

Diversity of the piRNA pathway for nonself silencing: worm-specific piRNA biogenesis factors

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The PIWI-interacting RNA (piRNA) pathway protects animal germline cells from transposable elements and other genomic invaders. Although the genome defense function of piRNAs has been well established, the mechanisms of their biogenesis remain poorly understood. In this issue of *Genes & Development*, three groups identify novel factors required for piRNA biogenesis in *Caenorhabditis elegans*. These works greatly expand our understanding of the piRNA pathway in worms, highlighting both its shared and its unique properties.

piRNAs: genome defenders in the germline

PIWI-interacting RNAs (piRNAs) are predominantly germline-specific small RNAs found in animals that are characterized by their 20- to 35-nucleotide (nt) length, a 2'-O-methyl modification at the 3' end, and a strong bias for uridine at the 5' end (1U). piRNAs bind to PIWI subfamily proteins of the Argonaute (Ago) family and guide them via base-pairing to target RNAs. Like many Ago family proteins, PIWI proteins possess endonucleolytic "slicer" activity and can induce post-transcriptional silencing via cleavage of complementary RNAs (Brennecke et al. 2007; Gunawardane et al. 2007; De Fazio et al. 2011; Reuter et al. 2011). On the other hand, some nuclear-localized PIWI proteins, such as fly Piwi and mouse Miwi2, are known to induce transcriptional silencing via either the heterochromatic histone mark H3K9me3 or DNA methylation in a manner that is independent of target cleavage (Aravin et al. 2008; Kuramochi-Miyagawa et al. 2008; De Fazio et al. 2011; Wang and Elgin 2011; Sienski et al. 2012).

The primary role of the piRNA pathway is to protect germline cells from genomic invaders such as transposons and other "nonself" elements, thereby ensuring transgenerational genome integrity. Indeed, the loss of PIWI proteins or piRNAs causes transposon activation and severe defects in gametogenesis (Cox et al. 1998; Deng and Lin

2002; Kuramochi-Miyagawa et al. 2004, 2008; Vagin et al. 2006; Aravin et al. 2007; Carmell et al. 2007; Houwing et al. 2007; Batista et al. 2008; Das et al. 2008; Li et al. 2009). While the genome defense function of piRNAs is well established and generally evolutionally conserved, organisms use piRNAs in remarkably flexible ways to achieve this function.

In fly ovaries and testes as well as in the prepachytene stage of mouse spermatogenesis, piRNAs are largely made from transposons and transposon-derived repetitive sequences, which target transposons themselves to silence (Fig. 1). In contrast, worms produce a pool of piRNAs—called 21U-RNAs in *Caenorhabditis elegans* (as they are precisely 21 nt in length with 5' uridine)—from intergenic and intronic regions of protein-coding genes (Ruby et al. 2006). 21U-RNAs are loaded into PRG-1, one of *C. elegans* PIWI proteins (Batista et al. 2008; Das et al. 2008; Wang and Reinke 2008). 21U-RNAs in PRG-1 tolerate several mismatches in the selection of their targets, allowing them to recognize virtually any sequence (Bagijn et al. 2012; Lee et al. 2012). On the other hand, the *C. elegans* Ago protein CSR-1 loads a broad repertoire of endogenous siRNAs named 22G-RNAs (22 nt in length with 5' guanine) that are complementary to thousands of protein-coding genes expressed in the germline (Claycomb et al. 2009). Strikingly, CSR-1 acts to protect the protein-coding genes from PRG-1-mediated silencing and activate their expression (Lee et al. 2012; Shirayama et al. 2012; Conine et al. 2013; Seth et al. 2013; Wedeles et al. 2013). Thus, while the fly and mouse prepachytene piRNA systems distinguish self and nonself sequences to silence their enemies specifically, the worm 21U-RNA system scans every sequence in preparation for any potential threats and instead uses "tagging" by CSR-1-loaded 22G-RNAs to avoid self-attack (Fig. 1). Mice produce an enigmatic class of piRNAs, called pachytene piRNAs, which arise from unannotated

