Editorial



# New rules for regulation of genes by piRNAs in *C. elegans*

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Transposable element (TE) insertions create new gene regulatory elements or new gene products, thus representing a major driver for the evolutionary adaptation and genome plasticity of organisms (1). However, integration of TEs can also provoke genomic instability by disrupting protein-coding genes and alter transcriptional regulatory networks. Thus, TEs must be tightly controlled, especially in germ cells, as uncontrolled transposition can be mutagenic and cause sterility, abortive development and genetic diseases (2,3). Considerable attention is recently given to the consequences of uncontrolled transposition in somatic tissues and to their potential consequences in cancers, aging and degenerative diseases (4-7). Methylation of nucleic acid (DNA and RNA), as well as small non-coding RNAs are known to play crucial roles to limit TE activity in both germline and somatic tissues (8). Indeed, the main mechanism that has evolved in the animal gonads to keep transposons under control is the piwi interacting RNA (piRNA) pathway (9,10). piRNAs are a class of small non-coding RNAs bound by members of the PIWI clade of Argonautes proteins that rely on certain sequence complementarity to recognize their targets, mostly transposons targets but not exclusively. This pathway is highly conserved in animal gonads and its misregulation leads to defects in gametogenesis and ultimately to sterility. A similar mechanism for transposon control exists in cells of animals and plants involving another class of small RNA, the so-called siRNA (short-interfering RNA). The roles of the piRNA and siRNA pathways to limit TEs expansion are not a matter of debates anymore. In addition, there is some evidence for a role of piRNAs in regulation of *Drosophila* mRNAs (11) and, since many piRNAs in mammal and worms do not have identified targets (12), there is still room for other piRNA functions.

In *C. elegans*, piRNAs loaded in PRG-1 recruits an RNA-dependent RNA polymerases (RdRP) on their complementary targets RNAs, leading to the synthesis of 22G-RNAs. These 22G-RNAs are eventually loaded onto a worm-specific Argonaute proteins (WAGOs) to induce silencing of complementary genes (13,14). It is noteworthy that since they are produced by a piRNA-recruited RdRP, 22G-RNAs pinpoint the initial target sites of piRNAs in the genome.

In a recent issue of *Science*, Zhang and colleagues gained insight into the piRNA targeting mechanism (15) in *C. elegans* by identifying targets of unique piRNAs and analyzing in details how these piRNAs select their targets.

In parallel they uncovered a mechanism whereby endogenous piRNAs can repress non-self RNA sequences and deciphered how, in contrast, a protective sequence inside the targeted RNA prevents self-genes silencing.

Zhang and colleagues first examined two *C. elegans* strains expressing a synthetic piRNA complementary to a GFP sequence, or carrying a deletion of an endogenous piRNA locus. Small RNA sequencing revealed the appearance or decline of these piRNAs and of the corresponding 22G-RNA populations. The authors thus identified piRNAs that were complementary to 6 (synthetic piRNA-GFP) and 11 (endogenous piRNA locus) RNA targets, respectively.

Analysing carefully those targets and their corresponding complementary piRNA triggers, they noticed that a short sequence of the piRNAs, from the second to the seventh

nucleotide, most often perfectly pairs with their RNA targets, as described before for the miRNA seed regions in animals and plants (16). This implies a critical role for the pairing of this so-called *piRNA seed* region in *C. elegans*. Additional observations suggested that base pairing outside of the seed region accommodates few mismatches and contributes to a lesser extent, as with miRNAs, to piRNA target recognition.

In *C. elegans*, transgenes carrying foreign sequences (GFP, mCherry) are frequently silenced, especially in the germline. The authors hypothesized that endogenous piRNAs are guided by the discovered pairing rules to recognize these foreign sequences. Under this assumption, the removal of piRNA targets sites in the transgenes should allow unsilencing of their expression. Using a prediction software based on the piRNA pairing rules, they predicted 17 piRNA targets sites in GFP sequences of a foreign silenced GFP::CDK-1 transgene. Accordingly, mutations of all these potential piRNA targets sites allowed the expression of the re-encoded GFP::CDK-1 transgene.

Next, the authors engineered a new synthetic piRNA to silence the re-encoded GFP::CDK-1 transgene. They were thus able to systematically analyze the effect of mutations of this synthetic piRNA on the GFP::CDK-1 expression. Their results confirmed that piRNA targeting in *C. elegans* prefers near perfect pairing at the piRNA seed region (2–7 nt) and tolerates mismatches outside the seed region.

Importantly, the authors provided two additional striking example of germline silencing of non-self RNA sequences by endogenous piRNAs: the mCherry::Anillin and the bacterial Cas9 transgenes. By re-encoding the mCherry::Anillin, the authors permitted its germline expression. Likewise, by mutating all predicted piRNA target sites in the Cas9 mRNA sequences, a re-encoded Cas9 transgene acquired the capacity to be expressed stably in the worm germline. Accordingly, the authors were able to edit the *C. elegans* genome using the CRISPR/Cas9 system in this transgenic line.

