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Acknowledgements

We thank D. Garbers for the *gcy-10::GFP* reporter plasmid; M. Han for *let-60* cDNAs and the *mek-2(ku114)* strain; M. Koga, Y. Ohshima, N. Hisamoto and K. Matsumoto for pEF1α::GFP; A. Fire for vectors; and C. Bargmann, T. Schedl and Y. Emori for their comments and advice. All other nematode strains used in this study were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

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An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells

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In a diverse group of organisms that includes *Caenorhabditis elegans*, *Drosophila*, planaria, hydra, trypanosomes, fungi and plants, the introduction of double-stranded RNAs inhibits gene expression in a sequence-specific manner[1–7]. These responses, called RNA interference or post-transcriptional gene silencing, may provide anti-viral defence, modulate transposition or regulate gene expression[1,6,8–10]. We have taken a biochemical approach towards elucidating the mechanisms underlying this genetic phenomenon. Here we show that 'loss-of-function' phenotypes can be created in cultured *Drosophila* cells by transfection with specific double-stranded RNAs. This coincides with a marked reduction in the level of cognate cellular messenger RNAs. Extracts of transfected cells contain a nuclease activity that specifically degrades exogenous transcripts homologous to transfected double-stranded RNA. This enzyme contains an essential RNA component. After partial purification, the sequence-specific nuclease co-fractionates with a discrete, ~25-nucleotide RNA species which may confer specificity to the enzyme through homology to the substrate mRNAs.

Although double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous biological contexts including *Drosophila*[11,12], the mechanisms underlying this phenomenon have remained mostly unknown. We therefore wanted to establish a biochemically tractable model in which such mechanisms could be investigated.

Transient transfection of cultured, *Drosophila* S2 cells with a *lacZ* expression vector resulted in β-galactosidase activity that was easily detectable by an *in situ* assay (Fig. 1a). This activity was greatly reduced by co-transfection with a dsRNA corresponding to the first 300 nucleotides of the *lacZ* sequence, whereas co-transfection with a control dsRNA (*CD8*) (Fig. 1a) or with single-stranded RNAs of either sense or antisense orientation (data not shown) had little or no effect. This indicated that dsRNAs could interfere, in a sequence-specific fashion, with gene expression in cultured cells.

To determine whether RNA interference (RNAi) could be used to target endogenous genes, we transfected S2 cells with a dsRNA corresponding to the first 540 nucleotides of *Drosophila cyclin E*, a gene that is essential for progression into S phase of the cell cycle. During log-phase growth, untreated S2 cells reside primarily in G2/M (Fig. 1b). Transfection with *lacZ* dsRNA had no effect on cell-cycle distribution, but transfection with the *cyclin E* dsRNA caused a G1-phase cell-cycle arrest (Fig. 1b). The ability of *cyclin E* dsRNA to provoke this response was length-dependent. Double-stranded RNAs of 540 and 400 nucleotides were quite effective, whereas

