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REVIEW

A multitasking Argonaute: exploring the many facets of *C. elegans* CSR-1

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Abstract While initial studies of small RNA-mediated gene regulatory pathways focused on the cytoplasmic functions of such pathways, identifying roles for Argonaute/small RNA pathways in modulating chromatin and organizing the genome has become a topic of intense research in recent years. Nuclear regulatory mechanisms for Argonaute/small RNA pathways appear to be widespread, in organisms ranging from plants to fission yeast, *Caenorhabditis elegans* to humans. As the effectors of small RNA-mediated gene regulatory pathways, Argonaute proteins guide the chromatin-directed activities of these pathways. Of particular interest is the *C. elegans* Argonaute, chromosome segregation and RNAi deficient (CSR-1), which has been implicated in such diverse functions as organizing the holocentromeres of worm chromosomes, modulating germline chromatin, protecting the genome from foreign nucleic acid, regulating histone levels, executing RNAi, and inhibiting translation in conjunction with Pumilio proteins. CSR-1 interacts with small RNAs known as 22G-RNAs, which have complementarity to 25% of the protein coding genes. This peculiar Argonaute is the only essential *C. elegans* Argonaute out of 24 family members in total. Here, we summarize the current understanding of CSR-1 functions in the worm, with emphasis on the chromatin-directed activities of this ever-intriguing Argonaute.

Keywords Small RNA · Argonaute · Gene regulatory pathway · Chromatin · *C. elegans*

Abbreviations

AGO	Argonaute
CDE-1	Co-suppression defective-1
CSR-1	Chromosome segregation and RNAi deficient-1
ChIP	Chromatin immunoprecipitation
Co-IP	Co-immunoprecipitation
DRH-3	Dicer-related helicase-3
dsRNA	Double-stranded RNA
EKL-1	Enhancer of KSR-1 lethality-1
EGO-1	Enhancer of GLP-1
Eri	Enhanced RNAi
FBF	fem-3 mRNA binding factor
HCP-3/CENP-A	Holocentromere protein 3/centromeric protein A
HRDE-1	Heritable RNA deficient-1
HTZ-1/H2AZ	Histone variant H2AZ homolog
ModENCODE	Model organism encyclopedia of DNA elements
PAZ	PIWI–Argonaute–Zwille
PIWI	P-element induced wimpy testes
Rde	RNAi deficient
RdRP	RNA-dependent RNA polymerase
RNAi/exoRNAi	Exogenous RNA interference

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SAGO	Secondary Argonaute
SLBP	Stem loop binding protein
WAGO	Worm Argonaute

Introduction

As the major effectors of small RNA-mediated gene regulatory pathways, Argonaute proteins are guided to their target transcripts by the sequence specificity provided by a small RNA binding partner. Notably, Argonaute superfamily proteins have been identified in each of the three domains of life, based on the presence of the P-element induced wimpy testes (PIWI) domain (Makarova et al. 2009; Hock and Meister 2008). The PIWI domain interacts with both guide RNA and target RNA and three key residues in the PIWI domain (DDE/H) confer endonuclease activity in those Argonautes, which have the capacity to degrade target RNAs or act as "slicers" (Liu et al. 2004; Song et al. 2004). Other key domains present in Argonautes are the PIWI–Argonaute–Zwille (PAZ) and Mid domains, which coordinate the 3′ and the 5′ end of the small RNA, respectively (Hutvagner and Simard 2008). Some Argonaute/small RNA gene regulatory pathways, such as the microRNA pathway, are highly conserved while others appear to function in a species-specific manner (Kim et al. 2009). Together, these pathways play roles in a variety of processes, from defending against viruses and other foreign nucleic acids to organizing chromosomes and modulating translation throughout development (Ketting 2011).

The nematode *Caenorhabditis elegans* has been a champion for characterizing small RNA-mediated gene regulatory pathways and dissecting the mechanisms of such pathways since the initial discoveries of microRNAs and RNA interference (RNAi) almost 20 years ago (Lee et al. 1993; Fire et al. 1998). The *C. elegans* genome encodes 24 Argonaute proteins and produces four major types of endogenous small RNAs (Fig. 1) (Grishok 2013). In combination, these pathways provide a versatile repertoire of gene regulatory activities that range in their outcome from translational inhibition to transcriptional attenuation and impact a variety of developmental processes (Fischer 2010). The *C. elegans* Argonaute protein superfamily is divided into three subcategories based on homology and the small RNAs with which they interact: PIWIs, which interact with the 21U or piRNAs; Argonautes, two of which have been shown to function with microRNAs and two of which have been shown to function with 26G-RNAs; and the worm Argonautes (WAGOs), which interact with 22G-RNAs (Yigit et al. 2006; Das et al. 2008; Batista et al. 2008; Grishok et al. 2001; Conine et al. 2010; Gu et al. 2009; Vasale et al. 2010; Shirayama et al. 2012; Claycomb et al. 2009; Han et al. 2009).

The WAGOs are the largest *C. elegans* Argonaute subgroup, with 14 members, and do not have direct homologs outside of nematodes (Yigit et al. 2006; Gu et al. 2009). Of the WAGOs studied thus far, all have been shown to interact with 22G-RNAs, which are not dependent on Dicer for their biogenesis (Yigit et al. 2006; Gu et al. 2009; Claycomb et al. 2009; Shirayama et al. 2012; Burton et al. 2011). Instead, 22G-RNAs are produced by RNA dependent RNA polymerases (RdRPs), using target transcripts as a template for synthesis (thus, 22G-RNAs display perfect complementarity to their targets) (Aoki et al. 2007; Gu et al. 2009; Pak and Fire 2007). 22G-RNAs possess a 5′ guanine triphosphate and fall into two major classes: Those that were isolated in association with WAGO-1 and target transposable elements, intergenic regions, pseudogenes, and some protein coding genes, and those that were isolated in association with chromosome segregation and RNAi deficient (CSR-1) and target germline-expressed protein coding genes (Gu et al. 2009; Claycomb et al. 2009).

With 14 members, perhaps it is not surprising that many of the WAGOs studied thus far appear to function in a redundant manner (Yigit et al. 2006; Gu et al. 2009). In fact, in a systematic analysis of mutants in each of the Argonaute genes, Yigit et al. (2006) revealed that only one WAGO-type Argonaute was essential under normal growth conditions: CSR-1. In subsequent years, this Argonaute has been implicated in a number of diverse and fascinating processes, ranging from the exogenous RNAi pathway, to nematode holocentromere formation and histone messenger RNA (mRNA) maturation, among others (Yigit et al. 2006; Claycomb et al. 2009; Avgousti et al. 2012). Its diverse roles and involvement in key developmental processes have made CSR-1 a topic of intense interest. Here, we discuss the many facets of the Argonaute CSR-1, with an emphasis on the chromatin-directed functions of this fascinating *C. elegans* Argonaute.

