CHAPTER THREE

Concepts and functions of small RNA pathways in *C. elegans*

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Abstract

A diversity of gene regulatory mechanisms drives the changes in gene expression required for animal development. Here, we discuss the developmental roles of a class of gene regulatory factors composed of a core protein subunit of the Argonaute family and a 21–26-nucleotide RNA cofactor. These represent ancient regulatory complexes, originally evolved to repress genomic parasites such as transposons, viruses and

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retroviruses. However, over the course of evolution, small RNA-guided pathways have expanded and diversified, and they play multiple roles across all eukaryotes. Pertinent to this review, Argonaute and small RNA-mediated regulation has acquired numerous functions that affect all aspects of animal life. The regulatory function is provided by the Argonaute protein and its interactors, while the small RNA provides target specificity, guiding the Argonaute to a complementary RNA. *C. elegans* has 19 different, functional Argonautes, defining distinct yet interconnected pathways. Each Argonaute binds a relatively well-defined class of small RNA with distinct molecular properties. A broad classification of animal small RNA pathways distinguishes between two groups: (i) the microRNA pathway is involved in repressing relatively specific endogenous genes and (ii) the other small RNA pathways, which effectively act as a genomic immune system to primarily repress expression of foreign or "non-self" RNA while maintaining correct endogenous gene expression. microRNAs play prominent direct roles in all developmental stages, adult physiology and lifespan. The other small RNA pathways act primarily in the germline, but their impact extends far beyond, into embryogenesis and adult physiology, and even to subsequent generations. Here, we review the mechanisms and developmental functions of the diverse small RNA pathways of *C. elegans*.

1. A general core of small RNA pathways: Argonaute proteins

Before we dive into the rich world of gene regulation by small RNAs, it is essential to introduce how these short stretches of RNA act. Small RNA molecules by themselves are powerless: their non-capped 5′ ends and non-polyadenylated 3′ ends render them easy targets for cellular RNA degradation pathways. However, bound to different members of the so-called Argonaute family of proteins, they form sequence-specific gene regulatory machines (Fig. 1A). Target specificity is mostly determined by the sequence of the small RNA molecule, while the type of output is set by the Argonaute protein, of which various paralogs are typically encoded in the genome. Hence, a better name for these pathways would be "Argonaute-pathways."

The Argonaute protein family is ancient, with members found in Archaea. The origin of this family of proteins likely lies in the need for a defense system against nucleic acid parasites, such as transposons, viruses and retroviruses. From this ancient cellular immune system, many different gene regulatory mechanisms have evolved. Yet, the basic architecture of the Argonautes that act in these different processes, and the way they bind their small RNA cofactors are deeply conserved. Target recognition can proceed via extensive base pairing between small RNA and the target, but in some cases, notably for miRNAs, only limited base pairing is required. Being

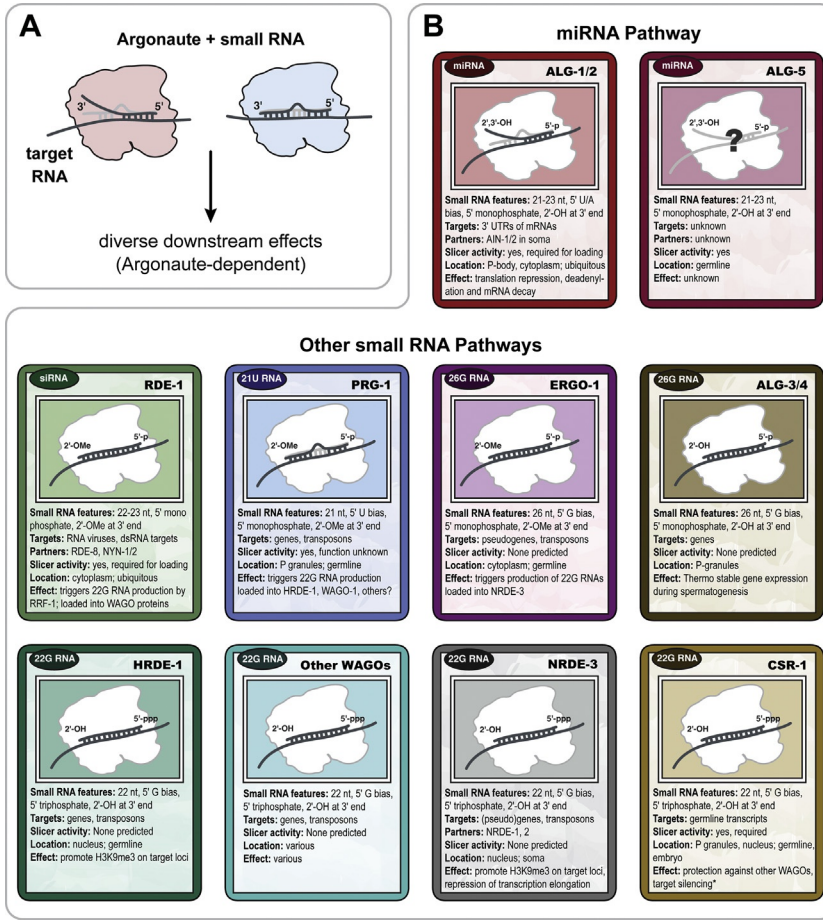

Fig. 1 The *C. elegans* Argonautes. (A) Schematic of the main components of eukaryotic small RNA pathways. An Argonaute protein is loaded with a short 21–26 nt long RNA that guides the complex to a target RNA through different extents of base complementarity (two classes of Argonautes are depicted, which represent these different binding modes). Recruitment of Argonautes typically has a repressive effect on the target RNA, achieved through different mechanisms that depend on the specific identity of the Argonaute protein. (B) Key distinguishing features of the different Argonaute proteins of *C. elegans* and their associated small RNAs are summarized. The Argonautes involved in miRNA-mediated repression and those involved in other pathways are separated given their different properties. The different types of small RNAs are defined by their modes of biogenesis and molecular features, which are further described in the text as the different classes are introduced.

related to the RNaseH family, many, but not all Argonautes can cleave their targets, provided the small RNA and its target pair extensively. We refer the interested reader to previously published reviews for more information on the biochemical properties of these proteins (Dueck & Meister, 2014; Meister, 2013; Swarts et al., 2014).

The *C. elegans* genome encodes 26 Argonaute genes, but some are likely pseudogenes and it is currently thought that 19 of these are functional. These fall into different classes and different types of Argonautes are loaded with distinct types of small RNA (Fig. 1B). These RNAs are characterized by specific molecular features that stem from specialized biogenesis pathways. Distinct Argonaute-small RNA complexes have different functions, but there is also frequent cross-talk among the different pathways. In this review we discuss the molecular mechanisms of known Argonaute-driven pathways in the nematode *C. elegans* and insights into how they affect development and some aspects of adult physiology.

2. The microRNA pathway (or the ALG-1/-2 pathway)

microRNAs (miRNAs) were the first endogenous small RNAs described in animals. The founding members, *lin-4* and *let-7*, were discovered in *C. elegans* as regulators of developmental timing (Lee, Feinbaum, & Ambros, 1993; Reinhart et al., 2000), but it became clear that hundreds of different miRNAs are widespread across metazoans and that their biogenesis and mechanism of action are highly conserved (recently reviewed in Ambros & Ruvkun, 2018). Two decades of research have made miRNAs arguably the best-known class of animal small RNAs to date, and we encourage the reader to explore different aspects of their biology in a number of other recent reviews (Ambros & Ruvkun, 2018; Bartel, 2018; Dexheimer & Cochella, 2020). Here, we give a brief overview of their molecular properties and mode of action, highlighting aspects relevant to further understand their contribution to *C. elegans* development.

2.1 miRNA biogenesis, target specificity and repressive function

miRNAs are 21–23 nt long, single-stranded RNAs that are defined primarily by their biogenesis (Ha & Kim, 2014; Treiber, Treiber, & Meister, 2019). Relevant to their roles in development, it is worth mentioning that they are encoded in the genome as RNA polymerase II-dependent precursors. Longer transcripts are processed through cleavage by the endonucleases

Drosha and Dicer, and mature miRNAs are loaded into the Argonautes ALG-1 and ALG-2 (Grishok et al., 2001). ALG-1 and ALG-2 are only partially redundant (Tops, Plasterk, & Ketting, 2006), most likely due to different expression patterns (Brown, Svendsen, Tucci, Montgomery, & Montgomery, 2017). A small subset of miRNAs in the germline are also loaded into ALG-5, but it is currently unknown whether ALG-5 targets mRNAs in a similar manner to ALG-1/2 (Brown et al., 2017).