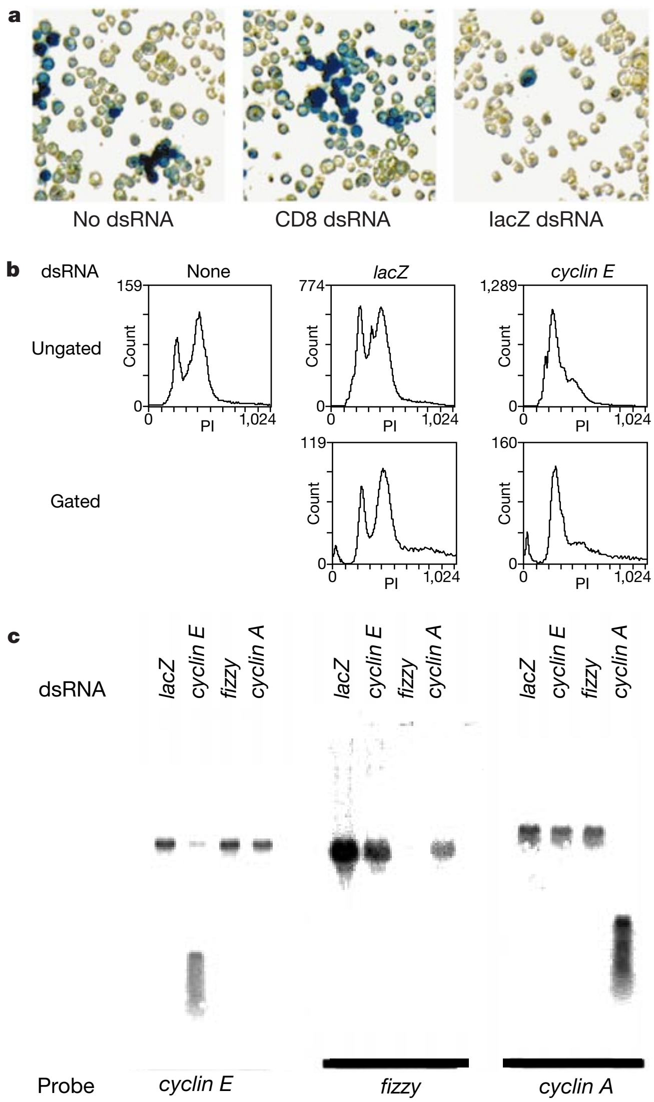

Figure 1 RNAi in S2 cells. **a**, *Drosophila* S2 cells were transfected with a plasmid that directs *lacZ* expression from the copia promoter in combination with dsRNAs corresponding to either human CD8 or lacZ, or with no dsRNA, as indicated. **b**, S2 cells were co-transfected with a plasmid that directs expression of a GFP–US9 fusion protein (12) and dsRNAs of either *lacZ* or *cyclin E*, as indicated. Upper panels show FACS profiles of the bulk population. Lower panels show FACS profiles from GFP-positive cells. **c**, Total RNA was extracted from cells transfected with *lacZ*, *cyclin E*, *fizzy* or *cyclin A* dsRNAs, as indicated. Northern blots were hybridized with sequences not present in the transfected dsRNAs.

dsRNAs of 200 and 300 nucleotides were less potent. Double-stranded *cyclin E* RNAs of 50 or 100 nucleotides were inert in our assay, and transfection with a single-stranded, antisense *cyclin E* RNA had virtually no effect (see Supplementary Information).

One hallmark of RNAi is a reduction in the level of mRNAs that are homologous to the dsRNA. Cells transfected with the *cyclin E* dsRNA (bulk population) showed diminished endogenous *cyclin E* mRNA as compared with control cells (Fig. 1c). Similarly, transfection of cells with dsRNAs homologous to *fizzy*, a component of the anaphase-promoting complex (APC) or *cyclin A*, a cyclin that acts in S, G2 and M, also caused reduction of their cognate mRNAs (Fig. 1c). The modest reduction in *fizzy* mRNA levels in cells transfected with *cyclin A* dsRNA probably resulted from arrest at a point in the division cycle at which *fizzy* transcription is low[14,15]. These results indicate that RNAi may be a generally applicable method for probing gene function in cultured *Drosophila* cells.

The decrease in mRNA levels observed upon transfection of specific dsRNAs into *Drosophila* cells could be explained by effects at transcriptional or post-transcriptional levels. Data from other systems have indicated that some elements of the dsRNA response may affect mRNA directly (reviewed in refs 1 and 6). We therefore sought to develop a cell-free assay that reflected, at least in part, RNAi.

S2 cells were transfected with dsRNAs corresponding to either *cyclin E* or *lacZ*. Cellular extracts were incubated with synthetic mRNAs of *lacZ* or *cyclin E*. Extracts prepared from cells transfected with the 540-nucleotide *cyclin E* dsRNA efficiently degraded the *cyclin E* transcript; however, the *lacZ* transcript was stable in these lysates (Fig. 2a). Conversely, lysates from cells transfected with the *lacZ* dsRNA degraded the *lacZ* transcript but left the *cyclin E* mRNA intact. These results indicate that RNAi ablates target mRNAs through the generation of a sequence-specific nuclease activity. We have termed this enzyme RISC (RNA-induced silencing complex).