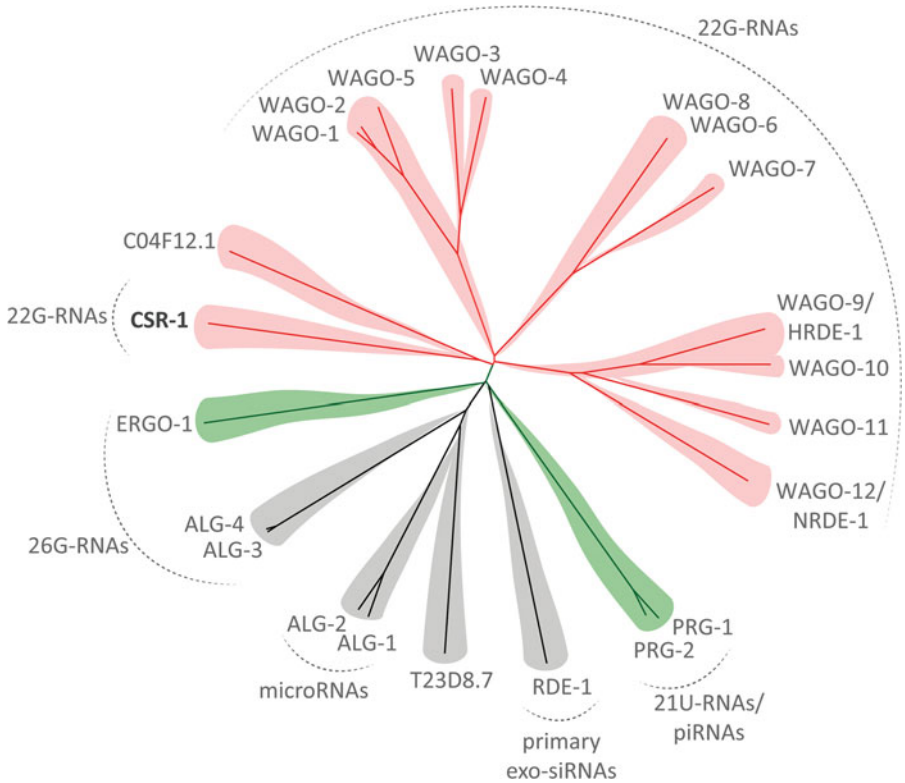

Fig. 1 A phylogenetic tree of the *C. elegans* AGO family. *C. elegans* possesses 24 different AGO proteins. The tree separates the AGO family members into three clades: members of the WAGO subclade are shown in *red*, members of the PIWI subclade are shown in *green*, and members of the Argonaute subclade are shown in *black*. The small RNA class that each AGO is known or predicted to associate with is indicated by a *curved dotted line*. The tree was generated with Phylodendron (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html) and is based on alignments of the full-length Argonaute protein sequences (obtained from Wormbase), generated using T-coffee. Argonaute pseudogenes were omitted from this tree

Chromatin regulatory roles for CSR-1

CSR-1 loss of function phenotypes

CSR-1 was so named by Yigit et al. for its mutant phenotype, in which loss of CSR-1 function leads to chromosome segregation defects and RNAi deficiency (see below). Mutation of *csr-1* results in penetrant hermaphrodite sterility. A few escaper embryos that are produced display embryonic lethality (Yigit et al. 2006). Cell biology studies of *csr-1* mutant embryos showed that mitotic chromosomes fail to properly condense and congress on the metaphase plate, kinetochores (the protein structures on chromosomes that serve as attachment sites for microtubules of the mitotic spindle) fail to orient to the poleward faces of chromosomes, and the aberrant mitotic division that ensues results in anaphase bridging, and aneuploidy (Yigit et al. 2006; Claycomb et al. 2009). In the adult hermaphrodite germline, loss of CSR-1 leads to a number of developmental and chromosomal defects, including reduced mitotic proliferation, resulting in an overall reduced number of germ cells. Increases in the number of leptotene- and zygotene-staged nuclei are concurrent with a decrease in the number of pachytene nuclei (leptotene and zygotene precede pachytene during meiotic prophase I), indicating difficulties in meiotic progression. The presence of large, diffuse germline nuclei in *csr-1* mutants is indicative of defects in chromatin condensation, similar to those observed in embryos (She et al. 2009). The oocytes of *csr-1* mutants display increased numbers of 4′,6-diamidino-2-phenylindole (DAPI) staining bodies, reflecting a loss of integrity of the homologous chromosome pairs, despite the fact that the chromosomes display no initial defects in pairing or synapsis (She et al. 2009; Claycomb et al. 2009). In somatic cells of *csr-1* mutant adults, we have observed that gut nuclei display

chromosome bridging and incomplete division, indicating a role for CSR-1 in differentiated somatic tissues (J. M. Claycomb, unpublished results). Together, these results reflect a requirement for CSR-1 in chromosome organization and segregation during both mitosis and meiosis.

One additional phenotype of *csr-1* mutants that may be related to defects in chromosome organization is a prominent and unique defect in the formation and/or maintenance of germ granules, called P granules in *C. elegans*. P granules carry mRNAs and proteins that function in germline differentiation, including a number of small RNA pathway proteins such as the Argonautes (Updike and Strome 2010; Claycomb et al. 2009; Batista et al. 2008). Normally associated with the nuclear envelope and nuclear pore complexes in the adult germline, P granules dissociate from the nuclear periphery and are localized in the cytoplasmic core of the syncytial germline in *csr-1* mutants (Claycomb et al. 2009; Updike and Strome 2009). Similar P granule aggregation defects are also present in *csr-1* mutant embryos (Updike and Strome 2010). Because of their close association with the nuclear pore, it is possible that anomalies in nuclear organization due to the chromosome and chromatin defects of *csr-1* mutants lead to the dissociation of P granules from the nuclear periphery. Notably, in a screen of over 15,000 genes by RNAi, Updike et al. only observed this phenotype upon loss of *csr-1* and *csr-1* pathway components (Updike and Strome 2009). Likewise, we have used this phenotype to successfully screen for additional bona fide CSR-1 pathway members (M. A. Francisco and J. M. Claycomb, in preparation), which indicates that this phenotype is extremely specific to CSR-1 and likely reflects an important aspect of CSR-1 activity that we have yet to fully understand.