Target specificity of ALG-1/2 relies on sequence complementarity, in particular to the 5′ region of the miRNA—encompassing nucleotides 2–8—also known as the seed sequence (reviewed in Bartel, 2009). Conservation analyses as well as structural and functional studies indicate that for most miRNAs seed-pairing is the main specificity determinant. However, different miRNAs also engage in varying degrees of pairing through their 3′ regions. Additional 3′ complementarity is particularly important in the presence of a weak seed match, either due to mismatches, G-U wobble pairing, or simply due to low CG content. In fact, *let*-7 targets two binding sites in the *lin-41* 3′ UTR, both of which have imperfect seed matches and extensive 3′ pairing (Reinhart et al., 2000; Vella, Reinert, & Slack, 2004). This makes *lin-41* an exceptional target of *let*-7 among the many possible targets with a seed match (Ecsedi, Rausch, & Grosshans, 2015); but it also makes *lin-41* a specific target for *let*-7 as opposed to the *let*-7 sisters (miR-48, miR-84 and miR-241), which share the same seed sequence but differ at their 3′ end (Abbott et al., 2005; Brancati & Grosshans, 2018; Broughton, Lovci, Huang, Yeo, & Pasquinelli, 2016; Zhang, Artiles, & Fire, 2015). Coupling a weak seed match with additional complementarity can more generally increase specificity for a certain miRNA-target pair (Didiano & Hobert, 2008; Garcia et al., 2011) and at the same time render the interaction more robust, for example to differences in miRNA concentration (Brancati & Grosshans, 2018).

Binding of miRISC to a target mRNA, typically to the 3′ UTR, represses translation and triggers deadenylation and ultimately mRNA decay (Bartel, 2018; Jonas & Izaurralde, 2015). Whether regulation at the protein or the mRNA level leads to functionally meaningful target repression remains a matter of debate and is likely affected by experimental conditions (recently reviewed in Ambros & Ruvkun, 2018; Dexheimer & Cochella, 2020). Both effects, however, have been linked to another protein component of miRISC, AIN-1 or its redundant homolog AIN-2 (Ding, Spencer, Morita, & Han, 2005; Zhang et al., 2007). AIN-1/2 belong to the widely conserved GW182 family of proteins, characterized by the presence of

multiple Gly-Trp dipeptides that allow interaction with Argonautes (Dexheimer & Cochella, 2020). GW proteins recruit the deadenylases CCR4-NOT and PAN2/3, which eventually trigger mRNA decay, but also proteins that interfere with cap-dependent translation (Amaya Ramirez, Hubbe, Mandel, & Bethune, 2018; Jonas & Izaurralde, 2015; Rasch, Weber, Izaurralde, & Igreja, 2020). Interestingly, in the *C. elegans* germline, ALG-1 does not interact with AIN-1 and tested miRISC targets are not destined for degradation (Dallaire, Frederick, & Simard, 2018). These targets are translationally repressed at least in part due to the action of the DEAD box helicase and P-granule component, GLH-1. miRISC-mediated translational repression independent of GW proteins has also been observed in *Drosophila* S2 cells (Fukaya & Tomari, 2012; Wu, Isaji, & Carthew, 2013). It therefore seems that targets of miRISC may succumb to different fates depending on miRISC composition, subcellular localization and potentially other variables.

2.2 The miRNA repertoire of *C. elegans*

The *C. elegans* genome encodes 145 high-confidence miRNAs (Fromm et al., 2020), out of which 83 can be grouped into 23 families based on their shared seed sequences, with families containing 2–8 members each (Alvarez-Saavedra & Horvitz, 2010). The remaining miRNAs are singletons, i.e. they have unique seed sequences and thus target specificity. A number of these have been implicated in different biological functions (Ambros & Ruvkun, 2018), but for the vast majority functions are still unknown. Members of a family are predicted to largely share target specificity; however, as shown for the *let-7* and the *let-7 sisters* (Abbott et al., 2005; Broughton et al., 2016; Ecsedi et al., 2015), this is not necessarily the case and will require experimental validation as more families are further studied. For a few families, members do act redundantly, revealing important developmental functions when all family members are ablated (Abbott et al., 2005; Alvarez-Saavedra & Horvitz, 2010; Shaw, Armisen, Lehrbach, & Miska, 2010). However, for most families, no obvious defects were observed even upon deletion of all family members and their biological functions remain unknown.

Most miRNAs in *C. elegans* are specific to the *Caenorhabditis* clade, but 20/23 seed-defined families are conserved beyond the genus, and 11/23 have at least one member that seems to have originated at the base of bilaterian animals (Fromm et al., 2020). In contrast, only about 1/3 of all

singletons seem to have a counterpart beyond the genus, and about half of these are conserved across Bilateria (Fromm et al., 2020). Among the miRNAs with recognized functions in development, there is an over-representation of highly conserved miRNAs, but this may reflect a bias in choice of focus of study rather than a lack of identifiable functions in the clade-specific miRNAs. In fact, a few *Caenorhabditis*-specific miRNAs have also been shown to have well-defined functions, and there is a trend for those functions to be more restricted to specialized cells (Drexel, Mahofsky, Latham, Zimmer, & Cochella, 2016; Johnston & Hobert, 2003). Most miRNAs in the animal kingdom are in fact specific to restricted clades; it is interesting to speculate that they may also play roles in cells with specialized functions.

Whereas a small fraction of miRNAs are expressed broadly, if not ubiquitously across the animal, most miRNAs have varying degrees of cell-type specificity (Alberti et al., 2018; Alberti & Cochella, 2017; Martinez et al., 2008). This is largely determined at the transcriptional level through the action of enhancers and promoters that control RNA pol II activity.

2.3 Biochemical versus biological targets

Given that miRNA targets are defined by their match to the relatively short seed sequence, each miRNA is predicted to target dozens of different mRNAs. Most of these mRNAs with matching sequences are indeed biochemical targets of miRISC: they are bound by miRISC, they are derepressed in the absence of specific miRNAs or the miRNA-associated protein machinery, and they are repressed if the targeting miRNA is overexpressed (Broughton et al., 2016; Grosswendt et al., 2014). However, a growing body of evidence points to only a few of these being functionally relevant targets, at least in the context of the measurable cellular or organismal functions of the miRNAs that have been studied to this extent (Drexel et al., 2016; Ecsedi et al., 2015; Finger et al., 2019; Mockly & Seitz, 2019; O'Hern et al., 2017; Sarin et al., 2007; Tran et al., 2019; Wightman, Ha, & Ruvkun, 1993). Whether miRNA-mediated repression of a target will be phenotypically consequential for a cell, or for the whole organism is determined by properties that define the regulatory contribution of any gene (miRNA or else), and can be summarized in three main points (Fig. 2):

1. *Dose sensitivity of the target*: The impact of miRNA-mediated repression on a target will depend on whether that target is repressed below its functional threshold or not. In a comparable situation, halving the dose of a