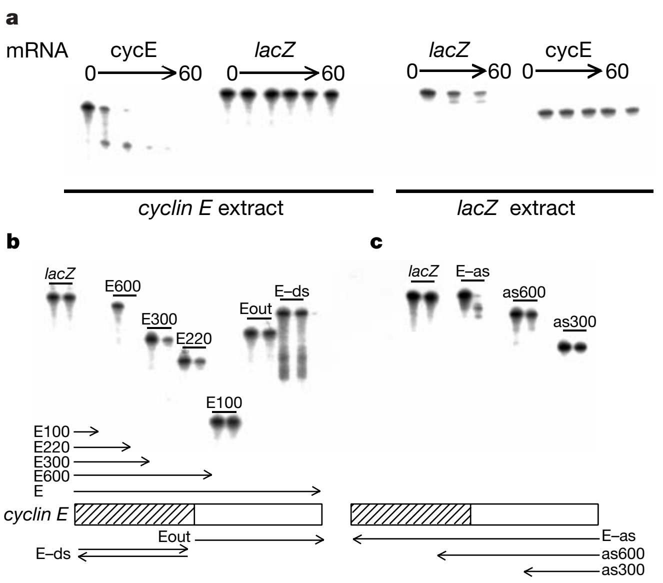

Figure 2 RNAi *in vitro*. **a**, Transcripts corresponding to either the first 600 nucleotides of *Drosophila cyclin E* (E600) or the first 800 nucleotides of *lacZ* (Z800) were incubated in lysates derived from cells that had been transfected with either *lacZ* or *cyclin E* (cycE) dsRNAs, as indicated. Time points were 0, 10, 20, 30, 40 and 60 min for *cyclin E* and 0, 10, 20, 30 and 60 min for *lacZ*. **b**, Transcripts were incubated in an extract of S2 cells that had been transfected with *cyclin E* dsRNA (cross-hatched box, below). Transcripts corresponded to the first 800 nucleotides of *lacZ* or the first 600, 300, 220 or 100 nucleotides of *cyclin E*, as indicated. Eout is a transcript derived from the portion of the *cyclin E* cDNA not contained within the transfected dsRNA. E-ds is identical to the dsRNA that had been transfected into S2 cells. Time points were 0 and 30 min. **c**, Synthetic transcripts complementary to the complete *cyclin E* cDNA (Eas) or the final 600 nucleotides (Eas600) or 300 nucleotides (Eas300) were incubated in extract for 0 or 30 min.

Although we occasionally observed possible intermediates in the degradation process (see Fig. 2), the absence of stable cleavage end-products indicates an exonuclease (perhaps coupled to an endonuclease). However, it is possible that the RNAi nuclease makes an initial endonucleolytic cut and that non-specific exonucleases in the extract complete the degradation process[16]. In addition, our ability to create an extract that targets *lacZ in vitro* indicates that the presence of an endogenous gene is not required for the RNAi response.

