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RNAa in action: From the exception to the norm

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Small RNA programmed Argonautes are sophisticated cellular effector platforms known to be involved in a diverse array of functions ranging from mRNA cleavage, translational inhibition, DNA elimination, epigenetic silencing, alternative splicing and even gene activation. First observed in human cells, small RNA-induced gene activation, also known as RNAa, involves the targeted recruitment of Argonaute proteins to specific promoter sequences followed by induction of stable epigenetic changes which promote transcription. The existence of RNAa remains contentious due to its elusive mechanism. A string of recent studies in *C. elegans* provides unequivocal evidence for RNAa's fundamental role in sculpting the epigenetic landscape and maintaining active transcription of endogenous genes and supports the presence of a functionally sophisticated network of small RNA-Argonaute pathways consisting of opposite yet complementary "yin and yang" regulatory elements. In this review, we summarize key findings from recent studies of endogenous RNAa in *C. elegans*, with an emphasis on the Argonaute protein CSR-1.

Keywords: Argonaute, epigenetic memory, gene expression, RNAa, RNAe

Abbreviations: RNAa, RNA activation; RNAe, RNA-induced epigenetic silencing; piRNA, Piwi-interacting RNA; RNAi, RNA interference; WAGO, worm-specific AGO; RISC, RNA induced silencing complex; RDRP, RNA-dependent RNA polymerase; LCE, lin-4 complementary element; miRNAa, miRNA induced RNAa; TSS, transcription start site

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The gene regulatory machinery composed of small RNAs and their Argonaute protein partners have typically been synonymous with repression of gene expression given their established roles in mRNA degradation, translational suppression and heterochromatin formation.[1-3] Remarkably, small RNAs also revealed an activating side[4] in 2006 with the observance of stable and specific gene induction in mammalian cells following transfection with promoter-targeted synthetic small RNAs, a phenomenon known as RNA activation (RNAa).[5] The underlying mechanism of RNAa remained elusive however, leading to concerns that the observed gene induction was caused by secondary effects of canonical RNA interference (RNAi). New findings from a series of recent studies in *C. elegans* reveal once again an activating side of the small RNA-Argonaute pathways[6-11] and establish RNAa as a regulatory mechanism of endogenous gene expression.

RISC as an Epigenetic Activator

Argonautes are a family of highly conserved proteins which are classified in most organisms into 2 major clades: the AGO and PIWI clades. In *C. elegans*, there is an expanded family of worm-specific AGOs (WAGOs). Short RNAs depend on different Argonautes for function by forming RNA/Argonaute complexes. This requirement distinguishes Argonaute-dependent small RNA pathways from those mediated by long noncoding regulatory RNAs which function independently of Argonaute proteins. The complexes formed by short RNAs and AGOs have traditionally been referred to as the RNA-induced silencing complex (RISC) given their role as mediators of canonical RNAi.

RNA-loaded RISC typically suppresses gene expression by catalyzing the degradation of complementary mRNAs. However, when targeted to selected promoter sequences RISC can induce localized activating epigenetic marks which promote transcription (RNAa).[5] RNAa was first observed in human cells following exposure to synthetic short dsRNAs designed to target gene promoters with perfect sequence complementarity.[5,12] Like

RNAi, RNAa is also an AGO-dependent process (predominantly AGO2 and AGO1) but displays distinct *in vitro* kinetics including delayed onset and persistent activity across several cell divisions. These features contrast sharply with those of RNAi and suggest that epigenetic mechanisms are involved. New findings in *C. elegans* now support the idea that RNAa is likely an evolutionarily conserved mechanism that utilizes such small RNA-Argonaute machinery.