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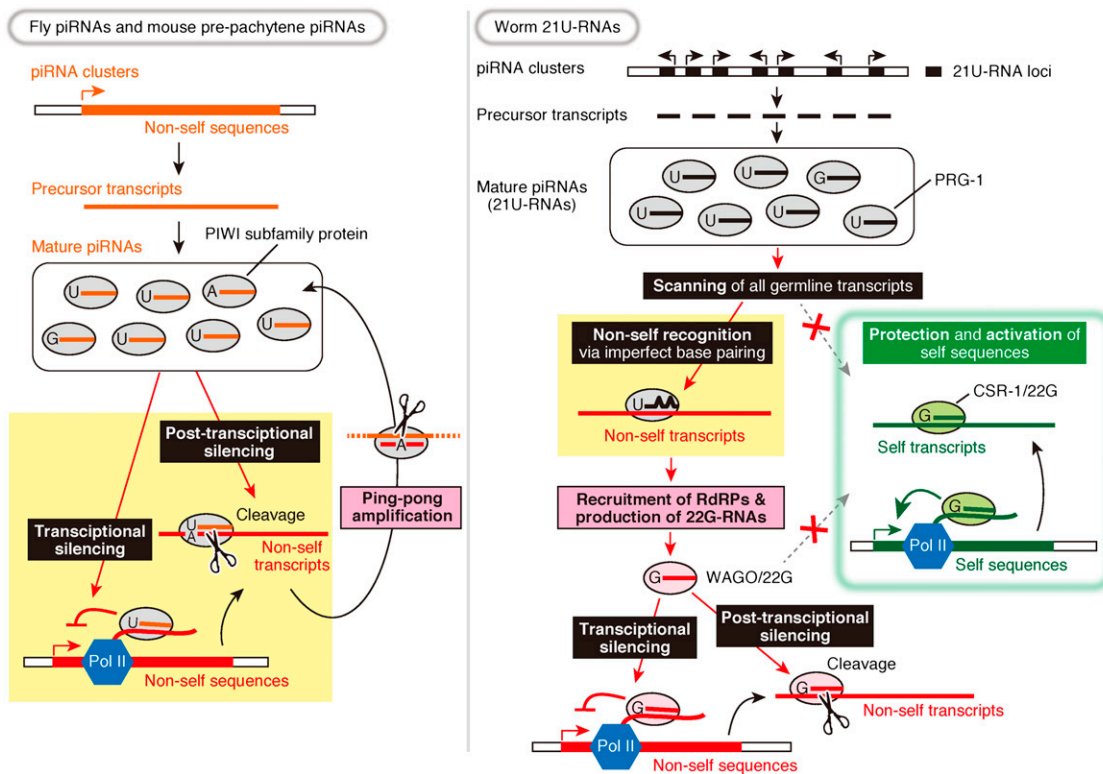


Figure 1. Similarities and differences between the piRNA pathway in flies and mice and that in worms. Fly and mouse piRNA clusters generate long single-stranded piRNA precursor transcripts from which multiple piRNAs are produced. The mature piRNAs are loaded into PIWI proteins that target transposon RNAs with sequence complementarity and silence them by cleavage (post-transcriptional silencing). The cleaved transposon RNAs become new piRNA precursors and amplify piRNAs against the transposons (ping-pong amplification). A subset of PIWI proteins is localized to the nucleus and induces transcriptional silencing. In contrast, each 21U-RNA locus codes a single 21U-RNA precursor in worms. 21U-RNAs in PRG-1 scan virtually all germline transcripts to detect potential harmful exogenous sequences, allowing several mismatches for recognition. To avoid self-attack, the CSR-1/22G-RNA pathway marks endogenous protein-coding genes for their protection from silencing and their activation. When the PRG-1/21U-RNA complex recognizes its targets, RNA-dependent RNA polymerases (RdRPs) are recruited, and another class of 22G-RNAs is produced. These 22G-RNAs are loaded into worm-specific Ago proteins (WAGOs) and silence their targets at both transcriptional and post-transcriptional levels. The role of piRNAs is different between flies/mice and worms (highlighted in yellow), but both achieve the same purpose of nonself silencing. Note that worms lack the ping-pong amplification but instead have the WAGO/22G-RNA pathway, thereby amplifying silencing signals (highlighted in pink).

