An RNA-Mediated Silencing Pathway Utilizes the Coordinated Synthesis of Two Distinct Populations of siRNA

Eleanor M. Maine[1,*]
[1]Department of Biology, Syracuse University, 107 College Place, Syracuse, NY 13244, USA
*Correspondence: emmaine@syr.edu
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In this issue of *Molecular Cell*, Gent et al. (2010) describe the participation of two siRNA populations, generated by two different RNA-directed RNA polymerases, in a pathway to silence expression of endogenous genes in *Caenorhabditis elegans*.

Small regulatory RNAs mediate transcriptional and posttranscriptional gene silencing in eukaryotes. In recent years, numerous classes of small RNAs have been implicated in transposon silencing, viral defense, developmental gene regulation, and other processes (see Carthew and Sontheimer, 2009; Siomi and Siomi, 2009). The core components of RNA-mediated silencing mechanisms are (1) a small RNA working in conjunction with (2) an Argonaute protein to target specific nucleic acid sequences. One class of small RNAs, called small interfering RNAs (siRNAs), was first identified for its role in exogenous RNA interference (RNAi), a form of gene silencing triggered by introduction of foreign double-stranded RNA (dsRNA) (Fire et al., 1998) (Figure 1A). Later, endogenous siRNAs were identified and linked to endogenous mechanisms of gene silencing.

It has been a challenge to understand the physiological importance of different classes of endogenous siRNAs. They may be produced by endonuclease cleavage of a longer dsRNA and/or by activity of an RNA-directed RNA polymerase (RdRP) on single-stranded RNA template. Different siRNAs may have different physical features (length, 5′ end structures) based on how they were generated. For example, siRNA produced via endonuclease cleavage has a 5′ monophosphate, whereas endonuclease-independent siRNA has a 5′ triphosphate. Also puzzling has been the functional relationships among different siRNA pathways. For example, extensive genetic analysis of the RNAi response in *C. elegans* identified two classes of mutants: (1) RNAi-defective mutants (*rde*) are relatively insensitive to exogenous dsRNA (presumably because a component of the core RNAi machinery is absent), and (2) enhanced RNAi (*eri*) mutants are hyperresponsive to exogenous dsRNA. Accumulation of (at least some) endogenous siRNAs requires both proteins that function in exogenous RNAi (e.g., Dicer endonuclease) and ERI proteins (e.g., the RdRP called RRF-3) (Lee et al., 2006; Duchaine et al., 2006; Yigit et al., 2006). Hence, it was proposed that endogenous and exogenous RNA silencing pathways share core components; when RRF-3 or another specialized component of the endogenous pathway is disabled, core RNAi components are available to engage more fully in the exogenous RNAi pathway, thus leading to an enhanced RNAi response.

Now, Gent et al. (2010) describe a novel mechanism whereby the coordinated activity of two different RdRPs silences gene expression in *C. elegans*. The authors employed genetic, deep sequencing, and bioinformatic approaches to define populations of siRNAs whose biogenesis depends on the activity of RRF-3 and other ERI factors. By comparing siRNAs isolated from wild-type, *rrf-3*, and germline-deficient mutants, they identified a large set of RRF-3-dependent siRNAs present in the germline and a rare set present in the soma. The authors chose to focus on somatic RRF-3 function and in particular on production of the most abundant RRF-3-dependent siRNAs, which are generated from a set of 23 "exemplary" mRNA templates. These siRNAs are also reduced in animals that are mutant for other *eri* genes and components of the Dicer complex. A subset of siRNAs targeting these 23 mRNAs was also reduced in animals that lack RRF-1, an RdRP known to function in somatic RNAi.

To determine whether RRF-3-dependent siRNAs promote gene silencing in the soma, as predicted, the authors constructed and characterized mRNA sequencing libraries from several mutants. Putative mRNA targets were indeed elevated in *rrf-3* and other *eri* and Dicer complex mutants as well as in mutants lacking RRF-1 and ERGO-1, an Argonaute protein implicated in ERI pathway function. Hence, all of these factors function to silence a common set of somatic genes.