CSR-1 22G-RNAs and other CSR-1 pathway components

Immunoprecipitation of CSR-1 complexes from whole adult hermaphrodite worms that possess embryos within (called "gravid adults"), coupled with small RNA cloning and Illumina sequencing revealed that CSR-1 associates with small RNAs of approximately 22 nucleotides and that possess a 5′ guanine triphosphate (Claycomb et al. 2009). The CSR-1 22G-RNAs are a distinct subset of the total set of 22G-RNAs that are complementary to over 4,000 germline-expressed protein coding gene transcripts (referred to as "targets") distributed along the length of each autosome (Gu et al. 2009; Claycomb et al. 2009). Although CSR-1 targets genes along the X chromosome, the relative number of CSR-1 targets is less on this chromosome likely due to the low overall number of germline-expressed genes present on the X chromosome (Claycomb et al. 2009). However, it bears noting that loss of the X chromosome during cell division is tolerated, while loss of autosomes is not (Albertson et al. 1997), such that the requirements for CSR-1 in X chromosome segregation may be less strict than for the autosomes.

In addition to CSR-1, several other components of the pathway have been identified by the common features of their loss of function phenotypes: embryonic chromosome segregation and kinetochore defects, germline chromatin and P granule defects, exoRNAi deficiency, and a loss of particular subsets of small RNAs, the 22G-RNAs. Thus far, CSR-1 pathway members include the RNA-dependent RNA polymerase, enhancer of GLP-one (EGO-1); the Dicer-related helicase (DRH-3); the Tudor-domain protein, enhancer of KSR-1 lethality (EKL-1); and the beta-nucleotidyl transferase, co-suppression defective (CDE-1) (Kim et al. 2005; Claycomb et al. 2009; van Wolfswinkel et al. 2009; Yigit et al. 2006; Duchaine et al. 2006; Rocheleau et al. 2008; Smardon et al. 2000; She et al. 2009; Robert et al. 2005). Together, EGO-1, DRH-3, and EKL-1 have been shown to form a complex that has been implicated in the synthesis of 22G-RNAs (Gu et al. 2009). DRH-3 and EKL-1 are required for nearly all 22G-RNAs produced in the worm (both WAGO-1 and CSR-1 type), while EGO-1 predominantly synthesizes CSR-1 pathway specific 22G-RNAs (Gu et al. 2009; Claycomb et al. 2009).

The flip side of small RNA synthesis is small RNA turnover. A subset of the CSR-1 22G-RNAs possesses a CDE-1-dependent 3′ poly-uridine tail, which is thought to contribute to the turnover of CSR-1 22G-RNAs (van Wolfswinkel et al. 2009). This notion is based on data whereby *cde-1* mutants display a higher steady state level of 22G-RNAs within CSR-1 complexes, concomitant with a dramatic reduction in small RNA species possessing a poly-U tail (van Wolfswinkel et al. 2009). Although CDE-1 has been shown to interact with EGO-1 by Co-IP, neither CDE-1 nor any of the other CSR-1 pathway factors for that matter has been shown to physically interact with CSR-1 (van Wolfswinkel et al. 2009; Claycomb et al. 2009). Together, these factors form a pathway that is thought to influence chromatin in the worm.

CSR-1 target regulation

Interestingly, several lines of experimentation indicate that CSR-1 does not generally downregulate the expression levels (mRNA or protein) of its target genes, despite possessing key residues to act as a "slicer" Argonaute (see below for more detail) (Aoki et al. 2007). That CSR-1 would not influence its target gene mRNA or protein expression levels is contrary to nearly every Argonaute studied thus far and adds to the intrigue surrounding CSR-1. For instance, microarray studies in *csr-1*, *drh-3*, and *cde-1* mutant backgrounds showed little or no change in steady state mRNA levels for the majority of CSR-1 target genes (Claycomb et al. 2009; van Wolfswinkel et al. 2009; Gu et al. 2009; Updike and Strome 2009; Aoki et al. 2007). Likewise, quantitative reverse transcription PCR analysis of several P granule components showed little change in their levels after *csr-1* knockdown by RNAi (Updike and Strome 2009). Western blot experiments examining protein levels for a handful of CSR-1 targets also showed no changes in adult hermaphrodites (Claycomb et al. 2009). Although the majority of transcripts do not change in *csr-1*, *drh-3*, or *cde-1* mutants, another study, which performed RNA-seq analysis, reported that the mRNA target levels for a handful of CSR-1 targets, some of which are involved in chromosome organization (including the polycomb protein MES-6, a chromodomain protein CEC-6, and a kinesin involved in spindle dynamics, KLP-7), are upregulated 2-fold or more in *ego-1* mutant adults (Maniar and Fire 2011). Examination of previous microarray data from *csr-1* mutants revealed that KLP-7 and MES-6 mRNAs show <1.7-fold change, while CEC-6 (T12E12.2) displays a 3-fold increase in mRNA levels. These data may reflect that *csr-1* and *ego-1* behave slightly differently with regard to regulating mRNA expression or may be attributable to technical differences in animal staging or genetic background (aside from the *csr-1* and *ego-1* mutations) for each experiment. Nonetheless, these data suggest that, while CSR-1 does not broadly cause the downregulation of target mRNAs, it may act differentially on a small number of targets to regulate their expression levels.

One type of target that exhibited decreased expression levels in *csr-1* mutants was the set of core histone transcripts. Histone RNA transcripts are regulated differently from most protein coding gene transcripts, in part due to a key stem loop structure in their 3′ untranslated region (UTR). The stem loop recruits factors that cleave sequences in the 3′ UTR downstream from the stem loop, which enables histone mRNA transport from the nucleus and its subsequent translation. In most organisms, the stem loop is recognized by stem loop binding protein (SLBP), which recruits U7 small nucleolar RNA (U7 snRNA). Cleavage and polyadenylation specificity factor 73 (CPSF73) is subsequently recruited to the transcript and cleaves the 3′ UTR downstream of the stem loop (Marzluff et al. 2008). In *C. elegans*, a key subset of the 3′ end processing machinery, U7 snRNA and an associated factor LSM11 are not present, thus raising the question as to how histone 3′ ends are processed in worms (Davila Lopez and Samuelsson 2008). Avgousti et al. provided a potential solution to this problem, when they reported that EGO-1-dependent 22G-RNAs were utilized by CSR-1 to recognize the 3′ UTR of histone RNAs, downstream of the stem loop. The authors proposed that CSR-1 (or another endonuclease, such as CPSF73, possibly recruited by CSR-1) was responsible for the cleavage of histone 3′ UTRs (Fig. 2b). They showed that loss of *csr-1*, *ego-1*, or *drh-3* leads to a decrease in processed histone mRNA and protein, while histone pre-mRNA levels increase. Avgousti et al. also drew a phenotypic parallel between the germline and embryonic lethal phenotypes of *csr-1* pathway mutants and phenotypes resulting from the loss of the SLBP, CDL-1. However, it is important to note that the loss of *cdl-1* does not result in the P granule defect that is characteristic of *csr-1* pathway mutants (Avgousti et al. 2012).