intergenic regions during postnatal spermatogenesis; their targets remain unclear (Girard et al. 2006; Grivna et al. 2006).

piRNA biogenesis in flies and mice

Compared with microRNAs and siRNAs, much less is known about the mechanism of piRNA biogenesis. piRNAs are generally processed from longer, single-stranded precursor RNAs. Transcription of piRNA precursors requires specific transcription factors, which vary according to species and the precise type of piRNAs. For example, mouse pachytene piRNAs are transcribed by A-Myb, a master regulator of male meiosis (Li et al. 2013), while transcription from the fly *flamenco* locus, a major unistrand piRNA cluster, requires the transcription factor Cubitus interruptus (Goriaux et al. 2014). On the other hand, piRNA production from dual-strand clusters in flies depends on Rhino (a variant of heterochromatin protein 1

[HP1]) and Cutoff (homologous to the yeast transcription termination factor Rai1) (Klattenhoff et al. 2009; Pane et al. 2011). In all of these cases, piRNA precursors are produced by RNA polymerase II as long, continuous single-stranded transcripts, which can span >100 kb.

Processing of these long piRNA precursors into mature piRNAs can be divided into two distinct modes: primary processing and ping-pong amplification. Primary processing involves the endonucleolytic cleavage of precursor RNAs by Zucchini/MitoPLD (Ipsaro et al. 2012; Nishimasu et al. 2012). Next, a subset of PIWI proteins (e.g., Aubergine in flies) preferentially incorporates 1U fragments with the aid of the Hsp90 chaperone machinery (Kawaoka et al. 2011; Olivieri et al. 2012; Preall et al. 2012; Izumi et al. 2013). Subsequently, their 3' ends are trimmed by a 3'-to-5' exonuclease named Trimmer (whose identity remains unknown) to the mature piRNA length (Kawaoka et al. 2011), followed by 2'-O-methylation by Hen1 (Horwich et al. 2007; Kirino and Mourelatos 2007; Ohara et al. 2007;

Saito et al. 2007). Thus, the 1U bias is a molecular signature of primary piRNAs.

Ping-pong amplification, or the secondary piRNA biogenesis pathway, drastically increases the production of piRNAs and is coupled with slicer-dependent destruction of active transposons. In this scheme, primary PIWI proteins first cleave their complementary target RNAs across from the 10th position of their 1U piRNA guides. The 3' fragments of this cleavage possess adenine at the 10th position (10A) and are then incorporated into another subset of PIWI proteins (e.g., Ago3 in flies) as new piRNA precursors. Finally, their 3' ends are matured by trimming and 2'-O-methylation, as in the primary processing pathway. The resultant secondary piRNAs can, in turn, cleave their complementary target RNAs (from which primary piRNAs originate), thereby fueling the amplification loop. Accordingly, primary and secondary piRNAs show precisely 10-nt overlaps at their 5' ends, with characteristic 1U and 10A signatures, respectively (Aravin et al. 2007, 2008; Brennecke et al. 2007; Gunawardane et al. 2007).

