CHAPTER ONE

Biology and Mechanisms of Short RNAs in *Caenorhabditis elegans*

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Contents

Abstract

The significance of noncoding RNAs in animal biology is being increasingly recognized. The nematode *Caenorhabditis elegans* has an extensive system of short RNAs that includes microRNAs, piRNAs, and endogenous siRNAs, which regulate development, control life span, provide resistance to viruses and transposons, and monitor gene duplications. Progress in our understanding of short RNAs was stimulated by the discovery of RNA interference, a phenomenon of sequence-specific gene silencing induced by exogenous double-stranded RNA, at the turn of the twenty-first century. This chapter provides a broad overview of the exogenous and endogenous RNAi processes in *C. elegans* and describes recent advances in genetic, genomic, and molecular analyses of nematode's short RNAs and proteins involved in the RNAi-related pathways.

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1. INTRODUCTION

Within the last 20 years, it has been recognized that short RNAs 20–30 nt in length and their protein cofactors of the ancient Argonaute family are integral components of most eukaryotic organisms. Some short RNAs, such as the microRNA *let*-7, are well conserved in animals (Pasquinelli et al., 2000), while others, such as endogenous siRNAs antisense to foreign RNA sequences and involved in genome surveillance, can be generated within the lifetime of an individual (reviewed in Billi, Freeberg, & Kim, 2012).

The nematode *Caenorhabditis elegans* represents an ideal organism for studies of short RNA biology. The first microRNAs to be discovered, *lin-4* (Lee, Feinbaum, & Ambros, 1993) and *let*-7 (Reinhart et al., 2000), were found in the course of rigorous genetic analysis of the *C. elegans* cell lineage, and the discovery of double-stranded RNA-induced gene silencing (RNA interference or RNAi) in *C. elegans* (Fire et al., 1998) precipitated the recognition of the connection between RNAi and miRNAs providing reference to related phenomena in plants and fungi. The short RNAs of a specific length (20–30 nt) and Argonaute proteins manifested the newly found connections (reviewed in Czech & Hannon, 2011; Hutvagner & Simard, 2008) and represented the culmination of the first decade of RNA-silencing research.

From that point, numerous avenues of short RNA studies emerged, and the second decade of RNAi-related research featured advances in biochemistry, structural biology, and genetics. miRNA-based regulation proved to be almost as common as regulation by transcription factors. Advances in deep sequencing technology brought short RNA discovery to new levels and enabled identification of another class of RNAs, piRNAs, which interact with the PIWI subfamily of Argonaute proteins (reviewed in Ishizu, Siomi, & Siomi, 2012). Deep sequencing reinforced genomic approaches in RNAi research in *C. elegans*, and the wealth of endogenous short RNAs (endo-siRNAs), distinct from miRNAs and piRNAs, that came out of these studies is truly remarkable (reviewed in Fischer, 2010). Endo-siRNAs are not unique to *C. elegans*; they exist in *Drosophila* and mammals as well (reviewed in Okamura & Lai 2008), but nematodes developed the most extensive collection of Argonaute proteins, studies of which are poised to discover new biological functions and mechanisms of gene regulation.

This review attempts to cover the breadth of RNAi-related research in *C. elegans* since 2005. For earlier reviews by this author, see Grishok and Mello (2002) and Grishok (2005). miRNAs have received more dedicated reviews than the other short RNAs discovered more recently. Therefore, a more in-depth discussion on miRNA can be found elsewhere (Abbott, 2011; Ambros, 2011; Kaufman & Miska, 2010; Ketting, 2010; Mondol & Pasquinelli, 2012; Pasquinelli, 2012; Ruvkun, 2008; Vella & Slack, 2005). Here, an overview of miRNA research includes their newly discovered biological functions in *C. elegans*, as well as recent advances concerning the molecular mechanisms governing miRNA expression and their role in gene regulation. A large portion of the review is dedicated to the biology of endogenous RNAi in *C. elegans*, including mechanistic insights into the biogenesis of various siRNA species, regulation of mRNA stability and transcription, RNAi inheritance, and connections to other pathways and factors affecting RNAi. The unique biogenesis of *C. elegans* piRNAs (21U-RNAs) is highlighted, as well as their cooperation with endo-siRNAs in genome surveillance. Finally, advances in our understanding of the systemic nature of RNAi in *C. elegans* are described.

2. miRNA FUNCTION

2.1. Biological effects of miRNAs

2.1.1 Developmental timing

Cell divisions and cell-fate decisions during *C. elegans* development follow a stereotypical lineage (reviewed in Ambros, 2011; Rougvie, 2005), which includes programs specific for sequential postembryonic larval stages L1, L2, L3, and L4 and terminal differentiation in the adult. Mutant screens have identified animals with abnormalities in developmental timing, that is, heterochronic mutants, some of which never progress to later developmental programs, while others execute them precociously (reviewed in Ambros, 2011; Rougvie, 2005). The discovery of the first miRNA, *lin-4*, was made during the process of cloning of a gene with a heterochronic mutant phenotype and with the realization that the product of the gene represents a noncoding RNA that exists as a longer hairpin (70 nt) or a shorter (~22 nt) species (Lee et al., 1993). Moreover, genetic analyses revealed that the *lin-14* gene, which itself is important in the heterochronic pathway, is negatively regulated by *lin-4*, and molecular characterization of *lin*-14 gain-of-function mutations pointed to the *lin-14* 3′UTR as the site of negative regulation by *lin-4* (Wightman, Ha, & Ruvkun, 1993). After the nature

of *lin-4* was revealed, several regions of partial complementarity between *lin-4* RNA and the *lin-14* 3′UTR were identified, and the paradigm of negative regulation by miRNA was born (Lee et al., 1993; Wightman et al., 1993). This seminal work by Victor Ambros, Gary Ruvkun, and their colleagues began to be fully appreciated only by the turn of the twenty-first century when another heterochronic gene, *let-7*, turned out to encode a short RNA (Reinhart et al., 2000), which was remarkably conserved in animals (Pasquinelli et al., 2000), and numerous other endogenous short RNAs with hairpin precursors were cloned from *C. elegans*, *Drosophila*, and mammalian cells (Lagos-Quintana, Rauhut, Lendeckel, & Tuschl, 2001; Lau, Lim, Weinstein, & Bartel, 2001; Lee & Ambros, 2001).

Although mutations in the *lin-4* and *let-7* genes caused obvious developmental phenotypes, extensive analyses of new miRNAs in *C. elegans* concluded that this is not generally true (Miska et al., 2007) and that many miRNAs, especially related ones, work redundantly (Abbott et al., 2005; Alvarez-Saavedra & Horvitz, 2010). For example, *let-7* family members *mir-48*, *mir-84*, and *mir-241* cooperate in repressing the transcription factor Hunchback-like (HBL-1) (Abbott et al., 2005; Li, Jones-Rhoades, Lau, Bartel, & Rougvie, 2005). Nevertheless, control of developmental timing remains the best understood biological function of miRNAs in *C. elegans* (reviewed in Ambros, 2011; Resnick, McCulloch, & Rougvie, 2010; Sokol, 2012), and *let-7* miRNA and its relatives are the most well characterized of all the miRNAs. Their *C. elegans* targets, such as LIN-28, LIN-41, and LET-60/Ras, proved to be conserved in mammals and enabled the recognition of *let-7* as a tumor suppressor and a prodifferentiation factor (reviewed by Ambros, 2011; Mondol & Pasquinelli, 2012).

2.1.2 Embryonic development

The similarity between the RNAi and miRNA pathways in *C. elegans* was recognized because downregulation by RNAi of Argonaute-like genes *alg-1* and *alg-2* resulted in heterochronic phenotypes that resembled the *let-7* mutant (Grishok et al., 2001). Interestingly, the most severe phenotype resulting from inactivation of *alg-1* and *alg-2* is embryonic lethality (Grishok et al., 2001; Vasquez-Rifo et al., 2012), which suggests that some miRNAs or their combination has a role in embryonic development.

Although initial analysis of deletion mutations in 87 miRNA genes identified few mutants with gross developmental abnormalities (Miska et al., 2007), it reported temperature-sensitive embryonic lethality associated with the *mir-35–41* miRNA cluster (Miska et al., 2007). Subsequent deletion of

all eight members of the *mir-35* family (*mir-35* through *mir-42*) revealed a more penetrant embryonic phenotype that was not temperature sensitive (Alvarez-Saavedra & Horvitz, 2010). Importantly, this family of miRNAs is expressed during both oogenesis and embryogenesis (Alvarez-Saavedra & Horvitz, 2010; Wu et al., 2010), and the lethality could be rescued by either maternal or early zygotic expression of the miRNAs (Alvarez-Saavedra & Horvitz, 2010). The targets of this miRNA family responsible for the developmental phenotypes could not be identified in suppressor screens and remain unknown.

Another group of related miRNAs required for embryonic development is the *C. elegans mir-51* family (Alvarez-Saavedra & Horvitz, 2010; Shaw, Armisen, Lehrbach, & Miska, 2010), which belongs to a broader miR-100 family defined by human miR-100. The miRNAs of the *mir-51* family, *mir-51–56*, are broadly expressed at all developmental stages (Lim et al., 2003; Shaw et al., 2010; Wu et al., 2010), consistent with their role in embryonic and postembryonic development (Alvarez-Saavedra & Horvitz, 2010; Shaw et al., 2010) and their genetic interaction with multiple developmental pathways (Brenner, Kemp, & Abbott, 2012). The earliest developmental phenotype of *mir-51* family mutants is an unattached pharynx, that is, a lack of attachment of the pharyngeal muscle to the mouth (Shaw et al., 2010). This phenotype was shown to be at least in part due to the misregulation of the *mir-51* target gene, *cdh-3* (Fat cadherin ortholog-3), which is presumed to interfere with the homophilic interactions of another Fat cadherin ortholog required for the maintenance of pharynx attachment, CDH-4 (Schmitz, Wacker, & Hutter, 2008).

As miRNAs of the same family often work redundantly (see earlier), it is easy to imagine that unrelated miRNAs may also cooperate in gene regulation or that miRNAs may act redundantly with other factors. Indeed, Abbot and colleagues found that mutations in 25 out of 31 tested miRNAs showed phenotypes, including embryonic lethality, in sensitized genetic backgrounds (Brenner, Jasiewicz, Fahley, Kemp, & Abbott, 2010).

Notably, in addition to *lin-4* and *let-7*, another miRNA, *lsy-6*, was identified in a forward mutant screen (Johnston & Hobert, 2003). This genetic screen was aimed at finding genes controlling asymmetry of the ASE gustatory neurons, which are morphologically symmetrical but exhibit functional asymmetry (Hobert, 2006). *lsy-6* miRNA is specifically expressed in the left ASE neuron, where it inhibits the transcription factor COG-1 and reinforces expression of asymmetric genes (Johnston & Hobert, 2003). It was discovered recently that *lsy-6* is the first gene to be expressed

asymmetrically in the ASE neurons and that this asymmetry is initiated by the TBX-37/38 transcription factors (Cochella & Hobert, 2012). These transcription factors act early in the ASEL but not in the ASER lineage and contribute to chromatin decompaction and "priming" of the *lsy-6* locus for subsequent full activation in ASEL several cell divisions later (Cochella & Hobert, 2012).

2.1.3 Postembryonic development

The miRNAs of the heterochronic pathway, *lin-4* and the *let-7* family, have a profound role in regulating postembryonic development (reviewed in Ambros, 2011; Mondol & Pasquinelli, 2012; Rougvie, 2005; Sokol, 2012). This was first recognized during studies of the defects in lateral hypodermal seam cell lineages (reviewed in Ambros, 2011; Rougvie, 2005). Later, the role of *let-7* family miRNAs in promoting the cessation of molting through inhibition of conserved nuclear hormone receptor genes was described (Hayes, Frand, & Ruvkun, 2006). This regulation takes place through both the heterochronic pathway targets of the *let-7* family, *lin-41* and *hbl-1*, and the direct inhibition of nuclear receptor genes by the *let-7* family miRNAs (Hayes et al., 2006). *lin-4* and the *let-7* family also have important roles during vulva development. The conserved EGF/RAS and Notch signaling pathways govern vulva specification during larval stage 3 (reviewed in Gupta, Hanna-Rose, & Sternberg, 2012). The *let-7* family was first implicated in the direct inhibition of *let-60*/RAS in studies of vulva development in *C. elegans* (Johnson et al., 2005). Moreover, it has been demonstrated recently that the timing of *lin-14* inhibition by *lin-4* in specific vulva precursor cells contributes to the precision of cell-fate specification (Li & Greenwald, 2010).

Also, the timing of nervous system development is controlled by *lin-4* as the negative regulation of *lin-14* and *lin-28* by *lin-4* in the hermaphrodite-specific neuron during larval stage 4 contributes to the timing of its axon elongation (Olsson-Carter & Slack, 2010).

In addition to loss-of-function studies, analyses of miRNA expression patterns using reporters combined with overexpression experiments yielded insight into the role of miRNAs in specific cells. Thus, *mir-61* transcription is directly activated by LIN-12/Notch in specific vulva precursor cells (Yoo & Greenwald, 2005). This miRNA was shown to promote LIN-12 expression in a positive-feedback loop by directly inhibiting its negative regulator *vav-1* (Yoo & Greenwald, 2005). The function of the conserved miRNA *mir-57* in regulation of positional cell-fate specification was

identified because of the intriguing posterior expression of an *mir-57* transcriptional reporter throughout embryonic and postembryonic development (Zhao et al., 2010). Although *mir-57* loss-of-function produced weak phenotypes, overexpression of *mir-57* resulted in robust posterior abnormalities (Zhao et al., 2010). Interestingly, *mir-57* was found to directly inhibit the Hox gene *nob-1*, while expression of *mir-57* was dependent on an earlier onset of *nob-1* expression in posterior lineages (Zhao et al., 2010). Another conserved miRNA, *mir-124*, is expressed specifically in ciliated sensory neurons and was shown to promote the mRNA expression signature associated with this type of neuron (Clark et al., 2010).

A number of postembryonic defects, including small body size and defective egg laying, have been reported in mutants lacking all members of the abundantly expressed *mir-58* family (*mir-58*, *mir-80*, *mir-81*, and *mir-82*) (Alvarez-Saavedra & Horvitz, 2010); the targets of this family of miRNAs remain to be found. Also, analyses of miRNA function in sensitized backgrounds have implicated a number of miRNAs in regulation of embryogenesis, adult viability, and the process of gonad migration (Brenner et al., 2010). Despite these efforts, it looks like much of the gene regulation by miRNAs during development remains to be revealed.

2.1.4 Physiology

There has been significant progress in elucidating the physiological roles of miRNAs in *C. elegans*. *mir-1* is an miRNA with conserved expression in muscle tissue. Its elimination in flies and mice is lethal (Sokol & Ambros, 2005; Zhao et al., 2007), while *C. elegans mir-1* mutants are viable. The viability of *mir-1* mutants in *C. elegans* enabled the dissection of its role in synaptic transmission at neuromuscular junctions (Simon et al., 2008). It was shown that *mir-1* modulates muscle sensitivity to acetylcholine through a direct regulation of the abundance of acetylcholine receptor subunits (Simon et al., 2008). Moreover, *mir-1* was implicated in acetylcholine release from neurons due to its nonautonomous role in regulating the transcription factor MEF-2 in the muscle and MEF-2-dependent retrograde signal from the muscle to the neuron (Simon et al., 2008).

The function of another conserved miRNA, *mir-34*, was connected to the regulation of DNA damage-induced cell death in *C. elegans* when *mir-34* mutant animals showed increased susceptibility to radiation in the soma and increased resistance to radiation in the germline (Kato et al., 2009). The mechanistic aspects of *mir-34* function in the DNA damage response are not known.

The role of the *mir-240/786* cluster in the regulation of the *C. elegans* defecation cycle was noted in the initial analysis of a panel of deletion mutants (Miska et al., 2007). A more detailed study of the phenotype revealed that *mir-786*, but not *mir-240*, was involved (Kemp et al., 2012). Moreover, the authors showed that *mir-240/786* is expressed in the posterior intestinal cells, where the pacemaker for the ~50-s calcium-induced defecation rhythm is located, and that *mir-786* directly regulates the fatty acid elongase *elo-2* to control the defecation cycle (Kemp et al., 2012). These results suggest that fatty acid composition in the posterior intestinal cells affects the pacemaker properties of these cells (Kemp et al., 2012).

2.1.5 Longevity and stress response

C. elegans is the first model organism where the genetic pathway of insulin/insulin-like growth factor 1 signaling was shown to negatively regulate life span by inhibiting the longevity-promoting transcription factor DAF-16/FOXO (reviewed in Lapierre & Hansen, 2012). Interestingly, the founding miRNA *lin-4* and its target *lin-14* were also implicated in the DAF-16-dependent control of aging in *C. elegans* either in parallel or through insulin signaling; *lin-4* mutants had a reduced life span, while *lin-14* loss-of-function extended it (Boehm & Slack, 2005). Moreover, *let-7* family members, *mir-84* and *mir-241*, were recently shown to participate in the DAF-16-dependent life span extension induced by germline ablation (Shen, Wollam, Magner, Karalay, & Antebi, 2012). This miRNA-dependent life span increase was due to direct inhibition of *lin-14* and *akt-1* and activation of DAF-16 and its transcriptional targets (Shen et al., 2012). These results are consistent with earlier findings implicating *lin-14* in the negative regulation of longevity (Boehm & Slack, 2005) and also with reports describing a general reduction in miRNA levels, including those of *lin-4* and the *let-7* family, during aging (de Lencastre et al., 2010; Ibanez-Ventoso et al., 2006; Kato, Chen, Inukai, Zhao, & Slack, 2011). In line with this, recent identification of a temperature-dependent mutant allele in the miRNA-processing factor *pash-1* enabled experiments in which miRNA levels were manipulated in adult animals. These experiments revealed that global miRNA reduction accelerates aging (Lehrbach et al., 2012).