To examine the substrate requirements for the dsRNA-induced, sequence-specific nuclease activity, we incubated a variety of *cyclin-E*-derived transcripts with an extract derived from cells that had been transfected with the 540-nucleotide *cyclin E* dsRNA (Fig. 2b, c). Just as a length requirement was observed for the transfected dsRNA, the RNAi nuclease activity showed a dependence on the size of the RNA substrate. Both a 600-nucleotide transcript that extends slightly beyond the targeted region (Fig. 2b) and an ~1-kilobase (kb) transcript that contains the entire coding sequence (data not shown) were completely destroyed by the extract. Surprisingly, shorter substrates were not degraded as efficiently. Reduced activity was observed against either a 300- or a 220-nucleotide transcript, and a 100-nucleotide transcript was resistant to nuclease in our assay. This was not due solely to position effects because ~100-nucleotide transcripts derived from other portions of the transfected dsRNA behaved similarly (data not shown). As expected, the nuclease activity (or activities) present in the extract could also recognize the antisense strand of the *cyclin E* mRNA. Again, substrates that contained a substantial portion of the targeted region were degraded efficiently whereas those that contained a shorter stretch of homologous sequence (~130 nucleotides) were recognized inefficiently (Fig. 2c, as600). For both the sense and antisense strands, transcripts that had no homology with the transfected dsRNA (Fig. 2b, Eout; Fig. 2c, as300) were not degraded. Although we cannot exclude the possibility that nuclease specificity could have migrated beyond the targeted region, the resistance of transcripts that do not contain homology to the dsRNA is consistent with data from *C. elegans*. Double-stranded RNAs homologous to an upstream cistron have little or no effect on a linked downstream cistron, despite the fact that unprocessed, polycistronic mRNAs can be readily detected[17,18]. Furthermore, the nuclease was inactive against a dsRNA identical to that used to provoke the RNAi response *in vivo* (Fig. 2b). In the *in vitro* system, neither a 5′ cap nor a poly(A) tail was required, as such transcripts were degraded as efficiently as uncapped and non-polyadenylated RNAs.

Gene silencing provoked by dsRNA is sequence specific. A plausible mechanism for determining specificity would be incorporation of nucleic-acid guide sequences into the complexes that accomplish silencing[19]. In accord with this idea, pre-treatment of

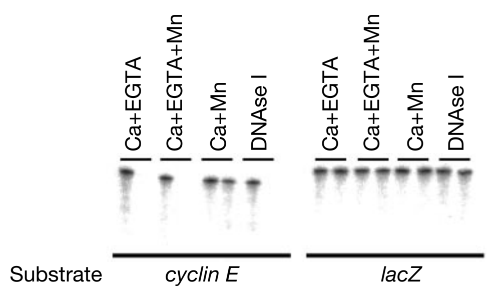

Figure 3 Substrate requirements of the RISC. Extracts were prepared from cells transfected with *cyclin E* dsRNA. Aliquots were incubated for 30 min at 30 °C before the addition of either the *cyclin E* (E600) or *lacZ* (Z800) substrate. Individual 20-μl aliquots, as indicated, were pre-incubated with 1 mM $CaCl_2$ and 5 mM EGTA, 1 mM $CaCl_2$, 5 mM EGTA and 60 U of micrococcal nuclease, 1 mM $CaCl_2$ and 60 U of micrococcal nuclease or 10 U of DNase I (Promega) and 5 mM EGTA. After the 30-min pre-incubation, EGTA was added to those samples that lacked it. Yeast tRNA (1 μg) was added to all samples. Time points were at 0 and 30 min.

extracts with a Ca^{2+}-dependent nuclease (micrococcal nuclease) abolished the ability of these extracts to degrade cognate mRNAs (Fig. 3). Activity could not be rescued by addition of non-specific RNAs such as yeast transfer RNA. Although micrococcal nuclease can degrade both DNA and RNA, treatment of the extract with DNAse I had no effect (Fig. 3). Sequence-specific nuclease activity, however, did require protein (data not shown). Together, our results support the possibility that the RNAi nuclease is a ribonucleoprotein, requiring both RNA and protein components. Biochemical fractionation (see below) is consistent with these components being associated in extract rather than being assembled on the target mRNA after its addition.

