator activity in absence of 21U RNAs? Within the so-called WAGO-clade, CSR-1 is the only Argonaute required by itself for fertility (Yigit et al., 2006), and the RdRP enzyme that makes CSR-1-bound 22G RNAs, EGO-1, has been identified as an enhancer of a germline proliferation defect (Qiao et al., 1995). Interestingly, CSR-1 stimulates gene expression (Claycomb et al., 2009), suggesting that loss of gene activity is more detrimental to germ cells than loss of silencing. We hypothesized that mutator-induced sterility may stem from inappropriate silencing of germ-cell-expressed genes. To test this we checked whether expression of endogenous genes is affected through single-worm RT-PCR analysis of a random set of germ-cell-specific transcripts. We found that our ability to detect transcripts from these genes differs among individual sterile animals (Figure S2A), suggesting that these genes may be stochastically, inappropriately silenced. Since this assay is blind to the specific germ-cell-defect individuals and may report

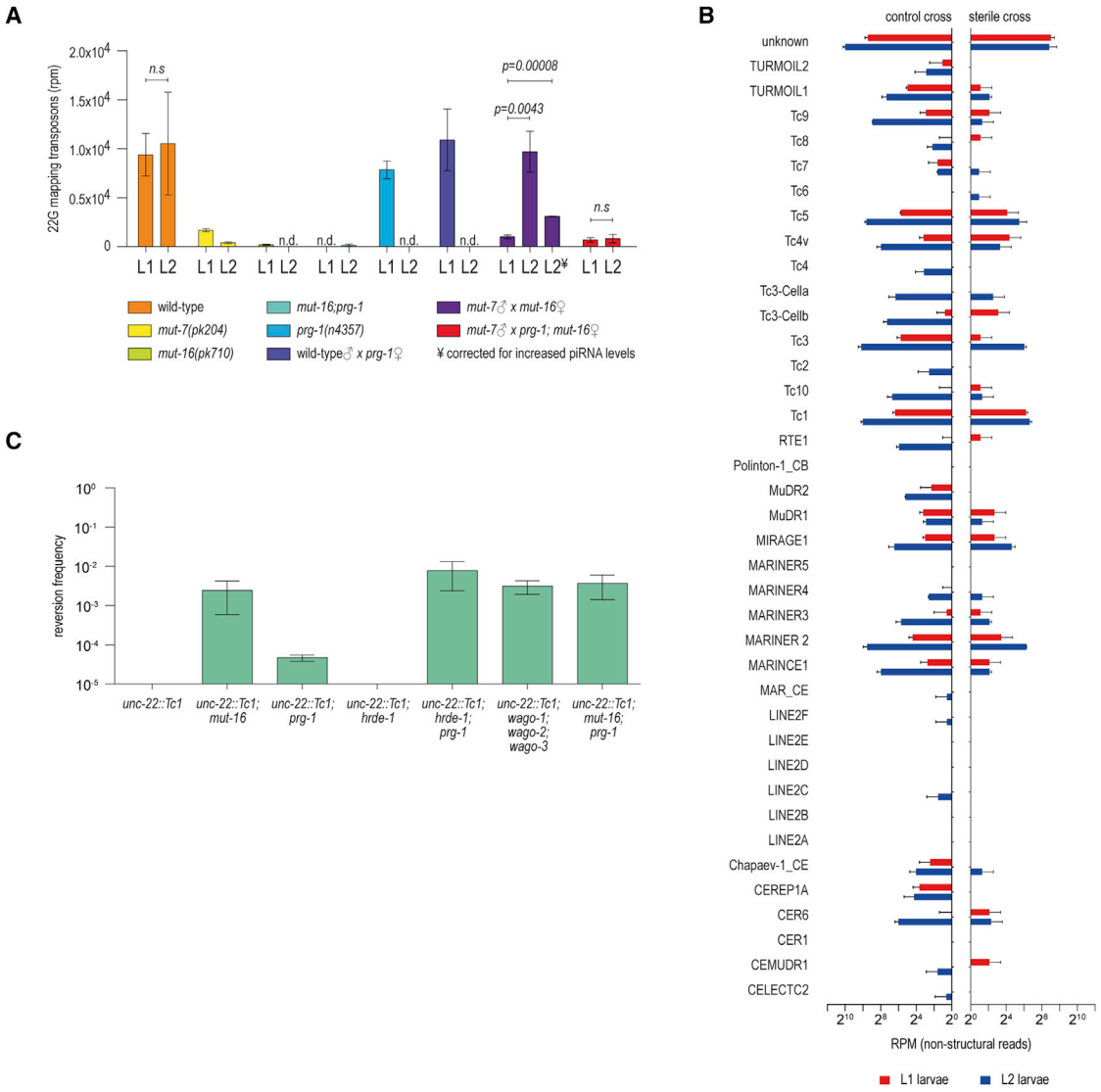

Figure 2. Maternal 21U RNAs Are Required to Re-establish Transposon Silencing

(A) Column chart showing levels of 22G small RNAs mapping anti-sense to transposons in L1 and L2 larvae of the indicated genotypes. Error bars represent SD between at least two biological duplicates. ¥: corrected for 21U RNA levels (see Experimental Procedures).

(B) Bar chart showing levels of 22G RNAs targeting various transposon families in larvae where the mutator pathway was reactivated in the presence (control cross; *mut-7*♂ × *mut-16*♀) or absence (sterile cross; *mut-7*♂ × *mut-16;prg-1*♀) of maternal 21U RNAs.