In addition to PIWI proteins, many other proteins are required for the production of piRNAs, including Tudor family proteins and ATP-dependent RNA helicases. Although the precise role of each factor is still unclear, their involvement in the piRNA biogenesis is fundamentally conserved between flies and mice (Malone et al. 2009; Reuter et al. 2009; Shoji et al. 2009; Vagin et al. 2009; Frost et al. 2010; Kuramochi-Miyagawa et al. 2010; Olivieri et al. 2010, 2012; Saito et al. 2010; Zheng et al. 2010; Handler et al. 2011; Liu et al. 2011; Watanabe et al. 2011; Ipsaro et al. 2012; Nishimasu et al. 2012; Preall

et al. 2012; Xiol et al. 2012; Pandey et al. 2013; Saxe et al. 2013).

piRNA biogenesis in *C. elegans*

Like fly and mouse piRNAs, precursors of *C. elegans* 21U-RNAs are first transcribed by RNA polymerase II. Unlike fly and mouse piRNAs, however, each 21U-RNA locus in *C. elegans* produces an independent short transcript of merely ~26 nt (Ruby et al. 2006; Cecere et al. 2012). 21U-RNAs can be divided into two groups: type I and the less abundant type II (Fig. 2; Gu et al. 2012). Type I 21U-RNA loci accumulate within two piRNA cluster regions on chromosome IV and have a conserved 8-nt motif (CTGTTTCA) located ~40 nt upstream of each locus. The motif is recognized by Forkhead (FKH) family transcription factors and is essential for type I 21U-RNA production (Ruby et al. 2006; Cecere et al. 2012). In contrast, type II 21U-RNA loci have no apparent upstream motif and are dispersed throughout the genome (Gu et al. 2012). Regardless of their types, precursors of 21U-RNAs begin precisely 2 nt upstream of mature 21U-RNAs (Gu et al. 2012). They are thought to first be decapped, the 5' 2-nt region is removed, and then they are loaded into PRG-1. Subsequently, the 3' end is believed to be shortened by ~3 nt to generate the mature 21-nt length and the 2'-O-methylated by HENN-1, like piRNAs are in other animals (Fig. 2; Billi et al. 2012; Kamminga et al. 2012; Montgomery et al. 2012).

Unlike flies and mice, *C. elegans* does not produce secondary piRNAs via the slicer-dependent ping-pong amplification loop. In fact, the slicer activity of PRG-1 is dispensable for target silencing (Bagijn et al. 2012; Lee