In plants, the phenomenon of co-suppression has been associated with the existence of small (~25-nucleotide) RNAs that correspond to the gene that is being silenced[19]. To address the possibility that a similar RNA might exist in *Drosophila* and guide the sequence-specific nuclease in the choice of substrate, we partially purified our activity through several fractionation steps. Crude extracts contained both sequence-specific nuclease activity and abundant, heterogeneous RNAs homologous to the transfected dsRNA (Figs 2 and 4a). The RNAi nuclease fractionated with ribosomes in a high-speed centrifugation step. Activity could be extracted by treatment with high salt, and ribosomes could be removed by an additional centrifugation step. Chromatography of soluble nuclease over an anion-exchange column resulted in a discrete peak of activity (Fig. 4b, *cyclin E*). This retained specificity as it was inactive against a heterologous mRNA (Fig. 4b, *lacZ*). Active fractions also contained an RNA species of 25 nucleotides that is homologous to the *cyclin E* target (Fig. 4b, northern). The band observed on northern blots may represent a family of discrete RNAs because it could be detected with probes specific for both the sense and antisense *cyclin E* sequences and with probes derived from distinct segments of the dsRNA (data not shown). At present, we cannot determine whether the 25-nucleotide RNA is present in the nuclease complex in a double-stranded or single-stranded form.

RNA interference allows an adaptive defence against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. Our data, and that of others[19], is consistent with a model in which dsRNAs present in a cell are converted, either through processing or replication, into small specificity determinants of discrete size in a manner analogous to antigen processing. Our results suggest that the post-transcriptional component of dsRNA-dependent gene silencing is accomplished by a sequence-specific nuclease that incorporates these small RNAs as guides that target specific messages based upon sequence recognition. The identical size of putative specificity determinants in plants[19] and animals predicts a conservation of both the mechanisms and the components of dsRNA-induced, post-transcriptional gene silencing in diverse organisms. In plants, dsRNAs provoke not only post-transcriptional gene silencing but also chromatin remodelling and transcriptional repression[20,21]. It is now critical to determine whether conservation of gene-silencing mechanisms also exists at the transcriptional level and whether chromatin remodelling can be directed in a sequence-specific fashion by these same dsRNA-derived guide sequences. □

Note added in proof: Recently, Tuschl *et al.* have reported the development of cell-free extracts from *Drosophila* embryos that can carry out RNAi (T. Tuschl, P. D. Zamore, D. P. Bartel and P. A. Sharp, *Genes Dev.* **13,** 3191–3197; 1999). Their results also indicate that the RNAi is accomplished at least in part by nuclease degradation of targeted mRNAs.

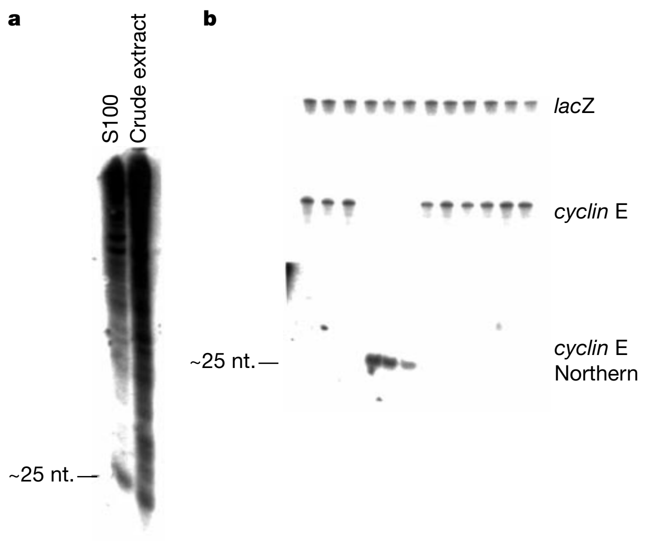

Figure 4 The RISC contains a potential guide RNA. **a**, Northern blots of RNA from either a crude lysate or the S100 fraction (containing the soluble nuclease activity, see Methods) were hybridized to a riboprobe derived from the sense strand of the *cyclin E* mRNA. **b**, Soluble *cyclin-E*-specific nuclease activity was fractionated as described in Methods. Fractions from the anion-exchange resin were incubated with the lacZ, control substrate (upper panel) or the cyclin E substrate (centre panel). Lower panel, RNA from each fraction was analysed by northern blotting with a uniformly labelled transcript derived from sense strand of the *cyclin E* cDNA. DNA oligonucleotides were used as size markers.