(C) Column chart depicting the reversion frequency of *unc-22::Tc1* in animals of the indicated genotype. Error bars represent SD among the values obtained from three experiments.

on secondary defects, we addressed this issue also through visual analysis of a germ-cell-specific fluorescent reporter transgene. We crossed a 21U-targeted mCherry transgene from a *mut-7* mutant male into *prg-1* or *prg-1;mut-16* double-mutant hermaphrodites. Consistent with what we published before (de Albuquerque et al., 2014), all cross offspring of the *prg-1* mutant hermaphrodites showed mCherry expression in the germline. Cross offspring of the *prg-1;mut-16* double-mutant hermaphrodites displayed stochastic silencing of mCherry, even in animals with apparently almost wild-type germline morphology (Figures 3A and S2B). We conclude that genes that are normally not targeted for silencing can be silenced by mutator activity in absence of both maternal 21U RNAs and mutator information from both parents, possibly triggering the observed sterility phenotype that develops in these animals.

CSR-1 and WAGO Pathways Recognize Mutual Targets

The above-described mutator-driven, ectopic silencing of genes suggests that Argonaute proteins that induce silencing, such as WAGO-1 and HRDE-1, have the potential to be effectively loaded with 22G RNAs derived from expressed genes. In other words, WAGO-1 and HRDE-1 should be able to accept 22G RNAs that are normally found enriched in CSR-1. To address this, we re-analyzed published HRDE-1 (Shirayama et al., 2012) and