Although miRNAs are generally less abundant in aged animals, some miRNAs, such as *mir-34*, *mir-71*, *mir-238*, *mir-239*, and *mir-246*, are upregulated (de Lencastre et al., 2010; Ibanez-Ventoso et al., 2006; Kato et al., 2011). All these miRNAs have been implicated in the control of longevity: *mir-71*, *mir-238*, and *mir-246* act to enhance life span (Boulias &

Horvitz, 2012; de Lencastre et al., 2010), while *mir-34* and *mir-239* inhibit longevity (de Lencastre et al., 2010; Yang et al., 2013). The increased life span of *mir-34* mutants depends on autophagy (Yang et al., 2013) and *mir-239* loss-of-function extends the life span in a DAF-16-dependent manner (de Lencastre et al., 2010). Interestingly, *mir-34* and *mir-71* were also upregulated during starvation-induced L1 diapause and in adults with post-dauer life history (Karp, Hammell, Ow, & Ambros, 2011). Increase in life span often correlates with enhanced resistance to stress, while short-lived animals are stress sensitive. Indeed, this correlation was shown for *mir-71*, *mir-238*, *mir-239*, and *mir-246* (Boulias & Horvitz, 2012; de Lencastre et al., 2010).

An in-depth analysis of *mir-71* function determined that *mir-71* is specifically required for life span extension induced by germ cell loss, but not for the increased longevity of animals with reduced insulin signaling (Boulias & Horvitz, 2012). Moreover, although *mir-71* is broadly expressed, it functions in neurons to promote the transcriptional activity of DAF-16 in the intestine of germline-deficient animals (Boulias & Horvitz, 2012).

As studies of miRNAs in aging continue to progress, more similarities between different species emerge. For in-depth reviews on the role of short RNAs in longevity, see Ibanez-Ventoso and Driscoll (2009), Smith-Vikos and Slack (2012), and Kato and Slack (2013), and for more general reviews on the biological functions of miRNAs in *C. elegans*, see Kaufman and Miska (2010) and Abbott (2011).

2.2. Biogenesis and molecular mechanisms of miRNAs

The topic of miRNA biogenesis and their molecular function in *C. elegans* and other species has been reviewed extensively over the years, with a number of excellent reviews published recently (Bartel, 2009; Fabian, Sonenberg, & Filipowicz, 2010; Hammell, 2008; Kai & Pasquinelli, 2010; Kim, Han, & Siomi, 2009; Krol, Loedige, & Filipowicz, 2010; Mondol & Pasquinelli, 2012; Turner & Slack, 2009), including several chapters in the book *Regulation of microRNAs, Advances in Experimental Medicine and Biology* (2010) (Grosshans & Chatterjee, 2010; Ketting, 2010; Lehrbach & Miska, 2010). A summary of the state of the field is provided below without references to primary literature, which can be found in the listed reviews.

miRNAs are generally encoded by RNA polymerase II-transcribed genes, which are subject to positive and negative regulation by specific *cis*-acting transcription factors. Interestingly, miRNAs and the transcription

factors that regulate them are often engaged in feedback loops. Primary miRNA transcripts (pri-miRNAs) are capped and polyadenylated; their processing in the nucleus is mediated by the RNase III family enzyme Drosha and its partner Pasha (DRSH-1 and PASH-1, respectively, in *C. elegans*). Some miRNAs are encoded in the introns of protein-coding genes and rely on splicing, not Drosha, for their initial processing in the nucleus. The product of the initial processing, pre-miRNA, is generally a ~70-nt hairpin structure and is further processed in the cytoplasm by another RNase III homolog, Dicer (DCR-1 in *C. elegans*). The product of Dicer processing is a ~22-bp RNA duplex, which consists of a mature miRNA and its complementary "miRNA-star." Generally, the 22-nt strand exhibiting weaker thermodynamics in base pairing at the 5′end is the mature miRNA. The mature miRNA gets incorporated into the RNA-induced-silencing complex (RISC) as a binding partner of an Argonaute protein. In *C. elegans*, 2 out of 26 Argonaute proteins, ALG-1 and ALG-2, interact with miRNAs (Table 1.1). How these proteins are recruited into the miRNA pathway as opposed to multiple other RNAi-related pathways is not clear, although the structural properties of the hairpin precursor were shown to have a role. Interaction with Argonaute proteins as well as engagement in base pairing with target mRNA protects miRNAs from degradation by exonucleases, such as XRN-2 in *C. elegans*. In recent years, regulation of miRNA stability was found to play an important role in the dynamic properties of miRNA-based gene silencing.

miRNAs regulate their target mRNAs through base pairing at 3′UTRs and by recruiting proteins that ultimately cause translational repression or deadenylation. The key interacting partners of Argonaute proteins that are essential for miRNA silencing are the *C. elegans* homologs of GW182: AIN-1 and AIN-2. miRNAs recognize their targets with imperfect complementarity, and interactions involving the "seed" region, that is nucleotides 2–8, in the mature miRNA occur most often, although other interaction modes have been described for biologically relevant miRNA/mRNA pairs as well. Because of the limited base pairing, computational predictions of valid miRNA targets include other parameters, such as the conservation and accessibility of target sites and the thermodynamics of miRNA–mRNA interaction. In addition to computationally predicted miRNA targets, genomic data on ALG-1 RNA-binding sites and AIN-1/AIN-2 interacting mRNAs are currently available for *C. elegans* researchers, and a targeted proteomic approach has been developed for validation of predicted targets.

Table 1.1 The list of *C. elegans* Argonaute proteins

Protein (Cosmid gene name)	Pathway/function	References
RDE-1 (K08H10.7)	Exo-RNAi, endo-RNAi Slicing of the passenger strand in primary siRNA duplex	Tabara et al. (1999), Steiner, Okihara, Hoogstrate, Sijen, and Ketting (2009), and Correa, Steiner, Berezikov, and Ketting (2010)
ERGO-1 (R09A1.1)	Endo-RNAi (Eri 26G, embryo) Slicing of the passenger strand in primary siRNA duplex	Yigit et al. (2006), Han et al. (2009), and Vasale et al. (2010)
ALG-1 (F48F7.1)	miRNA	Grishok et al. (2001), Hutvagner, Simard, Mello, and Zamore (2004), and Bouasker and Simard (2012)
ALG-2 (T07D3.7)	miRNA	Grishok et al. (2001), Hutvagner et al. (2004), and Bouasker and Simard (2012)
ALG-3 (T22B3.2)	Endo-RNAi (Eri 26G, sperm)	Han et al. (2009) and Conine et al. (2010)
ALG-4 (ZK757.3)	Endo-RNAi (Eri 26G, sperm)	Han et al. (2009) and Conine et al. (2010)
PRG-1 (D2030.6)	21U-RNA (piRNA)	Cox et al. (1998), Batista et al. (2008), Das et al. (2008), and Wang and Reinke (2008)
PRG-2 (C01G5.2)	21U-RNA (piRNA)	Cox et al. (1998), Batista et al. (2008), Das et al. (2008), and Wang and Reinke (2008)
CSR-1 (F20D12.1)	Slicer with secondary siRNAs Endo-RNAi (22G, antisense to genes) Germline and soma, nuclear and cytoplasmic	Yigit et al. (2006), Aoki, Moriguchi, Yoshioka, Okawa, and Tabara (2007), and Claycomb et al. (2009)
C04F12.1	Most closely related to CSR-1	Yigit et al. (2006) and Gu et al. (2009)

Continued

Table 1.1 The list of *C. elegans* Argonaute proteins—cont'd

Protein (Cosmid gene name)	Pathway/function	References
NRDE-3/ WAGO-12 (R04A9.2)	Nuclear exo-RNAi, secondary siRNAs Soma endo-RNAi (22G, ERGO-1 26G-dependent)	Yigit et al. (2006), Guang et al. (2008), and Gu et al. (2009)
HRDE-1/ WAGO-9 (C16C10.3)	Heritable exo-RNAi Nuclear endo-RNAi (22G, germline) 21U-initiated 22G	Yigit et al. (2006), Gu et al. (2009), Ashe et al. (2012), Buckley et al. (2012), and Shirayama et al. (2012)
WAGO-1 (R06C7.1)	Exo-RNAi, secondary siRNA Cytoplasmic endo-RNAi (22G, Eri, repeats), 21U-initiated 22G	Yigit et al. (2006), Gu et al. (2009), and Shirayama et al. (2012)
WAGO-2 (F55A12.1)	Exo-RNAi, secondary siRNA Endo-RNAi (22G, Eri, repeats)	Yigit et al. (2006) and Gu et al. (2009)
WAGO-3/ PPW-2 (Y110A7A.18)	Exo-RNAi, secondary siRNA Endo-RNAi (22G, Eri, transposons, repeats)	Vastenhouw et al. (2003), Yigit et al. (2006), and Gu et al. (2009)
WAGO-4 (F58G1.1)	Exo-RNAi, secondary siRNA Endo-RNAi (22G, Eri, repeats)	Yigit et al. (2006) and Gu et al. (2009)
WAGO-5 (ZK1248.7)	Exo-RNAi, secondary siRNA Endo-RNAi (22G, Eri, repeats)	Yigit et al. (2006) and Gu et al. (2009)
WAGO-6/ SAGO-2 (F56A6.1)	Exo-RNAi, secondary siRNA Endo-RNAi (22G, Eri, repeats)	Yigit et al. (2006) and Gu et al. (2009)
WAGO-7/ PPW-1 (C18E3.7)	Exo-RNAi, secondary siRNA Endo-RNAi (22G, Eri, repeats)	Tijsterman, Okihara, Thijssen, and Plasterk (2002), Yigit et al. (2006), and Gu et al. (2009)
WAGO-8/ SAGO-1 (K12B6.1)	Exo-RNAi, secondary siRNA Endo-RNAi (22G, Eri, repeats)	Yigit et al. (2006) and Gu et al. (2009)
WAGO-10 (T22H9.3)	Exo-RNAi, secondary siRNA Nuclear endo-RNAi (22G, Eri, repeats) 21U-initiated 22G	Yigit et al. (2006), Gu et al. (2009), and Shirayama et al. (2012)

Table 1.1 The list of *C. elegans* Argonaute proteins—cont'd

Protein (Cosmid gene name)	Pathway/function	References
WAGO-11 (Y49F6A.1)	Exo-RNAi, secondary siRNA Endo-RNAi (22G, Eri, repeats)	Yigit et al. (2006) and Gu et al. (2009)
T23D8.7	ALG-brunch of Argonautes	Yigit et al. (2006)
C14B1.7	WAGO-brunch of Argonautes	Yigit et al. (2006)
C06A1.4	Pseudogene?	Yigit et al. (2006)
M03D4.6	Pseudogene?	Yigit et al. (2006)

Identification of biologically relevant miRNA targets and pathways regulated by miRNAs is challenging, but it continues to grow quickly. Also, in addition to the main components involved in miRNA biogenesis and function, highlighted earlier, a number of their cofactors and modulators have been identified. The emerging picture is that silencing by miRNAs is affected by cell-specific factors that either enhance or relieve the effect of miRNAs by a variety of mechanisms. These include regulation of transcription, processing or stability of miRNAs, a cooperation between miRNAs and other RNA-binding proteins in the regulation of mRNA stability or translation, as well as regulation of alternative polyadenylation and, consequently, 3′UTR length, which often eliminates miRNA binding and silencing. Surprisingly, the nuclear function of *let*-7 miRNA and ALG-1 in promoting the processing of *let*-7 pri-mRNA has been described recently (Zisoulis, Kai, Chang, & Pasquinelli, 2012), and a role for zinc finger protein SOMI-1 and chromatin-remodeling factors in promoting *mir-84*-dependent gene regulation was reported (Hayes, Riedel, & Ruvkun, 2011). These results suggest that miRNAs may exist in complexes distinct from RISC and guide the activity of nuclear RNA-processing and, perhaps, that of chromatin-binding factors.

3. RNAi AND ENDOGENOUS siRNAs

Initial studies of RNAi in *C. elegans* that were conducted before 2005 (see earlier reviews by this author (Grishok, 2005; Grishok & Mello, 2002), and references therein) defined its key properties, such as dependence on dsRNA, systemic nature and heritability, and also discovered genes required

for RNAi. These studies also recognized that some of the genes, such as *rde-1* and *rde-4*, act to initiate the silencing response while others, such as *mut-7* and *rde-2*, work at more downstream steps, and that yet another group of factors, such as *sid-1*, is involved in the systemic transport of silencing. Also, at the turn of the twenty-first century, siRNAs were detected in *C. elegans*, Dicer-dependent processing of dsRNA to siRNAs was confirmed using *C. elegans* extracts, and amplification of silencing RNA agents was suggested by the discovery of the role of RNA-dependent RNA polymerases (RdRP) in RNAi. Moreover, during these years, the similarity between RNAi and other homology-dependent silencing phenomena was recognized, as well as the antagonistic relationship between RNAi and adenosine deaminases that act on RNA (ADARs). Finally, the identification of mutants, such as those inactivating RdRP *rrf-3*, which were more susceptible to exogenous RNAi, together with the discovery of endogenous short RNAs that were distinct from miRNAs, predicted the existence of endogenous RNAi-based mechanisms. Similar to the discovery of miRNAs, endo-siRNAs were initially found in *C. elegans* and later identified in flies and mammals.

3.1. Exogenous RNAi pathway

3.1.1 RDE-4 and Dicer

The upstream role of the *rde-4* gene in the RNAi pathway was validated by the molecular properties of its protein product. *rde-4* encodes a protein with two dsRNA-binding motifs (dsRBM1 and dsRBM2) and was shown to bind long dsRNA and to exist in a complex with Dicer (DCR-1), dicer-related helicase DRH-1 and Argonaute protein RDE-1 (Tabara, Yigit, Siomi, & Mello, 2002; Figure 1.1). This complex is involved in the initiation of the silencing response to exogenous dsRNA. Its existence was further validated by proteomic analyses of the interacting partners of DCR-1 (Duchaine et al., 2006) and by characterization of DCR-1-containing complexes using gel filtration (Thivierge et al., 2012). RDE-4 was shown to bind long dsRNA with much higher affinity than short (~20 bp) dsRNA species and to dimerize through its C-terminal domain (Parker, Eckert, & Bass, 2006). Interestingly, although dimerization of RDE-4 was not required for dsRNA binding, it was important for dsRNA processing, as shown by *in vitro* siRNA production assays with *rde-4* mutant extracts supplemented with recombinant RDE-4 (Parker et al., 2006). A subsequent *in vitro* study demonstrated that RDE-4 binds dsRNA cooperatively, and that dsRBM2 is important for dsRNA binding (Parker, Maity, & Bass, 2008). It also showed that the linker region between the two dsRBMs is required for dsRNA

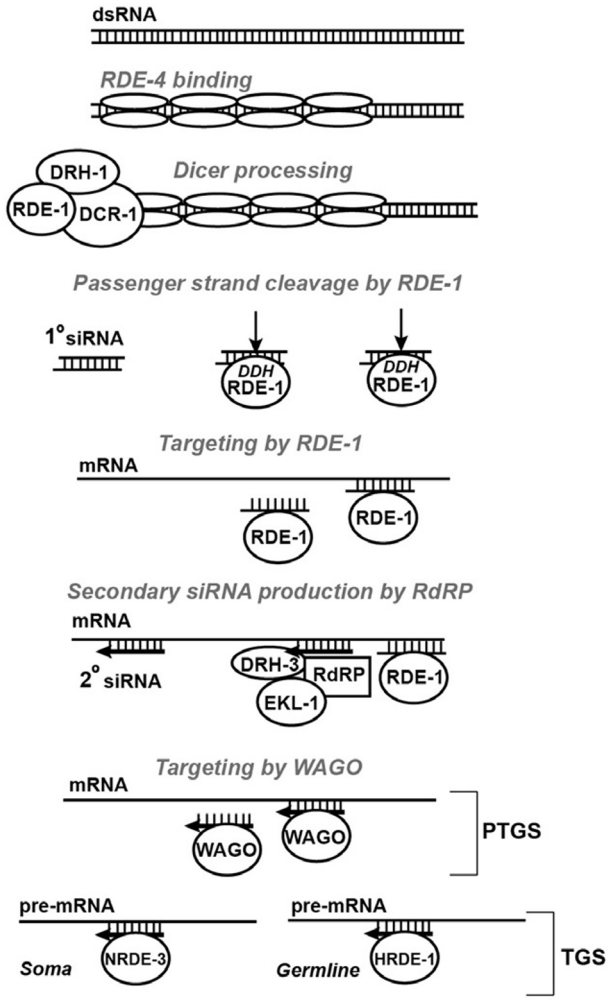

Figure 1.1 Schematic of the exogenous RNAi pathway, which responds to environmental or injected dsRNA. The RDE-4 dimer binds dsRNA followed by the Dicer complex containing Dicer-related helicase DRH-1 and Argonaute RDE-1. Dicer cleaves dsRNA to produce primary double-stranded siRNAs bound by RDE-1 (shown with catalytic amino acid triad DDH). RDE-1 cleaves the passenger strand in the siRNA duplex and is brought to the target mRNA by the guide strand. Targeting by RDE-1 stimulates secondary siRNA production by the RdRP (RRF-1 or EGO-1) complex using selected mRNA as a template. WAGO Argonautes bind to the secondary siRNAs and initiate posttranscriptional gene silencing (PTGS) through poorly understood mechanisms. Somatic NRDE-3/WAGO-12 binds to secondary siRNAs in the cytoplasm and brings them to the nucleus where they target pre-mRNA and induce transcriptional gene silencing (TGS). Nuclear Argonaute HRDE-1/WAGO-9 mediates TGS in the germline. Only factors with clear mechanistic roles are included in the schematic (here and in other figures).

processing and predicted that this region interacts with Dicer (Parker et al., 2008). *In vivo* studies confirmed the important functional roles of dsRMB2 and the linker region as well as their requirement for the interaction between RDE-4 and DCR-1 (Blanchard et al., 2011). It is thought that RDE-4 recognizes dsRNA and recruits Dicer and the rest of the complex for dsRNA processing into siRNAs (Parker et al., 2006; Figure 1.1). Interestingly, it was reported that when a high level of dsRNA is produced from transgenes, initiation of the silencing process does not require RDE-4 (Habig, Aruscavage, & Bass, 2008). It is not clear whether the level or the timing of RDE-4 expression is extensively regulated as it mediates RNAi at all developmental stages and in both somatic and germ cells. In the germline, a new protein, DEPS-1, which is required for the assembly of P-granules in germ cells, was found to promote *rde-4* mRNA and protein expression in this tissue and, consequently, efficient RNAi response in the germline (Spike, Bader, Reinke, & Strome, 2008). The role of DEPS-1 in control of *rde-4* is separate from its function in P-granule assembly. In general, RDE-4 acts nonautonomously in RNAi (Blanchard et al., 2011; Jose, Garcia, & Hunter, 2011). Therefore, its regulation in one tissue may affect the efficiency of RNAi in another and its regulation in the germline may affect the progeny.