In a somewhat remarkable experiment, overexpression of transgenes encoding histone mRNAs engineered to be independent of 3′ end cleavage was capable of rescuing the lethality normally associated with loss of *csr-1*, *ego-1*, or *drh-3*. This result suggests that the embryonic lethal phenotype associated with loss of the CSR-1 pathway may be attributable to decreases in histone levels (Avgousti et al. 2012). However, one important caveat to consider with regard to these experiments is that the reduction in *csr-1* and *ego-1* was achieved by RNAi, not by genetic lesion. Several subsequent attempts to rescue the *csr-1* mutant by histone transgene expression (both from the published Grishok lab strain known as AGK458, as well as by several newly generated single-copy transgenes from our lab) have been unsuccessful in our hands (V. Cheung and J.M. Claycomb, unpublished results). Despite the potential caveats to the complementation experiments, these data nonetheless present a plausible positive role for CSR-1 in the 3′ end processing of

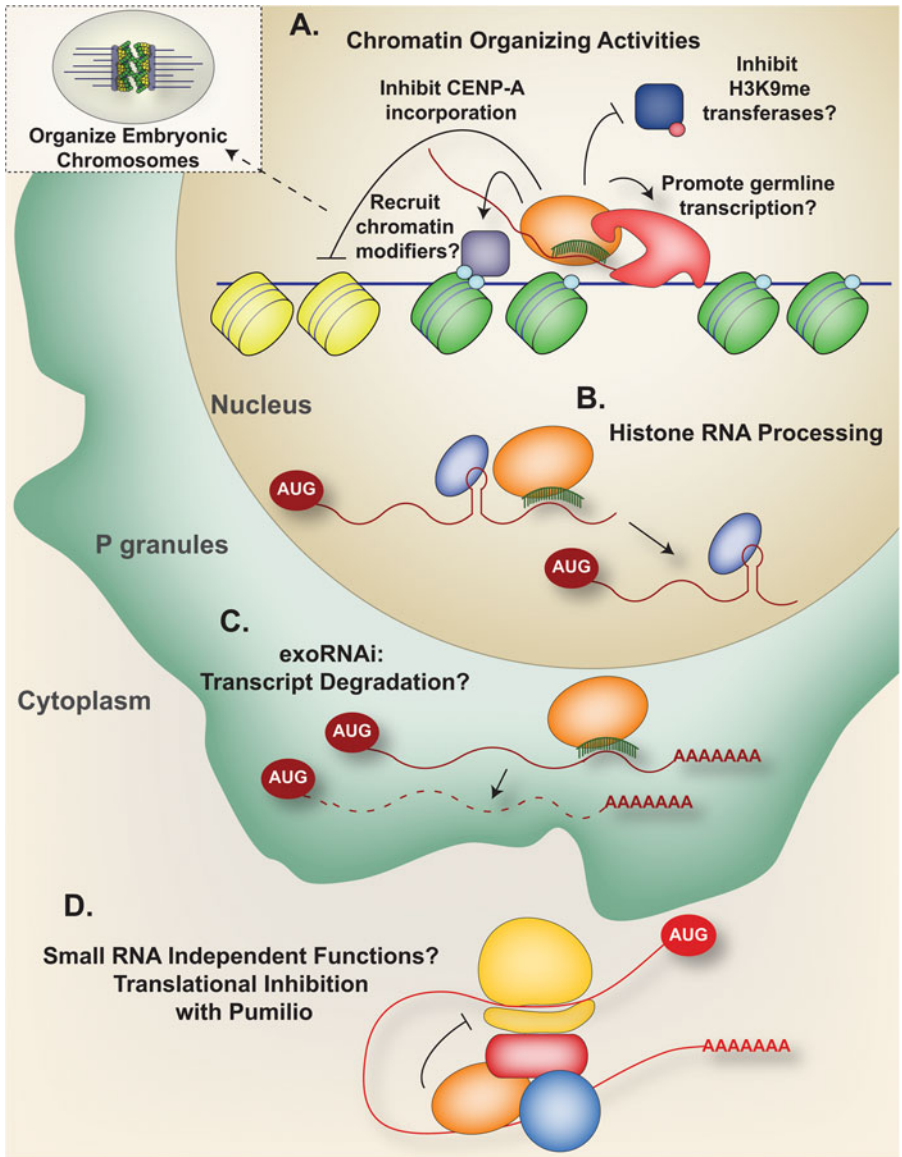

Fig. 2 Models for the various functions of CSR-1. A single germline nucleus is depicted, along with the perinuclear P granules and cytoplasm, as marked. **a** chromatin organizing activities of CSR-1 (*orange*) are depicted. CSR-1 (*orange*) is guided by 22G-RNAs (*green*) to its target gene transcripts (*red*) in the nucleus. Here, CSR-1 interacts with RNA polymerase II (*red*) to influence CSR-1 chromatin domains (green nucleosomes with *blue*, euchromatic histone modifications). Possible activities for CSR-1 include promoting germline transcription, inhibiting histone H3 lysine 9 methyltransferases (*blue*, with red methyl group), inhibiting CENP-A incorporation at inappropriate loci (yellow nucleosomes), and recruiting chromatin modifiers or histone methyltransferases that promote euchromatin (*purple*, with blue methyl group). These activities are likely to coordinately contribute to the organization of germline and embryonic chromosomes to promote proper chromosome segregation. **b** CSR-1 plays a role in histone RNA processing. The stem loop binding protein (SLBP, *indigo*) interacts with the stem loop of histone RNAs (*red*) to stabilize the RNA and recruit other factors. CSR-1 (*orange*) is guided to the 3′ UTR of histone pre-mRNA transcripts via 22G-RNAs (*green*) and may catalyze 3′ end cleavage or recruit an endonuclease to do so. **c** CSR-1 is required for germline exoRNAi. CSR-1 (*orange*) is guided to target transcripts (*red*) by 22G-RNAs generated from exogenously derived dsRNA. Because CSR-1 possesses three residues required for endonuclease activity, it is capable of cleaving and degrading target mRNAs. This activity could occur in the cytoplasm, the P granules, or the nucleus for that matter. **d** Thus far, one small RNA-independent function for CSR-1 (*orange*) has been described. CSR-1 has been shown to interact with Pumilio RNA binding protein family members (*blue*). Together, CSR-1 and Pumilio (FBF in *C. elegans*) form a ternary complex with the translation elongation factor eEIF1A (EFT-3, *red*) to inhibit the translation of FBF target mRNAs (*red*) in the germline

histone RNAs, and such positive roles for Argonautes in regulating gene expression are rare.