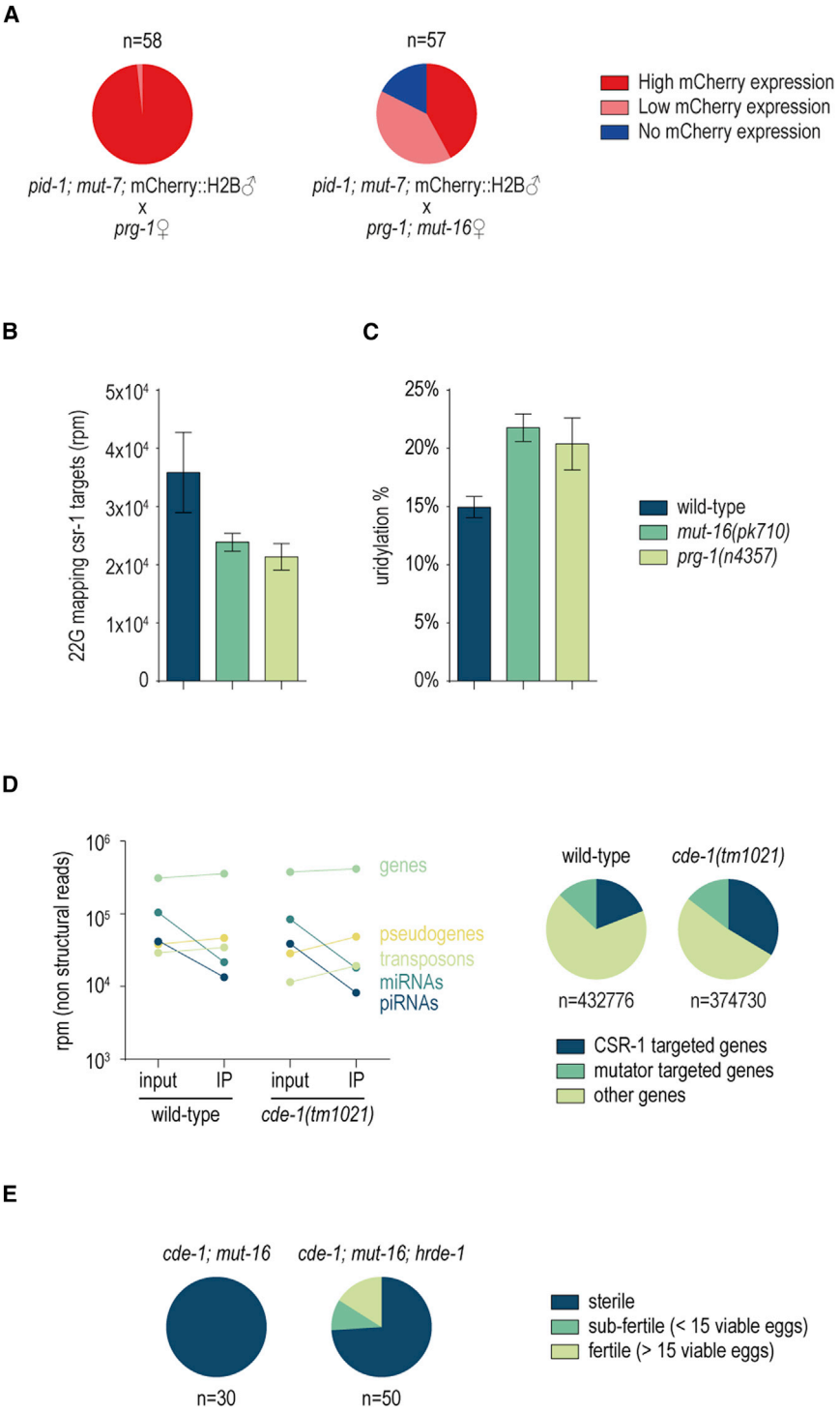

Figure 3. Loading of CSR-1-Type 22G RNAs into HRDE-1 and Inappropriate Gene Silencing in the Germline

(A) Pie chart showing the fraction of offspring that expresses a germline-specific mCherry::H2B transgene, from a control cross (left) and cross that results in sterile offspring (right).

(B) Levels of 22G RNAs mapping to protein-coding genes targeted by CSR-1 (Table S1). Error bars represent SD between at least two biological replicates.

(C) Fraction of 22G RNAs mapping to protein-coding genes targeted by CSR-1 that contain non-templated 3′ uridylation.

(D) Left panel depicts levels of 21U RNAs, miRNAs, and 22G RNAs anti-sense to genes, pseudogenes, and transposons in input and HRDE-1 immunoprecipitates from either wild-type or *cde-1(tm1021)* animals. Right panel shows the proportion of 22G RNAs mapping to genes annotated as CSR-1 targets and genes annotated as mutator targets in HRDE-1 immunoprecipitates.