While most of the biochemical and structural work on Dicer has been conducted in other systems, an important insight into the role of its helicase domain was achieved using the *dcr-1*(*mg375*) mutant with a point mutation in this domain (Pavelec, Lachowiec, Duchaine, Smith, & Kennedy, 2009; Welker et al., 2011, 2010). Although DCR-1 helicase mutations primarily affected endogenous siRNA levels (Pavelec et al., 2009; Welker et al., 2010) and did not seem to affect exogenous RNAi (Welker et al., 2010) in these *in vivo* studies, it was discovered later that the helicase domain is important for the generation of siRNAs from internal regions of long dsRNA with blunt termini because *dcr-1*(*mg375*) mutant extracts were deficient in this process (Welker et al., 2011). miRNA precursors do not require such processive action of Dicer, and miRNA levels were not affected by mutations in its helicase domain (Welker et al., 2010, 2011).

3.1.2 RDE-1 Argonaute protein

Argonaute proteins exist in complexes with short RNAs and function in RNA-mediated silencing processes (reviewed in Czech & Hannon, 2011; Hutvagner & Simard, 2008). There are 26 genes encoding Argonaute family proteins in *C. elegans* (Table 1.1), but only one, *rde-1*, was implicated in the

exogenous RNAi pathway through unbiased discovery in the initial screen for RNAi-deficient mutants (Tabara et al., 1999). Although RDE-1 is not required for dsRNA processing *per se* (Parrish & Fire, 2001), it interacts with the Dicer complex (Duchaine et al., 2006; Tabara et al., 2002) and binds to the primary siRNA duplex generated by Dicer (Yigit et al., 2006; Figure 1.1).

miRNAs are naturally processed from short hairpins with ~22 bp stems, where bulges disrupting pairing are common, and siRNAs are generated from long dsRNA precursors in exogenous RNAi. However, it has been shown that hairpin precursors with perfectly base-paired stems are efficiently processed into siRNAs in mammalian cells (reviewed in McManus, Petersen, Haines, Chen, & Sharp, 2002; McManus & Sharp, 2002) and in *C. elegans* (Sijen, Steiner, Thijssen, & Plasterk, 2007). There are no dedicated Argonaute proteins for RNAi and miRNAs in mammals, but there is a clear separation between Argonautes acting in the exo-RNAi and miRNA pathways in *C. elegans*: RDE-1 is specific for RNAi (Tabara et al., 1999), while ALG-1 and ALG-2 are specific for miRNAs (Grishok et al., 2001; Table 1.1). Interestingly, it was shown that perfect base pairing in siRNA hairpins was important for the loading of the processed product onto RDE-1, while precursors containing bulges were loaded onto ALG-1 (Jannot, Boisvert, Banville, & Simard, 2008; Steiner et al., 2007). However, this distinction is not perfect, as many natural miRNA precursors contain few mismatches and their resulting mature miRNAs have been found in complex with RDE-1 (Steiner et al., 2007). In fact, miRNAs comprise the majority of short RNAs bound to RDE-1 in wild-type worms that are not subject to exogenous RNAi (Correa et al., 2010). Despite the fact that RDE-1 preferentially binds to short RNAs produced from perfectly base-paired substrates, *let-7* miRNA generated in such a manner requires ALG-1 for its biological function (Jannot et al., 2008). Also, siRNAs targeting the gene *unc-22*, which were generated from a mismatched precursor suited for ALG-1 loading, induced the *unc-22*-silencing phenotype and required RDE-1 for their activity (Steiner et al., 2007). These results indicate that although the structure of the precursor predisposes short RNAs for loading onto Argonautes with appropriate functional roles, it does not determine the functional specificity of the pathway, which is determined by the Argonaute itself, possibly through its interacting partners. Indeed, it was shown that RDE-1 and ALG-1 reside in separate nucleoprotein complexes (Gu et al., 2007; Steiner et al., 2007). Moreover, although artificial siRNAs were produced from the hairpin precursor in *rde-1* and *rde-4* mutant

backgrounds, they remained in complex with Dicer (Sijen et al., 2007), suggesting that RDE-4 is required for siRNA loading even though it binds poorly to short dsRNAs (Parker et al., 2006).

Although all Argonaute proteins are capable of binding short RNAs, they differ in their RNase H-like capacity to cleave RNA, which is dependent on the DDH catalytic motif (reviewed in Czech & Hannon, 2011; Hutvagner & Simard, 2008). The catalytic activity of Argonautes from other systems is used in two steps in the RNAi process: (1) removal of the "passenger" strand in the siRNA duplex and (2) degradation of the mRNA target (reviewed in Czech & Hannon, 2011; Hutvagner & Simard, 2008). As RDE-1 was shown to act upstream in the RNAi process, it seemed unlikely that its "slicer" capacity would be used for mRNA degradation. Indeed, in experiments where RDE-1 proteins with mutations in the DDH motif were introduced in an *rde-1* mutant background, it was shown that this motif was required for the RNAi response specifically at the step of passenger-strand cleavage, and that mutant RDE-1 proteins were not capable of binding to the target mRNA (Steiner et al., 2009; Figure 1.1). As RDE-1 is absolutely required for exogenous RNAi and binds primary siRNAs generated by Dicer, it seems to guide the downstream components of the RNAi pathway, such as the RdRP complex, to the target mRNA without causing mRNA cleavage (Figure 1.1).

3.1.3 siRNA amplification and additional Argonautes

The dsRNA-induced silencing effect in *C. elegans* is remarkably strong, although the amount of injected dsRNA is limited (Fire et al., 1998). The possibility of RNAi amplification had been conjectured about early on in the RNAi field and was confirmed with the discoveries of the requirement of the RdRP genes, *ego-1* and *rrf-1*, for exo-RNAi (Sijen et al., 2001; Smardon et al., 2000). Early models envisioned the generation of long dsRNA by RdRPs using target mRNAs as templates and primary siRNAs as primers and the processing of these secondary dsRNA molecules by Dicer (Sijen et al., 2001). However, the discovery that secondary siRNAs are strictly target dependent but largely antisense to mRNA by Northern blotting (A. Grishok, P.D. Zamore and C.C. Mello, unpublished, Figure 1.2) suggested a different mechanism. Indeed, subsequent studies determined that secondary siRNAs are products of *de novo* synthesis by RdRPs (Figure 1.1) and carry 5′triphosphates (Pak & Fire, 2007; Sijen et al., 2007) and identified endogenous siRNA molecules with similar characteristics (Ambros, Lee, Lavanway, Williams, & Jewell, 2003; Lee, Hammell, & Ambros, 2006;

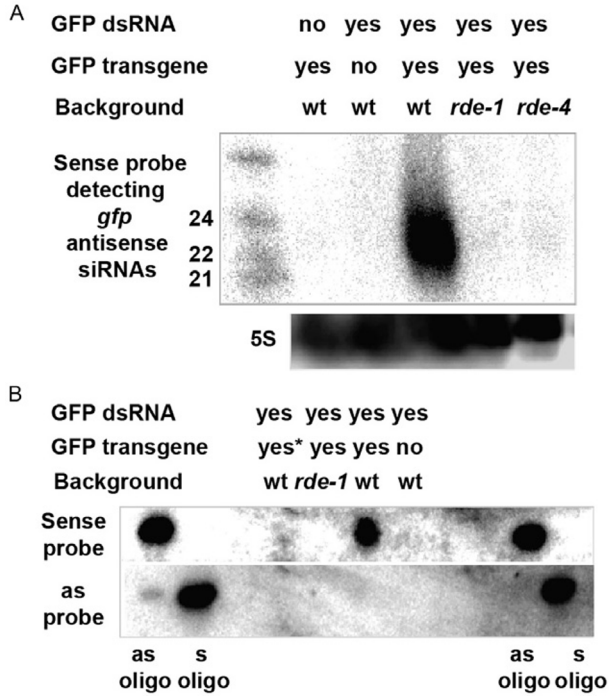

Figure 1.2 Target-dependent accumulation of antisense siRNAs during exo-RNAi. (A) Top: Northern blot detecting antisense *gfp* siRNAs in worms either containing or lacking the *pes-10::gfp* transgene and either exposed to *gfp* dsRNA or not. Only those worms that contain the transgene (target *gfp* mRNA) and that are exposed to *gfp* dsRNA show siRNA accumulation. *rde-1* and *rde-4* are required for the initiation of RNAi response and for secondary siRNA accumulation *in vivo*. Bottom: 5S RNA detection by northern blot is shown as a loading control. (B) Secondary siRNAs are antisense to the target (*gfp*) mRNA and are not detected by the probe designed to detect sense siRNAs. *gfp* sense and antisense RNA oligos are loaded for control of hybridization. Asterisk designates a transgenic strain *gsk-3*::GFP that shows a lower accumulation of siRNAs after dsRNA treatment compared to the *pes-10::gfp* strain (all other GFP transgene lanes in both panels). *Data presented in this figure are from the Ph.D. thesis of Alla Grishok, UMass Worcester, 2001.*

Pak & Fire, 2007; Ruby et al., 2006). Moreover, the production of secondary siRNAs by RdRP activity was recapitulated *in vitro* (Aoki et al., 2007). Interestingly, although secondary siRNAs are very efficient at promoting target mRNA degradation both directly (Aoki et al., 2007) and indirectly (Yigit et al., 2006), their competence in inducing tertiary siRNA production is limited (Montgomery et al., 2012; Pak, Maniar, Mello, & Fire, 2012). Therefore, the primary siRNAs are potent in promoting secondary siRNA production

but do not induce silencing themselves, while secondary siRNAs are efficient in executing silencing effects but have a limited capacity to induce indefinite siRNA amplification (Pak et al., 2012).

Although generation of secondary siRNAs was shown to be Dicer independent (Aoki et al., 2007), RRF-3 RdRP was discovered in Dicer immunoprecipitates (Duchaine et al., 2006). It turned out that some of the endogenous RNAi pathways require Dicer processing of dsRNA generated by RRF-3 (see discussion in Section 3.2). A recent biochemical analysis revealed that RdRPs RRF-1 and EGO-1, which are responsible for Dicer-independent production of siRNAs, exist in a complex with Dicer-related helicase DRH-3 (Aoki et al., 2007; Gu et al., 2009; Thivierge et al., 2012) and tudor domain protein EKL-1 (Gu et al., 2009; Thivierge et al., 2012; Figure 1.1), while the RRF-3 complex contains DRH-3, Dicer, and a distinct tudor domain protein, ERI-5 (Thivierge et al., 2012).

The Argonaute protein RDE-1 was implicated in the initiation of the RNAi response in *C. elegans* (Grishok, Tabara, & Mello, 2000; Tabara et al., 1999, 2002), while *Drosophila* Ago2 was identified in an RISC that mediates target mRNA degradation (Hammond, Boettcher, Caudy, Kobayashi, & Hannon, 2001). As the family of Argonaute genes is extensive in *C. elegans* (it includes 26 members, Table 1.1), this suggested that additional Argonaute proteins act downstream in the *C. elegans* RNAi process, similar to *Drosophila* Ago2. Indeed, multiple Argonaute proteins were found to contribute incrementally to the efficiency of RNAi, such that multiple Argonaute (MAGO) mutant strains, which contain up to 12 individual mutant Argonaute genes (e.g., MAGO12), are resistant to RNAi (Gu et al., 2009; Yigit et al., 2006). MAGO Argonautes (also called worm-specific Argonautes or WAGO) are found in complexes with secondary siRNAs produced by RdRPs (Gu et al., 2009; Yigit et al., 2006; Figure 1.1). These downstream Argonautes appear to be limiting because their overexpression leads to a higher sensitivity to RNAi (Yigit et al., 2006). Unexpectedly, WAGO family Argonautes do not contain the amino acid residues required for endonucleolytic activity; therefore, they must recruit additional cofactors for target mRNA degradation (Yigit et al., 2006).

Another Argonaute protein, CSR-1, was shown to promote the efficiency of exogenous RNAi in the germline (Yigit et al., 2006). However, it functions mainly in one of the endogenous RNAi pathways described later in this chapter, and its role in exo-RNAi may be indirect (Gu et al., 2009). Despite the fact that "slicing" by Argonaute proteins does not appear to play

a major role in silencing induced by exogenous RNAi, *C. elegans* extracts are fully capable of supporting slicing reactions with exogenously provided siRNAs (Aoki et al., 2007). siRNAs that mimic secondary molecules produced by RdRP were shown to be more efficient in *in vitro* slicing compared to those with primary siRNA features, and the CSR-1 Argonaute was found to be responsible for this Slicer activity (Aoki et al., 2007).

3.1.4 Mutator proteins

The *mut-7* and *rde-2/mut-8* genes were found to act downstream of primary siRNAs in the RNAi pathway (Grishok et al., 2000; Ketting, Haverkamp, van Luenen, & Plasterk, 1999; Ketting & Plasterk, 2000; Tijsterman, Ketting, Okihara, Sijen, & Plasterk, 2002). They belong to a larger group of "mutator" genes that are required for transposon silencing in the germline and for RNAi; mutations in this group of genes cause transposon mobilization and a high frequency of spontaneous mutations (Collins, Forbes, & Anderson, 1989). MUT-7 contains a 3′–5′ exonuclease domain and may participate in target mRNA degradation directly (Ketting et al., 1999). Biochemical analysis of the MUT-7 protein demonstrated that it is present in separate complexes in the nucleus and in the cytoplasm (Tops et al., 2005). The cytoplasmic complex also contains RDE-2 and was shown to increase in size upon dsRNA treatment (Tops et al., 2005). This increase in size was not observed in *rde-1* and *rde-4* mutants (Tops et al., 2005). Although the cytoplasmic MUT-7 complex appears to be distinct from the complex containing RdRP RRF-1 (Thivierge et al., 2012), it is interesting that specific cellular compartments called "*Mutator* foci," identified at the periphery of germline nuclei and containing six mutator proteins, also showed colocalization with RRF-1 (Phillips, Montgomery, Breen, & Ruvkun, 2012). The formation of *Mutator* foci was shown to be dependent on the glutamine/asparagine (Q/N)-rich protein MUT-16; *Mutator* foci also include MUT-7, RDE-2/MUT-8, nucleotidyl transferase MUT-2/RDE-3, DEAD-box RNA helicase MUT-14, and MUT-15/RDE-5 (Phillips et al., 2012). The *Mutator* foci were found to be adjacent to germline P-granules but distinct from them and were found not to depend on P-granule components for their stability (Phillips et al., 2012).

3.1.5 ABC transporters

dsRNA can be introduced into *C. elegans* by injection or by feeding dsRNA-expressing bacteria (environmental RNAi), and the RNAi-silencing process spreads systemically (see discussion in Section 5). Mutants

deficient in gene silencing initiated by dsRNA feeding but supporting silencing induced by dsRNA injection were thought to influence the uptake of dsRNA from the environment (Tabara et al., 1999; Tijsterman, May, Simmer, Okihara, & Plasterk, 2004). Surprisingly, the RNAi sensitivity of many of these mutants was shown to depend on the concentration of the dsRNA trigger rather than the method of its delivery, such that the defect in RNAi was revealed only at lower concentrations (Han, Sundaram, Kenjale, Grantham, & Timmons, 2008; Sundaram, Echalier, Han, Hull, & Timmons, 2006; Sundaram et al., 2008). One class of genes implicated in the efficiency of RNAi is the membrane-localized ATP-binding cassette (ABC) transporters, which use ATP to translocate small molecules across membranes (Sundaram et al., 2006, 2008). A mutation in the ABC transporter gene *haf-6* was isolated in a genetic screen (which also yielded *rde-1* and *rde-4*; Tabara et al., 1999) and was characterized later (Sundaram et al., 2006). Following this, 49 out of 61 ABC transporter genes were analyzed for their role in RNAi, and 10 of them, termed ABC_{RNAi}, were confirmed to promote the silencing process (Sundaram et al., 2006, 2008). The ABC domain of HAF-6 was shown to be important for its role in RNAi and for the localization of HAF-6 to membrane structures consistent with endoplasmic reticulum in the intestine and germline, two tissues where RNAi defects were observed in *haf-6* mutants (Sundaram et al., 2006).

Interestingly, mutants in ABC_{RNAi} transporters showed genetic interaction with the mutator class of RNAi-resistant mutants: double heterozygotes containing an ABC_{RNAi} mutation and a Mut mutation showed a second-site noncomplementation interaction that resulted in RNAi resistance (Sundaram et al., 2008). Similar genetic interaction was reported previously for *mut-7* and *rde-2/mut-8*, which encode components of the same complex (Tops et al., 2005). Consistently, ABC_{RNAi} mutants show defects in transposon silencing, that is, the mutator phenotype (Sundaram et al., 2008). The genes *rsd-2* and *rsd-6* were also initially thought to be involved in systemic RNAi silencing (Tijsterman et al., 2004), but were later described as dose-dependent mutants with mutator phenotypes (Han et al., 2008). RSD-2 localizes to multiple cellular compartments, including the nucleolus, and partially colocalizes with HAF-6 (Han et al., 2008). *haf-6*, *rsd-2*, and *rsd-6* mutants were shown to be required for the accumulation of some endogenous siRNAs and secondary siRNAs in the exo-RNAi pathway (Zhang et al., 2012). Intriguingly, HAF-6 localization was observed in the perinuclear region in germ cells (Sundaram et al., 2008); it would be

interesting to explore whether this localization overlaps with the recently found *Mutator* foci (Phillips et al., 2012).

3.1.6 RDE-10/RDE-11 complex

The RDE-10/RDE-11 complex is the most recently identified protein complex promoting exogenous RNAi (Yang et al., 2012; Zhang et al., 2012). This complex appears to be nematode-specific. The *rde-10* and *rde-11* genes were identified in genetic screens for RNAi-deficient mutants, and their protein products were found to be major interacting partners through proteomic analyses (Yang et al., 2012; Zhang et al., 2012). The RDE-10/RDE-11 complex has been shown to promote secondary siRNA amplification and mRNA degradation and to work in parallel with the nuclear RNAi pathway (Yang et al., 2012; Zhang et al., 2012). In addition, this complex appears to show preference for partially degraded mRNA (Yang et al., 2012). The RDE-10 protein has been found to associate with mRNA targeted by exo-RNAi, and this interaction was shown to be independent of *rrf-1* and *rde-11* but dependent on *rde-1* (Yang et al., 2012). Altogether, these results suggest that the RDE-10/RDE-11 complex engages target mRNA in response to RDE-1 and primary siRNAs but prior to RRF-1 and stabilizes partially degraded mRNA to promote the generation of secondary siRNAs (Yang et al., 2012; Zhang et al., 2012).