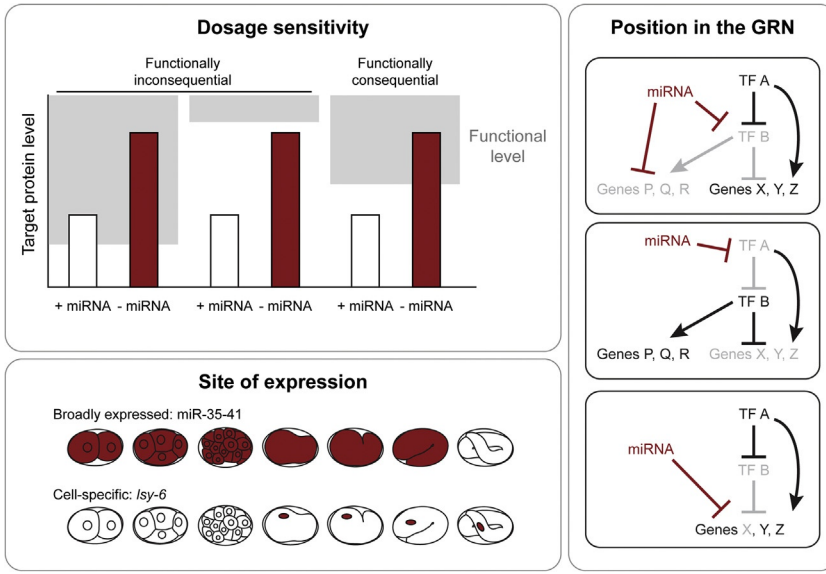

Fig. 2 Determinants of the cellular and organismal consequences of miRNA-mediated repression. *Dosage sensitivity*: the same twofold repression exerted by the miRNA in this example may have drastically different consequences depending on the thresholds that define the target's functional level (represented by the gray boxes). *Positions in the Gene Regulatory Network (GRN)*: a simple GRN is schematized composed of two transcription factors (TFs), their targets and a miRNA; black letters indicate the gene is expressed and gray indicates repression (either transcriptional or post-transcriptional). Top: repression of genes that should also be transcriptionally off may serve to de-noise gene expression. Middle: repression of the TF at the top of the GRN causes a switch in gene expression pattern. Bottom: repression of a terminal gene may affect an individual property of a cell without changing its identity. *Site of expression*: at the organismal level, a key determinant of the impact of a miRNA is given by the site and timing of expression; two miRNAs with extreme expression patterns (ubiquitous from early in embryogenesis versus specific to a single, post-mitotic neuron) are shown.

gene product for example by heterozygous deletion of a gene, may cause no effect in one case, but may result in defects related to haploinsufficiency in another (Pinzon et al., 2017).

2. *Position of the target in the cellular gene regulatory network*: The impact of repression will differ depending on whether the target is at the top of a cell's gene regulatory network (e.g. a transcription factor whose regulation will have multiple downstream effects) or at the end of the regulatory cascade (e.g. a terminal identity gene whose function defines only a specific property of a cell) (Ebert & Sharp, 2012).

3. *Site and time of action within the organism*: Impact at the organismal level will additionally be determined by when and where miRNA-mediated repression takes place. The consequences of a miRNA expressed ubiquitously in the early embryo are very different from those of a miRNA expressed in a single neuron at the end of embryonic development (Alberti & Cochella, 2017).

Finding the functionally relevant targets among the many possible regulatory interactions is essential to understand the contribution of miRNA-mediated repression to development and physiology (Fridrich, Hazan, & Moran, 2019). Studies in *C. elegans* have taken the lead relative to other experimental systems in this respect, thanks to the ability to perform rigorous genetic tests. In addition to testing whether a target is indeed derepressed in the absence of the miRNA, and whether lowering the dose of that target suppresses the phenotypes caused by loss of that miRNA, the advent of CRISPR/Cas9 has enabled the possibility to perform the gold standard test for RNA interactions: precise mutation of the seed matching sequence in a functionally relevant target or group of targets should phenocopy the loss-of-function phenotype of the miRNA (Drexel et al., 2016; Ecsedi et al., 2015; Finger et al., 2019; O'Hern et al., 2017; Tran et al., 2019) and restoring pairing to the target(s) by introducing the complementary mutations in the miRNA gene should rescue the phenotype (as long as those mutations do not disrupt miRNA biogenesis).

Here we reviewed the main components of the miRNA pathway and the determinants for their impact on the organism. In the second part of this review, we discuss the developmental and physiological roles of this and other small RNA pathways that we introduce next.

3. Other small RNA pathways

Besides miRNAs, a diversity of endogenous small RNAs operate in *C. elegans* (Almeida, Andrade-Navarro, & Ketting, 2019). To understand the developmental roles of mutants in these pathways, it is important to briefly describe a number of general, and some more specific concepts related to them.

3.1 General concepts

A major conceptual difference between miRNAs and other small RNAs is that these other small RNAs typically do not act as individual species that recognize a specific binding site in their target, but rather act as pools of small

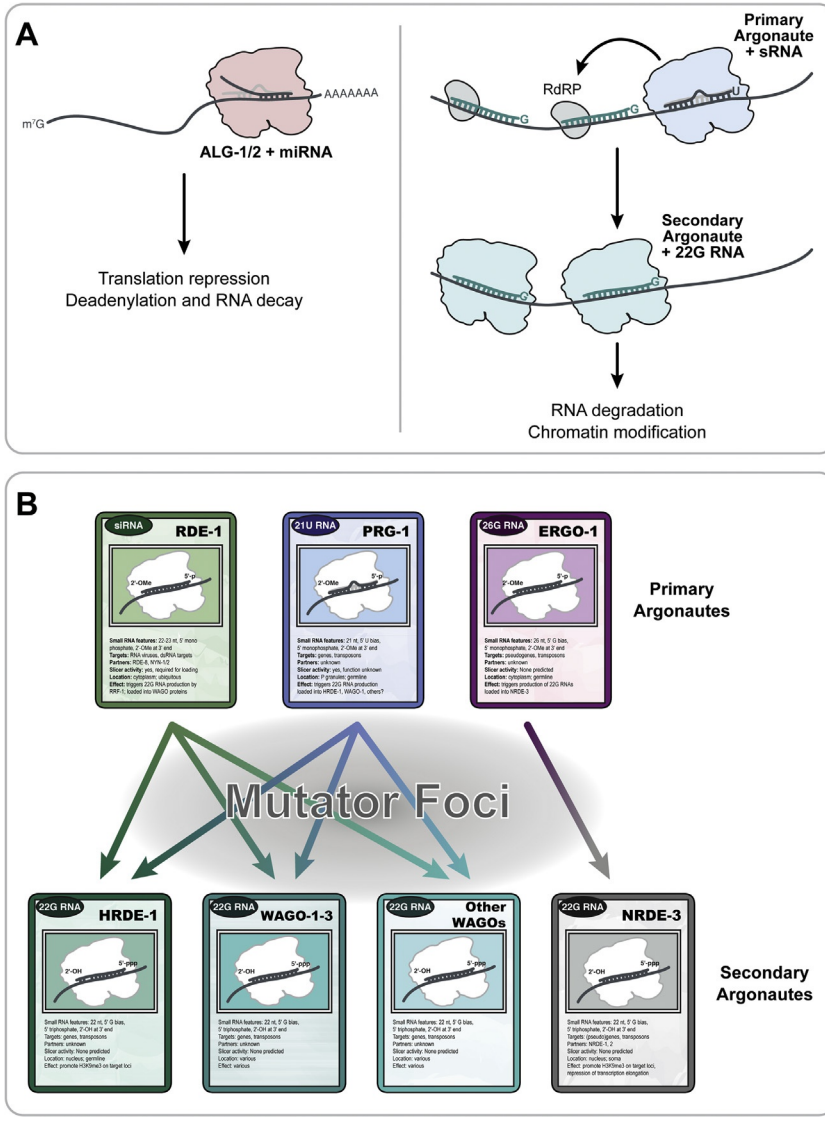

Fig. 3 Two distinct modes of small RNA-mediated regulation. (A) miRNA-mediated repression relies on specific small RNA/binding site interactions with one or few, typically conserved, binding sites in the 3′ UTR of the target transcript; this interaction triggers translation repression and mRNA decay. Other small RNA pathways rely on target recognition by a primary Argonaute carrying endogenous or exogenous primary small RNAs, activating RdRP-mediated production of secondary small RNAs (22G RNAs), which are loaded into different secondary Argonautes to exert various effects. This amplifies the original trigger based on the encountered targets. (B) Multiple small RNA triggers converge to Mutator foci where they activate secondary 22G RNA production by RRF-1. Secondary 22G RNAs are loaded into distinct secondary Argonautes.

RNAs that recognize many sites within one target RNA, sometimes covering the complete mRNA (Fig. 3A). This is likely related to the fact that many of these act as genome immune systems, warding off the expression of "non-self" genes, such as transposons, endogenous retroviruses and pseudogenes (Almeida, Andrade-Navarro, & Ketting, 2019; Ozata, Gainetdinov, Zoch, O'Carroll, & Zamore, 2019; Sarkies & Miska, 2013; Shabalina & Koonin, 2008). This requires a flexible population of small RNAs, which is not hard-wired in the genome but can adopt a multitude of different sequences, similar to antibodies in our immune system. This function is very likely one of the reasons that small RNA generating mechanisms evolve quickly (Ozata et al., 2019; Sarkies et al., 2015).

