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A balance between silencing foreign DNA and protecting self in *Caenorhabditis elegans*

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Abstract

Unrestrained transposon mobilization threatens genome integrity. To survive, organisms have evolved silencing pathways capable of distinguishing self from non-self. This review emphasizes *Caenorhabditis elegans* genome defense with a particular emphasis on systems-level detection of foreign DNA and the balance between silencing and protective pathways. Abundant small RNAs (piRNAs and siRNAs), aberrant DNA structures (e.g., introns), and heterochromatin domains largely mediate silencing. For example, CRISPR-based manipulation of endogenous piRNAs has elucidated precise targeting rules and a novel, conserved role in tuning endogenous germline gene expression. Protective pathways are only just becoming clear: small RNA pathways (CSR-1), deamination of endogenous dsRNA, and a pervasive, embedded DNA watermark (PATCs) can all counteract silencing to protect endogenous genes.

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Introduction

> *"Know the enemy and know yourself; in a hundred battles you will never be in peril. When you are ignorant of the enemy, but know yourself, your chances of winning or losing are equal. If ignorant both of your enemy and yourself, you are certain in every battle to be in peril."*
>
> — Sun Tzu, The Art of War (5th century BC)

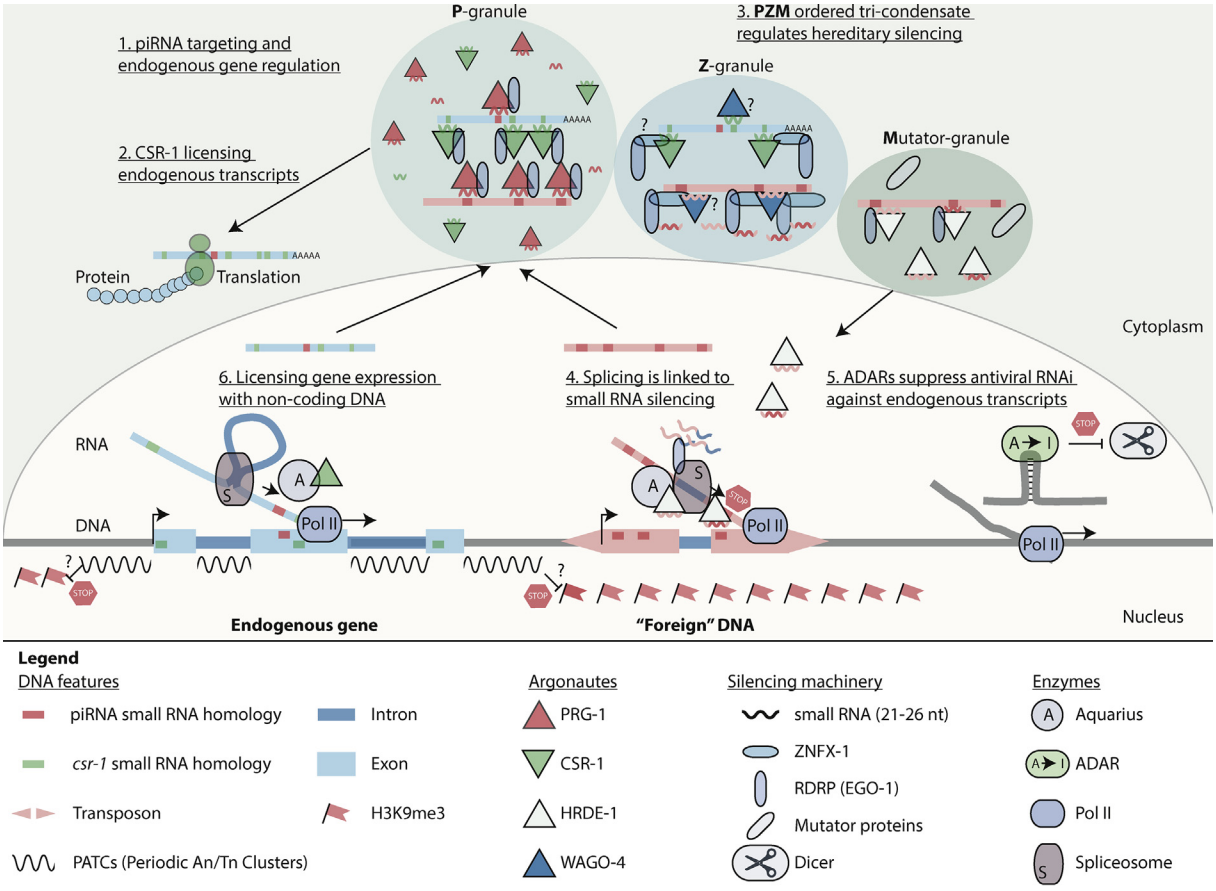
Mobile elements pose a challenge to organisms. Uncontrolled transposition can lead to gene inactivation or genome instability although organisms also co-opt transposons as a source of regulatory elements [1]. Organisms derive significant benefits from regulated and tissue-specific mechanisms to identify and silence transposons. At the same time, organisms need mechanisms to identify "self" to prevent irreversible silencing of endogenous genes. Several recent reviews cover the field of transgenerational gene regulation [2–4]. Therefore, this review rather narrowly focuses on recent studies relating to initial detection and the balance of silencing and activating pathways in the nematode *Caenorhabditis elegans* (Figure 1).