3.1.7 Transcriptional silencing induced by dsRNA

The mechanism of gene silencing induced by dsRNA in *C. elegans* is still poorly understood. It appears that posttranscriptional, cotranscriptional, and transcriptional mechanisms are involved and that some genes are preferentially silenced through mRNA degradation in the cytoplasm, while pre-mRNA degradation coupled with transcriptional silencing is prevalent for others. For example, RNAi-induced transcriptional silencing of the *elt-2::GFP/LacZ* reporter includes a decrease in pre-mRNA levels, reduction in RNA Pol II occupancy at the transgenic array, and a decrease in histone H4 acetylation (Grishok, Sinskey, & Sharp, 2005). In this system, the initiation of silencing was shown to be dependent on *rde-1* and *rde-4*, and down-regulation of a number of genes with predicted nuclear roles had an effect on the efficiency of silencing (Grishok et al., 2005).

Detailed analyses of dsRNA-induced silencing of nuclear transcripts such as *lir-1-lin-26* (Bosher, Dufourcq, Sookhareea, & Labouesse, 1999) and *lin-15b-lin-15a* polycistronic RNAs have been published by Kennedy and colleagues (Burkhart et al., 2011; Guang et al., 2010, 2008). Several mutants

that specifically affect nuclear RNAi (*nuclear RNAi defective* (*nrde*)) were identified in forward genetic screens, and the molecular functions of the corresponding gene products were investigated. Interestingly, an Argonaute protein, NRDE-3, was shown to be specifically required for nuclear RNAi (Guang et al., 2008; Figure 1.1). NRDE-3 is expressed in somatic cells, contains a nuclear localization signal (NLS), and localizes to the nucleus in an NLS-dependent and siRNA-dependent manner (Guang et al., 2008). NRDE-3 is also called WAGO-12 and belongs to the group of WAGO Argonautes (Gu et al., 2009; Table 1.1), consistent with its binding of secondary siRNAs produced by RdRP RRF-1 (Burkhart et al., 2011; Guang et al., 2008; Figure 1.1). NRDE-3 escorts siRNAs to the nucleus, where it binds target pre-mRNA and promotes the interaction between the nuclear serine/arginine-rich conserved protein NRDE-2 and pre-mRNA. The action of NRDE-3, NRDE-2, and the two additional nematode-specific proteins NRDE-1 and NRDE-4 leads to inhibition of pre-mRNA synthesis and depletion of the RNA Pol II ChIP signal downstream of the region targeted by dsRNA, while Pol II appears to accumulate at DNA sequences corresponding to the dsRNA (Burkhart et al., 2011; Guang et al., 2010). This effect of RNAi on transcriptional elongation is accompanied by an increase in histone H3 lysine 9 (H3K9) methylation near the dsRNA-targeted region (Burkhart et al., 2011; Guang et al., 2010). Notably, the target genes chosen for the exo-RNAi-induced silencing experiments that revealed the function of the NRDE system normally have very low levels of H3K9 methylation and therefore do not represent the endogenous targets of this pathway. Although NRDE-3 and NRDE-2 were shown to interact with pre-mRNA in an siRNA-dependent manner, these proteins do not associate with chromatin, while NRDE-1 does (Burkhart et al., 2011). Interestingly, mutation in *nrde-4* does not prevent the interaction of NRDE proteins with pre-mRNA, but it abolishes NRDE-1 recruitment to chromatin and H3K9 methylation (Burkhart et al., 2011). The NRDE system is likely to provide further insight into the mechanisms of dsRNA-induced H3K9 methylation and transcription inhibition.

3.1.8 Inheritance of dsRNA-induced gene silencing

dsRNA-induced gene silencing was originally shown to occur in animals injected with dsRNA and in their progeny, but it did not persist further (Fire et al., 1998). Moreover, mated *rde-1* and *rde-4* mutants injected with dsRNA were fully capable of transmitting the silencing signal to their

rde/+ progeny, which indicates that generation of primary siRNAs and target-dependent siRNA amplification in P0 is not required for the persistence of the silencing signal in the F1 generation (Tabara et al., 1999). These data are consistent with a persistence of the dsRNA that precedes primary siRNA production (see discussion in Section 5). The dsRNA is likely to be distributed to all cells in the F1 progeny as the zygote divides and to eventually lead to target mRNA-dependent siRNA amplification and silencing in cells expressing the target mRNA. A recent study found that the persistence of dsRNA-induced silencing for target genes expressed in the F1 larva, but not those expressed in the embryo, required the *nrde* pathway genes and that these genes promoted accumulation of NRDE-3-bound siRNAs (Burton, Burkhart, & Kennedy, 2011). Moreover, this study found that F1 animals exhibited a much higher level of NRDE-dependent H3K9 methylation at the dsRNA-targeted locus compared to their parents, which had been exposed to the dsRNA (Burton et al., 2011). These results indicate that the nuclear RNAi pathway and, specifically, the NRDE-3 Argonaute, which associates with secondary siRNAs, are predominantly responsible for silencing in somatic tissues of F1 animals.

Inheritance of RNAi beyond the F1 generation is qualitatively different from the persistence of RNAi in F1 because its initiation requires the function of *rde-1* and *rde-4*, and therefore primary siRNA production, siRNA amplification, or both, in the P0 animals injected with dsRNA (Grishok et al., 2000). The fact that this type of RNAi inheritance is mediated by an extragenic epigenetic factor supports the idea that siRNAs are inherited (Grishok et al., 2000). Moreover, as inheritance of dsRNA-induced silencing beyond the F1 generation has been demonstrated only for genes expressed in the germline (Alcazar, Lin, & Fire, 2008; Buckley et al., 2012; Grishok et al., 2000; Gu et al., 2012; Vastenhouw et al., 2006), secondary siRNAs generated on maternal transcripts and deposited into the zygote are the most likely agents of long-term RNAi inheritance.

Inheritance of RNAi beyond the F1 generation was first shown in experiments targeting essential genes and therefore selecting progeny inheriting less silencing material (Grishok et al., 2000). In a subsequent study, a detailed pedigree-based analysis of dsRNA-induced viability over generations was performed (Alcazar et al., 2008). This study identified a bottleneck in RNAi inheritance at the F4 generation, such that F4 animals survived well but rarely had viable progeny themselves (Alcazar et al., 2008). The properties of RNAi inheritance up to the F4 generation were

consistent with that of a diffusible epigenetic element that could be transmitted through both oocyte and sperm (Alcazar et al., 2008).

Genomic analysis was further used for studies of the connection between RNAi and chromatin and transgenerational RNAi inheritance (Gu, Pak, et al., 2012). This work clearly identified a dsRNA-induced H3K9 methylation mark at the several loci targeted by dsRNA in animals fed with dsRNA as well as in their F1 and F2 progeny. Interestingly, H3K9me3 could spread from the targeted locus and was detected up to 11 kb away from the dsRNA trigger region (Gu, Pak, et al., 2012). Notably, dsRNA-induced H3K9me3 required *rde-1*, WAGO secondary Argonautes, and *nrde-2*, even though the level of siRNA production was not altered in *nrde-2* mutant worms (Gu, Pak, et al., 2012). siRNAs were found to decrease in abundance in the F1 and F2 generations, although they were still present; therefore, this study is consistent with the idea of inherited siRNAs and suggests that they induce H3K9me3 (Gu, Pak, et al., 2012). Importantly, although the accumulation of siRNAs was detected 4 h after dsRNA exposure and increased further after 24 h, no H3K9 methylation was present at the target loci at that time, appearing only after 48 hours (Gu, Pak, et al., 2012). This significant lag period between the two events argues against immediate siRNA-guided modification of chromatin and suggests multiple intermediate steps in this process. Another study of dsRNA-induced heritable silencing of a single copy GFP reporter followed the persistence of silencing in more than 60% of the animals over four generations (Ashe et al., 2012). Although both sense and antisense siRNAs were identified in animals treated with dsRNA, only antisense siRNAs persisted to the F4 generation (Ashe et al., 2012). Consistent with the detection of H3K9 methylation at the loci targeted by RNAi described earlier, a mutation in the gene *set-25*, which encodes a putative H3K9 methyltransferase, alleviated the heritable silencing induced by dsRNA (Ashe et al., 2012).

Similar to the requirement of the *nrde* pathway genes *nrde-1*, *nrde-2*, and *nrde-4* for the persistence of dsRNA-induced silencing that targets somatic genes in the F1 generation, these genes are also required in F1 and later progeny for the silencing of germline-expressed genes (Ashe et al., 2012; Buckley et al., 2012). However, whereas the Argonaute NRDE-3 mediates F1 silencing in the soma, it is not required for germline silencing (Ashe et al., 2012; Buckley et al., 2012). Instead, another Argonaute named *heritable RNAi defective* (*hrde-1*, also known as *wago-9*; Gu et al., 2009; Table 1.1) was identified in genetic screens for RNAi inheritance-deficient mutants (Ashe et al., 2012; Buckley et al., 2012). HRDE-1/WAGO-9 shows

germline-specific nuclear expression (Ashe et al., 2012; Buckley et al., 2012; Shirayama et al., 2012); it is required for the inheritance dsRNA-induced silencing in the F2 and F3 generations (Ashe et al., 2012; Buckley et al., 2012) and for epigenetic silencing initiated by other means (see Section 4.2.2). HRDE-1 was shown to interact with secondary siRNAs (Figure 1.1) and to promote the association of NRDE-2 with pre-mRNA and the induction of H3K9me3 at the target locus (Buckley et al., 2012). The biological role of HRDE-1 and other genes involved in exo- and endo-RNAi is discussed in Section 3.3.3.

3.1.9 Chromatin modulators of the RNAi response

As many components involved in the exogenous RNAi pathway appear to be rate limiting, it is possible that factors affecting their abundance may influence the efficiency of the RNAi response. Certain proteins with predicted chromatin-binding properties and some known regulators of transcription are particularly important in modulating the sensitivity of *C. elegans* to dsRNA-induced silencing.

Shortly after the discovery of the potency of dsRNA in inducing gene silencing, large-scale RNAi-based screens started to be used for identifying gene function in *C. elegans*. In one such approach, when pools of dsRNA molecules targeting several genes were used in an initial screen, it was discovered that downregulation of genes with predicted chromatin function—*zfp-1* and *gfl-1*—as well as H3K36 methyltransferase MES-4 (Rechtsteiner et al., 2010) and two genes encoding Polycomb-group-related proteins compromised the efficiency of other dsRNAs from the same pool (Dudley, Labbe, & Goldstein, 2002).

Conversely, mutations in other genes encoding chromatin regulators, such as *C. elegans* Retinoblastoma (Rb) homolog *lin-35*, were shown to promote the efficiency of RNAi (Lehner et al., 2006; Wang et al., 2005). These negative regulators of RNAi belong to the genetically defined group of SynMuv (Synthetic Multivulva) B genes that act redundantly with the SynMuv A and SynMuv C groups to negatively regulate the inappropriate initiation of Ras signaling and ectopic vulvae development (Ceol & Horvitz, 2004; Cui et al., 2006; Ferguson & Horvitz, 1989). The core protein complex formed by the SynMuv B gene products is a repressive chromatin complex (dREAM/Muv B) (Harrison, Ceol, Lu, & Horvitz, 2006). This complex, along with other associated proteins, was shown to inhibit germline-specific fate in the somatic tissues of *C. elegans* (Unhavaithaya et al., 2002; Wang et al., 2005). Interestingly, expression of a number of

germline-enriched Argonaute proteins is enhanced in SynMuv B mutants (Grishok, Hoersch, & Sharp, 2008; Wu, Shi, Cui, Han, & Ruvkun, 2012), which suggests a potential reason for their enhanced susceptibility to RNAi (Figure 1.3).

There is a peculiar genetic relationship between the chromatin factors promoting RNAi, such as *zfp-1*, *gfl-1*, and *mes-4*, and the SynMuv B genes inhibiting RNAi efficiency. It was found that the ectopic vulvae formation seen in SynMuv B; SynMuv A double mutants is suppressed by mutations in a group of genes called SynMuv suppressors (Cui, Kim, & Han, 2006). Interestingly, *zfp-1*, *gfl-1*, and *mes-4* belong to the SynMuv suppressor group (Cui, Kim, & Han, 2006; Wang et al., 2005), and many other SynMuv suppressors have been found to promote RNAi (Cui, Kim, & Han, 2006). A mechanistic understanding of the relationship between SynMuv B genes and SynMuv suppressors is currently not clear. As ZFP-1 negatively modulates transcription (Mansisidor et al., 2011) and localizes to the promoters of SynMuv B genes (Mansisidor et al., 2011), it is possible that inhibition of *zfp-1* function leads to a diminished RNAi response due to enhanced expression of SynMuv B proteins and lower levels of Argonaute proteins (Figure 1.3). The effect of MES-4 in promoting Argonaute expression is likely to be direct (Gaydos, Rechtsteiner, Egelhofer, Carroll, & Strome, 2012; Kudron et al., 2013; Figure 1.3).

An interesting connection between miRNA function and regulation of RNAi efficiency has been discovered recently (Massirer, Perez, Mondol, & Pasquinelli, 2012). Although biologically relevant targets have not been

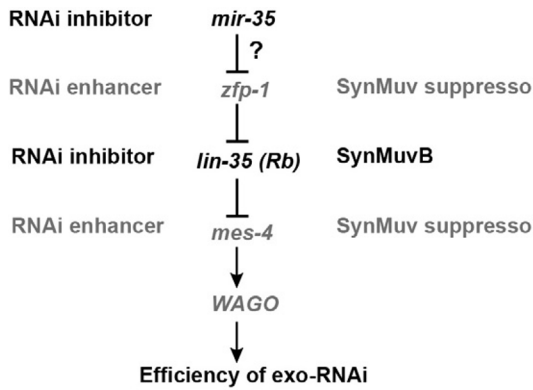

Figure 1.3 A hypothetical genetic pathway connecting chromatin-associated factors regulating exo-RNAi efficiency. Positive or negative connections between the components are based on the published data with the exception of negative regulation of *zfp-1* by *mir-35*, which is speculative (3′UTR of *zfp-1* contains a predicted *mir-35* binding site).

described for the abundant *mir-35* family expressed in the germline and embryo, a mutation eliminating a large number of *mir-35* family miRNAs, *mir-35–41*(*gk262*), led to enhanced susceptibility to exogenous RNAi (Massirer et al., 2012). This effect was due to lower levels of LIN-35/Rb in *mir-35* mutants because ectopic expression of LIN-35 rescued the RNAi hypersensitivity phenotype. Notably, maternal expression of LIN-35 was shown to be sufficient for the restoration of the normal RNAi response in animals lacking zygotic *lin-35* function (Massirer et al., 2012). It would be interesting to determine how the *mir-35–41*(*gk262*) mutation leads to reduced LIN-35 Rb levels and whether negative regulation by *mir-35–41* of SynMuv suppressors with the potential to inhibit *lin-35*, such as ZFP-1, can explain the relationship between *mir-35–41* and LIN-35 (Figure 1.3).

3.2. Genomic and molecular features of endogenous RNAi

Initial studies of RNAi in *C. elegans* predicted its antiviral role, while miRNAs appeared as endogenous regulators of gene expression. Then, the discovery of an enhanced RNAi response in the RdRP mutant *rrf-3* suggested an endogenous role for the putative secondary siRNAs generated by this enzyme and a competition between the exo- and endo-RNAi pathways (Simmer et al., 2002). Indeed, endogenous siRNAs antisense to protein-coding genes were discovered shortly thereafter (Ambros et al., 2003) and an active investigation of endogenous RNAi in *C. elegans* began. By 2012, a significant body of knowledge had been generated, and a SnapShot summary comparing endogenous RNAi machinery and mechanisms across species has been published recently (Flamand & Duchaine, 2012).

3.2.1 Discovery of endo-siRNAs

Sequencing experiments aimed at identifying more miRNAs by Ambros and colleagues also yielded reads antisense to 551 different protein-coding genes as well as reads matching retrotransposons and a peculiar locus on the X chromosome termed "X cluster" (Ambros et al., 2003). At that time, a prevalence of guanosine in the 5′ position of endo-siRNAs was noted (Ambros et al., 2003). Another sequencing report by the same group defined 1085 genes with matching antisense siRNAs (Lee et al., 2006). Initial analyses of the genetic requirements for endo-siRNA production determined that mutants compromised in exo-RNAi, such as *rrf-1*, *rde-3*, *mut-7*, and *mut-14*, as well as mutants with an enhanced RNAi response, such as *rrf-3* and *eri-1*, affected endo-siRNA accumulation (Lee et al., 2006). Interestingly, although both *rde-1* and *rde-4* are required for the exo-RNAi

response, only *rde-4* appeared to have a role in endo-RNAi (Lee et al., 2006).

The development of the new high-throughput pyrophosphate sequencing method (Margulies et al., 2005) enabled a deeper analyses of short RNA populations in *C. elegans* and further classification of endo-siRNAs (Pak & Fire, 2007; Ruby et al., 2006). A prevalent length of either 21–22 or 26 nt and a 5′G in both kinds of siRNAs was reported (Ruby et al., 2006). The features of the 26-nt class of endo-siRNAs included a 5′monophosphate and a modified 3′terminus (Ruby et al., 2006), whereas the 22-nt class was underrepresented in libraries generated by a 5′monophosphate-ligation-dependent protocol and contained a 5′triphosphate like the secondary siRNAs generated by RdRPs during exo-RNAi (Pak & Fire, 2007; Ruby et al., 2006). Many unique cloned sequences matched splice junctions, which supported their origin from RdRP amplification using mature mRNA as a template; however, intron-matching reads were also found, which suggests the possibility of nuclear RdRP-dependent amplification (Pak & Fire, 2007; Ruby et al., 2006).