A general concept that applies to these pathways is one of amplification: the identified target RNA is not just silenced or destroyed, but used as a source to generate more small RNAs (Ketting, 2011) (Fig. 3A). This is also akin to an adaptive immune reaction, in which a specific initial response is amplified to produce effective neutralization of the target. In *C. elegans* this is achieved by primary Argonautes that make their targets into templates for the production of secondary small RNAs by RNA-dependent RNA polymerases (RdRPs). Secondary small RNAs are loaded into secondary Argonautes.

Well-known primary Argonautes are RDE-1, PRG-1 and ERGO-1, binding to siRNAs, 21 U-RNAs and 26G-RNAs, respectively (Fig. 1). Primary Argonautes are phylogenetically closely related to Argonautes from other species. Secondary Argonautes on the other hand are more nematode specific, and for this reason are also named WAGOs (short for Worm-specific Argonautes) (Fig. 1). The small RNAs bound by WAGO proteins are collectively called 22G-RNAs, due to their length and presence of a G-nucleotide at the 5′ end. Interestingly, some secondary Argonautes can, directly or indirectly, induce production of 22G-RNAs that can thus be considered tertiary sRNAs (Sapetschnig, Sarkies, Lehrbach, & Miska, 2015). However, no dedicated tertiary Argonaute has been identified yet. Such amplification steps may serve multiple purposes, including compensation for limited amounts of primary small RNAs, enabling a systemic response and adaptation to sequence variations within targets. Additionally, amplification allows inheritance of the silencing signal across generations (Lev et al., 2019). Next, we describe key-characteristics of a number of small

RNA pathways, starting with the pathway that unleashed the small RNA revolution: RNAi driven by exogenous double-stranded RNA (*exo*-RNAi) (Fire et al., 1998).

3.2 The exo-RNAi pathway (or the RDE-1 pathway)

Exo-RNAi requires the primary Argonaute RDE-1 (Tabara et al., 1999). RDE-1 is loaded with short interfering RNAs (siRNAs) directly produced from the administered dsRNA, through the action of Dicer (Grishok et al., 2001; Ketting et al., 2001; Knight & Bass, 2001; Tabara, Yigit, Siomi, & Mello, 2002; Yigit et al., 2006). RDE-1 is a cleavage competent Argonaute protein, but this activity is dispensable for target silencing. Rather, its enzymatic activity is needed to remove one of the two siRNA strands following loading of an siRNA duplex (Steiner, Okihara, Hoogstrate, Sijen, & Ketting, 2009). How then does RDE-1 silence its targets? The NYN-domain nucleases, such as RDE-8, NYN-1 and NYN-2, are required for this (Tsai et al., 2015). Why target cleavage is handed over to another protein while RDE-1 itself could also cleave is unclear, but could be related to the fact that the targeted RNA needs to be marked as a template for an RdRP to generate the secondary 22G-RNAs, that are loaded into different WAGO proteins. Finally, we note that this pathway is an important weapon against exogenous pathogens, as it is required for virus control in *C. elegans* (Ashe et al., 2013; Sarkies, Ashe, Le Pen, McKie, & Miska, 2013). Developmental functions have not been reported thus far.

3.3 The PRG-1 pathway

Argonautes of the Piwi clade fulfill essential roles in the germline of many animals. PRG-1 is the main Piwi protein of *C. elegans*, and is associated with 21U-RNAs (Batista et al., 2008; Das et al., 2008; Wang & Reinke, 2008) (Fig. 1). This makes 21U-RNAs the piRNAs of *C. elegans*, but given their very different mode of biogenesis compared to piRNAs from *Drosophila* and mouse, we will use the name 21U-RNAs. In flies and mice, piRNAs are generated from large transcripts that can each be processed into a multitude of different piRNA molecules (Ozata et al., 2019). In contrast, the 21U-RNAs are generated from tens of thousands of miniature genes, that each produce a single 21U-RNA molecule (Ruby et al., 2006), which is processed from an approximately 28 nucleotide long precursor transcript (Gu et al., 2012). The factors involved in this process are currently being

identified from both forward and reverse genetic screens (de Albuquerque et al., 2014; Goh et al., 2014; Weick et al., 2014). First, transcription of these precursors requires a dedicated machinery. The many 21U-RNA producing loci cluster into foci within the nucleus (Weick et al., 2014), where a protein complex named USTC is involved in stimulating their transcription (Weng et al., 2019). This process of 21U-RNA transcription, and most notably its termination, bears resemblance to transcription of short nuclear RNAs (snRNAs) (Beltran et al., 2019), leading to the hypothesis that the dedicated machinery driving 21U precursor transcription evolved from already available mechanisms involved in transcription of snRNAs.

Interestingly, the downstream processing of 21U precursors also bears an evolutionary relationship to snRNA processing. Recently, a multi-subunit complex named PETISCO was identified as a potential platform for 21U precursor RNA processing, and one of its subunits was found to interact with snRNA processing factors named Gemins (Cordeiro Rodrigues et al., 2019). Interestingly, both USTC and PETISCO harbor subunits that appear to be specific to 21U-RNA biogenesis, as well as subunits with additional functions. Following 5′ precursor processing, which has not yet been mechanistically resolved, 21U-RNA intermediates are loaded into PRG-1 and further trimmed at their 3′ ends by the exonuclease PARN-1 (Tang, Tu, Lee, Weng, & Mello, 2016). Maturation is completed by 2′-*O*-methylation at the 3′ end by HENN-1 (Billi et al., 2012; Kamminga et al., 2012; Montgomery et al., 2012). This modification is known to stabilize small RNAs when they interact with their targets in a fully base-paired manner (Ameres et al., 2010).

Biochemically, PRG-1 can cleave its target transcripts, but like RDE-1, it does not seem to need this activity for regulation (Bagijn et al., 2012). In fact, many PRG-1 targets, including transposons but also endogenous genes, display a miRNA-type of binding, incompatible with target cleavage (Bagijn et al., 2012; Batista et al., 2008; Shen et al., 2018; Zhang et al., 2018). Like for RDE-1, this may relate to the need for PRG-1 to induce the production of secondary small RNAs (22G-RNAs) (Batista et al., 2008; Das et al., 2008). Different secondary Argonautes act downstream of PRG-1, resulting in potentially different regulatory outcomes following PRG-1 target recognition, ranging from mRNA de-stabilization to chromatin modification and transcriptional regulation.

Interestingly, PRG-1 mediated regulation may differ between the adult germline and the primordial germ cells (PGCs) in the embryo. First, maternally provided PRG-1, which functions in the PGCs, is required for

effective silencing in the adult germline (de Albuquerque, Placentino, & Ketting, 2015). In addition, embryonic and adult 21U-RNAs may differ in the way they interact with their targets: embryonic 21U-RNAs seem to base pair more extensively to their targets than adult 21U-RNAs. This statement is based on differential sensitivity of 21U-RNAs to loss of HENN-1 between embryonic and adult 21U-RNA populations (Svendsen et al., 2019). While this idea requires more work to be substantiated, such a developmentally dynamic output of an Argonaute protein would represent a novel aspect of regulation mediated by the Piwi class of Argonautes.

3.4 26G-RNA pathways

The 26G-RNAs represent another type of primary small RNA in *C. elegans*. They are not directly transcribed from the genome but rather made from an RNA template by the RdRP enzyme RRF-3 and further cleavage of the dsRNA intermediates by Dicer (Gent et al., 2010; Vasale et al., 2010; Welker et al., 2011). Both these proteins have been identified as part of a large complex known as ERIC, which appears to assemble in response to target RNA identification (Almeida et al., 2018; Thivierge et al., 2011). How targets for ERIC are specified is unclear, but it appears that defective splicing of a given transcript plays a role (Newman et al., 2018). Two types of 26G-RNA pools can be distinguished. One is bound to the primary Argonaute ERGO-1 (Gent et al., 2010; Han et al., 2009; Vasale et al., 2010), and a second pool is loaded into two paralogous Argonaute proteins, ALG-3 and ALG-4 (ALG-3/4 henceforth) (Fig. 1).