One other extremely intriguing positive role for CSR-1 has been proposed. Several recent studies have demonstrated a role for piRNAs in the recognition of foreign nucleic acid in the germline (Shirayama et al. 2012; Ashe et al. 2012; Buckley et al. 2012; Luteijn et al. 2012; Bagijn et al. 2012; Lee et al. 2012). piRNA recognition of an invading nucleic acid target leads to the production of 22G-RNAs that act in several steps to silence the foreign nucleic acid at both the transcriptional and posttranscriptional level. Subsequently, this pathway transmits an epigenetic memory of the foreign nucleic acid to the next generation, using the secondary 22G-RNAs and the accumulation of heterochromatin modifications on the foreign gene. In this process, over 40,000 piRNA sequences (Gu et al. 2012), which are thought to pair with targets via incomplete complementarity (similar to microRNAs), are capable of recognizing a vast repertoire of nucleic acid sequences, including those transcripts the worm intends to express normally (Lee et al. 2012; Bagijn et al. 2012). A key question resulting from these findings is this: How does the worm protect its own germline transcripts from the potentially overzealous silencing activities of the piRNA genome surveillance pathway? One possible answer lies in CSR-1, which targets greater than 80% of germline-expressed genes and could somehow mark these transcripts as self, possibly even at the level of chromatin modification.

Chromatin-directed activities of CSR-1

Immunolocalization studies of CSR-1 showed that it is localized to meiotic chromosomes in the oocyte, mitotic chromosomes in the embryo, and to P granules in both the germline and embryo (Claycomb et al. 2009). Based on its mutant phenotype and localization pattern, a model for CSR-1 function in the nucleus emerged: CSR-1 is guided by its 22G-RNA partners to complementary target gene loci where it may impact chromatin composition. Indeed, in additional biochemical experiments, CSR-1 was shown to physically interact with chromatin by subcellular fractionation and by chromatin immunoprecipitation (ChIP). ChIP analysis shows that CSR-1 is enriched at its target gene chromosomal loci, but not at loci targeted by other Argonautes, such as WAGO-1 (which interacts with a different subset of 22G-RNAs). Furthermore, CSR-1 is not enriched at loci that are not the targets of any known small RNA pathway. Using genetics and RNAse treatment experiments, the enrichment of CSR-1 at target loci was determined to be dependent on both 22G-RNAs and the target transcripts themselves, suggesting that CSR-1 may interact with nascent transcripts at its target gene loci (Claycomb et al. 2009). Indeed, our own recent Co-IP experiments have demonstrated that CSR-1 associates with RNA polymerase II, reinforcing the notion that CSR-1 is recruited to nascent transcripts (C. J. Wedeles, J. M. Claycomb, in preparation).

CSR-1 target genes are found in domains dispersed along the chromosomes and are estimated to comprise about 10–15% of the total 100 Mb genome (Claycomb et al. 2009; Claycomb 2012). A survey of ModENCODE data (Gerstein et al. 2010), along with our own ChIP experiments revealed that CSR-1 chromatin domains are characterized by histone modifications that are typically associated with euchromatin (Table 1), such as histone H3 lysine 4 methylation (H3K4me, H3K4me2, and H3K4me3), histone H3 lysine 36 methylation (H3K36me2 and H3K36me3), histone H3 lysine 9 acetylation, and histone H4 lysine 8 and lysine 16 acetylation. Likewise, the histone variant HTZ-1 (H2AZ), which has been shown to be enriched at the majority of germline-expressed genes, is also enriched at CSR-1 targets (Table 1). Histone modifications typically associated with heterochromatin are not enriched or even present for that matter in CSR-1 chromatin domains (Table 1). For instance, histone H3 lysine 9 methylation (H3K9me, H3K9me2, and H3K9me3) and histone H3 lysine 27 methylation (H3K27me) are depleted at CSR-1 targets (C. J. Wedeles and J. M. Claycomb, in preparation).

Perhaps, these histone modification patterns are not surprising, as we know that CSR-1 target genes are expressed in the germline and would anticipate that euchromatin-associated histone modifications would be present at these loci. However, these patterns are notable because all other Argonaute pathways associated with modifying chromatin lead to the deposition of repressive histone marks (van Wolfswinkel and Ketting 2010). Furthermore, we have evidence to suggest that, despite the overall decreases in histone levels in *csr-1* mutants reported by Avgousti et al., CSR-1 *participates* in modulating euchromatin and is not simply correlated with euchromatic histone modifications. Our observations include that *csr-1* mutants display altered patterns of histone modifications as measured

Table 1 Histone modifications enriched/depleted in CSR-1 chromatin domains

Histone modification	Type of modification	Enriched/depleted	Method used
Histone variant HTZ-1/H2A.Z	Euchromatin	Enriched	ModENCODE
H3K4me2	Euchromatin	Enriched	ModENCODE, ChIP
H3K4me3	Euchromatin	Enriched	ModENCODE, ChIP
H3K9ac	Euchromatin	Enriched	ChIP
H3K27ac	Euchromatin	Enriched	ModENCODE
H3K36me2	Euchromatin	Enriched	ModENCODE, ChIP
H3K36me3	Euchromatin	Enriched	ModENCODE, ChIP
H4K8ac	Euchromatin	Enriched	ChIP
H4K16ac	Euchromatin	Enriched	ModENCODE
H3K9me	Heterochromatin	Depleted	ModENCODE, ChIP
H3K9me2	Heterochromatin	Depleted	ModENCODE, ChIP
H3K9me3	Heterochromatin	Depleted	ModENCODE, ChIP
H3K27me	Heterochromatin	Depleted	ModENCODE
H3K79me3	Mixed	Depleted	ChIP
H4K20me	Mixed	Mixed	ModENCODE

Histone modifications were identified as being 2-fold or more enriched at CSR-1 target genes relative to several nonsmall RNA targeted loci throughout the genome by ChIP and/or showing 2-fold or greater enrichment at CSR-1 target genes in ModENCODE genome browser surveys. Those modifications marked as depleted display <2-fold enrichment by ChIP or in ModENCODE genome browser surveys.

by ChIP (also see below), and we have identified chromatin factors that physically associate with CSR-1 (C. J. Wedeles, M. F. Francisco and J. M. Claycomb, in preparation). Furthermore, studies of chromatin after passage through the dauer state, an extreme developmental state that serves to protect the worm from adverse environmental conditions, revealed that *csr-1* mutants have altered patterns of histone H3K4me3 and H4 acetylation relative to wild-type worms subjected to the same conditions (Fig. 2a) (Hall et al. 2013).