Bioinformatic analysis of the siRNA sequence data revealed an intriguing pattern: RRF-3 and RRF-1 were responsible for synthesis of two distinct siRNA populations, the 26G and 22G RNAs, respectively. *rrf-3* mutants lacked essentially all 26G siRNAs and a subset of 22G RNAs. *rrf-1* mutants lacked the same subset of 22G RNAs but had a normal 26G RNA population. 26G and 22G siRNA populations have distinct structural features, consistent with their being produced via different mechanisms. 26G RNAs are 26 nt in length and contain a 5′ monophosphate consistent with endonuclease cleavage. They are relatively rare among the total endogenous siRNA pool in wild-type somatic tissues. The much more abundant 22G RNAs are 22 nt in length and contain a 5′ triphosphate group, suggesting they are direct RdRP products and have not undergone

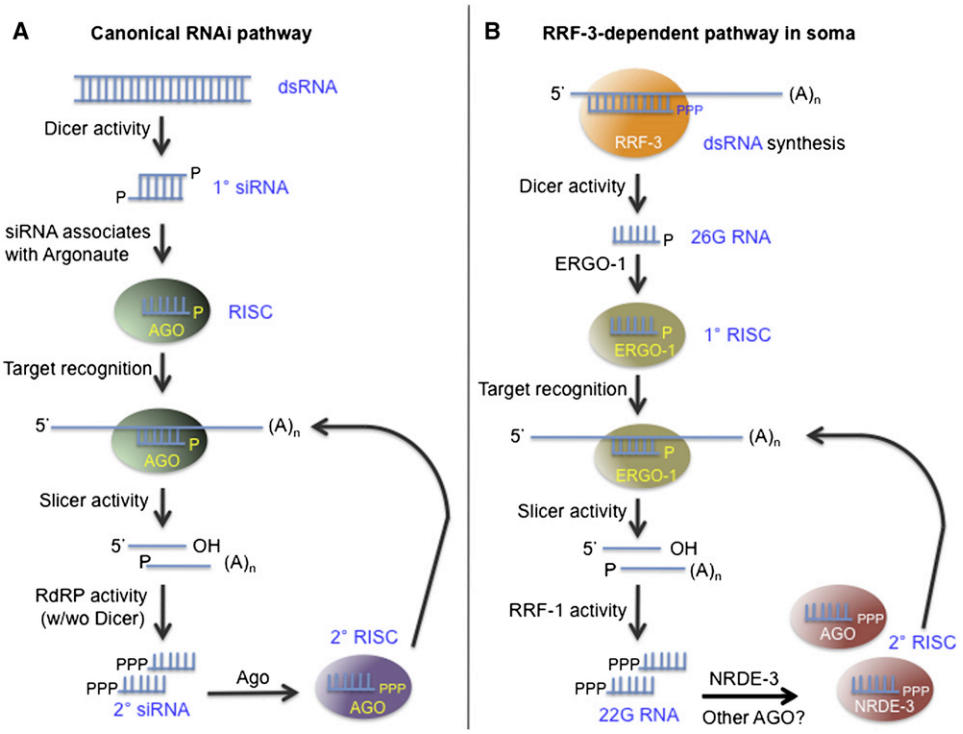

Figure 1. Comparison of the Canonical RNAi Mechanism and the Proposed Somatic ERI Pathway

(A) Dicer endonuclease cleaves foreign dsRNA to generate primary (1°) siRNAs that associate with Argonaute protein (AGO) to produce an RNA-induced silencing complex (RISC). RISC is targeted to and cleaves mRNA that is complementary in sequence to the antisense siRNA strand. The silencing response is amplified in organisms containing RdRP, which generates antisense RNA from targeted mRNA templates. Secondary (2°) siRNAs recruit additional RISC to (intact) target mRNAs, thereby amplifying the RNAi response. *C. elegans* 2° siRNAs contain a 5′ triphosphate; in other species, 2° siRNAs may be products of Dicer activity and consequently contain 5′ monophosphate.

(B) RRF-3 utilizes specific mRNA templates to generate dsRNA, which is cleaved by Dicer to generate 26G RNAs. ERGO-1 associates with these 26G RNAs to form RISC, which presumably cleaves mRNA targets. In a secondary phase, RRF-1 produces 22G RNAs from the same mRNA species. These 22G RNAs associate with NRDE-3 (and perhaps other Argonautes), forming secondary RISC complexes that further target mRNA for silencing.

endonuclease cleavage. Both populations are predominantly antisense to mRNA sequences, consistent with synthesis from mRNA templates, and their sequences begin at a guanine residue corresponding to a common cysteine in the template.

The authors propose a two-step model wherein RRF-3 and RRF-1 sequentially produce 26G and 22G RNAs to target a set of mRNAs for degradation (Figure 1B). First, RRF-3/RdRP generates dsRNA from mRNA templates, which is cleaved by Dicer to form 26G siRNAs. The antisense siRNA strand associates with ERGO-1 and may target the same pool of mRNAs for degradation. Second, for some of these RRF-3 targets, RRF-1/RdRP utilizes the cleaved mRNA species as template, producing 22G siRNAs. These secondary siRNAs participate in a secondary round of RNA silencing that is important for effectively silencing the target gene. Evidence suggests NRDE-3/Argonaute participates at this step.