A recent report in *Drosophila* showed that lack of expression of a chimeric inducible promoter UASt is caused by endogenous piRNAs from the hsp70 locus in female germ cells. Using the same strategy as Zhang *et al.*, DeLuca and Spradling (17) removed the hsp70 sequences from the UASt promoter, giving rise to UASz, an inducible promoter that works very efficiently in germline cells of *Drosophila*.

As silencing of foreign sequences by endogenous piRNAs appears to be a widespread phenomenon in *C. elegans*, Zhang and colleagues next wondered whether genes normally expressed in the germline have evolved mechanisms to avoid silencing by endogenous piRNAs. It is noteworthy that previous studies have shown that most transcripts if not all in *C. elegans* germline are targeted by two different Argonautes proteins: association of WAGO Argonaute with 22G-RNAs silences target transcripts, whereas association of CSR-1 Argonaute with 22G-RNA confers silencing-resistance to the target transcripts, allowing their expression in the germline (18,19).

Using the piRNA pairing rules described above, the authors predicted that every gene expressed in the germline contains at least one piRNA target site. However, about a half of these genes is indeed silencing-resistant (CSR-1 targets) whereas the other half is as expected silenced (WAGO-1 targets). What are the causes of these distinct regulations in the germline? The authors first noticed that the germline-silenced genes contain more predicted piRNA sites than the germline expressed genes. However, when the authors engineered and expressed additional synthetic piRNAs against the silencing-resistant genes (*pie-1*, *nop-1*, *cdk-1*, and *oma-1*), these genes remained surprisingly unsilenced, ruling out the possibility that only the number of piRNA target sites could explains the difference between silenced and silencing-resistant genes in the germline. These results confirmed that expressed germline genes exhibit a resistance to piRNA-mediated gene silencing in *C. elegans* as shown before (13,20,21). Zhang and colleagues demonstrate however that those self-genes escape piRNA silencing even if they present piRNA targeting sites.

As aforementioned, CSR-1 Argonaute-associated 22G-RNAs were proposed to be involved in a mechanism of licensing gene expression in the germline (13). However, the *nop-1* gene, which contains piRNA targeting sites, remained unsilenced in CSR-1 knock-down conditions.

In order to explain this discrepancy with the literature and to challenge the hypothesis of an inter-generational relay of CSR-1/22G-RNAs by chromatin marks deposited on target genes, the authors removed the endogenous copy of the *nop-1* gene using CRISPR/Cas9. They re-introduced a copy of the *nop-1* gene, at the original or at another location in the genome. However, whether reintroduced to its original or to an ectopic site, the *nop-1* gene remained unsilenced in the gonads. Thus, there is no clear evidence that epigenetic mechanisms such as chromatin marks are involved in licensing gene expression in the germline.

The authors finally investigated whether specific

 

nucleotide sequences in genes could provide resistance to piRNA silencing in the germline. Previously, repeats of a 10 nt motif called periodic adenine/thymine clusters (PATCs) were found in introns and promoters of some genes expressed in the germline of *C. elegans* (22) as well as in transgenes inserted in repressive chromatin domains (23). Computational analysis by Zhang and colleagues confirmed that the number of PATCs sequences inside genes are positively correlated with their expression in the germline. Moreover, they found an inverse correlation between 22G-RNA production around piRNA target sites in genes and the number of PATCs in these genes (high 22G-RNA levels if <10 PATCs; low 22G-RNA levels if >50 PATCs). These observations strongly suggested that PATCs negatively affect in *cis* the ability of piRNAs to induce and/or maintain 22G-RNAs production at their target sites.

To confirm experimentally that PATCs are bona fide signals that block silencing activity triggered by piRNAs, the authors replaced one intron of a silenced mCherry transgene with a PATC-containing intron from the *smu-1* gene, an endogenously expressed gene in the *C. elegans* germline. Strikingly, this intron swapping allowed stable expression of mCherry transgene. Accordingly, small RNA sequencing revealed that fewer mCherry antisense 22G-RNAs were produced by this PATCs-engineered mCherry transgene.

Altogether, Zhang and colleagues' findings demonstrate that, when reaching a critical number, PATCs act in *cis* to counteract 22G-RNA production and to promote gene-resistance to piRNA silencing.

Works of the Craig Mello's group supports most of the conclusions of Zhang and colleagues (15). Thus, the pairing rules of piRNAs as well as the silencing of non-self RNAs were confirmed by (24), and the capacity of *C. elegans* genes to escape from piRNA silencing in the germline and be finely regulated was analysed in details by (25,26). Altogether, the four publications demonstrate that the mode of action of piRNAs in worms diverged from the ones in other animals. However, common function looks conserved in the animal kingdom: piRNAs control the correct development of gonads by regulating finely gene expression and protect these essential organs over generations against invasions by non-self genes.

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