3.2.2 Distinction between WAGO- and CSR-1-associated 22G-endo-siRNAs

The application of the Illumina/Solexa deep sequencing platform stimulated the discovery of additional short RNAs in many organisms, including *C. elegans*. An earlier described class of endogenous 21–22 nt RNAs was significantly expanded and called 22G-RNAs (Gu et al., 2009). 22G-RNAs are most abundant in the germline, although somatic 22G have also been described. The two major classes of 22G-RNAs are defined by their interacting Argonaute proteins: the majority of 22G-RNAs antisense to protein-coding genes exist in complex with CSR-1 (Claycomb et al., 2009; Gu et al., 2009), while the WAGO family (required for the exogenous RNAi response) interacts with 22G-RNAs targeting transposons, pseudogenes, cryptic loci, and a few coding genes (Gu et al., 2009; Figures 3.4–3.6). 22G have features consistent with them being RdRP products, and they require RdRPs for their accumulation. RRF-1 and EGO-1 act redundantly in generating germline-enriched WAGO-22G-RNAs; somatic 22G-RNAs require RRF-1, and CSR-1-bound 22G are dependent only on EGO-1 (Claycomb et al., 2009; Gu et al., 2009). Dicer-related helicase DRH-3 and tudor-domain protein EKL-1 are required for the production of both classes of 22Gs and interact with RdRPs (Aoki et al., 2007; Gu et al., 2009), consistent with their characterization as the core RdRP module

components (Thivierge et al., 2012; Figures 3.4–3.6). Interestingly, sequencing of short RNAs from non-null *drh-3* mutants with mutations in the putative helicase domain revealed the persistence of 22G-RNAs antisense to 3′UTR sequences, suggesting that RdRPs are recruited to these regions and that the helicase activity of DRH-3 is required for their access to more upstream target mRNA sequences (Gu et al., 2009). The abundance of WAGO-22G-RNAs is also dependent on RDE-3 and MUT-7 (Gu et al., 2009). In addition, the essential role of the MUT-16 protein in the formation and/or stability of both germline and somatic 22G-RNAs of the WAGO class was recently described (Zhang et al., 2011).

WAGO-22G-RNAs are more abundant than CSR-1 22G-RNAs, and they have a clear silencing effect on their targets, including suppression of transposon mobilization (Gu et al., 2009; Zhang et al., 2011). Although CSR-1 was shown to be associated with chromatin at regions targeted by its cofactor 22G-RNAs (Claycomb et al., 2009), only modest changes in gene expression were detected in *csr-1*(−) worms, which contrasts with their severe sterility phenotype (Claycomb et al., 2009; She, Xu, Fedotov, Kelly, & Maine, 2009; Yigit et al., 2006) and with the strong defects in chromosome segregation in animals treated with *csr-1* dsRNA (Claycomb et al., 2009; Yigit et al., 2006). Interestingly, the majority of genes corresponding to the antisense 22G-RNAs that are enriched in CSR-1 immunoprecipitation samples showed reduction in their expression in *csr-1*(−) sterile adults (Avgousti, Palani, Sherman, & Grishok, 2012; Claycomb et al., 2009). At the same time, analyses of 22G-RNA and mRNA abundance in *ego-1*(−) sterile worms revealed that ∼300 germline genes were modestly upregulated in the absence of EGO-1-dependent 22G-RNAs (Maniar & Fire, 2011).

The abundance of CSR-1 22G-RNAs is controlled by the germline-specific nucleotidyltransferase protein CDE-1 (van Wolfswinkel et al., 2009). CDE-1 specifically uridylates CSR-1 22G-RNAs and interacts with RdRP EGO-1, but not with CSR-1 (van Wolfswinkel et al., 2009). Although the abundance of CSR-1 22G-RNAs is increased in *cde-1* mutants, these endo-siRNAs do not function properly, which is evident by the similarity of the chromosome segregation defects of *csr-1* and *cde-1* mutant worms (van Wolfswinkel et al., 2009). The function of the WAGO pathway in transposon silencing and exo-RNAi is also compromised in *cde-1* mutants, which suggests that CDE-1 is involved in proper separation of the 22G pathways (van Wolfswinkel et al., 2009).

Interestingly, a recent comparison of short RNA populations and their targets in four related nematodes revealed a higher than average conservation of genes targeted by CSR-1 22G-RNAs, whereas WAGO targets showed poor conservation and produced more siRNAs than conserved CSR-1 targets (Shi, Montgomery, Qi, & Ruvkun, 2013).

3.2.3 26G- and 22G-endo-siRNAs of the ERI pathway

The first *e*nhanced *R*NA*i* (*eri*) gene, *eri-1*, was identified by mutant alleles with increased sensitivity to exogenous RNAi (Kennedy, Wang, & Ruvkun, 2004). The *eri-1* gene encodes two protein isoforms, both of which contain a nucleic acid-binding SAP domain and a 3′-to-5′ exonuclease domain (Gabel & Ruvkun, 2008; Kennedy et al., 2004). Initially, the increased exo-RNAi response in *eri-1* mutants was thought to be due to a suppressed degradation of siRNAs (Kennedy et al., 2004). Later, ERI-1 was recognized as a component of a specific endogenous RNAi pathway that is required for the production of certain endo-siRNAs and that competes with exo-RNAi (Duchaine et al., 2006; Figure 1.4). Also, ERI-1 was found among proteins coimmunoprecipitating with Dicer in an unbiased proteomic approach (Duchaine et al., 2006). Only the longer ERI-1b isoform functions in endo-RNAi, while the shorter ERI-1a isoform was shown to have a conserved role in 5.8S rRNA processing (Gabel & Ruvkun, 2008). Interestingly, the RdRP RRF-3, which also restricts exo-RNAi (Simmer et al., 2002), was also identified in the complex with Dicer (Duchaine et al., 2006), and two other *eri* genes, *eri-3* and *eri-5*, encode Dicer complex components (Duchaine et al., 2006). Recent biochemical analyses revealed that the Dicer–RRF-3 core complex contains DRH-3 and Tudor domain protein ERI-5, which interacts with Dicer directly (Thivierge et al., 2012; Figure 1.4). The full list of ERI–Dicer-1 complex (ERIC) components identified by proteomic analyses also includes ERI-3, ERI-1, and RDE-4 (Thivierge et al., 2012).

The molecular function of ERIC is to produce a longer species of endogenous siRNAs called 26G-RNAs (Gent et al., 2010; Han et al., 2009; Vasale et al., 2010; Figure 1.4). Two classes of 26G-RNAs have been identified: Class I is present in purified sperm, while Class II is most abundant in oocytes and embryos and diminished in expression during postembryonic development (Han et al., 2009; Figure 1.4). Depletion of both types of 26G-RNAs affects the expression of their target genes, although the effect of depleting Class II 26G-RNAs persists much longer in development than the 26Gs themselves (Han et al., 2009). The fact that the ERI pathway also affects

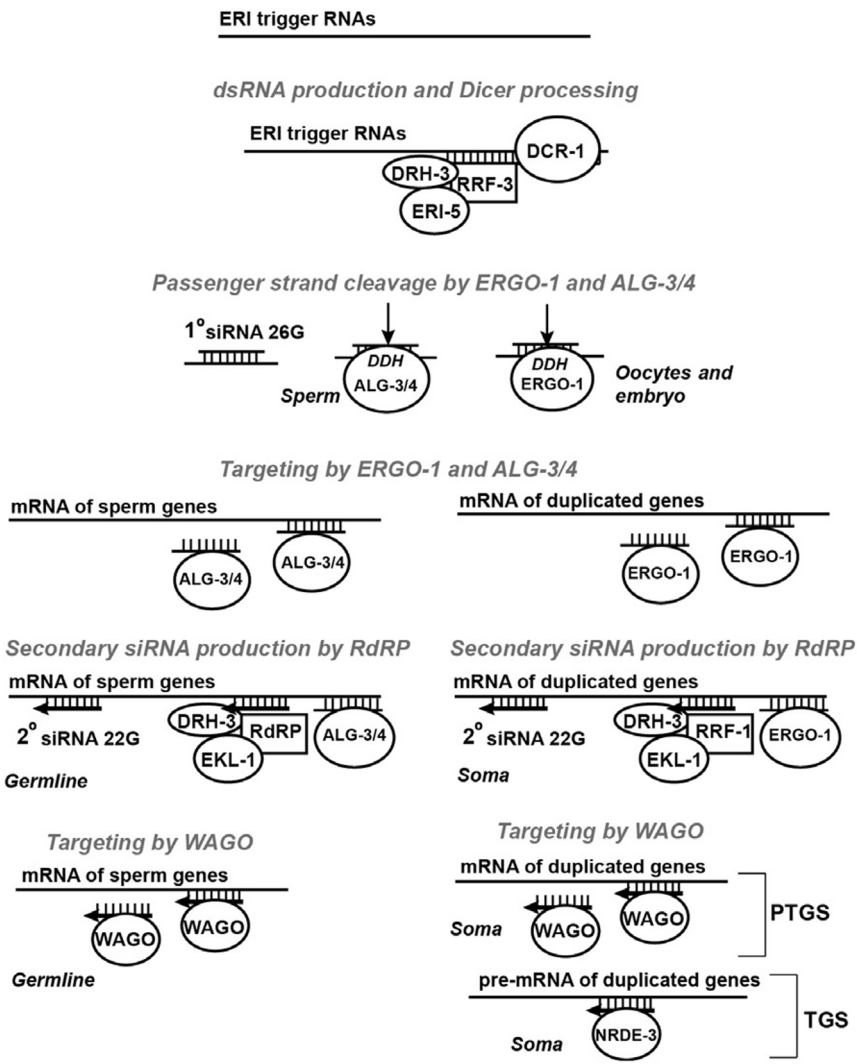

Figure 1.4 ERI-dependent endogenous RNAi pathways. Specific messages are selected as templates for RdRP RRF-3 by unknown mechanisms that include recognition of duplicated genes. dsRNA produced by the RRF-3 complex is cleaved by Dicer to produce 26G-duplex siRNAs where the passenger strand is ~19 nt. Two types of 26G-RNAs are recognized: sperm-specific 26G bound by ALG-3/4 and oocyte/embryo-specific 26G bound by ERGO-1. ERGO-1 and likely ALG-3/4 cleave the passenger strand in the 26G-siRNA duplex. mRNAs targeted by 26G-RNAs are used as templates for secondary 22G-RNA production; 22G-RNAs are incorporated into complexes with WAGO Argonautes which stimulate PTGS and TGS. Only a fraction of WAGO-bound 22G-RNAs is dependent on the ERI pathway.

production of some 22G-RNAs (Duchaine et al., 2006; Gu et al., 2009) suggested a connection between 26G- and 22G-RNAs. Indeed, analysis of the short RNAs found in complex with ERGO-1 Argonaute (Yigit et al., 2006) identified Class II 26G-RNAs (Vasale et al., 2010; Figure 1.4), and a subset of 22G-RNAs was found to be associated with 26G-RNA-producing loci (Vasale et al., 2010). Importantly, *rrf-1* and WAGO were required for the accumulation of these 22G-RNAs (Figure 1.4), but not for the accumulation of corresponding 26G-RNAs, which required *rrf-3* and *ergo-1*. Therefore, a two-step pathway involving two rounds of RdRP amplification (by RRF-3 and RRF-1) and two Argonaute types (primary-ERGO-1 and secondary-WAGO) emerged (Vasale et al., 2010; Figure 1.4). The accumulation of 22G-RNAs corresponding to Class I spermatogenesis-specific 26G-RNAs is dependent on ALG-3/4 Argonautes expressed in the germline region that undergoes spermatogenesis (Conine et al., 2010); therefore, the molecular function of ALG-3/4 is likely to be similar to that of ERGO-1 (Figure 1.4). An independent study based on deep sequencing of short RNAs from *rrf-3* mutant animals that identified somatic target genes regulated by the ERI pathway suggested a similar two-step model of 26G-RNA-dependent 22G-RNA production (Gent et al., 2010). Notably, 26G-RNAs, unlike 22G-RNAs, were found to be modified at their 3′ends (Ruby et al., 2006; Vasale et al., 2010). HENN-1 RNA methyltransferase was implicated in methylating the 3′ ends of the ERGO-1 class of 26G-RNAs, and this methylation had a stabilizing effect on these RNAs (Billi et al., 2012; Kamminga et al., 2012; Montgomery et al., 2012). In the absence of HENN-1, uridylation of 26Gs was frequently observed (Kamminga et al., 2012).

The somatic Argonaute protein NRDE-3, which binds to secondary siRNAs and translocates to the nucleus in an siRNA-dependent manner, has also been connected to the ERI pathway (Figure 1.4). NRDE-3 is localized to the nucleus in wild-type worms (Guang et al., 2008) but loses nuclear localization in mutants with defective ERI-dependent 22G production, including *eri-1*, *rde-4*, *ergo-1* (Guang et al., 2008), *eri-9*, *dcr-1*(*mg375Eri*) (Pavelec et al., 2009), and *eri-6/7* (Fischer et al., 2011). Moreover, silencing of a GFP-sensor regulated by the ERI-dependent 22G-siR-1 was shown to require NRDE-3 (Montgomery et al., 2012). Given the connection between 26G and 22G production, these results are consistent with the endogenous role of NRDE-3 in repression of 26G-RNA-target genes (Burkhart et al., 2011; Guang et al., 2008). Notably, initiation of GFP-sensor silencing by the NRDE-3-dependent 22G-siR-1 tolerated some degree of

mismatching (Montgomery et al., 2012). *mut-2* and *mut-7* are also required for the nuclear localization of NRDE-3 (Guang et al., 2008), which suggests that they have a role in the ERI pathway. Indeed, *mut-16*, *mut-2*, and *mut-7* were shown to be essential for the accumulation of ERGO-1 26G-RNAs, and *mut-16* was partially involved in the production of secondary 22G-RNAs of the ALG-3/4 class (Zhang et al., 2011).

An initial survey of the loci targeted by ERGO-1-dependent 22G-RNAs revealed that they are preferentially localized to chromosome arms and enriched in gene duplications (Vasale et al., 2010; Figure 1.4). A more extensive investigation of ERGO-1 targets was conducted in a study of the ERI-6/7 helicase, which is encoded by two adjacent genes whose pre-mRNAs fuse in a rare *trans*-splicing event (Fischer, Butler, Pan, & Ruvkun, 2008). ERI-6/7 is homologous to helicases that act with short RNAs in plants and animals. Similar to ERGO-1, ERI-6/7 is required for the production of Class II 26G-RNAs abundant in oocytes and embryos and for that of their dependent 22G-RNAs, which are abundant in somatic tissues (Fischer et al., 2011). This temporal shift in the peaks of abundance of Class II 26Gs and their corresponding 22Gs suggests that RRF-3-dependent 26G production and RRF-1-dependent 22G generation are separated in time and that RRF-3 acts in the embryo, while RRF-1 acts postembryonically (Fischer et al., 2011). Interestingly, bioinformatic analyses of 78 genes matching *eri-6/7*-dependent siRNAs revealed groups of related genes that are poorly conserved in other species and have few introns (Fischer et al., 2011). These analyses are consistent with the ERGO-1 26G branch serving to silence recently duplicated or horizontally acquired genes (Fischer et al., 2011). This conclusion is further supported by a recent high-throughput sequencing study of the evolution of short RNAs using four related nematode species: *C. elegans*, *Caenorhabditis briggsae*, *Caenorhabditis remanei*, and *Caenorhabditis brenneri* (Shi et al., 2013). Although 26G-RNA sequences themselves are not conserved, it is remarkable that embryo-specific ERGO-1 26G-RNA-target genes showed only 2–12% conservation when considering the higher than average conservation of sperm-specific 26G-RNA-target genes (Shi et al., 2013). This study also detected conservation in the localization of ERGO-1 26G-RNA-producing loci to gene-poor chromosome arms (Shi et al., 2013).

The distinctive feature of 26G-RNA biogenesis is its Dicer dependence, which is in contrast to the Dicer-independent biogenesis of 22G-RNAs (Figure 1.4). The sequences of 26G-RNAs are not consistent with a processive generation by Dicer using long dsRNA substrates produced by

RRF-3 (Fischer et al., 2011; Vasale et al., 2010). Although 26G-RNAs are mostly 26 nt reads antisense to mRNAs in wild-type worms, stabilization of the passenger-strand short RNAs antisense to 26Gs was detected in *ergo-1* mutants, which is consistent with slicing of the passenger strand by ERGO-1 (Fischer et al., 2011). Interestingly, the passenger strand of the 26G duplex is ~19 nt, such that ~3–4 nt 5′ and 3′ overhangs of the longer 26G strand are present (Fischer et al., 2011; Figure 1.4).

The involvement of the helicase domain of Dicer in endo-siRNA production was demonstrated with the *dcr-1*(*mg375*) mutation, which was identified in genetic screens for ERI mutants (Pavelec et al., 2009). A more extensive study using transgenes with point mutations in the helicase domain, which were introduced into a *dcr-1*(−) null background, confirmed that this domain is essential for endo-siRNA production and demonstrated that it specifically affects 26G-RNA levels genome wide (Welker et al., 2010). An increased number of 26G reads was noted in *C. elegans* mutants of ADARs (Warf, Shepherd, Johnson, & Bass, 2012). The predominant 26G duplex RNA structures identified by this study contained 3 nt 3′ overhangs consistent with findings from studies of Dicer processing of long dsRNA (Welker et al., 2011; Figure 1.4). Therefore, the 3′ end of 26G-RNA results from Dicer cleavage, and it was suggested that the 3′–5′ nuclease ERI-1 is involved in the processing of the 3′end of the 19-nt-long 26G passenger strand, which would result in more variability in the 5′ overhangs of the 26G duplex (Warf et al., 2012). The precise mechanism of 26G-RNA biogenesis involving the Dicer, ERI-1, and RRF-3 module remains to be elucidated.

Although RDE-1 and RDE-4 are equally required for exo-RNAi, RDE-4 has a much larger role in endo-RNAi. RDE-4 is required for 26G production (Vasale et al., 2010) and also appears to have a connection to EGO-1-dependent siRNAs (Maniar & Fire, 2011). However, the precise molecular function of RDE-4 in endo-RNAi is not clear. RDE-1 has a role in the production of most abundant somatic 22G-RNAs corresponding to the Y47H10A.5 gene, which is initiated by the *mir-243* miRNA that RDE-1 binds (Correa et al., 2010), and in the production of a few other 22G-RNAs. Notably, these RDE-1-dependent siRNAs are also depleted in *rde-10* and *rde-11* mutants, which otherwise affect few endo-siRNAs (Zhang et al., 2012).