This model provides an explanation for the enhanced RNAi phenotype of *rrf-3* mutants. When RRF-3 is inactive, 26G RNA siRNAs are not produced, freeing RRF-1 to participate in exogenous RNAi. It is worth noting that the authors also detected many mRNA templates for which 26G and 22G RNA production was uncoupled, i.e., RRF-3 produced 26G RNAs from these mRNA templates, but this activity was not required for production of the corresponding 22G RNAs. In these cases, an RRF-3-independent mechanism for 22G RNA production must exist.

The data raise many intriguing mechanistic and functional questions. Do mRNAs contain a signal sequence to attract specific RdRP(s)? What mechanism links RRF-1 activity to RRF-3-targeted mRNAs? Does silencing occur solely at the level of the message or also at the chromatin level? Given that *rrf-3* and other *eri* mutants have relatively subtle somatic phenotypes, what is the physiological/developmental importance of somatic gene silencing via the ERI pathway? In contrast to the soma, *eri* mutants have striking defects in germline development, particularly in spermatogenesis (Gent et al., 2009; Pavelec et al., 2009; and references therein). This is not surprising, given that most 26G RNAs are in fact produced in the germline, as reported by Gent et al. (2010) and another recent study by Han et al. (2009). It seems likely that a two-step RdRP mechanism is active in the germline, perhaps utilizing EGO-1, an RdRP active in germline RNAi, in the second step. Indeed, the implications of this work extend beyond *C. elegans* to the many organisms that contain multiple RdRPs and hence may utilize similar

multistep RdRP mechanisms to regulate developmental gene expression.

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The Tango of Histone Marks and Chaperones at Replication Fork

Karina B. Falbo[1] and Xuetong Shen[1,*]
[1]Department of Carcinogenesis, Science Park Research Division, MD Anderson Cancer Center, Smithville, TX 78957, USA
*Correspondence: snowshen@mac.com
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Nucleosomes are disassembled during DNA replication. How histone modifications and histone chaperones collaborate to reassemble nucleosomes on replicated DNA is explored by Jasencakova et al. (2010) and Burgess et al. (2010) in this and a recent issue of *Molecular Cell*, respectively.

The complexity of chromatin structure requires that a multiplicity of factors work together at the replication fork to ensure accurate inheritance of both the DNA sequence and the epigenetic information contained in histones. Although the mechanisms by which DNA replicates itself are well established, relatively little is known about how the chromatin state is duplicated during replication. The transmission of chromatin states at replication forks involves multiple processes such as nucleosome disassembly/assembly, positioning of nucleosomes, and establishment of histone posttranslational modification (PTM) patterns. While previous studies have identified many of the key players in these processes, including histone chaperones and histone PTMs, the molecular mechanisms by which these histone PTMs tango with histone chaperones around the replication fork remain obscure (Groth et al., 2007b; Ransom et al., 2010). Burgess et al. (2010), in a recent issue, and Jasencakova et al. (2010), in this issue, provide novel molecular insights into how histone chaperones interact with histone PTMs during replication.

Histone PTMs are essential for proper duplication of the chromatin structure. In yeast, newly synthesized histone H3 is acetylated at K56 by Rtt109, a modification that is important for nucleosome assembly and genome stability during DNA replication. The histone chaperone Asf1 is needed for acetylation of H3K56, which mediates the binding of H3 to other histone chaperones, Rtt106 and CAF1 (Ransom et al., 2010). Other acetylation marks on histone H3 at K9, K14, K18, K23, and K27 have also been implicated in nucleosome assembly (Li et al., 2008). However, the identity of the acetyltransferase (HAT) responsible for the acetylation and the function of these acetylation marks in addition to H3K56Ac is unclear. Burgess et al. (2010) have now shown that in *Saccharomyces cerevisiae*, Gcn5 promotes replication-coupled nucleosome assembly, in part by acetylating the histone H3 tail, which in turn regulates the binding of H3 to CAF1. From synthetic genetic interactions, the authors first noted that the double mutant *gcn5 rtt109* is more sensitive to DNA damaging agents, suggesting Gcn5 works in a parallel nucleosome assembly pathway with Rtt109/H3K56Ac under replication stress. They further showed that Gcn5 achieves this role mainly through acetylating the tails of H3. Interestingly, although the two H3 acetylation events by Rtt109 and Gcn5 are largely independent, acetylation of H3 tails by Gcn5 is functionally required for efficient loading of H3K56Ac acetylated by Rtt109 to replication forks. The authors discovered that the association between histone H3 and CAF1 was dramatically reduced in the absence of Gcn5 or Gcn5-mediated H3 tail acetylation, providing an elegant