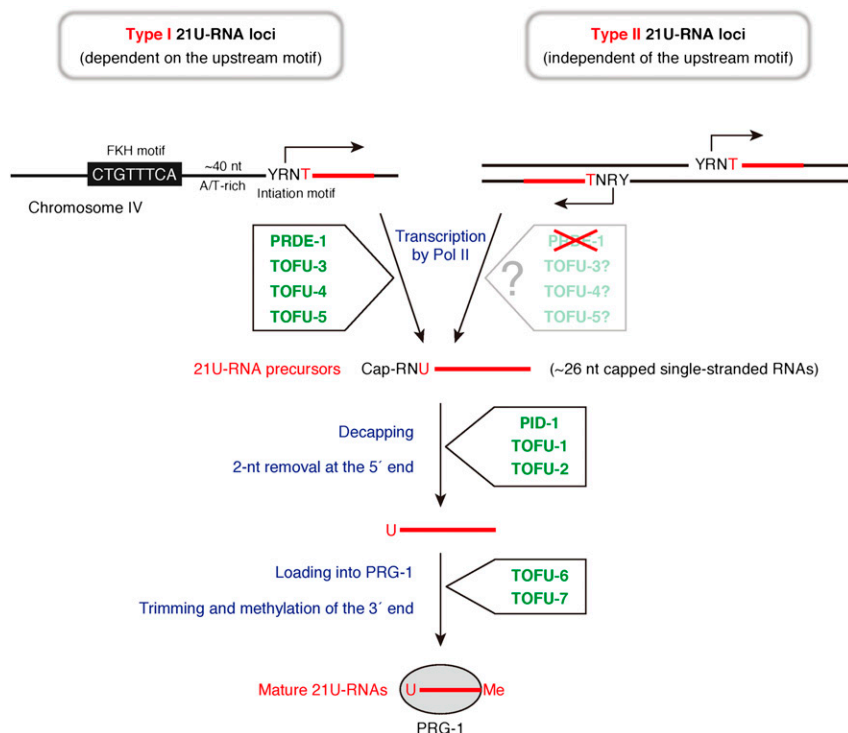


Figure 2. Model of the 21U-RNA biogenesis and novel factors required for each process. *C. elegans* 21U-RNA loci are divided into two groups, type I and type II, according to the presence of the upstream conserved 8-nt motif, which is recognized by FKH family transcription factors. Type I 21U-RNA loci have this motif and are clustered on chromosome IV. On the other hand, type II 21U-RNA loci lack the upstream motif and frequently present in both directions on the genome. The YR motif (Y = C or T; R = A or G) is important for efficient transcription initiation. Each 21U-RNA locus produces ~26-nt capped 21U-RNA precursors. After initial processing, including decapping and removal of 2 nt at the 5' end, the precursor RNA is thought to be loaded into PRG-1, and its 3' end is further processed by trimming. The newly identified 21U-RNA biogenesis factors (green) and the steps where they function are shown.

et al. 2012). Instead, target recognition by 21U-RNAs in PRG-1 recruits RNA-dependent RNA polymerases and triggers massive production of secondary siRNAs (22G-RNAs). These 22G-RNAs are loaded into worm-specific Agos (WAGOs), which include the nuclear Ago protein HRDE-1 (WAGO-9) and mediate target silencing at both post-transcriptional and transcriptional levels (Fig. 1; Das et al. 2008; Bagijn et al. 2012; Lee et al. 2012). The self-protective CSR-1/22G-RNA pathway can counteract both types of silencing by WAGO-loaded 22G-RNAs (Lee et al. 2012; Shirayama et al. 2012; Conine et al. 2013; Seth et al. 2013; Wedeles et al. 2013). Strikingly, once genes are either silenced by WAGO/22G-RNAs or activated by CSR-1/22G-RNAs, their silent/active status can be stably inherited over generations and thereby mediates an epigenetic memory (Ashe et al. 2012; Luteijn et al. 2012; Shirayama et al. 2012; Conine et al. 2013; Seth et al. 2013; Wedeles et al. 2013). Thus, *C. elegans* uses two types of 22G-RNAs with precisely opposite functions: CSR-1-loaded 22G-RNAs for self-protection and activation upstream of 21U-RNAs and WAGO-loaded 22G-RNAs for nonself silencing downstream from 21U-RNAs (Fig. 1).

Novel piRNA biogenesis factors in *C. elegans*

Twenty-One-u Fouled Ups (TOFUs)

While an understanding of the worm piRNA (21U-RNA) pathway is emerging, little is currently known about factors required for their production. In fact, most of piRNA biogenesis factors identified in other animals, including the endonuclease Zucchini/MitoPLD, are not conserved in worms. In this issue of *Genes & Development*, three groups (de Albuquerque et al. 2014; Goh et al. 2014; Weick et al. 2014) report on their discoveries of novel factors that are involved in the production of 21U-RNAs in *C. elegans*.

Goh et al. (2014) performed an RNAi screen covering >80% of annotated *C. elegans* protein-coding genes and identified 22 genes that affect mature 21U-RNA levels (named TOFUs). More than half of identified TOFU genes have no apparent orthologs in other animals. Interestingly, genome-wide RNAi screenings for piRNA factors in flies did not hit existing TOFU homologs (Czech et al. 2013; Muerdter et al. 2013). To determine how each TOFU functions, Goh et al. (2014) focused on the top seven TOFUs with the strongest reduction in mature 21U-RNA levels (TOFU1–7) and analyzed 21U-RNA precursor levels upon their depletion. The investigators observed a clear accumulation of ~26-nt 21U-RNA precursors in the absence of TOFU-1 and TOFU-2, indicating that these two factors are required for processing after precursor RNA production (Fig. 2). Because both proteins have an ATP-binding domain and TOFU-2 has similarity to the DEAD-box helicase DDX1 in mammals, these are expected to function in ATP-dependent processes, perhaps involving rearrangement of RNA–protein complexes. On the other hand, depletion of TOFU3–5 resulted in a significant reduction of precursor RNAs. Therefore, these factors are required for the production or stability of 21U-RNA precursors (Fig. 2). TOFU-3