The Missing "Yang" in the Small RNA-Argonaute Regulatory Network of *C. elegans*

The complexity of the small RNA world uncovered in *C. elegans* surpasses that found in all other organisms thus far. In addition to miRNAs, there is an even larger small RNA system in *C. elegans* which consists of Piwi-interacting RNAs (piRNAs) and their secondary small RNAs (endo-siRNAs)[13] which are generated by piRNA targeting of mRNAs. One sub-type of these secondary RNA species is the 22G-RNAs so called because of their 22 nt length and a preferred 5′G residue.[14] The *C. elegans* genome encodes for about 30,000 piRNAs which are primarily expressed in the germline and then processed into 21 nt RNAs through multiple steps (**Fig. 1**). These 21 nt mature piRNAs typically possess a 5′U and are thus termed 21U-RNA.[15] Once bound by the Piwi protein PRG-1, 21U-RNA guides PRG-1 to mRNA sequences through imperfect base-pairing where PRG-1 further recruits EGO-1, an RNA-dependent RNA polymerase (RdRP) to amplify the silencing signal by synthesizing 22G-RNAs which are antisense to the mRNA templates (**Fig. 1**). This process requires DRH-3 (dicer-related helicase 3) and several additional factors.[14] These 22G-RNAs can then be loaded by a WAGO (WAGO1/9/10) protein to form a 22G-RNA/WAGO complex which silences foreign sequences such as transposons, pseudogenes and aberrant transcripts either at the post-transcriptional or epigenetic level. The latter nuclear silencing mechanism has been termed RNA-induced epigenetic silencing (RNAe)[14,16] which initiates transcriptional gene silencing that is then maintained across generations through the recruitment of other epigenetic factors such as H3K9 methyltransferase and heterochromatin protein 1 (HP1).[17] RNAe thus serves as a surveillance mechanism to maintain the integrity of the germline genome.[14]

Given the large number of piRNAs that do not require strict base-pairing for target binding, any mRNA could theoretically be targeted by piRNAs for silencing.[18] Indeed, 50% of 22G-RNAs found in *C. elegans* target endogenous mRNAs.[14] This raises the question: how are 'self' RNAs distinguished from 'non-self' RNAs and protected from RNAe-mediated silencing? Interestingly, 22G-RNAs antisense to "self" RNAs interact with a different Argonaute protein, CSR-1 (chromosome segregation and RNAi deficiency 1), to form the 22G-RNA/CSR-1 complex which then binds nascent transcripts in a sequence-specific manner to associate with local chromatin[19] (**Fig. 1**). Although CSR-1 has been demonstrated to possess slicer activity *in vitro,*[20] there is no evidence to suggest that *in vivo* it cleaves the transcripts which it binds.[19] It is therefore plausible that the 22G-RNA/CSR-1 pathway is responsible for marking and protecting "self" RNAs from being silenced by the 22G-RNA/WAGO pathway, as has been suggested by Beth et al.[7] Previous studies have already shown that perturbation of this pathway causes defects in chromosome segregation, histone pre-mRNA processing and sterility.[6,19,21] New studies[7-9,22] have now proved that the 22G-RNA/CSR-1 system can do more than just passively defending "self" RNAs; it can actively promote their expression via epigenetic mechanisms as discussed below (**Fig. 1**).

RNAa in *C. elegans*

In the work by Wedeles et al.,[9] the authors first observed that CSR-1 interacts with RNA polymerase II (RNAP II) and is recruited to its target gene loci via nascent transcripts. To further assess the functions of CSR-1, they then employed an *in vivo* RNA tethering assay in which a gfp transcript containing phage lambda box b RNA hairpins (gfp::boxb) is expressed under the control of a germline promoter, and a fusion CSR-1 protein containing a phage lambda N anti-termination protein fragment (CSR-1::λN) is also expressed in the same cells. In this system, CSR-1 protein can be specifically tethered to the gfp transcript due to the ability of the Lambda N peptide to recognize box b hairpin RNA.[23] The authors found that in germline, tethering CSR-1 to gfp can stably protect the gfp transgene from piRNA-induced silencing. Moreover, tethering CSR-1 to an already silenced gfp can reactivate its expression *in trans*. Even more interesting is that in the continued presence (a few generations) of gfp::boxb and CSR-1::λN in the same strain, *cdk-1:gfp,* an allele silenced by RNAe (referred to as RNAe allele), could be activated although no CSR-1 is tethered to its mRNA. As suggested by the authors, it is possible that small RNAs derived from the gfp::boxb allele target CSR-1 to and activate the expression of cdk-1;fgp. Together, these analyses revealed that the CSR-1 pathway functions to maintain active germline gene expression by opposing the piRNA surveillance mechanism.