(E) Pie chart depicting fertility of *cde-1;mut-16* and *cde-1;mut-16;hrde-1* mutant animals.

Also see Figures S2 and S3.

WAGO-1 (Gu et al., 2009) immunoprecipitation (IP) data and found significant amounts of 22G RNAs from typical CSR-1 target genes. To check whether these 22G RNAs represent truly WAGO-1- or HRDE-1-bound 22G RNAs, we made use of the fact that CSR-1-bound 22G RNAs show higher 3′ non-templated uridylation than 22G RNAs bound by other Argonautes (van Wolfswinkel et al., 2009). The uridylation frequencies of typical CSR-1-bound 22G RNAs from the WAGO-1 and HRDE-1 IP datasets are lower than in CSR-1 IPs (Figure S3) (Claycomb et al., 2009), suggesting that these 22G RNAs do not stem from CSR-1 contamination but reflect genuine WAGO-1- and HRDE-1-bound 22G RNAs. Consistent with this, a moderate but significant drop of 22G RNAs derived from CSR-1 target genes can be observed upon loss of either *mut-16* or *prg-1* (Figure 3B), accompanied by increased uridylation frequencies of CSR-1-pathway 22G RNAs (Figure 3C). Given that CSR-1 operates independently of mutator genes (Gu et al., 2009), these data suggest that a non-CSR-1-bound pool of 22G RNAs derived from typical CSR-1 target genes is lost upon loss of PRG-1 or MUT-16.

The reverse is also true. In previously described CSR-1-bound 22G-RNA populations (Claycomb et al., 2009), we detect significant amounts of 22G RNAs from genes that are not considered to be typical CSR-1 target genes (Figure S3). Importantly, these 22G RNAs show similar uridylation rates compared to 22G RNAs derived from genes considered to be true CSR-1 targets (Figure S3), indicating that they are indeed bound by CSR-1 and do not stem from non-CSR-1-bound 22G RNA contaminations. These findings indicate that CSR-1 and WAGO-Argonautes do not bind 22G RNAs from unique genes. Rather, a gene is characterized by a certain ratio in which its 22G RNAs are represented in CSR-1 and WAGO-Argonaute proteins.