3.4.1 The ERGO-1 pathway

ERGO-1 is expressed in the female germline and in embryos. Its bound 26G-RNAs are methylated by HENN-1, and loss of HENN-1 destabilizes these 26G-RNAs both in the gonad as well as in the embryo (Billi et al., 2012; Kamminga et al., 2012; Montgomery et al., 2012), suggesting that ERGO-1 target recognition could be similar in both stages. ERGO-1 targeting triggers secondary 22G-RNA production (Gent et al., 2010; Zhang et al., 2011), which is channeled at least in part to the somatically-expressed, nuclear, secondary Argonaute, NRDE-3 (Gent et al., 2010). The molecular steps involved in this hand-over are practically unknown, but we do know that the ERGO-1 pathway draws on limited resources involved in 22G-RNA production, that are shared with *exo*-RNAi

(Duchaine et al., 2006; Gu et al., 2009; Lee, Hammell, & Ambros, 2006). Targets of ERGO-1 are often pseudogenes and recent gene duplications (Fischer et al., 2011; Gent et al., 2010; Han et al., 2009; Vasale et al., 2010), suggesting that this pathway may relay information on potentially problematic genes between germline and soma.

3.4.2 The ALG-3/4 pathway

The male germline expresses 26G-RNAs that bind to ALG-3/4 (Almeida, de Jesus Domingues, & Ketting, 2019; Conine et al., 2010, 2013). Even though they are clearly involved in establishing robust spermatogenesis-related gene expression, there is a dire lack of molecular details regarding ALG-3/4 function. In contrast to ERGO-1 bound 26G-RNAs, these are not methylated by HENN-1, and in many cases may not directly trigger 22G-RNA biogenesis. Conversely, it has been proposed that ALG-3/4 can positively regulate their targets transcriptionally via the 22G-RNA binding Argonaute CSR-1 (Conine et al., 2013). Positively regulated targets are mostly genes that are spermatogenesis specific. Other targets, however, experience repression via ALG-3/4; it is currently unclear how ALG-3/4 may generate such different output on different sets of target genes. Finally, this pathway is heavily affected by temperature (Conine et al., 2010, 2013).

In summary, the 26G-RNAs represent a versatile set of primary small RNAs, with many different, albeit still rather poorly defined biological functions. Next, we will focus on the secondary small RNAs of *C. elegans*, the 22G-RNAs.

3.5 22G-RNA pathways

Secondary 22G-RNAs are synthesized by RdRPs on RNA templates defined by primary (or secondary) Argonautes. This results in amplification of the silencing, as well as in a spreading of the 22G-RNA pool along the gene body, from 3′ to 5′ (Sijen et al., 2001). Currently, two 22G-RNA generating RdRPs are known: EGO-1 and RRF-1 (Maniar & Fire, 2011; Sijen et al., 2001), but for neither enzyme we know much about how they are activated, and how their products are processed and loaded into specific Argonaute proteins. EGO-1 and RRF-1 can to some extent act redundantly, but in normal conditions they cater different pathways. EGO-1 is involved in generating small RNAs loaded into the Argonaute CSR-1 (Fig. 1), while RRF-1 synthesizes small RNAs for Argonaute proteins that

are grouped in the so-called Mutator pathway. Generally, the Argonautes that load 22G-RNAs are part of a large branch of nematode-specific Argonautes: the WAGO-sub-family.

3.5.1 CSR-1 bound 22G-RNAs

These 22G-RNAs are made by the RdRP EGO-1, mostly from genes that are expressed in the germline (Claycomb et al., 2009; Maniar & Fire, 2011). How EGO-1 targets are selected is currently unknown. In the germline, CSR-1 appears not to silence its targets and several studies have identified CSR-1 as a "licensing factor": CSR-1 may identify genes as "self," and license their expression (Claycomb et al., 2009; Seth et al., 2013; Wedeles, Wu, & Claycomb, 2013). It has been proposed that this activity is needed to counteract PRG-1: given the ability of PRG-1 and 21U-RNAs to recognize targets with limited complementarity, they can potentially target many genes, including those that need to be expressed. Indeed, in the absence of CSR-1, PRG-1 binds to such targets more frequently (Shen et al., 2018). However, other studies appear to be inconsistent with this "licensing" model. First, CSR-1 is an active endonuclease that will cleave its target RNAs (Aoki, Moriguchi, Yoshioka, Okawa, & Tabara, 2007). Second, CSR-1 does have a repressive effect on certain targets and in fact, the chromosome segregation defect of *csr-1* mutant embryos is caused by mild overexpression of one such target (Gerson-Gurwitz et al., 2016). Third, a number of studies have shown that PRG-1 actually confers specificity to 22G-RNA activity, *preventing* erroneous gene silencing by 22G-RNAs (de Albuquerque et al., 2015; Phillips, Brown, Montgomery, Ruvkun, & Montgomery, 2015). Finally, CSR-1 is found in nematodes that have lost PRG-1 during evolution (Sarkies et al., 2015). A main function for CSR-1 in direct contrast to PRG-1 seems hard to reconcile with these findings. Currently, a satisfying model explaining these apparently contradictory observations has not been put forward. Part of the problem may be that we still lack clear mechanisms of how PRG-1, CSR-1 and other Argonautes and RdRPs, act at the molecular level. Another mostly ignored part of the puzzle, is the fact that CSR-1 and PRG-1 are both continuously expressed from the germline into early embryos. Different mechanisms, target repertoires, or both, may exist for PRG-1 and/or CSR-1 at different stages of development. If such differences indeed exist, our current views on the functionalities of these pathways are likely to be convoluted.

3.5.2 Mutator dependent 22G-RNAs

This class of 22G-RNAs depends on a group of proteins that was genetically identified as required for transposon silencing. Hence their name: Mutator proteins, or MUT, as increased transposition rates in these mutants result in the appearance of spontaneous mutant phenotypes. Driven by the intrinsically disordered protein MUT-16, the Mutator proteins assemble together in phase-separated condensates called Mutator foci, which also contain the RdRP RRF-1, but not EGO-1 (Phillips, Montgomery, Breen, & Ruvkun, 2012; Uebel et al., 2018). Interestingly, also the NYN-nuclease RDE-8 is found in Mutator foci, supporting the idea that this nuclease couples primary Argonaute activity to triggering RRF-1 function (Tsai et al., 2015). Detailed molecular functions for the MUT proteins have long remained unclear, but recently MUT-2/RDE-3 was shown to add polyUG tails onto the 3′ ends of mRNA fragments that undergo *exo*-RNAi, and that such polyUG tails are sufficient to trigger an exo-RNAi response by recruiting RRF-1 (Shukla et al., 2020). It is not yet clear whether polyUG tails play a role downstream of all primary Argonautes that activate RRF-1, but this seems an attractive hypothesis. 22-G RNAs made in Mutator foci are bound by many different Argonautes (Fig. 3B).

3.5.3 Initiation and maintenance of 22G-RNA populations

Primary Argonautes trigger de novo establishment of 22G-RNA populations against a diversity of target RNAs. However, maintenance of these 22G-RNA populations can, at least in some cases, become independent of the primary Argonaute. This is best established for PRG-1: a state of gene silencing that was initiated by PRG-1, but maintained in the absence of PRG-1 is referred to as RNAe (RNA induced epigenetic silencing), and can be inherited very stably across dozens of generations (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Also exogenously triggered RNAi can be similarly inherited (Gu et al., 2012). The 22G-RNAs associated with this persistent silencing have been dubbed tertiary 22G-RNAs (Sapetschnig et al., 2015), as they no longer depend on a primary Argonaute, but rather appear to be induced by secondary Argonautes. Two Argonautes required for inheritance of 22G-RNA-mediated silencing, seemingly involved in triggering and/or binding tertiary 22G-RNAs are WAGO-4 (Wan et al., 2018; Xu et al., 2018) and HRDE-1 (Ashe et al., 2012; Buckley et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Interestingly, HRDE-1 drives a nuclear RNAi response that leads to deposition of H3K9-trimethylation at targeted loci, and this has the potential to

also silence nearby genes as heterochromatin domains may expand or otherwise impact neighboring loci.