Another demonstration of the ability of the CSR-1 pathway to modulate chromatin involves the role of CSR-1 in a pathway that silences unpaired chromatin during meiosis via the formation of heterochromatin. This pathway serves to protect gametogenesis from aberrant checkpoint activation due to unpaired chromatin, as occurs in the case of some transgenes and the unpaired X chromosomes of males (male *C. elegans* have only a single X chromosome, while hermaphrodites possess two X chromosomes) (Maine 2010). Cell biology studies have demonstrated that loss of *csr-1* pathway function leads to an overall increase in H3K9me2 acquisition in the germline, where the modification spreads onto autosomes, which normally have very low levels of H3K9me2. Concomitant with this overall increase in H3K9me2, there is a decrease in the level of H3K9me2 on the unpaired X chromosome of males (van Wolfswinkel and Ketting 2010; She et al. 2009; Maine et al. 2005). Our Western blot data reflect the same phenomenon: *csr-1* mutants display global increases in H3K9me2 (C. J. Wedeles and J. M. Claycomb, in preparation). Thus, it was proposed that the CSR-1 pathway acts to either recruit H3K9 methyltransferase activity to the unpaired X or deflect this activity from acting on the autosomes (Maine 2010; van Wolfswinkel and Ketting 2010). Due to the large proportion of CSR-1 targets on the autosomes and relative dearth of CSR-1 targets on the X chromosome, it is likely that the CSR-1 pathway contributes to the recruitment of methyltransferases or other factors to the autosomes to exclude the activity of H3K9-histone methyltransferases (Fig. 2a) (Claycomb et al. 2009). The global misregulation of chromatin in *csr-1* pathway mutants could also contribute to the overall organization of chromosomes during subsequent embryonic divisions. Collectively, evidence is mounting to support the notion that CSR-1 can influence the composition of histone modifications in the worm at various developmental stages. It will be important to determine which genomic sequences incur CSR-1-

dependent changes in histone modifications going forward.

The CSR-1 targeted chromatin domains are directly adjacent to and inversely correlate with the histone H3 variant HCP-3/CENP-A. This histone variant serves as an epigenetic mark for the formation of centromeric nucleosomes in eukaryotes. In embryos lacking CSR-1, CENP-A is disorganized and kinetochores fail to properly orient toward the spindle poles, suggesting that the CSR-1 pathway could contribute to the organization of CENP-A throughout the genome (Claycomb et al. 2009). It is important to note that *C. elegans* chromosomes are holocentric, which means that kinetochores are localized along the entire length of each chromosome (Albertson and Thomson 1982; Maddox et al. 2004; Dernburg 2001; Melters et al. 2012). Although superficially distinct from monocentromeres, which most model organisms possess, the holocentric kinetochores of *C. elegans* are largely constructed from the orthologs of kinetochore proteins in monocentric organisms, including CENP-A (Buchwitz et al. 1999; Oegema et al. 2001; Kitagawa 2009). CENP-A has been shown to bind at a low density within protein coding gene domains that encompass approximately half of the genome (Gassmann et al. 2012). Gassmann et al. demonstrated that patterns of transcription dictate where CENP-A may be incorporated; that is, CENP-A is excluded from regions where germline transcription occurs, such as the CSR-1 target domains. It is important to keep in mind that, although CENP-A binding patterns have been elucidated, which of these chromosomal regions possess kinetochore activity has yet to be determined.

How does CSR-1 contribute to patterns of CENP-A incorporation? There are several possibilities (Fig. 2a). Because CSR-1 may influence the composition of chromatin at its targets and promote the expression of its germline target genes, it is possible that CSR-1 could influence the sites of CENP-A incorporation indirectly, by its relationship to germline transcription. In this manner, CSR-1 chromatin could exist as a type of euchromatic boundary element. Another possibility is that CSR-1 chromatin contributes to centromeric chromatin within the holocentromeres (i.e., CSR-1 chromatin constitutes a portion of the centromere). Many studies of monocentromeres have revealed a role for transcription and euchromatic histone modifications on histone H3 within the core centromere and to a lesser extent in pericentromeric chromatin. These histone modifications and centromeric transcription are required for the recruitment of kinetochore proteins and the formation of functional kinetochores (Chan et al. 2012; Sullivan and Karpen 2004; Stimpson and Sullivan 2011; Hall et al. 2012). In this model, CSR-1 chromatin domains are consistent with a euchromatic portion of the holocentromere. Chromatin and genomic tools are becoming increasingly more sophisticated in *C. elegans*, enabling researchers to approach these questions mechanistically in vivo. Clearly, the role of CSR-1 in holocentromere formation is a topic that is sure to be of much interest going forward.

A role for CSR-1 in exogenous RNAi

Exogenous RNA interference or exoRNAi involves the introduction of dsRNA by feeding worms *Escherichia coli* expressing dsRNA, soaking worms in dsRNA or injecting dsRNA into the worm. Once inside the cells of the worm, dsRNA is processed by Dicer into primary short interfering RNAs (siRNAs), and loaded onto the Argonaute RDE-1. In subsequent steps of the pathway, additional small RNAs (secondary siRNAs with characteristics of 22G-RNAs) are generated by RNA-dependent RNA polymerases (RdRPs) and loaded onto secondary (and perhaps even tertiary) WAGO-type Argonautes, including SAGO-1/WAGO-6 and SAGO-2/WAGO-8, and HRDE-1/WAGO-9, which are thought to elicit the gene-silencing outcome (Duchaine et al. 2006; Tabara et al. 1999; Yigit et al. 2006; Ashe et al. 2012; Buckley et al. 2012; Shirayama et al. 2012).