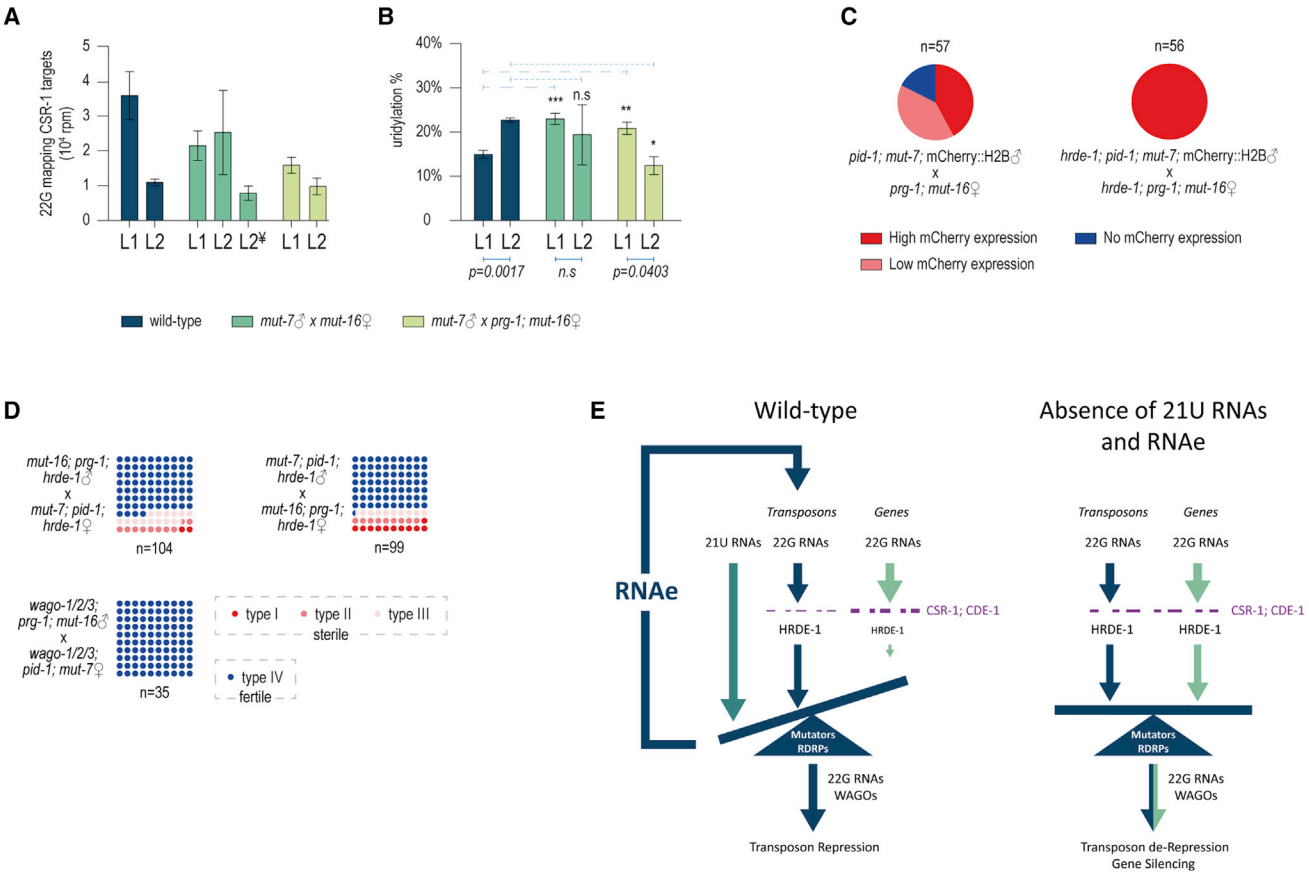

Figure 4. HRDE-1 and WAGO-1/WAGO-2/WAGO-3 Drive Mutator-Induced Sterility

(A) Levels of 22G RNAs mapping to protein-coding genes targeted by CSR-1 (Table S1). Error bars represent SD between at least two biological replicates. ¥: corrected for 21U RNA levels (see Experimental Procedures).

(B) Fraction of 22G RNAs mapping to protein-coding genes targeted by CSR-1 that contain non-templated 3′ uridylation in L1 and L2 larvae of the indicated genotype. p values where calculated using the two-tailed t test and assuming a normal distribution. Error bars represent SD between at least two biological replicates.

(C) Pie chart showing the fraction of the offspring expressing a germline-specific mCherry::H2B transgene, from the two indicated crosses. The left panel reflects the same data as depicted in Figure 3A, right panel.

(D) Quantitation of the observed germline defects in the *hrde-1* mutants and in *wago-1/wago-2/wago-3* triple mutants, where the mutator pathway was re-activated in the absence of functional 21U RNAs. Each dot represents 1%.

(E) Model describing how mutator-induced sterility can develop. See main text for more detailed description.

Also see Figure S4.

To further probe this balance, we checked whether increasing the amount of 22G RNAs from typical CSR-1 targets can result in increased loading of HRDE-1 with such 22G RNAs. Since disruption of *cde-1* leads to an over-accumulation of CSR-1-type 22G RNAs (van Wolfswinkel et al., 2009), we sequenced 22G RNAs from HRDE-1 immunoprecipitates from wild-type and *cde-1* mutant animals. This revealed that in *cde-1* mutant animals, the fraction of CSR-1-target-derived 22G RNAs in HRDE-1 is higher than in wild-type animals (Figure 3D). Interestingly, loss of MUT-16 in a *cde-1* mutant animal triggers sterility that can be partially rescued by loss of HRDE-1 (Figure 3E), strongly suggesting that in a *cde-1;mut-16* double mutant, HRDE-1 may be effectively silencing CSR-1 target genes. We conclude that CSR-1, WAGO-1, and HRDE-1 are loaded with 22G RNAs from each other's target genes and that a disturbed loading balance among the various Argonaute proteins can have significant effects on germ cell development.