3.2.4 Competition between ADARs and endogenous RNAi

There are two *C. elegans* genes encoding ADARs: *adr-1* and *adr-2* (Tonkin et al., 2002). Although only ADR-2 contains a catalytic domain and active

editing function, ADR-1 modulates the activity of ADR-2 *in vivo* (Knight & Bass, 2002; Tonkin et al., 2002). It was shown earlier that somatic expression of repetitive transgenes was silenced in ADAR mutants in an *rde-1*- and *rde-4*-dependent manner (Knight & Bass, 2002). Therefore, a similar competition between endogenous RNAi and ADARs for dsRNA substrates can be expected. Indeed, deep sequencing analyses identified a number of low-to-moderate copy inverted repeat regions with a dramatic increase in short RNA reads in ADAR mutants; consistently, transcripts from such loci were found to be multiply edited (Wu, Lamm, & Fire, 2011). Interestingly, although a corresponding decrease in mRNA levels was often observed, histone messages remained unchanged in ADAR(−) animals despite dramatic increases in short RNAs corresponding to some histone loci (Wu et al., 2011).

The effect of ADARs on the biogenesis of short RNAs was examined in another study (Warf et al., 2012). Although the levels of many miRNAs were increased in the absence of ADARs, most of these effects were found to be indirect, likely due to decreased sequestering of pri-miRNAs by ADARs from Drosha processing; only a couple miRNAs were found to be edited by ADARs (Warf et al., 2012). Surprisingly, generation of endo-siRNAs, which were selected by a 5′monophosphate-dependent sequencing protocol, was predominantly suppressed in the ADAR mutants, with ~40% of annotated loci producing fewer antisense siRNAs (Warf et al., 2012). These results are more consistent with the indirect effects of ADAR loss. Conversely, production of many Dicer-dependent 26G-antisense RNAs and their complementary 19 nt passenger strands was increased in the ADAR mutants, consistent with the competition between Dicer and ADARs for the dsRNA precursors of 26G-RNAs (Warf et al., 2012). As ADARs localize to the nucleus (Hundley, Krauchuk, & Bass, 2008), it is most plausible that the RRF-3-dependent synthesis of 26G-RNA precursors takes place there (Warf et al., 2012).

3.3. Biological functions of RNAi

3.3.1 Antiviral defense

The initiation of the RNAi response by dsRNA immediately suggested a natural antiviral role for this gene-silencing phenomenon (Fire et al., 1998). Moreover, the apparent lack of obvious developmental and physiological defects in *rde-1* and *rde-4* mutants further supported this idea (Tabara et al., 1999). This view of exogenous RNAi as a short RNA-based antiviral immunity was further confirmed by using transgenic animals expressing

Flock house virus, a plus-strand RNA animal nodavirus (Lu et al., 2005), as well as infections of *C. elegans* primary embryonic cell cultures with vesicular stomatitis virus, a rhabdovirus infecting insects and mammals (Schott, Cureton, Whelan, & Hunter, 2005; Wilkins et al., 2005).

An exciting development in studies of RNAi as an antiviral response came with the discovery of natural viruses infecting *C. elegans* and *C. briggsae* (Felix et al., 2011). Novel nodavirus-related RNA viruses, Orsay and Santeuil, were isolated from stably infected wild isolates of *C. elegans* and *C. briggsae*, respectively. Infection by these viruses resulted in clear morphological defects in the intestinal cells, which did not have a significant effect on animals, besides a lower rate of progeny production. A horizontal transmission of these viruses and a high specificity to *Caenorhabditis* species (*C. elegans* or *C. briggsae*) was described, as well as an efficient elimination of viruses by bleaching; no vertical transmission of the virus was detected in the progeny of bleach-treated parents. Consistent with an antiviral role of the exogenous RNAi pathway, individual *C. elegans* mutants defective for RNAi (*rde-1*, *rde-2*, *rde-4*, and *mut-7*) exhibited higher levels of viral RNA expression and more significant infection symptoms compared to the laboratory wild-type strain N2 (Felix et al., 2011). Also, deep sequencing of short RNA populations from infected animals revealed that 2% of all the unique sequences mapped to the viral RNA segments, and secondary 22G-RNAs were readily identified among these reads. Interestingly, a wild *C. elegans* isolate, JU 1580, which harbors the Orsay virus, was found to be resistant to RNAi targeting somatic but not germline genes (Felix et al., 2011). Other natural isolates of *C. elegans* also showed a variable sensitivity to somatic RNAi (Felix et al., 2011), and a wild-type isolate resistant to germline RNAi had been described earlier (Tijsterman, Okihara, et al., 2002). This natural variation in the RNAi responses could indicate a widespread coevolution of nematodes and nonlethal RNA viruses.

3.3.2 Silencing of transposons and repetitive elements

The first indication of the role of RNAi in genome surveillance came with the discovery that RNAi-deficient mutants often display increased rates of transposon mobilization in the germline, a mutator phenotype (Ketting et al., 1999; Sijen & Plasterk, 2003; Tabara et al., 1999; Tijsterman, Ketting, et al., 2002; Vastenhouw et al., 2003). The genome surveillance system in *C. elegans* comprises two 22G-RNA classes: the ERI/Dicer-dependent class and the ERI/Dicer-independent class (Gu et al., 2009; Figures 3.4 and 3.5). The 22G-RNAs of the ERI pathway target gene duplications and possibly horizontally transferred genes, as described in

Section 3.3, (Fischer et al., 2011; Vasale et al., 2010; Figure 1.4). Meanwhile, germline-enriched Dicer-independent 22G-RNAs bound by WAGO-1 and other WAGO Argonutes silence transposons, abberant transcripts, and cryptic loci (Gu et al., 2009; Figure 1.5). The generation of the WAGO

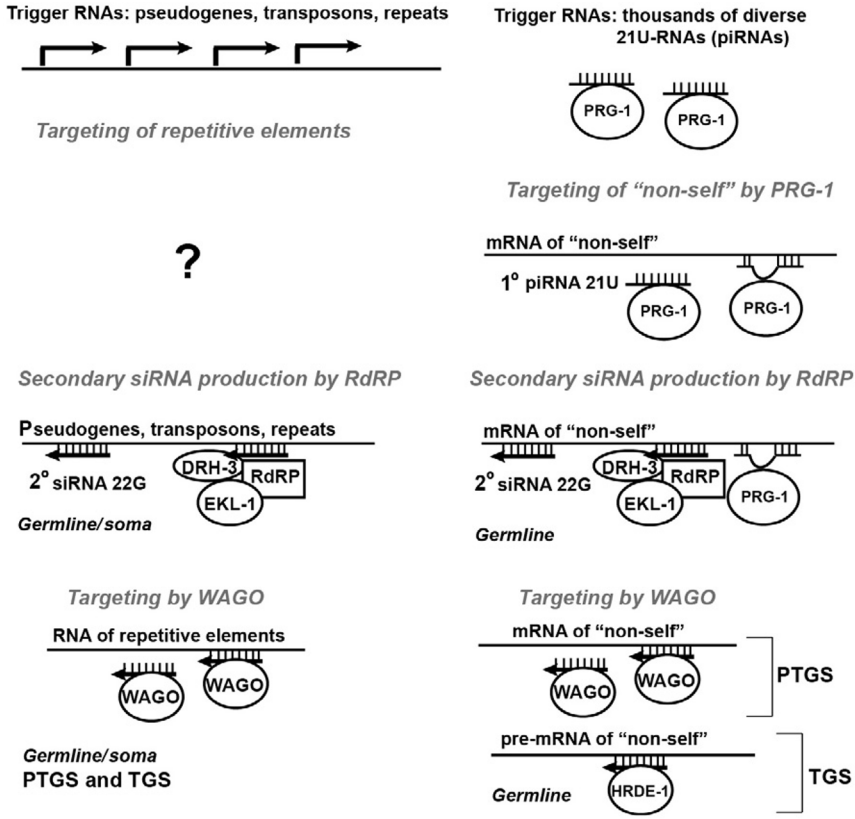

Figure 1.5 Repetitive regions and foreign genomic elements are targeted by the WAGO-22G-RNAs. Left: The initial steps promoting Dicer-independent 22G-RNA generation on repeats, pseudogenes, and transposons are poorly understood, but these 22G-RNAs are among the most abundant ones in *C. elegans*. The question mark refers to the possibility that some unknown Argonaute or a combination of Argonaute proteins bind to non-canonical primary siRNAs produced in a Dicer-independent manner and select mRNAs to be targeted by RdRP. It is also possible that some secondary siRNAs bound by WAGO are inherited by germline progenitor cells and stimulate RdRP activity on WAGO targets in the next generation; this system would then be Dicer and primary siRNA-independent, unlike the pathways shown in Figure 1.1, Figure 1.4 and Figure 1.5 (right panel). Right: 21U-RNAs bound by PRG-1 target foreign mRNA sequences (interaction does not require perfect complementarity) to promote RdRP-dependent production of WAGO-22G-RNAs. Notably, nuclear Argonaute HRDE-1 is responsible for initiating stable silencing of "non-self" sequences.

family 22G-RNAs is initiated in many cases by *C. elegans* piRNAs, and a combination of both types of short RNAs appears to have a more general role in recognizing foreign sequences distinct from endogenous genes (Figure 1.5; see Section 4.2.2). Importantly, the mutator proteins have been connected to the genome surveillance function of both the 26G–22G and piRNA-22G pathways (Gu et al., 2009; Zhang et al., 2011).

3.3.3 A summary of the global effects of 22G-RNAs on the expression of endogenous genes

The identification of endogenous short RNAs antisense to protein-coding genes poses a question concerning their relevance in gene regulation. To address this question, microarray analyses of gene misregulation in various mutant backgrounds have been performed (Asikainen, Storvik, Lakso, & Wong, 2007; Claycomb et al., 2009; Conine et al., 2010; Gent et al., 2009; Grishok et al., 2008; Gu et al., 2009; Lee et al., 2006; Maniar & Fire, 2011; van Wolfswinkel et al., 2009; Welker, Habig, & Bass, 2007). The general conclusion from these analyses is that the relationship between short RNAs and the gene loci from which they are generated, mostly by the action of RdRPs, is complex and specific to the endo-RNAi pathway to which these short RNAs belong. The WAGO family of 22G-RNAs has a strong negative effect on gene expression, which includes posttranscriptional, cotranscriptional, and transcriptional mechanisms in both the soma and the germline (Asikainen et al., 2007; Buckley et al., 2012; Conine et al., 2010; Duchaine et al., 2006; Fischer et al., 2011; Gent et al., 2010, 2009; Gu et al., 2009; Guang et al., 2008; Han et al., 2009; Lee et al., 2006; Pavelec et al., 2009; Vasale et al., 2010; Yigit et al., 2006; Zhang et al., 2011). However, as WAGO-22G-RNAs are largely dedicated to genome surveillance, they do not affect a large number of protein-coding genes and are most notably involved in spermatogenesis, with ALG-3/4 (Conine et al., 2010; Han et al., 2009), and maintenance of germline viability, with HRDE-1 (Buckley et al., 2012). It remains to be seen whether the germline mortality phenotype of the *hrde-1* mutant and some other *nrde* mutants is due to the gradually increased mobilization of transposons or due to the misregulation of protein-coding genes. On the other hand, the effects of the CSR-1 22G-RNAs on gene expression are modest (Claycomb et al., 2009; Maniar & Fire, 2011; van Wolfswinkel et al., 2009), though they represent the majority of short RNAs antisense to protein-coding genes, and the CSR-1 pathway mutants have the strongest developmental defects, described in Section 3.3.5. A recent study analyzed the effects of the RNAi pathways on changes in gene expression and

chromatin modifications in adult animals that had experienced a stress-resistant and nonaging dauer stage (postdauer worms) compared with those that had experienced a normal life cycle (Hall, Chirn, Lau, & Sengupta, 2013). This work suggested a potential role for CSR-1 in somatic gene regulation, which was revealed by a significant increase in the level of H3K4 methylation in postdauer *csr-1* hypomorph mutants compared to wild-type postdauer worms (Hall et al., 2013).

In addition to gene silencing, some endo-siRNAs in *C. elegans* have a positive role in gene regulation (Figure 1.6). It was discovered recently that CSR-1 22G-RNAs have a role in promoting the processing of 3′UTRs of

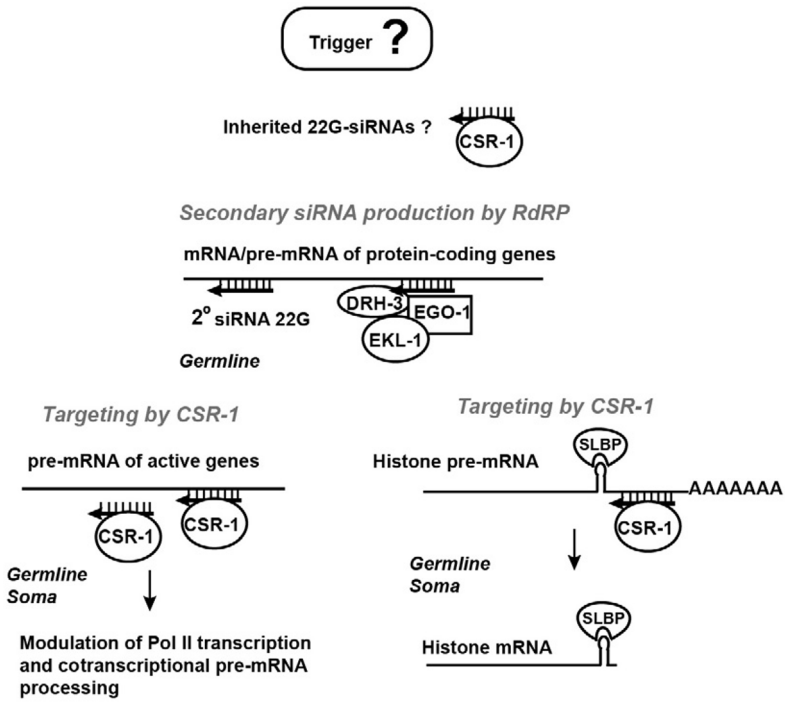

Figure 1.6 Biogenesis and function of CSR-1 22G-RNAs antisense to protein-coding genes. Triggers stimulating RdRP (EGO-1) recruitment to actively transcribed germline genes are not known at this time (question mark). The possibilities include currently unidentified primary siRNAs and their co-factor Argonautes or heritable secondary siRNAs produced by EGO-1 in the previous generation and deposited into germline precursor cells. CSR-1-bound 22G-RNAs modulate Pol II transcription of their targets and appear to positively regulate transcription in the germline. In addition to stimulating histone gene transcription, CSR-1 siRNAs promote histone mRNA biogenesis, possibly by guiding direct cleavage by CSR-1 after the conserved stem-loop. The stem loop in histone pre-mRNA and mRNA is recognized by the conserved stem-loop-binding protein (SLBP), which is required for proper histone mRNA biogenesis, nuclear export, and translation.

histone pre-mRNAs and that the deficiency in this process significantly contributes to the lethal and sterile phenotypes of the CSR-1 pathway mutants (Avgousti et al., 2012). Whether the majority of CSR-1 22G-RNAs targeting other genes also have an effect on pre-mRNA processing is not clear but presents an intriguing possibility. Consistent with this idea, a recent study of the evolutionary history of proteins in 85 genomes, which aimed to find genes with phylogenetic profiles similar to that of *C. elegans* RNAi-related factors, revealed a deep connection between RNAi and splicing (Tabach et al., 2013). Moreover, a significant number of splicing factors identified in this manner were shown to affect RNAi-dependent silencing of transgenes (Tabach et al., 2013).

3.3.4 Endogenous RNAi and adaptation to environment

Although the role of RDE-4 in exo-RNAi is understood relatively well (see Section 3.1.1), its contribution to endogenous RNAi pathways is defined less clearly. RDE-4 functions with RDE-1 in the rare cases when this Argonaute is involved in gene regulation (Correa et al., 2010; Gu et al., 2009), and it also coimmunoprecipitates with ERI-1 and ERI-5 (Thivierge et al., 2012) and is required for 26G production (Vasale et al., 2010). Moreover, *rde-4* is required for the maximum accumulation of 22G-RNAs produced by the germline RdRP EGO-1 (Maniar & Fire, 2011).

Microarray studies performed on *rde-4*-null worms identified a connection to regulation of stress response (Grishok et al., 2008; Mansisidor et al., 2011; Welker et al., 2007). Consistently, *rde-4* mutants have a decreased life span (Mansisidor et al., 2011; Welker et al., 2007) and an increased sensitivity to oxidative stress, pathogens (Mansisidor et al., 2011), and elevated temperatures (Blanchard et al., 2011). The signature of gene misregulation in *rde-4* mutant L1-L2 larvae significantly overlaps with that of *zfp-1* loss-of-function mutants (Grishok et al., 2008). ZFP-1 is a chromatin-binding protein enriched at promoters of highly expressed genes (Avgousti et al., 2012; Mansisidor et al., 2011). Intriguingly, only gene sets upregulated in the *zfp-1* and *rde-4* mutant worms (but not the downregulated gene sets) are enriched in endo-siRNA targets (Grishok et al., 2008). Although the majority of genes with increased expression in the *zfp-1* and *rde-4* mutants match CSR-1 22G-RNAs, the *pdk-1* gene, which encodes a conserved insulin signaling kinase whose upregulation is responsible for the shortened life span and the increased sensitivity to oxidative stress of the *zfp-1* and *rde-4* mutants, is a WAGO-22G target (Mansisidor et al., 2011). WAGO-22G-RNAs largely match the *pdk-1* promoter, which contains repeats that appear to

support the production of dsRNA (Mansisidor et al., 2011). Interestingly, many genes upregulated in *rde-4* mutants contain repeats in their promoters (Mansisidor et al., 2011). It is possible that the production of specific 22G-RNAs from repetitive elements that match genes with adaptive function is naturally selected to "fine-tune" their expression. It is equally possible that modulation of genes without repeats in their promoters by CSR-1 22G-RNAs is also subject to natural selection.