is a SUMO-related protease, and its mammalian homolog, SENP7, is known to directly interact with HP1 α and regulate its localization to pericentric heterochromatin (Maison et al. 2012). TOFU-5 has a Swi3, Ada2, N-CoR, and TFIIB (SANT) domain, a DNA-binding domain structurally related to that of Myb and found in various transcription regulatory factors. Thus, these factors presumably play a role in the transcription of precursors of abundant type I 21U-RNAs, although the investigators did not explicitly examine whether TOFU3–5 are also involved in the production of type II 21U-RNAs, a small population of 21U-RNAs that does not have the conserved upstream motif. Depletion of the other two TOFUs (TOFU-6 and TOFU-7) decreased mature 21U-RNA levels twofold to threefold but did not have any substantial effect on precursor RNA levels. Accordingly, the investigators speculate that TOFU-6 and TOFU-7 function downstream from initial processing of 21U-RNA precursors, e.g., PRG-1-loading or localization of 5' removed precursors (Fig. 2). Since TOFU-6 and TOFU-7 have an RNA recognition motif (RRM) and a hnRNP K homology (KH) domain, respectively, these factors presumably bind to precursor RNAs. Furthermore, TOFU-6 has a Tudor-like domain, implying a commonality with fly and mouse piRNA biogenesis pathways.

Goh et al. (2014) also found seven suppressor genes of 21U-RNA production from their screen. Intriguingly, one of them is CSR-1, the Ago protein that loads 22G-RNAs to protect protein-coding transcripts, including *prg-1* mRNA, from 21U-RNA-mediated silencing, as described above (Lee et al. 2012; Shirayama et al. 2012; Conine et al. 2013; Seth et al. 2013; Wedeles et al. 2013). Although 22G-RNAs in CSR-1 are thought to bind and mark self transcripts without causing their silencing, the investigators observed an elevation of *prg-1* mRNA in *csr-1* RNAi and *csr-1* mutants. Up-regulation of *prg-1* mRNA and a representative 21U-RNA was also seen in the mutants of other CSR-1/22G-RNA pathway factors, EGO-1 (an RNA-dependent RNA polymerase that synthesizes CSR-1-type 22G-RNAs) and EKL-1 (a Tudor domain protein required for the production of both CSR-1- and WAGO-type 22G-RNAs) (Claycomb et al. 2009; Gu et al. 2009). The investigators discuss the possibility that the CSR-1/22G-RNA pathway normally represses the *prg-1* mRNA level, while a previous study showed no significant change in the protein level of PRG-1 in the *csr-1* mutant (Claycomb et al. 2009). Although further analysis is warranted, the notion that the CSR-1/22G-RNA pathway negatively regulates the PRG-1/21U-RNA pathway highlights the intricate relationship between the two pathways.