Impact of Mutator-Induced Sterility on 22G RNAs from CSR-1 Target Genes

We next analyzed the 22G RNA content of L1 and L2 larvae that develop mutator-induced sterility. The various small RNA pools, including 21U RNAs and different 22G RNA classes, overall behaved as can be expected from a loss of mutator activity or PRG-1 (Figure S4). We then focused on 22G RNAs derived from typical CSR-1 target genes and used uridylation frequencies of 22G-RNA pools as a proxy for their physical association with either CSR-1 (high uridylation) or other Argonautes (low uridylation).

In wild-type animals, uridylation frequencies of typical CSR-1 22G RNAs increase when animals develop from L1 into L2 larvae (Figure 4A). At the same time, the abundance of these 22G RNAs

drops strongly (by ~70%) (Figure 4B). In contrast, during the same developmental step, a significant decrease in uridylation of CSR-1 22G RNAs is observed in animals that display mutator-induced sterility, accompanied by only a small drop in 22G-RNA abundance (~35%) (Figures 4A and 4B). A possible explanation for these observations could be that in animals that develop mutator-induced sterility, the 22G RNAs from typical CSR-1 targets are in fact not bound by CSR-1 but by another Argonaute protein, triggering ectopic gene silencing and sterility.

HRDE-1 Is Required to Trigger Mutator-Induced Sterility

The nuclear protein HRDE-1 is a good candidate to be loaded with such CSR-1-target-derived 22G RNAs, because HRDE-1 has been shown to be downstream of mutator proteins and PRG-1 (Ashe et al., 2012; Buckley et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Furthermore, we have shown that HRDE-1 can be readily loaded with 22G RNAs that normally load into CSR-1 (Figure S3) and that mutation of *hrde-1* can partially rescue the sterility of *mut-16;cde-1* mutants (Figure 3E). We thus tested whether HRDE-1 is required for the above-described, undue, stochastic silencing of a germline-expressed mCherry reporter transgene during mutator-induced sterility. Indeed, in absence of HRDE-1, this silencing is no longer observed (Figure 4C). In fact, the sterility phenotype itself is also largely rescued by loss of HRDE-1 (Figure 4D), and, consistent with these data, Phillips et al. (2015; in this issue of *Developmental Cell*) demonstrate that immuno-purified HRDE-1 binds more 22G RNAs from CSR-1 targets during mutator-induced sterility. Interestingly, a *wago-1/wago-2/wago-3* triple mutation also rescues the sterility (Figure 4D). These combined results provide strong evidence that Argonaute-driven silencing drives the mutator-induced sterility phenotype.

DISCUSSION

Multiple Argonaute Proteins Drive Tc1 Silencing in *C. elegans*

We demonstrate a requirement for PRG-1 in the establishment of de novo transposon 22G RNAs and show that *prg-1*, *hrde-1*, and *wago-1/wago-2/wago-3* are all involved in executing Tc1 silencing. An interesting possibility is that the WAGO-1/WAGO-2/WAGO-3 proteins reflect the actual silencing Argonautes and that PRG-1 and HRDE-1 only bring targeting specificity into the mutator foci that drive 22G-RNA biogenesis. In this light, PRG-1 could be seen as the provider of hard-wired (i.e., genome-encoded) silencing information, while HRDE-1 provides epigenetic memory of silencing. These findings extend the functional parallel between the *C. elegans* PRG-1 pathway and piRNA activity in other animal species, including the importance of the maternal piRNA pool (Le Thomas et al., 2014a, 2014b).