Using a different genetic approach, Seth et al.[7] first examined the role of CSR-1 in the process of epigenetically transmitted and RNA-induced transactivation (also named as RNAa). A previous study by the same group has found that when a worm strain carrying an RNAe transgene, gfp::cdk-1, which is silenced via the RNAe pathway as described above, is crossed with a strain carrying oma-1::gfp transgene which is resistant to RNAe, the latter transgene could activate the expression the RNAe transgene gfp::cdk-1 in the F1 progeny.[17] In the new study, the authors found that oma-1::gfp RNAa allele failed to activate the gfp::ckd-1 RNAe allele when *CSR-1* is inactivated by either RNAi or genetic mutation, indicating that *CSR-1* is required for RNAa.[7]

To find out whether RNAa could stably activate an RNAe transgene, the authors set up a series of crossing experiments to establish double-transgenic lines expressing 2 transgenes with either RNAa or RNAe activity, then separated the 2 transgenes by outcrossing to a wild-type

strain. They found that conversion of a silent RNAe allele to a permissive state requires multiple generations of continued exposure to RNAa. For example, the expression of gfp::cdk-1, the RNAe transgene, was activated in the presence of oma-1::gfp, the RNAa transgene, but disappeared when the RNAa transgene was crossed away in the next generation. When they cultured the double-transgenic strain oma-1::gfp;gfp::cdk-1 for over 10 and 30 generations before separating the 2 transgenes, they observed respectively one full generation and 10 generations of RNAe gene expression in the absence of RNAa allele. These data suggest that RNAa can counteract the effect of RNAe and be epigenetically transferred across generations, albeit with diminishing efficacy.