It seems likely that factors other than Argonaute proteins can trigger RdRP activity. For instance, no Argonaute has been found to activate EGO-1. Indeed, there are suggestions in the literature that other RNA binding proteins (RBPs) could be alternative triggers. For instance, components of the nonsense mediated decay pathway have been implicated in 22G-RNA generation (Gu et al., 2009). Also splicing may play a role: the intron-binding protein EMB-4/Aquarius is required for RNAi heritability and interacts with HRDE-1 (Akay et al., 2017; Tyc et al., 2017) and mutations in the splicing factor RNP-2/U1A affect targeting of transcripts by WAGO proteins (Newman et al., 2018). Curiously, histone mRNAs have recently been identified as preferred templates for RdRP activity, especially in absence of PRG-1 (Barucci et al., 2020). Given that the affected histone mRNAs are neither spliced nor poly-adenylated, this observation may again point at a role for splicing, but also for poly-adenylation in regulating potential Mutator activity (Barucci et al., 2020). Finally, RNA structure and RNA modifications may also affect which RNAs are used as RdRP templates in different contexts (Fischer & Ruvkun, 2020; Reich, Tyc, & Bass, 2018; Zhou et al., 2017; Zhu et al., 2018).

3.5.4 Specificity in 22G-RNA pathways

Different Argonaute proteins, and likely other factors, can activate Mutator foci activity, and an elegant model for RRF-1 activation has been proposed (Shukla et al., 2020). Yet, an important question remains unanswered: how can this singular activity generate different types of output? Clearly, PRG-1, RDE-1 and ERGO-1 dictate different pathways, yet they all require secondary 22G-RNA production in Mutator foci (Fig. 3B). Another way to phrase this question would be: How can the type of input that triggered Mutator activity be passed on to the correct downstream secondary Argonaute? While it is fair to state that basically we do not understand how this works, some reasonable working models can be put forward.

First, the recurrent theme of *primary Argonaute proteins not cleaving their targets* may be relevant to this question. If a primary Argonaute would simply cleave and release its target, it would be difficult to distinguish, for instance, a PRG-1-cleaved from an RDE-1-cleaved target. By using an additional nuclease (e.g. RDE-8), the primary Argonaute may remain associated with the target, steering downstream processes. Second, spatio-temporal separation between different pathways could play a role but has thus far not been

systematically addressed. To tackle this issue, a systematic analysis of Argonaute expression and localization will be required. Argonaute-interacting factors that regulate their loading and/or activity may also be differentially expressed and could provide specificity to the different small RNA pathways. Indeed, proteins that specifically regulate the loading of NRDE-3 during embryogenesis were recently identified (Lewis et al., 2020). Finally, distinct subcellular organization seems to play an important role in regulating *C. elegans* small RNA pathways. This rising topic is discussed next.

4. Subcellular organization of small RNA pathways

Small RNA pathways are intimately connected within membraneless organelles, which form by liquid-liquid phase separation (LLPS) (for reviews on LLPS, see Banani, Lee, Hyman, & Rosen, 2017; Shin & Brangwynne, 2017). It was recognized early on that miRNA-regulated mRNAs often associate with P-bodies (Liu et al., 2005; Parker & Sheth, 2007), even if silencing itself does not seem to depend on it (Eulalio, Behm-Ansmant, Schweizer, & Izaurralde, 2007). More recently, human Argonaute2 and its partner TNRC6 were shown to trigger LLPS and this accelerated deadenylation of targeted mRNAs (Sheu-Gruttadauria & MacRae, 2018). It seems possible that ALG-1/2 together with AIN-1/2 may trigger similar effects. Given that mRNAs themselves can also strongly affect LLPS (Lin, Protter, Rosen, & Parker, 2015; Zhang et al., 2015), it may help to select relevant targets for miRNAs. However, it remains an open question whether LLPS associated with miRNAs serves some specific function, or is just a consequence of the biophysical properties of the proteins involved.

Other small RNA pathways also segregate into LLPS domains. For instance, Mutator activity critically depends on the scaffolding protein MUT-16, which drives the formation of Mutator foci with LLPS characteristics (Phillips et al., 2012; Uebel et al., 2018). Furthermore, there is increasing evidence that distinct, but spatially closely connected LLPS domains may exist to allow sequestration of specific activities. For instance, P-granules are thought to shield mRNAs from Mutator activity, thereby preventing mistargeting of these mRNAs by 22G-RNAs (Dodson & Kennedy, 2019; Lee et al., 2020; Ouyang et al., 2019). A third type of LLPS domain are so-called Z-granules, named after the protein ZNFX-1 (Wan et al., 2018). The function of ZNFX-1 is not clear, but it interacts with RdRPs and affects 22G-RNA profiles, suggesting that it somehow affects Mutator activity (Ishidate

et al., 2018). So far, only one additional component of Z-granules has been described: the Argonaute WAGO-4, which is involved in RNAi inheritance (Wan et al., 2018; Xu et al., 2018). Finally, a fourth type of LLPS granule, SIMR-foci, has recently been described, and functionally placed between PRG-1 target recognition and 22G-RNA production (Manage et al., 2020). Precise molecular functions of these LLPS domains are still unclear, but a role for segregation of distinct activities into separate spatial compartments, such as the various 22G-RNA pathways, is an intriguing option.

In the next section, we focus on the roles that these pathways play during *C. elegans* development and adult physiology.

5. Developmental functions of small RNA pathways

5.1 Germline development and fertility

miRNAs are essential for the production of viable germ cells. The first observations supporting this came from ablation of the miRNA biogenesis machinery: loss of zygotic Drosha or its co-factor Pasha/DGCR8 results in completely sterile adults (Denli, Tops, Plasterk, Ketting, & Hannon, 2004; Grishok et al., 2001). Loss of Dicer has a similar effect (Ketting et al., 2001; Knight & Bass, 2001) but given its function in other small RNA pathways, this effect cannot be directly attributed to miRNAs. The sterility in the absence of miRNA function is most prominently caused by defects in ovulation, likely related to oocyte maturation (Rios, Warren, Olson, & Abbott, 2017). Interestingly, miRNAs are primarily required in the somatic gonad for this function, and do not seem to be essential in the germ cells themselves (Rios et al., 2017). Similarly, Dicer function is required in the somatic gonad for normal fertility (Drake et al., 2014); and ALG-1/2 activity is required in the distal tip cell (DTC) of the gonad to ensure normal levels of germ cell proliferation (Bukhari et al., 2012).

Even though multiple miRNAs are present in both the maternal and paternal germlines (Diag, Schilling, Klironomos, Ayoub, & Rajewsky, 2018; Minogue, Tackett, Atabakhsh, Tejada, & Arur, 2018; Stoeckius, Grun, & Rajewsky, 2014), a clear function in the germline has thus far only been uncovered for miR-35–41, which constrain the amount of germ cell apoptosis that is induced upon DNA damage (Doll, Soltanmohammadi, & Schumacher, 2019; Tran et al., 2019). This is mediated through the direct repression of a positive regulator of MAPK, *ndk-1*, and the trigger of apoptosis, *egl-1*, providing a thresholding activity that ensures that cell death

only occurs above a certain level of MAPK activity (Tran et al., 2019). MiRNAs are also necessary for spermatogenesis: miR-35–41 mutants have less spermatids and this seems to be due to activity of these miRNAs in the germline (McJunkin & Ambros, 2014). Moreover, the germline specific ALG-5 is necessary for the timing of the switch from spermatogenesis to oogenesis, although the mechanism behind this is unknown (Brown et al., 2017).

The 21U, 26G and 22G-RNA pathways display a clear bias for expression within germ cells, extending into early embryogenesis. Hence, it is not surprising that the phenotypes associated with changes in these pathways often relate to fertility and embryonic viability. Strangely enough, with the exception of CSR-1, none of the pathways, defined by individual Argonaute proteins, has proven to be strictly essential for fertility (Yigit et al., 2006). In part, this may be explained by redundancy between Argonaute proteins, but even a 12-fold knockout of these proteins did not result in acute sterility (Yigit et al., 2006). Consistent with this, Mutator mutants are also not sterile. However, at 25 °C sterility arises, at least partially due to defects in spermatogenesis (Ketting, Haverkamp, van Luenen, & Plasterk, 1999; Tabara et al., 1999), a phenotype they share with *alg-3/4* mutants (Conine et al., 2010, 2013). Recent work has begun to shed light on defects in the female germline in Mutator mutants: in animals lacking MUT-16, somatic genes are ectopically expressed within the germline, suggesting that germ cell identity becomes compromised, possibly due to defective chromatin organization caused by failing nuclear RNAi (Rogers & Phillips, 2020). Given that germline gene expression in *C. elegans* largely depends on post-transcriptional mechanisms (Merritt, Rasoloson, Ko, & Seydoux, 2008), it is possible that many "soma-specific" genes can be activated in the relaxed chromatin environment following heat stress. Why this effect is linked to heat stress is not clear, but it is interesting to note that heat-response elements in *C. elegans* are associated with transposable elements (Garrigues, Tsu, Daugherty, & Pasquinelli, 2019), which are among the main targets of the 22G-RNAs.