Methods

Cell culture and RNA methods

S2 (ref. 22) cells were cultured at 27 °C in 90% Schneider's insect media (Sigma), 10% heat inactivated fetal bovine serum (FBS). Cells were transfected with dsRNA and plasmid DNA by calcium phosphate co-precipitation[23]. Identical results were observed when cells were transfected using lipid reagents (for example, Superfect, Qiagen). For FACS analysis, cells were additionally transfected with a vector that directs expression of a green fluorescent protein (GFP)–US9 fusion protein[13]. These cells were fixed in 90% ice-cold ethanol and stained with propidium iodide at 25 μg ml^{-1}. FACS was performed on an Elite flow cytometer (Coulter). For northern blotting, equal loading was ensured by over-probing blots with a control complementary DNA (RP49). For the production of dsRNA, transcription templates were generated by polymerase chain reaction such that they contained T7 promoter sequences on each end of the template. RNA was prepared using the RiboMax kit (Promega). Confirmation that RNAs were double stranded came from their complete sensitivity to RNAse III (a gift from A. Nicholson). Target mRNA transcripts were synthesized using the Riboprobe kit (Promega) and were gel purified before use.

Extract preparation

Log-phase S2 cells were plated on 15-cm tissue culture dishes and transfected with 30 μg dsRNA and 30 μg carrier plasmid DNA. Seventy-two hours after transfection, cells were harvested in PBS containing 5 mM EGTA washed twice in PBS and once in hypotonic buffer (10 mM HEPES pH 7.3, 6 mM β-mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing *Complete* protease inhibitors (Boehringer) and 0.5 units ml^{-1} of RNasin (Promega). Cells were disrupted in a dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000*g* for 20 min. Supernatants were used in an *in vitro* assay containing 20 mM HEPES pH 7.3, 110 mM KOAc, 1 mM $Mg(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1 mM DTT. Typically, 5 μl extract was used in a 10 μl assay that contained also 10,000 c.p.m. synthetic mRNA substrate.

Extract fractionation

Extracts were centrifuged at 200,000*g* for 3 h and the resulting pellet (containing ribosomes) was extracted in hypotonic buffer containing also 1 mM $MgCl_2$ and 300 mM KOAc. The extracted material was spun at 100,000*g* for 1 h and the resulting supernatant was fractionated on Source 15Q column (Pharmacia) using a KCl gradient in buffer A (20 mM HEPES pH 7.0, 1 mM dithiothreitol, 1 mM $MgCl_2$). Fractions were assayed for nuclease activity as described above. For northern blotting, fractions were proteinase K/SDS treated, phenol extracted, and resolved on 15% acrylamide 8M urea gels. RNA was electroblotted onto Hybond N+ and probed with strand-specific riboprobes derived from cyclin E mRNA. Hybridization was carried out in 500 mM $NaPO_4$ pH 7.0, 15% formamide, 7% SDS, 1% BSA. Blots were washed in 1 × SSC at 37–45 °C.

Received 26 November 1999; accepted 26 January 2000.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank C.Velinzon and L. Rodgers for assistance with flow cytometry. Materials and advice were provided by A. Krainer, J. Yin and A. Nicholson. D.B. is supported by the Hugh and Catherine Stevenson Fund. G.J.H. is a Pew Scholar in the Biomedical Sciences. This work was supported in part by grants from the NIH (G.J.H.) and the US Army Breast Cancer Research Program (G.J.H.).