Rules for monitoring the genome for invaders using an endogenous oligo library

piRNAs are small RNAs that form a complex with PIWI Argonautes and have primarily been characterized for their ability to silence transposons in the germline. The piRNA pathway is conserved across model organisms (worms, flies, zebrafish, and mice) although "details" such as small RNA length, biogenesis, and amplification mechanism vary and evolve rapidly [5]. In *C. elegans*, piRNAs are individually transcribed as a diverse "oligo library" with at least 16,000 unique piRNAs that are 21 nucleotides long and form a complex with the PRG-1 PIWI Argonaute [6]. The sequence diversity of piRNAs and their ability to recognize targets via partial base pairing had hampered efforts to determine precise rules for piRNA:target binding [7,8].

Two recent publications revealed that piRNA binding resembles miRNA binding. Shen et al. [9] isolated PIWI complexes and identified ~200,000 target mRNAs directly bound by piRNAs. They determined that piRNA binding correlates with binding energy and not piRNA abundance; for example, deletion of a rare piRNA targeting an endogenous gene (*xol-1*, see below) had a larger effect than deleting a piRNA with >100-fold higher expression also targeting *xol-1*. Zhang et al. [10] deduced binding rules by modifying a single endogenous piRNA and determining changes in secondary small interfering RNAs (siRNAs) generated by piRNA targeting. Both studies converged on similar rules: piRNAs bind via a 7-nucleotide seed region with almost no mismatch tolerance, a 5-nucleotide central region that tolerates mismatches, a 6-nucleotide 3′ supplemental pairing region with moderate mismatch tolerance, and no apparent binding of the leading U base in piRNAs [9,10]. These precise binding rules will allow systems level prediction of endogenous genes under

Figure 1



Overview of the main silencing and protective pathways in the *C. elegans* germline. Schematic of known silencing and protective pathways that germ cells utilize to distinguish between foreign DNA, such as transposable elements, and endogenous genes. 1. piRNAs participate in the initial identification of foreign DNA through microRNA-like pairing rules and regulate the dosage of at least one endogenous gene [9,10]. 2. The CSR-1 Argonaute counter-balances the piRNA pathway to license endogenous genes for expression [20]. 3. A novel gene (ZNFX-1) defines a novel perinuclear liquid-like granule that is necessary for hereditary transgene silencing [31]. 4. RNAi and splicing pathways co-evolve [34]; recent studies show physical and genetic interactions between the splicing machinery and silencing pathways [37,38]. 5. Basal transcription generates double-stranded RNA which can trigger the antiviral RNAi pathway; adenosine deaminases that act on RNAs (ADARs) suppress the cellular response against endogenous dsRNAs [44]. 6. Endogenous germline genes located in repressive genomic regions contain a distributed class of non-coding DNA named Periodic An/Tn Clusters (PATCs). Transplanting PATCs into transgenes prevents silencing suggesting the non-coding DNA may identify endogenous genes [53].

piRNA regulation and *a priori* determination of protection against foreign DNA sequences, such as transposons and transgenes [11].