As its name suggests, in addition to its chromosome segregation defect, CSR-1 is required to raise a fully potent gene silencing response upon the introduction of exogenous dsRNA. Mutants in *csr-1* display an incompletely penetrant RNAi deficiency (Rde) against germline and early embryo gene targets, with about 70% of the worms in a population being affected (Yigit et al. 2006). This phenotype was subsequently rescued by expression of a 3xFlag::CSR-1 transgene expressed in the germline (Claycomb et al. 2009). A role for CSR-1 in somatic RNAi is less prominent, as Yigit et al. observed no phenotype when feeding *E. coli* expressing dsRNA directed against the muscle specific gene *unc-22* to *csr-1* worms, while Aoki et al. reported a subtle RNAi deficiency when soaking worms in dsRNA directed against the gut-expressed gene *Y57G11C.15* (Yigit et al. 2006; Aoki et al. 2007). Like CSR-1, the other factors of the CSR-1 pathway also display RNAi deficiency when mutated, due to defects in the generation of secondary small RNAs in response

to exogenous dsRNA (Duchaine et al. 2006; Gu et al. 2009; Yigit et al. 2006; Smardon et al. 2000; van Wolfswinkel et al. 2009; Claycomb et al. 2009). Together, these results demonstrate a requirement for CSR-1 in exo-RNAi, predominantly in the germline.

How does CSR-1 contribute to exoRNAi and at which step(s) in exoRNAi does CSR-1 act (Fig. 2c)? The function(s) of CSR-1 in RNAi may or may not be the same as its chromosome-organizing activities and could involve either or both isoforms of the Argonaute (see below). Owing to the fact that CSR-1 interacts with 22G-RNAs, it is anticipated that it acts in secondary or tertiary steps in the RNAi process. Due to the fact that CSR-1 can act in the nucleus, it is possible that it could function at the level of downregulating transcription of a target gene during an RNAi response. Such a role has recently been described for the WAGO HRDE-1/WAGO-9 (Ashe et al. 2012; Buckley et al. 2012; Shirayama et al. 2012).

CSR-1 retains the three key "slicer" residues (DDE/H), which are required for endonuclease activity on a target mRNA. Aoki et al. (2007) have demonstrated that CSR-1 can cleave mRNA in vitro when loaded with 22G-type triphosphate small RNAs, but this activity was abrogated in a D769A slicer-dead mutant. Furthermore, extracts made from *csr-1(fj54)* mutants were incapable of cleavage activity against an RNA target in vitro. Interestingly, CSR-1 displayed the most endonuclease activity in vitro when loaded with small RNAs that were completely complementary to target transcripts (Aoki et al. 2007), suggesting a potential "rule" for CSR-1 activity where complete complementarity elicits slicing of target transcripts. This phenomenon would be consistent with observations from other organisms (Brodersen and Voinnet 2009). We also anticipate that this in vitro activity likely reflects the endogenous activity of CSR-1 on its targets because the 22G-RNAs associated with CSR-1 match protein coding target mRNA sequences with complete complementarity (Claycomb et al. 2009). This does not preclude the possibility that interaction with other factors could modulate CSR-1 nuclease activity, as is the case for other Argonautes. Together these results suggest that CSR-1 could act as a slicer in RNAi to degrade transcripts, and this could occur either in the cytoplasm or nucleus (Fig. 2c).

Finally, one additional possibility for how CSR-1 could contribute to germline RNAi involves P granules. Many RNAi and endogenous small RNA factors, including CSR-1 itself, DRH-3, EGO-1, PRG-1, and WAGO-1 localize to P granules (Claycomb et al. 2009; Batista et al. 2008; Gu et al. 2009). Because of this, P granules are thought to be involved in germline RNAi responses and have been proposed to be sites of small RNA biogenesis or involved in germline transcript degradation. As described above, *csr-1* pathway mutants display a striking and unique P granule localization defect, in which the P granules dissociate from the nuclear periphery and are instead found in the cytoplasmic core of the syncytial germline (Claycomb et al. 2009; Updike and Strome 2009; Vought et al. 2005). Despite this dramatic defect, small RNA factors (aside from CSR-1) continue to localize to the aberrant P granules of *csr-1* mutants (M. A. Francisco and J. M. Claycomb, unpublished results), suggesting that these factors could still function in their respective small RNA pathways. It could be that close association with the nucleus is required for the appropriate trafficking of mRNAs into these granules. Although Updike and Strome used the RNA dye SYTO-14 to show that the aberrant *csr-1* P granules accumulated RNA (Updike and Strome 2009), they did not probe the sequences of the RNA present in the P granules; thus, it is possible that inappropriate RNA, and not the normal target transcripts of resident small RNA pathways, accumulates in the *csr-1* P granules. At the apex of P granule formation is DEPS-1, a nematode specific protein that is required for the recruitment of all examined subsequent factors to P granules. Notably, loss of *deps-1* also leads to Rde, supporting the notion that the complete loss of P granules leads to Rde (Spike et al. 2008). The same may or may not hold true for the *csr-1*-dependent P granule disruption.

Small RNA-independent roles for CSR-1?

In mass spectrometry experiments, which used Pumilio, an RNA binding protein and translational repressor (called FBF in *C. elegans*), as bait, Friend et al. (2012) identified both isoforms of CSR-1 as FBF-interacting proteins. Structural analysis demonstrated that the Mid and PIWI domains of CSR-1 were required for its interaction with FBF, while PAZ was dispensable. In vivo germline reporter assays, using GFP possessing the 3′ UTR of the FBF target *gld-1*, revealed that GFP (as a proxy for *gld-1*) was inappropriately expressed at an earlier stage of germline

development when *csr-1* or *fbf* were lost. One caveat to these experiments is that many FBF targets, including those examined (*cye-1* and *gld-1*) are CSR-1 target genes, and it is possible that they are regulated in a CSR-1/22G-RNA-dependent manner as well (Friend et al. 2012).

The authors went on to provide insights into how FBF and CSR-1 could inhibit the expression of FBF targets. FBF, CSR-1, and the translation elongation factor EFT-3 (an eEIF1A homolog) form a ternary complex, where FBF and CSR-1 act to inhibit the GTPase activity of EFT-3 to attenuate translation elongation (Fig. 2d). Notably, this complex and its activity appear to be conserved in humans, as AGO1-3, PUM2, and eEIF1A form a complex that acts to attenuate translation (Friend et al. 2012).

Because of its key role in binding small RNAs, the fact that the PAZ domain was not required for the FBF-CSR-1 interaction or activity of the complex suggests that the FBF-mediated aspects of CSR-1 function are independent of small RNAs. Furthermore, the authors suggest that this may be a more widespread means of regulating specific subsets of transcripts, as there are 11 Pumilio family proteins and 24 AGOs present in the worm (Friend et al. 2012). Although this is the first described small RNA-independent role for CSR-1, it opens the possibility that there are others yet to be revealed.