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A genetic link between co-suppression and RNA interference in *C. elegans*

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Originally discovered in plants[1,2], the phenomenon of co-suppression by transgenic DNA has since been observed in many organisms from fungi[3] to animals[4–7]: introduction of transgenic copies of a gene results in reduced expression of the transgene as well as the endogenous gene. The effect depends on sequence identity between transgene and endogenous gene. Some cases of co-suppression resemble RNA interference (the experimental silencing of genes by the introduction of double-stranded RNA)[8], as RNA seems to be both an important initiator and a target in these processes[9–13]. Here we show that co-suppression in *Caenorhabditis elegans* is also probably mediated by RNA molecules. Both RNA interference[14,15] and co-suppression[16] have been implicated in the silencing of transposons. We now report that mutants of *C. elegans* that are defective in transposon silencing and RNA interference (*mut-2*, *mut-7*, *mut-8* and *mut-9*) are in addition resistant to co-suppression. This indicates that RNA interference and co-suppression in *C. elegans* may be mediated at least in part by the same molecular machinery, possibly through RNA-guided degradation of messenger RNA molecules.

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We tested whether the MUT-7 protein, a putative 3′-5′ exoribonuclease required for transposon silencing and RNA interference (RNAi)[14], is also required for co-suppression in *C. elegans*. Co-suppression in *C. elegans* has been reported for a number of genes, including *fem-1*. As described previously[7], wild-type animals bearing a highly repetitive transgene containing multiple copies of the complete *fem-1* gene show a feminization of the germline, phenocopying loss-of-function mutations of the *fem-1* gene (Table 1). It has been shown that this effect depends on the presence of the *fem-1* promoter region[7]. When this region is not present, no feminization is observed, indicating that RNA is a mediator in co-suppression. We placed the same *fem-1* transgene in a *mut-7* mutant background and found that this feminization was no longer observed (Table 1). This result indicates that the RNA-mediated co-suppression effect of the *fem-1* transgene has a genetic basis and that it requires a protein (MUT-7) that is also involved in the processes of RNAi and transposon silencing. Thus, the aberrant RNA molecules that have been postulated in co-suppression[10,17] might be double-stranded RNA (dsRNA) molecules, also involved in RNAi[8].

To test whether the dependence of co-suppression on *mut-7* is general, we analysed two other genes for which co-suppression effects have been described: *gld-1* (ref. 6) and *mrt-2* (S. Ahmed and J. Hodgkin, personal communication). *Gld-1* co-suppression leads to an absence of oocytes and a tumorous germline, whereas *mrt-2* co-suppression results in hypersensitivity to ionizing radiation (which is consistent with the loss-of-function phenotypes of both genes[18,19]). Again, we find that the observed co-suppression effects

Table 1 Co-suppression of *fem-1*

	No. of animals with phenotype	
Genotype	Feminized	Wild type
Wild type; *pKEx1534*	28	2
mut-7(pk204); pkEx1534	0	27
rde-1(ne219); pkEx1539	30	1

Feminization of the germline by transgenes containing the *fem-1* gene. pkEx1534 was generated by injection of *fem-1* plasmid DNA into *mut7(pk204)* animals. This resulted in several non-co-suppressed transgenic lines (one containing the transgene pkEx1534). Restoration of *mut-7* gene function results in feminization of the germline. Injection of the same DNA into *rde-1(ne219)* animals results in lines displaying high levels of feminization.

Table 2 Co-suppression of *gld-1*

	No. of animals with phenotype	
Genotype	Tumorous germline	Wild type
mut-7(pk204)/+; pkEx1533	32	4
mut-7(pk204); pkEx1533	4	35
Complete promoter in wild type*	7	0
Complete promoter in *rde-1(ne219)**	4	0
Deleted promoter in wild type*	0	3†
Promoter only in wild type*	0	11

Induction of a 'tumorous germline' phenotype[18] by a *gld-1* multicopy transgene (pkEx1533), containing the complete *gld-1* promoter.
*The number of stable non-co-suppressed lines (designated wild type) or co-suppressed lines (tumorous germline) after injection is given; 31 (complete promoter in wild type), 22 (complete promoter in *rde-1(ne219)*), 32 (deleted promoter) and 107 (promoter only) F_1 transgenic animals were analysed.
† These three lines have no tumorous germline and produce oocytes. The strains produce some unfertilized eggs, indicative of a sperm defect, probably caused by a lower dosage of GLD-1 protein[18].