Loss of CSR-1 activity results in acute sterility (Maine et al., 2005; Vought, Ohmachi, Lee, & Maine, 2005; Yigit et al., 2006). A molecular phenotype observed in these mutants is the heterochromatinization of non-paired chromatin (She, Xu, Fedotov, Kelly, & Maine, 2009), such as the X-chromosome in males. Additionally, P-granule size and attachment to the nuclear periphery are affected (Andralojc et al., 2017; Updike & Strome, 2009). Another problem of *csr-1* mutant germlines is defective gene

regulation, with both mild germline gene repression (Claycomb et al., 2009), but also gain of gene expression (Campbell & Updike, 2015). It is currently unclear which of these phenotypes, if any, relates directly to CSR-1 activity, or whether they are secondary manifestations of another, yet unidentified problem.

5.2 Maternal effects on development

Both maternal and paternal miRNAs and other classes of small RNAs are contributed to the zygote (Diag et al., 2018; Minogue et al., 2018; Stoeckius et al., 2014). Loss of the miRNA biogenesis enzymes or ALG-1/2 from the germline causes embryonic lethality (Drake et al., 2014; Grishok et al., 2001; Rios et al., 2017), but it is currently unknown whether this is due to lack of maternal miRNAs or the requirement of these proteins for production and function of zygotically-expressed miRNAs. To date, the only maternal miRNAs known to play a role in the embryo belong to the miR-35–42 family, which is both maternally deposited and zygotically produced. Complete loss of the miR-35–42 family results in fully-penetrant embryonic lethality (Alvarez-Saavedra & Horvitz, 2010), but a partial loss-of-function (deletion of miR-35–41) causes reduced penetrance, temperature-sensitive embryonic lethality and a number of pleiotropic defects in fecundity and sex determination (Benner, Prothro, & McJunkin, 2019;McJunkin & Ambros, 2014 ; McJunkin & Ambros, 2017). Whereas either maternal or zygotically provided miR-35–41 are sufficient to produce viable embryos, both the maternal and the zygotic doses are necessary for normal fecundity and sex determination (McJunkin & Ambros, 2014; McJunkin & Ambros, 2017). It remains to be resolved however, whether the maternal pool is necessary to provide miRNA function during early embryogenesis, before zygotic transcription begins, or if it is necessary to produce the full dosage of miRNA necessary at later stages of embryogenesis.

Many primary and secondary Argonautes are strongly expressed in the female germline, and still clearly detectable in embryos (Batista et al., 2008; Buckley et al., 2012; Claycomb et al., 2009; Das et al., 2008; Gu et al., 2009; Wan et al., 2018). These are also relevant for the embryo, as maternal effects of 22G-RNAs and 21U-RNAs on germline development have been well documented. For instance, embryos from *csr-1* mutant mothers display chromosome segregation defects (Claycomb et al., 2009). This embryonic effect appears to find its cause in CSR-1 repressing at least one specific target (Gerson-Gurwitz et al., 2016). CSR-1 also appears to affect the onset of embryonic transcription (Fassnacht et al., 2018), although

direct targets for this process have not been identified. Other maternal effects relate to the maintenance of proper 22G-RNA pools: maternal, but also paternal small RNAs are needed to keep Mutator activity focused on the correct target transcripts (de Albuquerque et al., 2015; Phillips et al., 2015) (Fig. 4). Additionally, there is an intriguing flow of information from

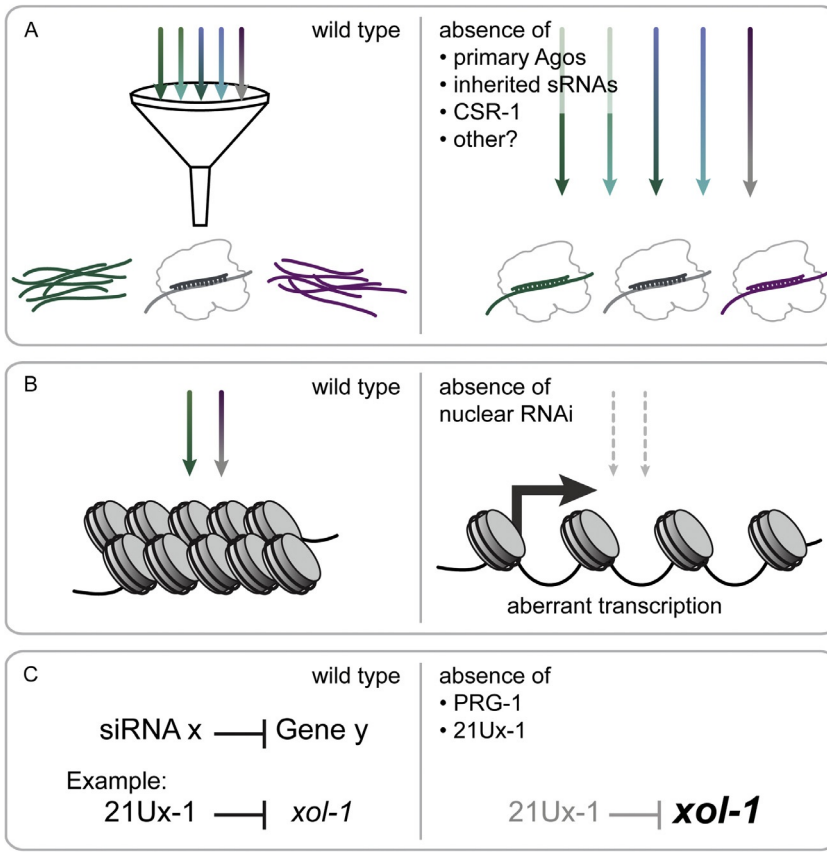

Fig. 4 Molecular consequences of small RNA pathway mutations. (A) Multiple factors provide specificity to the Mutator foci-related amplification, preventing erroneous silencing of "self" transcripts. A commonly observed consequence of mutations in small RNA pathway components is a loss of this specificity leading to aberrant silencing. (B) Loss of nuclear RNAi pathways (e.g. HRDE-1 and NRDE-3) leads to loss of repressive chromatin on target loci. Such chromatin rearrangements can affect neighboring loci as well and potentially more distant loci as well due to redistribution of chromatin modifiers. (C) 21U-RNAs typically function as part of a genomic immune system to silence "non-self" sequences. However, one 21U-RNA gene, 21Ux-1, has been selected as a specific repressor of a key player in the sex determination pathway, *xol-1*. In this case, 21Ux-1 acts conceptually similarly to a miRNA and may provide a glimpse of how these small RNA-guided pathways evolve to acquire endogenous functions.

the germline into the soma via ERGO-1 and NRDE-3 (Guang et al., 2010), although no developmental defects associated with loss of NRDE-3 function on the soma have been observed thus far. It is important to note that components of small RNA pathways may have additional functions in other processes that also affect early embryonic development. For instance, mutants in most subunits of the recently identified PETISCO complex, needed for 21U-RNA biogenesis, display a Mel (maternal effect lethal) phenotype, which is not related to 21U-RNA function but to additional functions of this complex (Cordeiro Rodrigues et al., 2019; Zeng et al., 2019). Clearly, a lot of work is still required before we can dissect out the direct links between these small RNA pathways and early embryonic development.