Gene regulatory roles for piRNAs outside of genome defense

piRNAs have primarily been characterized for their conserved role in transposon silencing and detection of foreign DNA (e.g., transgenes) (reviewed in Ref. [12]). However, few *C. elegans* piRNAs have obvious sequence homology to transposable elements, and only one transposon is derepressed by inactivating the piRNA pathway [6,13]. To identify functions for piRNAs in *C. elegans*, Tang et al. [14] recoded the most abundant piRNA and identified 16 endogenous gene targets, none of which were transposable elements. The target most affected by the loss of the X-linked piRNA was *xol-1*, a gene involved in sex determination [15]. Loss of the piRNA doubled the dosage of XOL-1 and de-regulated the sex-determination pathway (synthetic lethality and masculinization) [14]. By recoding piRNAs to target a fluorescent reporter that normally resists silencing, Seth et al. found that piRNAs can tune gene expression: targeting by three piRNAs was required to completely silence the transgene [16]. Given the large sequence diversity of piRNAs and flexible targeting rules, it is intriguing to consider whether piRNAs fine-tune many processes in the germline via post-transcriptional regulation of expression. In sum, these results [9,14,16] have

the significant implication that in addition to germline defense, piRNAs target endogenous genes and fine-tune their expression.

Maintaining a balance – silencing and activating genes by small RNA pathways

Dynamic systems require both activating and repressive pathways to maintain homeostasis; if a cell only has pathways to silence genes, the system risks getting trapped in the silent state. In germ cells, the role of piRNAs in repressing foreign DNA is well established [12], whereas a proposed activating role for CSR-1 small RNAs [8,17] is less well understood. CSR-1 is an essential Argonaute protein involved in chromosome segregation [18,19], which makes definitive experiments difficult, but studies implicate CSR-1 in transactivation of transgenes [20], protection from piRNA-mediated silencing [21], and promoting sense transcription [22] in the germline.

Analysing a broad set of piRNA:target hybrids, Shen et al. [9] demonstrated that piRNA sequence homology did not trigger siRNA production in transcripts also targeted by CSR-1, possibly by CSR-1 preventing PRG-1 binding or by preventing the production of WAGO siRNAs. Some observations, however, suggest a more complex interaction between the PRG-1 and CSR-1 pathways. First, in fertilized oocytes CSR-1 appears to down-regulate—or "tune"—many transcripts, demonstrating that CSR-1 can have a repressive role [23] either directly or indirectly. Second, for CSR-1 to protect from silencing over generations, it seems at least one copy of the endogenous gene would be necessary to generate complementary small RNAs. However, when endogenous genes with *csr-1* small RNA homology are re-introduced as transgenes, they are resistant to silencing, even in the absence of the original endogenous gene [10,16]. These observations are puzzling and difficult to reconcile with the proposed role for *csr-1* in preventing silencing. As proposed by Yu and Moazed [24], it is possible activating and repressive roles of CSR-1 are a result of spatial separation in cellular compartments by trafficking of effector proteins and small RNA populations.

Quality control of germline transcripts in perinuclear liquid-like condensates

Where do the PRG-1 and CSR-1 pathways interact to surveil germline transcripts? PRG-1 and CSR-1 primarily localize to perinuclear germline granules (Nuage or, in *C. elegans*, P granules) juxtaposed to nuclear pores. Mature mRNAs (spliced and polyadenylated) transit through P-granules [25] before translation in the cytoplasm or destruction in perinuclear "mutator" foci [26]. Therefore, P granules are well-positioned to function as a sorting station where PRG-1 and CSR-1 surveil transiting mRNAs and regulate the balance between transcription and silencing [27,28]. Phillips et al. [27] showed that PRG-1 and memory of piRNA targets (via maternal and paternal inheritance of siRNAs) are required for correct transcript sorting and normal transcription. Reactivating the endogenous RNAi pathway to determine how cells generate a memory of expression, PRG-1 targets were inappropriately expressed, and CSR-1 targets were silenced [27]. Similarly, de Albuquerque et al. [28] showed that memory of piRNAs is required for germline development by silencing transposable elements and preventing inappropriate silencing of endogenous germline genes. The signal for gene silencing is transmitted from P-granules back to the nucleus via HRDE-1, a nuclear Argonaute which mediates transgenerational silencing by guiding repressive chromatin modifications (H3K9me3) [29].