piRNA-induced silencing defective-1 (pid-1)

de Albuquerque et al (2014) performed ethyl methanesulfonate (EMS) screening with a 21U-RNA sensor and identified PID-1. PID-1 is an uncharacterized germline-specific small protein with no known orthologs in other animals. In *pid-1* mutants, mature 21U-RNAs derived from both type I and type II loci were markedly reduced, but precursor RNAs were accumulated. Thus, PID-1 is a general

factor that is required for 21U-RNA biogenesis, acting in a process downstream from precursor RNA production (Fig. 2). In *pid-1* mutants, a small amount of mature 21U-RNAs with normal properties are still generated, and the structure of accumulated precursor RNAs is also normal. These observations suggest that PID-1 deletion slows down the processing rate of precursor RNAs. PID-1 mostly localizes to the cytoplasm, but a small fraction is present in the nucleus. In addition, PID-1 has both a putative nuclear localization signal (NLS) and a nuclear export signal (NES). Based on these protein features, de Albuquerque et al. (2014) discuss the possibility that PID-1 is involved in the transport of precursor RNAs to the processing sites. Given that PID-1 has no apparent RNA-binding motif, additional factors are expected to participate in this process. Further characterization of PID-1 and its potential link to other factors will be needed to understand the exact function of PID-1.

piRNA silencing defective 1 (prde-1)

Weick et al. (2014) also conducted EMS mutagenesis screening using a 21U-RNA sensor and identified PRDE-1. PRDE-1 has a protein kinase-like domain, but amino acid residues critical for ATP binding in the kinase domain are not conserved. No clear orthologs of PRDE-1 are found in other animals. PRDE-1 is exclusively expressed in germline cells and localizes to specific nuclear foci. Using DNA-FISH in combination with immunostaining, the investigators revealed that PRDE-1 associates with the 21U-RNA cluster regions on chromosome IV. In *prde-1* mutants, a pronounced reduction of mature type I 21U-RNAs and their precursor RNAs was observed without apparent effect on type II 21U-RNAs. Thus, PRDE-1 is a factor specifically required for production of type I 21U-RNA precursors (Fig. 2). Considering its characteristic localization to chromosome IV, the investigators discuss a model in which PRDE-1 plays a role in recruiting RNA polymerase II to the FKH motif. Future studies examining the relationship between PRDE-1 and FKH transcription factors will clarify the exact role of PRDE-1 in type I 21U-RNA biogenesis.

Weick et al. (2014) compared the change in gene expression between *prg-1* and *prde-1* mutants and revealed the functional difference between type I and type II 21U-RNAs. Focusing on genes up-regulated in the *prg-1* mutant but not in the *prde-1* mutant, Weick et al. (2014) discovered a significant enrichment in innate immune genes. These findings provide insights into the evolution of the two types of 21U-RNAs and the regulation of gene expression by the 21U-RNA pathway. Their data also demonstrate that type II 21U-RNAs act upstream of WAGO/22G-RNAs, similar to type I 21U-RNAs.

Concluding remarks

Taken together, these three studies elucidate novel factors required for 21U-RNA production, representing the first step in understanding the mechanism of 21U-RNA biogenesis. Identification of PRDE-1, a factor exclusively

required for type I 21U-RNA production, underscores that there are two distinct classes of piRNAs in worms. Most of the identified 21U-RNA biogenesis factors do not have orthologs in other species, perhaps reflecting the elegant interplay between the 21U-piRNA pathway and the two distinct 22G endogenous siRNA pathways as a means to specifically and efficiently silence nonself genes in worms. Moreover, the genes identified by the three screenings did not overlap, implying that there are even more 21U-RNA-related factors to be revealed. Obviously, the next challenge is to clarify the role of each factor and the connections among them. Also of worthwhile study is the regulation of gene expression by the piRNA pathway in worms. As indicated by Weick et al. (2014) the two types of 21U-RNAs target different sets of genes, with type II 21U-RNAs specifically repressing innate immune genes. In this regard, it would be interesting to examine whether the production of type II 21U-RNAs is in turn regulated by innate immune responses. Given that the CSR-1/22G-RNA pathway negatively regulates the 21U-RNA production as reported by Goh et al. (2014), further studies focusing on the regulation of the piRNA pathway and its relationship with other small RNA pathways will be important to understand the biological significance of gene expression control by the piRNA pathway in *C. elegans*.

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