Additional considerations regarding CSR-1 function

Multiple isoforms of CSR-1

One underaddressed facet of CSR-1 biology is the fact that there are two isoforms of CSR-1 encoded by the *csr-1* gene (Claycomb et al. 2009). The long isoform possesses an additional 163 amino acids at its N terminus, which is, as far as has been reported, the only difference between the two isoforms of the protein. This N terminal region is rich in arginine and glycine (R/G) motifs, presenting the possibility for posttranslational modification by methylation, a common feature of PIWIs in other species that facilitates the interaction of Tudor domain proteins (such as EKL-1, for instance, although no physical interaction between EKL-1 and CSR-1 has been reported) (Vagin et al. 2009). As noted by Updike et al., the R/G motif is also found in proteins that localize to P granules, as CSR-1 was shown to do (Updike and Strome 2009; Claycomb et al. 2009). The R/G motif may also function as an RNA interaction motif or as a protein–protein interaction motif (Corley and Gready 2008). Certainly, the extended N terminal region could serve as a docking site for various long isoform-specific cofactors or regulatory proteins, but this has yet to be proven. The long isoform also possesses a putative nuclear localization signal (NLS) within the N terminal region, which could lead to its enrichment in the nucleus.

Translation of the short isoform begins at a methionine near the 5′ end of the second exon of the long isoform; thus, the short isoform is lacking both the R/G rich region and the putative NLS (Claycomb et al. 2009). The lack of a putative NLS makes it tempting to hypothesize that the long isoform is the only isoform enriched in the nucleus; however, this has yet to be clearly demonstrated. Both isoforms possess the three key catalytic residues in the PIWI domain and thus could function as "slicers." Owing to the use of an antibody that recognizes both forms of CSR-1, the 22G-RNAs identified in association with CSR-1 could be present in either or both types of CSR-1 complexes (Claycomb et al. 2009). The N terminus is the least highly conserved region of the CSR-1 protein between *Caenorhabditid* species, although both isoforms appeared to be encoded in the species we examined (see below). Thus far, distinct functions or subsets of 22G-RNAs have not been attributed to each isoform, but we do know that there are differences in the timing of each isoform's expression throughout development that could also contribute to differential activity. Clearly, elucidating the differences (and similarities) between CSR-1 isoforms will be an interesting topic of future study.

The evolution and conservation of CSR-1

CSR-1 can be identified by homology searches in clade V nematodes (of which *Caenorhabditid* species are representatives), and in clade III nematodes (a clade possessing such human parasitic nematodes as *Loa loa* and *Ascaris lumbricoides*) (Buck and Blaxter 2013; Dalzell et al. 2011). Nematodes of these groups possess holocentromeres, but there is not a strict correlation between holocentric nematodes and the presence of CSR-1. For instance, the analysis to date of clade IV nematodes suggests that they are holocentric, yet it is

difficult to identify a homolog of CSR-1 in species of this group (Melters et al. 2012). Interestingly, a nonessential paralog of CSR-1 (in *C. elegans*), C04F12.1, appears to be more highly conserved than CSR-1 among clades III–V; perhaps, this Argonaute assumes some of the roles of C.e.CSR-1 in other organisms (Buck and Blaxter 2013; Dalzell et al. 2011). In fact, C04F12.1 like CSR-1 is found in association with germline chromatin in large-scale proteomic studies, and our own cellular fractionation studies (M. Z. Wu and J. M. Claycomb, in preparation), strongly suggesting that it has nuclear functions (Chu et al. 2006).

CSR-1 orthologs and 22G-RNAs have been identified in *Ascaris suum* (Clade III), *Caenorhabditis briggsae*, *Caenorhabditis remanei*, and *Caenorhabditis brenneri* (Clade V) (Wang et al. 2011; Shi et al. 2013). During embryonic development, *Ascaris* chromosomes undergo a process called chromosome diminution, where large portions of the chromosomes are eliminated from the somatic genome (Goday and Pimpinelli 1993). In ciliated protozoans such as *Tetrahymena*, a PIWI Argonaute and small RNAs are involved in a process that marks portions of the genome for elimination (Wang et al. 2011; Kataoka and Mochizuki 2011). Thus, CSR-1 was proposed to be involved in this process in *Ascaris*; guided to loci by its accompanying 22G-RNAs, CSR-1 could either protect or denote portions of the genome to be eliminated. However, a subsequent study showed no correlation between the small RNAs of *Ascaris* and the portions of the genome that were eliminated or retained (Wang et al. 2012). Regardless, the presence of CSR-1 and 22G-RNAs in *Ascaris* provides the possibility of interesting regulatory activities that have yet to be explored.

Examination of small RNA populations and target genes in three *Caenorhabditid* species using the set of C.e.CSR-1 target genes as a reference revealed that CSR-1 target genes are more highly conserved when compared to all genes on average or WAGO-1 target genes (Shi et al. 2013). In fact, they determined that 85% of C.e.CSR-1 targets had a 1:1 ortholog in at least one of the three species examined, while only 60% of WAGO-1 targets had a 1:1 ortholog. In addition, 50–70% of C.e.CSR-1 target genes generate small RNAs in the three *Caenorhabditid* species, while only 20–30% of WAGO-1 targets generate small RNAs, even though WAGO-1 small RNAs are of greater abundance than CSR-1 small RNAs in all four *Caenorhabditid* species (Shi et al. 2013). The authors note that a high degree of conservation of CSR-1 targets would be expected if CSR-1 functioned in a protective manner against the piRNA surveillance pathway, as proposed by Shirayama et al. (2012). Our own studies of *C. briggsae* CSR-1 have revealed that *C.b.csr-1* is an essential gene, and its reduction by RNAi leads to chromosome segregation defects, suggesting functional conservation in nematodes that are separated by 20–80 million years of evolution (M. Z. Wu and J. M. Claycomb, in preparation).

Conclusion

Clearly, the *C. elegans* Argonaute CSR-1 is a fascinating member of this large, conserved protein family. CSR-1 plays key roles in both the cytoplasm (such as exoRNAi) and in the nucleus (such as processing histone transcripts and regulating histone modifications) throughout nematode development. Most of the activities of CSR-1 discovered so far require the CSR-1 22G-RNAs; however, one small RNA-independent activity of CSR-1 in inhibiting transcription has been revealed recently. Unveiling the mechanisms by which CSR-1 modulates chromatin and regulates germline transcription will be a topic of intensive research in the coming years. For instance, there are most likely a number of additional chromatin factors, which play roles downstream of CSR-1 in this pathway; perhaps, these will emerge from re-examination of chromatin factors implicated in RNAi-related processes.

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