Emphasizing the role of perinuclear granules in surveilling transcripts, two independent genetic screens for animals defective in transgenerational silencing isolated a conserved helicase-domain protein (ZNFX-1) that is localized to P-granules where it interacts with components of silencing and activating pathways [30,31]. Ishidate et al. [30] showed that ZNFX-1 is necessary to generate a balanced response against germline mRNAs, with siRNAs distributed across the full transcript. In the absence of ZNFX-1, some repressed transgenes are desilenced over time, whereas other stably expressed transgenes begin to variegate [30], suggesting that ZNFX-1 is necessary for balanced transmission of epigenetic memories of silencing and expression. By looking at the subcellular localization of ZNFX-1, Wan et al. [31] identified a novel perinuclear granule (Z-granule). Z-granules are dynamic, liquid-like condensates: in early development, Z-granules fuse with P-granules, in the late germline, the two granules separate into independent compartments, and in adult germ cells form ordered tri-condensate (PZM) granules with P granules and mutator foci [31]. These observations exemplify how cells can use dynamic compartmentalization via liquid-like condensates to regulate complex RNA processing pathways that scan germline mRNAs.

Splicing and gene silencing are linked by small RNA pathways

What tell-tale features of foreign DNA do cells use to initiate silencing responses? piRNAs scan primary sequences for partial complementarity, but additional gene features are probably also important for distinguishing between endogenous and foreign genes. One clear example is from the yeast *Cryptococcus neoformans* where sub-optimal introns stall spliceosomes resulting in siRNAs generated at splice junctions [32]. The splicing machinery also plays a role in gene silencing in *C. elegans*. For example, RNAi and splicing pathways co-evolve and perturbing the splicing machinery can

desilence transgenes [33], although one concern is whether the activating effect is direct or indirect. Also, mRNAs silenced by worm-specific Argonautes (WAGOs) are not fully spliced, with 22G siRNAs mapping to predicted introns [34]. 2° siRNAs mapping to introns suggest the presence of a nuclear RNA-dependent RNA polymerase (RDRP) templating siRNAs from immature transcripts. The RdRP is likely EGO-1 which, together with the Argonaute CSR-1, is necessary for heterochromatin assembly on unpaired DNA in the germline [35,36].

Several recent publications [37–39] demonstrate functional interactions between splicing and silencing in *C. elegans* using biochemical and genetic approaches. Akay et al. [37] identified proteins by mass-spectrometry that interact with a nuclear Argonaute pathway necessary for hereditary transposon silencing (HRDE-1). One interaction partner was the conserved RNA helicase protein (Aquarius, EMB-4) that directly links a nuclear Argonaute involved in transgenerational silencing (HRDE-1) and the transcriptional machinery, which explains how small RNAs can co-transcriptionally generate repressive modifications [37]. Interestingly, transgene silencing in the absence of Aquarius depends on the number of introns in a sensor transgene (GFP). The authors propose that introns form a barrier to gene silencing that the RNA helicase can overcome [37]. Tyc et al. [39] isolated Aquarius as an interaction partner for CSR-1 and showed that Aquarius binds only to introns of CSR-1 targets (licensed, or "non-repressed" genes) and to introns and exons of HRDE-1 targets (repressed genes). The authors suggest germ cells may use this binding pattern as a molecular signature to sort transcripts into silencing and activating pathways [39]. Newman et al. [38] directly perturbed the splicing machinery via viable loss-of-function alleles in U1 and U2 small nuclear ribonucleic proteins (snRNPs). U1 snRNP activity is necessary for targeting poorly conserved gene transcripts with divergent introns, and targeted transcripts that are bound to stalled spliceosomes. These siRNAs map to exon-intron junctions providing evidence that targeting is initiated co-transcriptionally or from incompletely processed transcripts [38].

Endogenous transcripts are protected from RNA interference by deamination

Adenosine deaminases that act on RNA (ADARs) bind to double-strand RNA (dsRNA) and edit adenosines (A) to inosine (I) (reviewed in Ref. [40]). Editing can have several effects: amino acid substitutions, modified splice junctions, and destabilization of dsRNA structures formed from inverted repeat sequences. dsRNA is the primary trigger of RNAi, and there is increasing evidence to support interactions between ADAR and RNAi pathways: First, transgenes are silenced by RNAi in somatic cells in the absence of ADARs [41]. Second, chemotaxis defects in ADAR mutants are rescued by impairing the RNAi pathway [42]. Third, ADARs prevent dsRNA from regions of the genome with low-level basal transcription from triggering the RNAi pathway [43].