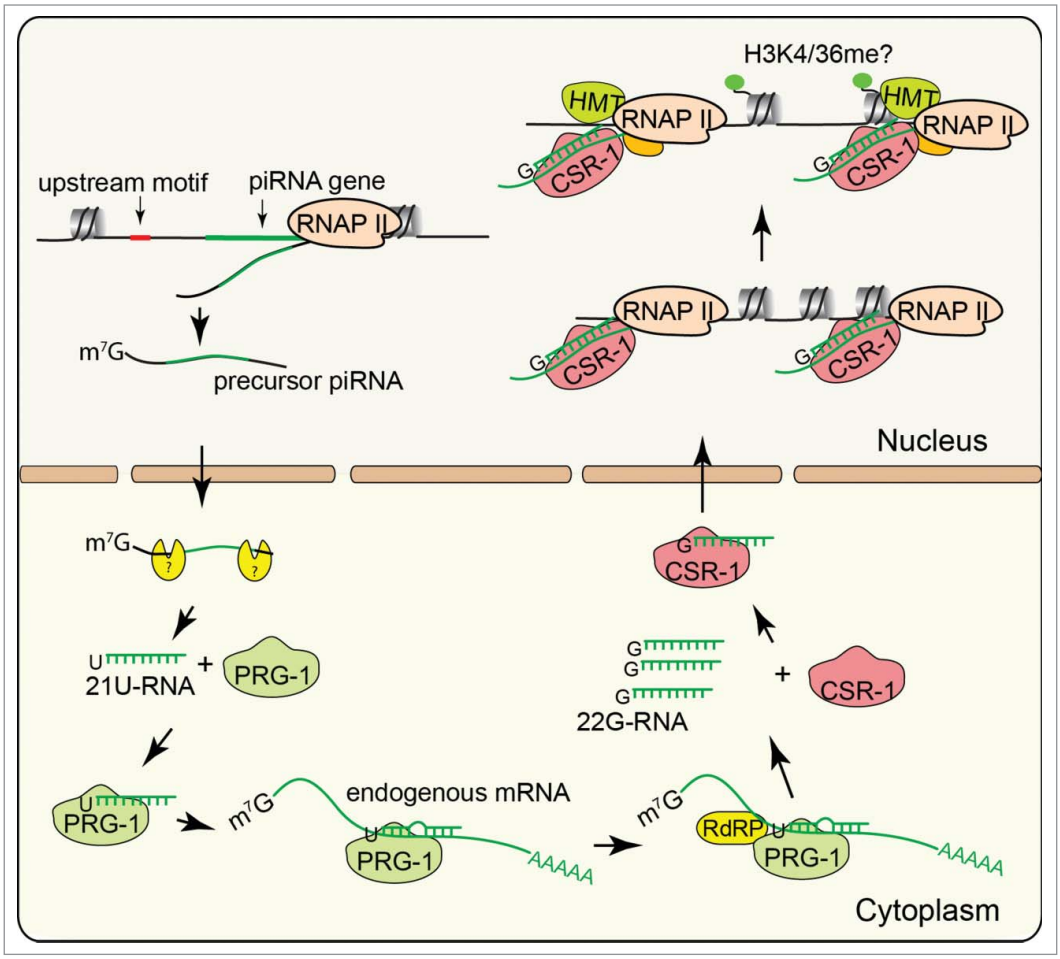

Figure 1. RNAa in *C. elegans*. piRNA genes encoded in the genome as clusters are individually transcribed in a process that requires a conserved upstream motif. The resulted piRNA precursors are then processed into mature 21U-RNAs in the cytoplasm through several undefined steps. The 21U-RNAs form a complex with PRG-1 protein and guide the complex to non-perfectly matched targets on endogenous mRNAs where PRG-1 further recruits RdRP to generate secondary 22G-RNAs. After being loaded by CSR-1, the 22G-RNAs then target CSR-1 to cognate nascent transcripts to interact with RNAP II and local chromatin where CSR-1 can further recruit histone modifying enzymes such as histone methyltransferases (HMTs) to promote epigenetic activation (H3K4me or H3K36me3).

In another study also from the Mello group, Conine et al. further examined the functional role of RNAa mediated by the CSR-1/22G-RNA pathway in spermatogenesis in *C. elegans*. The group has previously shown that ALG3/4 Argonautes (paralogous AGO-clade members) can engage 26G-RNAs antisense to mRNAs expressed during spermatogenesis.[24] In the new study, they showed that the primary function of the ALG3/4 pathway is to promote the transcription of spermiogenesis genes; as evidenced by increased H3K4me2 at their promoters, a chromatin modification associated with active transcription.[8] Both *ALG3/4* double and *CSR-1* single mutants resulted in partial temperature-dependent sterility, suggesting these 2 systems function in the same pathway. Indeed, *csr-1*, *alg-3* and *alg-4* triple mutant males showed defects in spermatogenesis leading to complete sterility at 25°C. They further showed that the CSR-1/22G-RNA pathway shares most target genes with the ALG3/4 26G-RNA pathway, and promotes expression of ALG-3/4 target mRNAs. Despite their similar target genes and function, ALG-3/4 proteins are expressed only during spermatogenesis, whereas CSR-1 protein is expressed beyond spermatogenesis and remains abundant in sperms. They then asked whether the 2 pathways might cooperate to propagate epigenetic memory of male-specific gene expression from one generation to the next via RNAa. By repeated backcrossing heterozygous hermaphrodites to homozygous mutant males, they found that the crossing led to a progressive loss of fertility which can be rescued by mating to wild-type males. These data suggest that the CSR-1/22G-RNA mediated RNAa pathway is capable of transmitting a transgenerational epigenetic signal.

RNAa mediated by the 22G-RNA/CSR-1 pathway operates not only in germ cells but also in somatic cells as shown in the study by Cecere et al.[10] By profiling nascent transcripts using global run-on sequencing (GRO-seq) in wild-type worms and a strain hypomorphic for *csr-1*, the authors found that 22G-RNA target genes were significantly enriched among genes whose transcription was decreased in the *csr-1* hypomorphic strain compared to

wild-type worms. These genes are represented by genes highly expressed at the particular developmental stages of worms (later L3 and early L4) used in the analysis, including genes expressed ubiquitously and germline-specifically. The downregulation of these genes appears to be 22G-RNA dependent since they are also downregulated in a *drh-3* mutant strain which depleted 22G-RNAs. Interestingly, transcripts antisense to those downregulated genes were globally increased in both *csr-1* and *drh-3* mutants, and are genes that do not express or express low at later L3 and early L4 stages. They further showed that CSR-1 is enriched in euchromatic regions and interacts with RNAP II in an RNA-dependent manner. These findings suggest that 22G-RNA targets CSR-1 to nascent sense transcripts where CSR-1 interacts with RNAP II to stabilize its transcription while restricting RNAP II transcription in the opposite direction. Together, this study not only corroborates the observations from the Claycomb and Mello groups but also suggests that the CSR-1/22G-RNA pathway functions to globally reinforce the transcription of actively transcribed genes expressed at certain developmental stages. How CSR-1 promotes RNAP II transcription of its target genes remains to be elucidated.