Parental Memory of RNAe

This study, as well as previous studies (Alcazar et al., 2008; Ashe et al., 2012; Grishok et al., 2000; Luteijn et al., 2012; Shirayama et al., 2012; Stoeckius et al., 2014), clearly demonstrates that mutator-dependent silencing information inherits through both the paternal and maternal lineages. It is, however, not clear how this memory is precisely transmitted. It seems likely that this occurs in the form of 22G RNAs, but how these small RNAs drive self-renewal is unclear. Given that mutator proteins are clustered in foci (Phillips et al., 2012), one scenario is that certain Argonaute proteins, including PRG-1 and HRDE-1, are capable of targeting mRNAs to these mutator foci, even though at steady state neither PRG-1 nor HRDE-1 has been reported to be in these foci. Since mutator foci are very small, this could be due to a lack of resolution in the experiments. Alternately, such targeting of transcripts to mutator foci may be indirect. For example, chromatin changes induced by HRDE-1 may lead to routing of transcripts from HRDE-1-targeted loci into mutator foci. Such mechanisms have been proposed to act in small-RNA-related chromatin pathways in *Drosophila* and fission yeast (Keller et al., 2012; Klattenhoff et al., 2009; Zhang et al., 2014).

Molecular Mechanism behind Mutator-Induced Sterility

Our results suggest that inappropriate targeting of CSR-1 target transcripts, i.e., mRNAs that are expressed in germ cells, by mutator activity, followed by loading of silencing-inducing Argonaute proteins with the resulting 22G RNAs, leads to sterility (see Figure 4E for a model). Interestingly, our data indicate that also in wild-type animals, mutators act on CSR-1 targets, although apparently this does not result in silencing of these targets. The easiest explanation for this is that in wild-type animals the small number of CSR-1-type 22G RNAs that is loaded into Argonaute proteins that drive silencing does not suffice to trigger silencing (also see next section). We propose that sterility develops only when 22G-RNA production from CSR-1 target genes is amplified, for example, through mis-directed mutator activity, triggered by absence of both parental 22G-RNA populations and maternal 21U RNAs. Various Argonaute proteins can execute the silencing leading to sterility. Whether these serve redundantly or act in more specialized settings is currently unclear. A second mechanism through which typical CSR-1-target 22G RNAs can be increased is through loss of CDE-1. These can trigger sterility, through HRDE-1, when "regular" mutator-driven 22G RNAs are removed, indicating that the ectopic mutator activity is but one mechanism to disrupt the balance between gene silencing and gene activity.

CSR-1: Gene Activation or Protection from Silencing?

How does CSR-1 counteract silencing? This issue has not been fully resolved. Some experiments have indicated that CSR-1 associates with chromatin (Claycomb et al., 2009; Wedeles et al., 2013), and hence a role for CSR-1 in maintaining open chromatin seems plausible. However, whether this is a direct effect of CSR-1 on chromatin or whether such chromatin effects are secondary remains unresolved. CSR-1 is also found on P granules, which are cytoplasmic structures. Therefore, CSR-1's role in maintaining gene expression might also depend on cytoplasmic activities. We propose that an important function of CSR-1 may be to de-stabilize 22G RNAs in a target RNA-dependent manner, similar to what has been described for miRNAs (Ameres et al., 2010). This is inspired by our finding that the number of CSR-1-bound 22G RNAs drop abruptly when animals develop from L1 to L2 stage, when the primordial germ cells (PGCs) become transcriptionally more active, accompanied by a rise in uridylation frequency. We demonstrated before that uridylation of these 22G RNAs by CDE-1 suppresses their abundance (van

Wolfswinkel et al., 2009), and we now show that at least a fraction of this expanded pool of 22G RNAs is bound by HRDE-1. Hence, target-dependent 22G-RNA de-stabilization might prevent the loading of 22G RNAs into HRDE-1, or similar Argonaute proteins, thus repressing silencing activities on CSR-1-targets. Such a mechanism could provide a silencing threshold, ensuring that a minimum level of 22G-RNA production needs to be achieved before silencing takes effect. Mutator activity may have evolved for that very purpose. Future experiments aimed at the loading of individual Argonaute proteins under different experimental conditions will be required to further test these hypotheses.