In a recent paper, Bass and colleagues determined the role of ADARs in antagonizing inappropriate silencing of endogenous transcripts by coupling high-throughput sequencing and dsRNA immunoprecipitation [44]. More than 1000 genomic regions produce transcripts that are A-to-I edited; in the absence of ADARs (*adr-1*; *adr-2*), these highly edited regions generate siRNAs, and the corresponding genes are downregulated in an RNAi-dependent manner [44]. Thus ADARs prevent the cell from mounting an RNAi response against endogenous transcripts. In mice, ADARs also prevent antiviral response to endogenous dsRNAs (e.g. Ref. [45]) suggesting a conserved role for ADARs.

A watermark protects endogenous genes from silencing

Large-scale differences between genomes of different organisms may also encode information about self and non-self. The *C. elegans* genome is enriched for AT-rich regions where runs of As and Ts are spaced by ~10 base pairs, corresponding to one turn of the DNA helix [46,47]. AT-rich sequences comprise ~10% of the genome and are named Periodic An/Tn clusters (PATCs) [48]. PATCs are strongly enriched in introns of germline-expressed genes that reside in repressive chromatin, suggesting a role for PATCs in protecting endogenous genes from silencing and perhaps allowing aggressive silencing of foreign DNA [48]. Despite their location in "globally" repressive chromatin domains, PATCs are anti-correlated with a repressive histone mark (H3K9me3) on a gene-by-gene basis [49]. Gene reporters introduced as multi-copy extrachromosomal arrays are almost always silenced in the germline [50], except for two examples of gene reporters each with high levels of PATCs [51].

We recently demonstrated that single-copy transgenes [52] are more frequently silenced in the germline within repressive genome domains [53]. Stochastic silencing, which depends on piRNAs [17], was almost entirely abolished by engineering transgenes to contain endogenous or synthetic PATC-rich DNA introns [53]. Similarly, Zhang et al. [10] showed that an mCherry transgene could be made refractory to silencing either by depleting piRNA binding sites or by incorporating PATC-rich introns. These results demonstrate that the PATC DNA structures are functionally important, perhaps by identifying transgenes as "self".

How might PATCs prevent silencing? AT-tracts can position nucleosomes on DNA and influence transcription by increasing DNA accessibility [54]. In *C. elegans*,

the A_n/T_n face of PATC-rich DNA is protected from endonuclease digestion [55], and mutations accumulate in phase with A_n/T_n clusters [53], indicating that nucleosomes are positioned relative to PATCs *in vivo* which may enable transcription from repressive domains.

Although it is likely that much DNA of the genome has no specific role ("junk DNA"), PATCs are seemingly random DNA sequences that take up a significant fraction of the genome, yet appear to play a well-defined biological role. Precisely because of the abundance and distribution of PATCs, it would be challenging for foreign DNA to mimic a PATC "watermark" embedded in promoters, introns, and 3′UTRs. PATCs are mostly specific to *Caenorhabditis* species [53], but many other periodic, non-random DNA sequences have been identified, including in humans, with no function assigned yet [56]. In one intriguing example, *Pristionchus pacificus* nematodes (lacking PATCs) can only be transformed with *P. pacificus* genomic DNA, possibly because of features distinguishing foreign genomic DNA from endogenous DNA [57]. There may, therefore, be more "large-scale" mechanisms of distinguishing self from foreign.

Conclusion

In conclusion, *C. elegans* employs a multitude of interlinked mechanisms to distinguish between self and foreign to maintain genome integrity, while maintaining endogenous gene expression. At present, pathways that detect and silence foreign DNA, such as siRNAs and chromatin modifications, are better understood with recent work increasingly elucidating protective mechanisms. Moreover, in what is likely a general theme, repressive pathways, such as the piRNA pathway, may have been co-opted to regulate endogenous gene expression. It will be interesting to follow the convergence of biochemical, genetic, and synthetic biology (e.g., *de novo* genome synthesis in bacteria [58], yeast [59], and human [60]) approaches in the coming years to understand how cells defeat their enemies in hundreds of battles.

Conflict of interest statement

Nothing declared.

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