3.3.5 Developmental roles of the CSR-1 22G-RNA pathway

The severe germline defects in mutants of RdRP EGO-1 provided the first indication of the relevance of RNAi to the regulation of developmental processes (Smardon et al., 2000). The *ego-1*(−) germline abnormalities included (1) a reduced proliferation of germ cells in the mitotic zone, a phenotype observed when GLP-1/Notch signaling is defective; (2) a premature entry of germ cells into meiosis and a slow progression through early meiotic prophase; and (3) the presence of oocytes with abnormal morphology and apparent chromatin condensation defects (Smardon et al., 2000; Vought, Ohmachi, Lee, & Maine, 2005). Moreover, embryos produced by the *ego-1* mutant mothers were noted to arrest in development after only a few cell divisions (Smardon et al., 2000; Vought et al., 2005). The *ego-1* activity was also implicated in the accumulation of H3K9 methylation on the unpaired and heterochromatin-rich single X chromosome in meiotic male germline and unpaired extrachromosomal fragments in hermaphrodites (Maine et al., 2005). In addition, abnormalities in the nuclear pore complex morphology in germ nuclei and in the germ cell-specific P-granule size and distribution were observed in *ego-1*(−) animals (Vought et al., 2005). In a survey of RNAi-related mutants, *csr-1*, *drh-3*, and *ekl-1* were identified as having phenotypes similar to *ego-1*(−) (She et al., 2009). Notably, Dicer mutants did not share these phenotypes (Maine et al., 2005). Several other studies reported chromosomal abnormalities in the oocytes and chromosome segregation defects in the embryos of *drh-3* (Claycomb et al., 2009; Duchaine et al., 2006; Nakamura et al., 2007), *csr-1* (Claycomb et al., 2009; Yigit et al., 2006), *ego-1*, *ekl-1* (Claycomb et al., 2009), and *cde-1* mutants (van Wolfswinkel et al., 2009). Further, P-granule defects were noted in the *csr-1* mutant (Claycomb et al., 2009) and uncovered in animals treated with *drh-3*, *csr-1*, and *ego-1* dsRNA in an unbiased RNAi screen for genes affecting P-granule function (Updike & Strome, 2009). These phenotypic analyses match well with the genomic studies separating CSR-1 and WAGO-22G pathways (see Section 3.2.2).

The cloning of short RNAs from the CSR-1 immunoprecipitates discovered its association with 22G-RNAs antisense to protein-coding genes (Claycomb et al., 2009; Figure 1.6). It was proposed that all these 22G-RNAs act together to specify chromatin features and architecture of holocentric *C. elegans* chromosomes (Claycomb et al., 2009). However, it was discovered recently that canonical histone proteins are severely depleted in the *csr-1*, *drh-3*, and *ego-1* mutant animals and that histone mRNAs are not processed properly in these mutants (Avgousti et al., 2012; Figure 1.6). Moreover, the overexpression of histone locus containing one copy of each core histone gene from a transgenic array significantly rescued the embryo lethality induced by the depletion of *csr-1* and *ego-1* by RNAi (Avgousti et al., 2012). These results suggest that histone depletion is a major contributing factor to the embryonic lethality seen in the CSR-1 pathway mutants and may also help explain many other germline abnormalities resulting from the defects in chromatin condensation. Despite this, P-granule defects in the RNAi mutants are not due to histone depletion (Avgousti et al., 2012).

Histone pre-mRNAs do not contain introns, but they require a specific 3′end processing event: a cleavage after the conserved stem-loop (reviewed in Marzluff, Wagner, & Duronio, 2008). In most organisms, there is a dedicated U7 snRNA, which base-pairs with a conserved sequence downstream of the stem-loop and recruits the cleavage complex (reviewed in Marzluff et al., 2008). The U7 snRNA and the conservation of its complementary downstream sequence are missing in nematodes (Davila Lopez & Samuelsson, 2008), but there are EGO-1-dependent 22G-RNAs complementary to the region downstream of the stem loop (Avgousti et al., 2012; Figure 1.6). As CSR-1 binds to histone mRNA and pre-mRNA (Avgousti et al., 2012) and is proficient in secondary siRNA-guided endonucleolytic cleavage (Aoki et al., 2007), it is most plausible that CSR-1 is an endonuclease which is responsible for the processing of histone pre-mRNA in *C. elegans* (Figure 1.6). However, it is also possible that CSR-1 acts to recruit the processing complex.

Interestingly, the germline function of CSR-1 also includes an interaction with the PUF (Pumilio/FBF) protein FBF-1 in the distal germline and the repression of translational elongation of FBF-1 target mRNAs (Friend et al., 2012). It is not clear whether this role of CSR-1 requires 22G-RNAs as the relevant phenotypes were less robust in the RdRP complex mutants: *ego-1*, *drh-3*, and *ekl-1* (Friend et al., 2012). Another example of the developmental role of the CSR-1 pathway genes is their requirement,

redundantly with the KSR-1 scaffolding protein, in the specification of the excretory duct, worm's renal system, which occurs during embryogenesis (Rocheleau et al., 2008). Which 22G-RNA-target genes are relevant for this process and whether 22G-RNAs act positively or negatively to regulate them are not known. As there are two isoforms of the CSR-1 protein and one of them is expressed in somatic tissues (Claycomb et al., 2009), additional contributions of CSR-1 and 22G-RNAs to gene regulation during postembryonic development are likely to be discovered.

3.3.6 Cooperation between RNAi and Rb

Although the exo-RNAi pathway mutants display few obvious phenotypes, it is possible that a more careful examination could reveal the contribution of this pathway to the development and/or fitness of nematodes. The importance of *rde-4* for normal longevity and stress resistance has been discussed earlier (see Section 3.3.4). In addition, genetic studies that were conducted before the classification of endo-siRNAs revealed the redundant roles of the exo-RNAi factors and Rb in the negative regulation of nuclear divisions in the intestinal cells at the early L2 stage (Grishok & Sharp, 2005; Ouellet & Roy, 2007). Although the RNAi mutants did not exhibit an increase in nuclei number, double mutant combinations of the presumptive *lin-35* (Rb) null alleles with *rde-4*, *rde-1*, or *mut-7* nulls significantly enhanced the supernumerary nuclei phenotype of *lin-35*(−) (Grishok & Sharp, 2005; Ouellet & Roy, 2007).

The nuclear divisions in the intestine are very sensitive to cyclin E dosage (Grishok & Sharp, 2005), and the supernumerary phenotype is suppressed by mutations in cyclin E (Grishok & Sharp, 2005). Cyclin E mRNA expression is elevated in *lin-35*(−) worms (Grishok & Sharp, 2005; Ouellet & Roy, 2007), and its level is further enhanced in the *lin-35; rde-4* double mutant strain (Grishok & Sharp, 2005). The cyclin E gene (*cye-1* in *C. elegans*) is a known conserved target of transcriptional repression by Rb, and an antisense RNA overlapping exon 1 of *cye-1* was detected (Grishok & Sharp, 2005). Therefore, regulation of this gene by Rb and RNAi is likely to be direct. The possibility of dsRNA production at the *cye-1* locus is consistent with the involvement of RDE-1 in the regulation of nuclear divisions. Additional phenotypes associated with the combination of *lin-35* and *rde-4* null mutations include egg laying defects, extra vulva protrusions, and gonad migration defects (Grishok & Sharp, 2005). As *rde-4* appears to have a role in multiple RNAi pathways (exo-RNAi, ERI, CSR-1), it remains to be seen which other RNAi mutants will have similar phenotypes when

combined with *lin-35*(−) and misregulation of which target genes is responsible for the phenotypes.

3.3.7 The role of ALG-3/4 endo-siRNAs in sperm development

The temperature-sensitive sterility and "High Incidence of Males" (Him) phenotypes, presumably associated with defects in sperm development, were reported in early studies of the mutator and Eri genes (Duchaine et al., 2006; Kennedy et al., 2004; Ketting et al., 1999; Simmer et al., 2002). These signature phenotypes were also found in the compound mutant strains of WAGO Argonautes (Gu et al., 2009; Yigit et al., 2006) and in the viable loss-of-function *drh-3* mutants (Gu et al., 2009). Consistently, the microarray analysis of L4-stage *rrf-3* and *eri-1* mutants identified the misregulation of genes required for sperm function (Asikainen et al., 2007).

Genetic analyses of the Eri mutants separated the enhanced RNAi sensitivity phenotype from the sperm-related sterility and Him phenotypes such that *eri-1*, *eri-3*, *eri-5*, *rrf-3*, and *dcr-1*(*mg375Eri*) mutant animals exhibited both phenotypes, whereas *ergo-1* and *eri-9* mutants did not show sterility and Him phenotypes but had a reduction in brood size at 25 °C (Pavelec et al., 2009). Also, *eri-6/7* mutants were not overtly sterile at elevated temperatures (Fischer et al., 2008) with some reduction in brood size (Fischer et al., 2011). These phenotypic analyses fit well with the genomic studies discussed earlier (see Section 3.2.3) that identified two classes of 26G-RNAs, only one of which was detected in sperm (Han et al., 2009; Figure 1.4). Therefore, the upstream Eri genes that correspond to the RRF-3 RdRP module (Thivierge et al., 2012) are required for both types of 26G-RNAs (Fischer et al., 2011; Han et al., 2009; Vasale et al., 2010) and are expected to have pleiotropic phenotypes, while other factors, such as the redundant Argonaute proteins ALG-3 and ALG-4, are required for the production of only sperm-specific 26G- and 22G-RNAs (Conine et al., 2010; Han et al., 2009; Figure 1.4). The phenotypes of the MAGO12 mutant strain are consistent with its role with ALG-3/4 26G-RNAs (Conine et al., 2010; Yigit et al., 2006). Since the Mutator mutants are Him, it could be expected that they act with sperm-specific 22G-RNAs. However, Mutators are not required for the production of sperm-specific 26G-RNAs, only partially affect ALG-3/4-dependent 22G levels, and are only partially rescued by male mating (Zhang et al., 2011).

The detailed analyses of 26G-related sperm development phenotypes were conducted in several studies (Conine et al., 2010; Gent et al., 2009; Pavelec et al., 2009). First, it was established that temperature-dependent

sterility was associated with defects in sperm, not oocytes (Conine et al., 2010; Gent et al., 2009; Pavelec et al., 2009). The X-chromosome nondisjunction leading to the increased incidence of males (Him phenotype) was also connected to spermatogenesis (Gent et al., 2009). Although early events in germ cell development appeared to be unaffected in the Eri mutants (Pavelec et al., 2009), multiple abnormalities in spermatogenesis and spermiogenesis were described, including cell division defects (Conine et al., 2010; Gent et al., 2009; Pavelec et al., 2009), defects in spermatid activation (which affects sperm motility) (Conine et al., 2010; Pavelec et al., 2009), as well as sperm-related paternal effects leading to abnormal mucrotubule structures in early embryos and resulting embryonic lethality (Gent et al., 2009).

Although misregulation of many sperm-specific genes was reported in the mutants affecting ALG-3/4 endo-siRNAs (Conine et al., 2010; Gent et al., 2009; Han et al., 2009; Pavelec et al., 2009), the connection between the misregulated genes and the phenotypes is not clear. Understanding how regulation of specific genes by endogenous RNAi contributes to development and fitness is the next challenge for the ALG-3/4 class of short RNAs and for endo-siRNAs antisense to protein-coding genes in general.

4. 21U-RNAs, *C. elegans* piRNAs

piRNAs are the third major class of endogenous short RNAs, which is specific to animals, unlike miRNAs and endo-siRNAs that exist in both animals and plants. piRNAs interact with the PIWI subfamily of the Argonaute proteins. They were initially described in *Drosophila*, where they play a major role in transposon silencing utilizing both posttranscriptional and transcriptional mechanisms (reviewed in Ishizu et al., 2012; Sabin, Delas, & Hannon, 2013). Another distinct feature of piRNAs is their preferential expression in germ cells. Sterility phenotypes of animals lacking piRNAs are due to the secondary effects of transposon mobilization in *Drosophila* (reviewed in Khurana & Theurkauf, 2010), but it is not clear whether this is also true for nematodes and mammals and whether piRNAs participate in regulation of endogenous genes.

4.1. Biogenesis of 21U-RNAs

21U-RNAs were discovered as a separate class of short RNAs with unique features that included 5′ uridine, 21-nt length, an apparent 3′end

modification, and the existence of two upstream genomic motifs (Ruby et al., 2006). One motif with the CTGTTTCA consensus is located at an invariant position preceding the 21U sequence and a smaller YRNT motif is present immediately upstream and ends with the 5′U of the 21U-RNA (Ruby et al., 2006; Figure 1.7). The 21U-RNA sequences themselves were found to be very diverse and the 21U-RNA-producing loci mapped to two broad regions on chromosome IV (Ruby et al., 2006). Although piRNAs in other species are longer (~26 nt), 21U-RNAs were recognized as functional equivalents of piRNAs due to their association with the *C. elegans* PIWI homolog, PRG-1 (Figure 1.5), and the dramatic reduction of 21U-RNA expression in *prg-1*(−) animals (Batista et al., 2008; Das et al., 2008; Wang & Reinke, 2008). Also, production of 21U-RNAs was shown to be Dicer-independent (Batista et al., 2008; Das et al., 2008). At the same time, the significance of the upstream motif in 21U-RNA expression was suggested by the higher abundance of 21U-RNAs with upstream motifs that

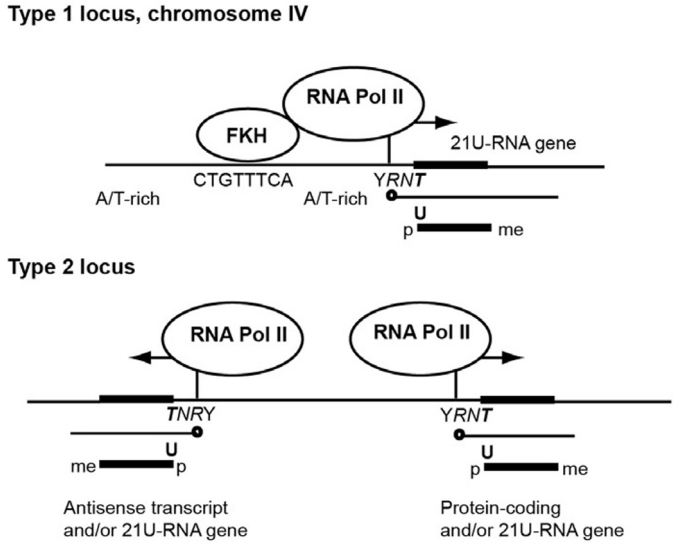

Figure 1.7 Biogenesis of 21U-RNAs (piRNAs). Each 21U-RNA is produced from a separate transcript defined by the YRNT sequence, where R (-2 relative to U) is the first nucleotide in the nascent 21U-RNA precursor. Two types of the 21U-RNA loci have been described: type 1 loci are prevalent on chromosome IV and contain nucleosome-depleted Pol II promoters with the CTGTTTCA DNA cis-element recognized by Forkhead transcription factors (FKH), and type 2 loci are present genome-wide and correspond to transcription start sites of protein-coding genes and other transcripts located in close proximity to promoters of protein-coding genes.

match closer to the consensus sequence (Batista et al., 2008). 21U-RNAs appear to be restricted to the germ tissue (Batista et al., 2008; Das et al., 2008; Wang & Reinke, 2008) and are expressed in both male and female germline (Batista et al., 2008; Das et al., 2008). Moreover, separate groups of male-enriched and female-enriched 21U-RNAs were recognized recently (Billi et al., 2013), and this separation also exists in other nematodes (Shi et al., 2013). Although genomic distributions of male-enriched and female-enriched 21U-RNAs are similar in *C. elegans*, these sub-groups are produced from different chromosomes in *C. briggsae* suggesting their different evolutionary history (Shi et al., 2013).

In other animals, numerous piRNAs are generated from one long precursor transcript (reviewed in (Ishizu et al., 2012)). However, the conserved motif associated with each individual 21U-RNA strongly suggested their autonomous expression in *C. elegans* (Ruby et al., 2006). Indeed, the upstream region is depleted of nucleosomes, which is a prominent feature of the promoters, and Pol II enrichment was only detected there in animals with germline tissue (Cecere, Zheng, Mansisidor, Klymko, & Grishok, 2012). Moreover, the CTGTTTCA requirement for 21U-RNA expression was demonstrated in experiments where this motif was deleted (Cecere et al., 2012) or scrambled (Billi et al., 2013; Figure 1.7). Furthermore, the sufficiency of the core motif for 21U-RNA expression was shown with single copy minimal expression cassettes integrated at genomic sites far away from the 21U clusters (Billi et al., 2013). A specific recognition of the CTGTTTCA consensus sequence by the Forkhead family of transcription factors was demonstrated *in vitro*, and binding of a Forkhead protein to the upstream regions of several 21U-RNAs was shown *in vivo* (Cecere et al., 2012; Figure 1.7). A decrease in 21U-RNA expression was correlated with the depletion of Forkhead proteins, many of which appear to act redundantly in promoting 21U-RNA expression (Cecere et al., 2012). Notably, the consensus **C**TGTTTCA sequence is enriched at male-specific 21U-RNAs promoters, whereas female-specific 21U-RNAs do not show a bias for C at the first position of the 8-mer (Billi et al., 2013). The 5′ cytidine was shown to be important for the biased expression of 21U-RNAs during spermatogenesis, although oogenesis-biased expression of 21U-RNAs cannot be explained by the differences in the 8-mer sequence (Billi et al., 2013). Although there is a notable Pol II enrichment at the 21U-RNA promoters (Cecere et al., 2012), it is orders of magnitude lower compared to the promoters of actively expressed germline genes (Cecere et al., 2012). Also, mRNA expression of many Forkhead protein-encoding genes shows

germline enrichment, but the corresponding protein expression appears to be very low (Cecere et al., 2012). The 5′ nucleotide in the Forkhead-specific DNA consensus sequence does not have a role in the interaction with the DNA-recognizing alpha helix of the Forkhead transcription factors, but it may provide a stabilizing contact with additional loop regions of the proteins (reviewed in (Obsil & Obsilova, 2011)) and therefore may determine the preferential binding of some male germline-enriched Forkhead family members to the **C**TGTTTCA consensus.

The 21U 8-mer upstream consensus sequence could not be detected as part of the precursor RNA by 5′-RACE experiments (Cecere et al., 2012) and by studies utilizing deep sequencing methods for the genome-wide annotation of long and short 5′ capped transcripts (Gu et al., 2012). Instead, the 5′ends of the 21U-RNA precursors map precisely to the purine (R) in the YRNT motif such that the nascent transcripts have two additional nucleotides preceding the first U of the mature 21U-RNA (Cecere et al., 2012; Gu, Lee, et al., 2012; Figure 1.7). The majority of 21U-RNA precursors appear to be ~26 nt long (Gu, Lee, et al., 2012), although longer ones also exist (Cecere et al., 2012; Gu, Lee, et al., 2012). Therefore, the biogenesis of 21U-RNAs must include the removal of the cap and two nucleotides at the 5′end (Cecere et al., 2012; Gu, Lee, et al., 2012), and trimming and methylation at the 3′end (Billi, Alessi, et al., 2012; Kamminga et al., 2012; Montgomery et al., 2012; Figure 1.7).