EXPERIMENTAL PROCEDURES

Sample Preparation for Small RNA Sequencing

C. elegans L1 larvae were obtained by bleaching gravid adults and hatching the eggs in M9. L1 cross-offspring larvae were obtained by single picking 200 eggs to an unseeded nematode growth media (NGM) plate, bleaching those eggs to remove any bacteria that were carried along, and allowing the eggs to hatch overnight. L1 larvae were then re-suspended in M9. Cross offspring were identified through the p*unc-119*::GFP transgene brought in via the male. L2 larvae were picked and washed in M9 buffer for each sample.

Total RNA Isolation

One hundred fifty L1 or 50 L2 *C. elegans* larvae were washed in M9 buffer (22 mM Na_2HPO_4, 33 mM KH_2PO_4, 86 mM NaCl, and 1 mM $MgSO_4$) and digested in lysis buffer (200 mM NaCl, 100 mM Tris [pH 8.5], 50 mM EDTA, 0.5% SDS, and 200 ug/mL Proteinase-K) for 3 hr at 65°C followed by 15 min at 95°C to denature the Prot-K. Lysate was then incubated with DNase I (NEB) for 30 min at 37°C. Total RNA was then isolated using TRIZOL-LS according to manufacturer instructions and dissolved in 8 uL of H_2O.

Library Preparation, Sequencing, and Data Analysis

Detailed procedures are described in the Supplemental Experimental Procedures. Sequencing data are available at GEO: GSE68988.

WAGOs IP Data Analysis

Sequencing data from CSR-1 IP, WAGO-1 IP, and WAGO-9 IP were obtained from Claycomb (Claycomb et al., 2009), Gu (Gu et al., 2009), and Shiryama (Shirayama et al., 2012), respectively. The raw reads in FastQ format were filtered from 5′ barcodes 3′ adaptor sequences using a custom python script, mapped, and processed to the *C. elegans* genome reference WS224, as mentioned before, with the exception that any gene was allowed to be in several "target categories."

Transposon Excision Analysis

For each analyzed genotype, mutant worms carrying the *unc-22::Tc1* insertion were singled into a 6 cm NGM plate seeded with 100 μl of OP50 and grown at 20°C. Plates were scored for wild-type moving worms at three different time points: when the total number of worms per plate was around 50, when the total number of worms per plate was around 100, and when the plate was starved, to which we estimated the total number of worms per plate to be 1,000. Transposition frequencies at each time point were calculated using the following formula: $f = -\ln[(T - R) / T] / N$, where T = total number of plates scored, R = number of plates with revertants, and N = number of worms in the plate. Each time point was considered as a biological replicate.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.07.010.

AUTHOR CONTRIBUTIONS

B.F.M.A. designed, executed, and interpreted experiments and performed computational analysis. M.P. planned, executed, and interpreted experiments. R.F.K. designed the study, interpreted results, and wrote the manuscript with input from B.F.M.A. and M.P.

ACKNOWLEDGMENTS

We thank IMB Core Facilities, in particular Emil Karaulanov and Chung-Ting Han, for support in library preparation, sequencing, bioinformatic support, and media preparation. We thank members of the Ketting group for stimulating discussions. M.P. was supported by a Boehringer Ingelheim Fonds PhD Fellowship. This work was further supported by a Deutsche Forschungsgemeinschaft grant KE 1888/1-1 (Project Funding Programme; R.F.K.) and a grant from Fundação para a Ciência e Tecnologia ([FCT]SFRH/BD/51001/2010; B.F.M.A.).

Received: December 15, 2014
Revised: April 27, 2015
Accepted: July 16, 2015
Published: August 13, 2015

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