miRNAa in *C. elegans*

Naturally occurring miRNAs may also have the ability to induce RNAa of genes whose promoters contain imperfect binding sites for the miRNAs,[25] a phenomenon which can be referred to as miRNA-induced RNAa (miRNAa). Until very recently, miRNAa has only been observed in cultured mouse and human cells.[25-27]

Turner et al. now showed that lin-4, the first miRNA discovered in *C. elegans* and important for the timing of stem cell fate decisions,[28] can activate its own transcription by binding to its own promoter.[11] By analyzing the promoter sequence of lin-4, the authors found a putative lin-4 complementary element (LCE) conserved in the lin-4 promoters of several nematode species. Deletion of the LCE in the lin-4-GFP reporter transgenic animals resulted in decreased transcriptional activity of the lin-4 promoter suggesting that the LCE is a functional cis-regulatory element potentially trans-regulated by lin-4. To test this idea, the authors went on to cross the GFP reporter line into a strain with mutated lin-4 that had no production of endogenous wild-type lin-4. Compared to the wild-type strain, the mutant animals had 3 times less GFP transcriptional activity with decreased RNAP II association with lin-4 promoter. Furthermore, overexpression of lin-4 in the lin-4-GFP reporter animals resulted in increased lin-4 transcriptional activity compared to control animals. These results point to a model in which mature lin-4 functions as a trans-acting factor and binds to the conserved LCE in its promoter to enhance promoter transcription by recruiting RNAP II and possibly additional protein factors. Therefore, the work by Turner et al. provided the first example of miRNAa that functions *in vivo* and to regulate transcripts other than mRNA. Future experiments are needed to identify the Argonaute proteins and chromatin factors involved in this process and the prevalence of miRNAa in *C. elegans*. Similar positive feedback loop regulation has been demonstrated with another well-known miRNA, let-7,[29] although a non-transcriptional mechanism that leads to enhanced maturation of let-7 seems to be responsible for the regulation.[29]

Potential RNAa Regulatory Networks in Human Cells

Accumulating evidence supports an intricate interplay between the small RNA machinery and chromatin in mammalian cells.[30] In analogy to *C. elegans* RNAe and RNAa, this additional layer of epigenetic regulation may constitute an epigenetic memory transmitted by small RNAs across cell divisions and may play important roles in cell physiology and disease. For example, cancer cells may exploit RNAa to sustain active expression of genes necessary for growth and survival. A recent study of genome-wide AGO-chromatin interaction carried out in human cancer cells by our group suggests that AGO1 interacts with RNAP II and binds to thousands of promoters of actively transcribed genes.[31] Similar to the effects of CSR-1 binding on chromatin in *C. elegans*[22] the impact of AGO1-promoter interactions is largely positive on gene expression.[31] The interactions appear to be small RNA/miRNA dependent since they can be affected by perturbing miRNA biogenesis[31] Apart from miRNAs, small RNAs that are equivalent to the *C. elegans* 22G-RNAs and capable of mediating AGO1 binding have yet to be identified in mammalian cells. We speculated that nascent promoter transcripts may directly recruit AGOs to chromatin,[30] given the spatial overlapping of AGO1 binding sites on chromosomes[31] and the origin of previously identified promoter transcripts known as TSSa-RNAs (transcription start site-associated RNAs)[32] or tiRNAs (transcription-initiation RNAs).[33] In this regard, a very recent study[34] has identified a new class of non-canonical miRNAs which are originated from the transcription start site (TSS) of hundreds of protein coding genes as single stranded transcripts and form hairpin structures which is processed by Dicer into 21–24 nt miRNAs termed TSS-miRNAs. Importantly, TSS-miRNAs are bound by AGO2,[34] suggesting that they could potentially be involved in RNAa by mediating AGO-chromatin interactions.

Conclusions

Initially, the finding that the RNAi machinery is also involved in gene upregulation appeared to be counterintuitive. A cascade of recent *C. elegans* studies corroborates this concept by showing concrete evidence for the existence of RNAa in worm germline and somatic cells. Collectively, these studies demonstrate a positive role for an endogenous Argonaute pathway in gene regulation. While the detailed molecular mechanisms for how 22G-RNA/CSR-1 complex activates target genes await to be solved, it will be very interesting to examine whether similar piRNA-initiated RNAa pathway exists in mammalian cells and whether other small RNAs such as TSS-miRNAs could target

Argonautes to chromatin loci to maintain active expression of gene pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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