Interestingly, additional *prg-1*-dependent 21U-RNAs produced from many chromosomes were recognized in later studies (Gu, Lee, et al., 2012; Kato et al., 2011). These 21U-RNAs are not associated with the conserved 8-mer motif (Gu, Lee, et al., 2012; Kato et al., 2011; Figure 1.7). However, the YRNN motif, where R represents the first transcribed nucleotide, was shown to be the general feature of Pol II transcription start sites in *C. elegans*, and Gu and co-authors found that the sites containing U as the third transcribed nucleotide (YRNT) produced 21U-RNAs (Gu, Lee, et al., 2012; Figure 1.7). In this study, 42% of the sense reads for ~26-nt 21U-RNA precursors corresponded to the 5′ends of longer mRNA transcripts (Gu, Lee, et al., 2012). Also, 21U-RNAs of this second, 8-mer-independent, type (Type 2) were generally enriched within 1000 bp upstream of the 5′ends of transcript annotations (Gu, Lee, et al., 2012). Currently, it is not clear how transcription of these Type 2 21U-RNAs is related to their neighboring or overlapping protein-coding genes and whether the ~26 nt 21U-RNA precursors arise from promoter-proximal Pol II pausing as has been suggested (Gu, Lee, et al., 2012).

4.2. Biological functions of 21U-RNAs

4.2.1 Function in fertility

The phenotype resulting from *prg-1*(*RNAi*) treatment was noted before the discovery of 21U-RNAs (Cox et al., 1998). It included a reduction in the size of the mitotic proliferation zone in RNAi-treated animals and a decrease in the number of progeny produced (Cox et al., 1998). In later studies, a decrease in both mitotic and meiotic germ nuclei was reported, although the change in the mitotic zone was more pronounced (Batista et al., 2008). Interestingly, this defect in *prg-1* mutant worms, as well as the decrease in 21U-RNA abundance, is not temperature-dependent, but the sterility of *prg-1*(−) animals is (Batista et al., 2008; Wang & Reinke, 2008; Yigit et al., 2006). It appears that the gametes in *prg-1* mutants are affected by a temperature-sensitive process (Batista et al., 2008; Wang & Reinke, 2008), and a defect in sperm activation may be one of the abnormalities leading to sterility (Wang & Reinke, 2008). Microarray profiling of dissected gonads from wild-type and *prg-1* mutant males detected a decrease in the expression of spermatogenesis genes (Wang & Reinke, 2008), but no dramatic changes in gene expression were detected using whole animals (Batista et al., 2008). A notable exception was the reversion of Tc3 transposon silencing and an increase in Tc3 mobilization in *prg-1* mutants (Batista et al., 2008; Das et al., 2008). Tc3 silencing was shown to be associated with an accumulation of endo-siRNAs that required *prg-1* function (Batista et al., 2008; Das et al., 2008), and a more extensive connection between *prg-1* and endo-siRNAs has been described recently (see Section 4.2.2). Importantly, a careful examination of *prg-1* mutant worms revealed their progressive sterility in generations due to germline mortality (E. Miska and S. Ahmed, personal communication). This phenotype bears some similarity to that of *hrde-1* mutant worms (discussed in Section 3.3.3) and could strengthen the biological significance of the connection between *C. elegans* piRNAs and endo-siRNAs.

4.2.2 Connection between 21U-RNAs, 22G-RNA production, and stable silencing of foreign DNA

Although the endogenous target genes regulated by 21U-RNAs were not immediately obvious, the use of single copy GFP-sensor transgenes confirmed the competence of 21U-RNAs in inducing gene silencing (Bagijn et al., 2012; Lee et al., 2012; Luteijn et al., 2012). Perfect complementarity between the 21U-RNA and its target site was not required for the initiation of silencing, as two mismatches were tolerated (Bagijn et al., 2012;

Lee et al., 2012). Consistently, PRG-1 proteins with mutations in the catalytic site were efficient at inducing silencing (Bagijn et al., 2012; Lee et al., 2012). Interestingly, an abundant production of antisense 22G-RNAs complementary to sequences 5′ upstream of the 21U-RNA GFP-sensor target site was detected (Bagijn et al., 2012; Lee et al., 2012; Luteijn et al., 2012). This 22G generation and the process of the reporter silencing itself were shown to be dependent on PRG-1 and the RNA methyltransferase HENN-1, which are involved in 21U-RNA biogenesis, on nuclear RNAi pathway genes *nrde-1*, *nrde-2*, *nrde-4*, and *hrde-1/wago-9*, and on other components required for secondary siRNA production: RdRPs EGO-1 and RRf-1, RdRP complex component DRH-3, and mutator genes *mut-7*, *rde-2/mut-8*, *mut-14*, and *rde-3/mut-2* (Ashe et al., 2012; Bagijn et al., 2012; Lee et al., 2012; Luteijn et al., 2012). Importantly, PRG-1 was shown to be required for the initiation but not for the maintenance of reporter silencing, which was very stable across generations (Bagijn et al., 2012; Lee et al., 2012; Luteijn et al., 2012), much more stable than previously described exo-RNAi-induced heritable silencing (see Section 3.1.8). Consistently, two SET domain-containing predicted histone methyltransferase genes and the gene *hpl-2*, which encodes a *C. elegans* homolog of Heterochromatin Protein 1(HP1), were implicated in 21U-RNA sensor silencing but not in 22G-RNA production (Ashe et al., 2012), and H3K9 methylation was shown to be enriched at the silenced sensor sequences (Luteijn et al., 2012).

The phenomenon of 21U-RNA-induced transgene silencing discussed above has also been described for single copy transgenes not designed to be PRG-1-dependent silencing reporters (Shirayama et al., 2012). This type of silencing could be transmitted in a dominant fashion through crosses to affect other single copy transgenes that were otherwise stably expressed (Shirayama et al., 2012); such transmission in crosses was also a feature of the 21U reporter silencing (Luteijn et al., 2012). Other features of this phenomenon included dependence on *prg-1* for the initiation of silencing, but not for its maintenance, and dependence on *rde-3*, *mut-7*, *hpl-2*, and *wago-9/hrde-1* (Shirayama et al., 2012), in agreement with the studies described above. In addition, Polycomb and Trithorax complex-related factors were implicated in silencing, as well as the cytoplasmic WAGO-1 and nuclear WAGO-10 (Shirayama et al., 2012). The silencing mechanism appeared to combine both posttranscriptional and transcriptional regulation and was associated with an increase in H3K9 methylation (Shirayama et al., 2012). An interesting feature of this system is that efficient 22G-RNA production was detected near sites with partial 21U-RNA complementarity,

which were not designed but identified later (Shirayama et al., 2012). The model suggested by these studies postulates that the diversity of 21U-RNAs makes them analogous to antibodies in the immune system, the diversity of which allows for the identification of any possible foreign targets distinct from "self" (Shirayama et al., 2012; Figure 1.5). The important question is how the organism defines "self" and protects it from silencing. It has been noted that transgene sequences corresponding to the endogenous genes targeted by the CSR-1 system accumulate fewer 21U-RNA-dependent 22G-RNAs. Therefore, it was suggested that CSR-1 22G-RNAs may play this antisilencing role (Luteijn et al., 2012; Shirayama et al., 2012). Curiously, there are experimental data consistent with the possibility that antisilencing also requires *prg-1* activity (Luteijn et al., 2012). In any case, it is very important to determine whether the presumptive role of CSR-1 22G-RNAs as the "maintenance of self" system (Shirayama et al., 2012) is linked to their role in regulating endogenous gene expression (likely transcriptional modulation or pre-mRNA processing).

A search for endogenous gene regulation by 21U-RNA-dependent 22G-RNAs identified a fraction of WAGO-22G-RNAs depleted in *prg-1* mutant animals (Bagijn et al., 2012; Lee et al., 2012). Importantly, these 22G-RNAs were distinct from those dependent on 26G-RNAs of the Eri pathway (Lee et al., 2012). Sequences with partial complementarity to existing 21U-RNAs could be identified next to these *prg-1*-dependent 22G loci, identifying them as 21U-RNA-dependent 22G-RNAs (Bagijn et al., 2012; Lee et al., 2012; Figure 1.5). A few genes were found to be negatively regulated by these 22G-RNAs (Bagijn et al., 2012; Lee et al., 2012), although the physiological consequences of this regulation are not clear. Computational prediction of the target sequences antisense to 21U-RNAs, which allowed mismatches, suggested that they are depleted in protein-coding genes (Bagijn et al., 2012), especially CSR-1 targets (Lee et al., 2012). A more recent study, which analyzed spermatogenesis-enriched and oogenesis-enriched 21U-RNAs separately, concluded that the predicted targets of the former were significantly depleted of spermatogenesis genes, while no bias was noted for the predicted targets on the latter class (Shi et al., 2013).

Although the transgene-silencing studies described earlier indicate that 21U-RNAs have the potential to silence foreign DNA elements, it is surprising that so little evidence of this can be found in the *C. elegans* genome. Also, none of the recent studies explained the fertility defects observed in *prg-1* mutant worms and the poor rescue of this phenotype by the

catalytically dead PRG-1 protein (Lee et al., 2012), which is very efficient in promoting transgene silencing. Until the mechanistic connection between PRG-1 and its targets clearly explains the progressive sterility phenotype of *prg-1* mutant worms, it is too early to conclude that the role of 21U-RNAs in the biology of *C. elegans* is understood.

5. SYSTEMIC FEATURES OF RNAi

Historically, the ability of dsRNA-induced silencing to spread from cell to cell represented a significant feature of the RNAi phenomenon in *C. elegans* (Fire et al., 1998). The systemic nature of RNAi was also supported by the ability to induce silencing by feeding nematodes dsRNA-expressing bacteria (Timmons & Fire, 1998). However, subsequent studies distinguished "systemic RNAi" from "environmental RNAi" induced by feeding as some mutants were found to be competent in the former and deficient in the latter (reviewed in Whangbo & Hunter, 2008; Zhuang & Hunter, 2012). This section will discuss both types of processes.

5.1. dsRNA import channel SID-1 and the features of mobile RNA species

A screen for systemic RNAi defective (Sid) mutants identified many alleles in the gene *sid-1*, which encodes a conserved protein with multiple transmembrane domains and is required for dsRNA import into cells (Feinberg & Hunter, 2003; Shih, Fitzgerald, Sutherlin, & Hunter, 2009; Shih & Hunter, 2011; Winston, Molodowitch, & Hunter, 2002). The features of the dsRNA import by SID-1 were mostly identified in *Drosophila* S2 cells expressing the *C. elegans* protein (*Drosophila* lacks SID-1 homologs) (Feinberg & Hunter, 2003; Shih et al., 2009; Shih & Hunter, 2011). It was determined that SID-1 is likely to multimerize and to form a dsRNA-gated channel selective for dsRNA (Shih et al., 2009; Shih & Hunter, 2011; Figure 1.8). dsRNA transport through SID-1 occurs by passive diffusion (Feinberg & Hunter, 2003; Shih et al., 2009), and proteins required for the initiation of RNAi prevent the export of imported dsRNA (Shih et al., 2009; Shih & Hunter, 2011). Interestingly, it was shown that transport of dsRNA molecules with single-stranded regions, such as miRNA precursors, is supported by SID-1 (Shih & Hunter, 2011).

Elegant mosaic analyses in *C. elegans* utilizing mutant rescue in specific cells revealed that *sid-1* is not required for the export of systemic silencing RNAs from varying cell types (Jose, Smith, & Hunter, 2009). This study also

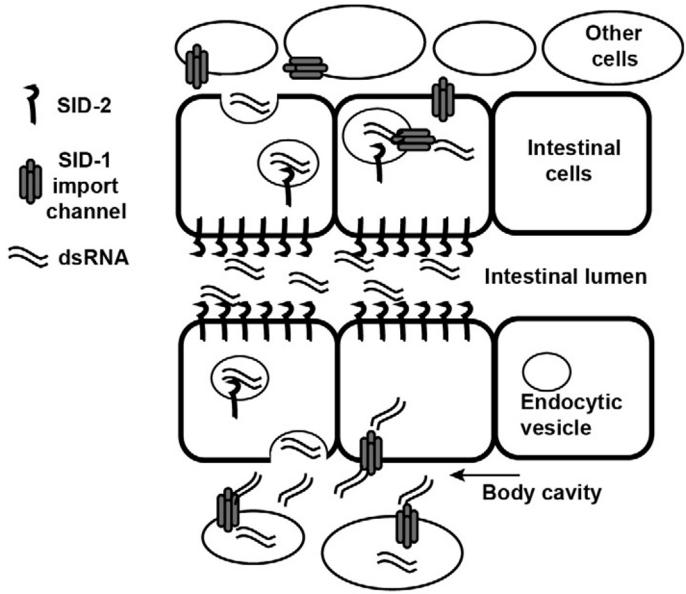

Figure 1.8 Systemic transport of ingested dsRNA. dsRNA is ingested with bacteria and accumulates in the intestinal lumen. SID-2 transmembrane protein is present on the apical side of intestinal cells facing the lumen and is required for the endocytosis of dsRNA. SID-1 is a dsRNA-specific channel composed of several subunits. SID-1 is required for the import of the dsRNA into the cells from endocytic vesicles and from the intercellular space (body cavity). Intestinal cells deficient in SID-1 can support endocytosis-dependent dsRNA transport from the intestinal lumen to body cavity, but SID-1 is required for further import into other cells. *This figure is based on figure 6 in McEwan, Weisman, and Hunter (2012).*

showed that multicopy transgenes readily generate such silencing signals, which spread from cell to cell via SID-1-dependent import (Jose, Smith, & Hunter, 2009). Earlier genetic studies demonstrated that *rde-4* and *rde-1* functions are not required for the generation of the systemic signal, which suggested that long dsRNA could be transported (Tabara et al., 1999). The competence of long dsRNA in systemic transport was further confirmed in mosaic analyses (Jose et al., 2011). This work also revealed the existence of additional mobile RNA species that require *dcr-1* and *rde-4*, but not *rde-1*, for their production, which suggests that double-stranded primary siRNAs, not the single-stranded siRNA products generated after passenger-strand cleavage by RDE-1, are mobile (Jose et al., 2011). Also, it was found that secondary siRNAs generated by RdRP could not be transported (Jose et al., 2011). The nonautonomous role of RDE-4 and the

cell-autonomous role of RDE-1 in RNAi were also observed in an earlier study (Blanchard et al., 2011). Surprisingly, the putative nucleotidyltransferase MUT-2 (RDE-3) was required for the generation of a mobile RNA signal; therefore, there is a possibility that primary siRNAs generated after Dicer cleavage can be modified (Jose et al., 2011).

5.2. Additional factors required for systemic and environmental RNAi

5.2.1 SID-2

SID-2 is a transmembrane protein found only in the apical membrane of intestinal cells facing the intestinal lumen (Figure 1.8) and specifically required for the environmental, but not systemic, RNAi response (Winston, Sutherlin, Wright, Feinberg, & Hunter, 2007). Similar proteins exist in other nematode species, such as *C. briggsae* and *C. remanei*, but only *C. elegans* SID-2 is competent in supporting environmental RNAi and can enable this response in *C. briggsae* (Nuez & Felix, 2012; Winston et al., 2007) and *C. remanei* (Nuez & Felix, 2012) when expressed from a transgene. Interestingly, sensitivity to environmental RNAi was observed in several other *Caenorhabditis* species (Nuez & Felix, 2012; Winston et al., 2007), and their phylogenetic analysis is consistent with the convergent evolution of this feature (Nuez & Felix, 2012).

SID-2 is required for the import of environmental dsRNA into intestinal cells, but its function is not sufficient for this import, which also requires SID-1 (Winston et al., 2007). Recent studies conducted in *C. elegans* and *Drosophila* S2 cells suggest a role for SID-2 in the initial uptake of dsRNA from the intestinal lumen by endocytosis and the subsequent requirement of SID-1 for the transport of dsRNA from the endocytic vesicles and/or the body cavity (Figure 1.8; McEwan et al., 2012).

5.2.2 SID-3

SID-3 is a conserved tyrosine kinase related to the Cdc-42-associated kinase (Ack) family and required for the efficient import of dsRNA (Jose, Kim, Leal-Ekman, & Hunter, 2012). It is widely expressed in a variety of *C. elegans* cells, where it shows a punctate pattern of expression. Interestingly, the cell-autonomous RNAi process is not impaired, and even enhanced, in *sid-3* mutant cells (Jose et al., 2012). The kinase domain of SID-3 is required for its function, which suggests that signaling events can modulate the efficiency of dsRNA import into cells (Jose et al., 2012).

5.2.3 SID-5

Mutations in *sid-5* were shown to reduce the efficiency of systemic RNAi (Hinas, Wright, & Hunter, 2012). SID-5 is widely expressed and associates with endosomes; however, unlike *sid-3*, *sid-5* is not required for the import of the silencing signal but appears to affect its export (Hinas et al., 2012). Although the SID-1 channel is required for dsRNA import into cells that execute silencing, a *sid-1*-independent transport of environmental silencing signals across the intestine has been observed (Jose et al., 2009). Such signals, that is, dsRNA, are taken up by SID-2-dependent endocytosis and thought to be released from endocytic vesicles into the body cavity without entry into the intestinal cells (Jose et al., 2009; Figure 1.8). SID-5 was implicated in this *sid-1*-independent transport across the intestine, which is consistent with SID-5's localization to endosomes (Hinas et al., 2012). It is likely that SID-5 generally contributes to systemic RNAi by facilitating the dsRNA export process from a variety of cells.

6. OUTLOOK

This chapter has highlighted the tremendous importance of short RNAs for *C. elegans* biology: short RNAs are used to regulate development, physiology, life span, and stress resistance; they provide an antiviral response and control gene duplications, silence transposons, and modulate expression of endogenous genes by a variety of mechanisms. It is certain that studies of RNAi-related processes in *C. elegans* will continue to thrive and that a comprehensive review of this kind will probably not be possible in the future. It can be expected that a better mechanistic understanding of posttranscriptional and transcriptional gene regulation by short RNAs will be achieved, and that it will be connected in due course to insights about the biological functions of miRNAs, endo-siRNAs, and 21U-RNAs. More often than not, insights from biological systems like *C. elegans* become very relevant to higher organisms and ultimately lead to a better understanding of life.

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