5.3 Transgenerational developmental effects

The importance of inherited 22G-RNAs becomes clearly visible when primary Argonaute input is removed. In such a scenario, inherited 22G-RNAs are crucial to maintain/perpetuate specificity of the small RNA pathways and prevent the biogenesis of toxic, off-target 22G-RNAs that render the animal directly fully sterile (de Albuquerque et al., 2015; Phillips et al., 2015). A recurring phenotype related to different small RNA pathways that likely relates to this effect is the so-called mortal germline (Mrt) phenotype (Buckley et al., 2012; Simon et al., 2014; Wan et al., 2018), in which sterility develops over multiple generations (Ahmed & Hodgkin, 2000). At least for the PRG-1 pathway, this sterility is not connected to major genomic damage. Rather, it seems to be an epigenetic issue: *prg-1* mutants may slowly, over the course of generations, loose proper control of many loci, such as but not limited to transposons. At the same time, such mutants may gain silencing at loci that should normally be expressed. One such deregulation event that contributes to the Mrt phenotype is the progressive, aberrant silencing of histone mRNAs by 22G-RNAs in the absence of PRG-1 (Barucci et al., 2020). Interestingly, the Mrt phenotype can be reset to a large extent by starvation (Simon et al., 2014), suggesting an intriguing link between nutrition signaling and small RNA pathways. Indeed, later studies showed that the sterility observed in the Mrt phenotype is related to diapause: a developmentally arrested state triggered by starvation (Heestand, Simon, Frenk, Titov, & Ahmed, 2018). Heritable effects of 22G-RNA

populations have also been described to relay environmental information such as heat stress, starvation or chemotaxis, and may travel from somatic to germline tissues (Devanapally, Ravikumar, & Jose, 2015; Ni et al., 2016; Posner et al., 2019; Rechavi et al., 2014). Possibly due to mechanisms that limit inheritance (Houri-Ze'evi et al., 2016) these effects only last 2–3 generations, much shorter than the PRG-1 initiated RNAe effect described earlier. To fully understand these effects, it will be essential to clarify how inheritance exactly takes place at the molecular level, and how it is interpreted in the embryo (also see chapter "Can brain activity transmit transgenerationally?" by Rechavi of this issue).

5.4 Sex determination

As mentioned above, the miR-35 family has also been shown to play a role in sex determination. In the absence of miR-35–41, XX embryos aberrantly activate part of the male gene expression program, and when combined with a sensitized background this causes penetrant masculinization of XX animals (Benner et al., 2019; McJunkin & Ambros, 2017). The function of miR-35–41 is embedded within the well-established sex determination pathway through the repression of at least two RNA binding proteins, ultimately affecting the pathway both upstream and downstream of the gene that drives male sexual fate, *her-1* (Benner et al., 2019; McJunkin & Ambros, 2017).

Generally speaking, piRNAs (and 21U-RNAs in *C. elegans*) have a clear role in transposon control and tend not to have evolutionary conserved functions in the regulation of specific genes. However, one specific 21U-RNA (21Ux-1) has been identified as a repressor of a key sex-determining gene, *xol-1* (Fig. 4). Hermaphrodites lacking 21Ux-1 are thus sensitized to defects on both dosage compensation and sex determination (Tang et al., 2018). This link between PRG-1 and *xol-1* is also found in *C. briggsae*, and piRNAs also play a role on sex determination in silk-moths (Kiuchi et al., 2014). These findings support a role for piRNAs in gene regulation in the germline beyond transposon control. It is yet unclear how general such specific functions of individual 21U-RNAs in *C. elegans* are. Possibly, many such examples await discovery, but it is also possible that the case of 21Ux-1 is a relatively rare example of a small RNA evolving toward a miRNA-like function in a developmental context.

5.5 Embryogenesis: Morphogenesis and cell specification

miRNAs play a number of roles during embryonic development, yet their contribution is still not fully defined due to challenges in completely removing miRNAs from embryos: zygotic mutants in Drosha, Pahsa or DGCR8 can be obtained from heterozygous mothers, but these embryos develop normally due to the maternal contribution of miRNAs or biogenesis factors (either in protein or mRNA form) (Denli et al., 2004; Grishok et al., 2001). Maternally rescued animals grow to adulthood but are sterile. Loss of zygotic ALG-1/2 results in morphogenesis defects and fully-penetrant embryonic lethality (Vasquez-Rifo et al., 2012). However, even in this case, maternal contribution of ALG-1/2 cannot be excluded and thus whether miRNAs also play earlier roles in embryogenesis remains to be explored.

Additional insight has come from studying loss-of-function mutants for individual miRNAs or miRNA families (Alvarez-Saavedra & Horvitz, 2010; Miska et al., 2007). These systematic mutant analyses revealed two miRNA families with functions during embryogenesis: the miR-35–42 and miR-51–56 families (Alvarez-Saavedra & Horvitz, 2010; Shaw et al., 2010). Both of these families are maternally contributed as well as broadly transcribed in the embryo. For the miR-35–42 family, both maternal and zygotic contributions must be eliminated to uncover the fully-penetrant embryonic lethality at the onset of morphogenesis (Alvarez-Saavedra & Horvitz, 2010), a defect that looks relatively similar to that caused by loss of ALG-1/2 (Vasquez-Rifo et al., 2012). For the miR-51–56 family, the maternal contribution is not sufficient and zygotic mutants obtained from heterozygous mothers display defects at later stages of morphogenesis (Alvarez-Saavedra & Horvitz, 2010; Shaw et al., 2010). The molecular bases for these defects in morphogenesis and failure to complete embryogenesis are still unknown. In addition to the functions in the germline described above, the miR-35–42 family controls apoptosis in the embryo through repression of *egl-1*, in conjunction with the miR-58 family (Sherrard et al., 2017). Derepression of *egl-1* in the absence of these miRNAs results in the premature death of the mother cells of some of the cells normally destined to die. By itself, this function does not explain the fully-penetrant embryonic lethality of miR-35–42 mutants, but it provides a piece of the puzzle to understand the contribution of this pleiotropic miRNA family to development. Similarly, the function of the miR-51–56 family remains mostly unexplained. One of the defects observed in animals lacking these miRNAs is an unattached pharynx; this is at least in part related to repression

of the Fat cadherin CDH-3 in the arcade cells, but this does not explain the broader morphogenesis defects (Shaw et al., 2010).

In contrast to these abundant and broadly expressed miRNA families, which are essential for embryogenesis, most miRNAs in *C. elegans* are individually dispensable to generate viable embryos (at least under laboratory conditions) (Miska et al., 2007) and seem to be expressed with varying degrees of cell-type specificity. Many miRNAs may thus play roles in the development or function of differentiated cells (Alberti & Cochella, 2017). *C. elegans* provides an advantage for the study of such cell-specific functions as miRNA expression can be determined with single-cell resolution and molecular and functional assays can be designed to assess the contribution of any gene to specific cells, within the context of the whole animal. A comprehensive list of miRNAs studied in *C. elegans*, their functions and targets has recently been compiled (Ambros & Ruvkun, 2018). Here we focus on a few themes that have emerged from the study of a still limited number of miRNAs that act in embryogenesis:

1. *Broadly expressed miRNAs may act as timers and/or thresholders*: It is intriguing to think what the function of a broadly expressed repressor during embryogenesis might be. The miR-35 family is practically ubiquitously expressed during embryogenesis, but then rapidly decays around the time of hatching (Stoeckius et al., 2009; Wu et al., 2010). Such temporal specificity may point to a function as a timer, preventing the premature occurrence of events that should happen at the end of embryogenesis. A non-exclusive possibility is that broadly expressed repressors could act as thresholders, generating binary patterns (in space and/or time) out of graded target expression. It has been proposed that the miR-35 family could act to prevent the premature execution of the sex determination pathway (McJunkin & Ambros, 2017). Given that the levels of the miR-35 family only go down at the end of embryogenesis and sex determination happens relatively early, a timer-like function of miR-35 would require the temporal thresholding of one or more targets. The function of the miR-35 family as a repressor of *egl-1* in the germline and in the soma (together with miR-58) also seems to fit the thresholder model (Sherrard et al., 2017; Tran et al., 2019) (Fig. 5).
2. *Cell-specific miRNAs can act in different manners*: Spatial specificity of expression enables additional functions for genetic repressors such as miRNAs. Two such functions (more extensively discussed in Alberti & Cochella, 2017) are exemplified by: (i) *lsy-6*, which acts as a genetic switch through the repression of a transcription factor in the left ASE neuron,

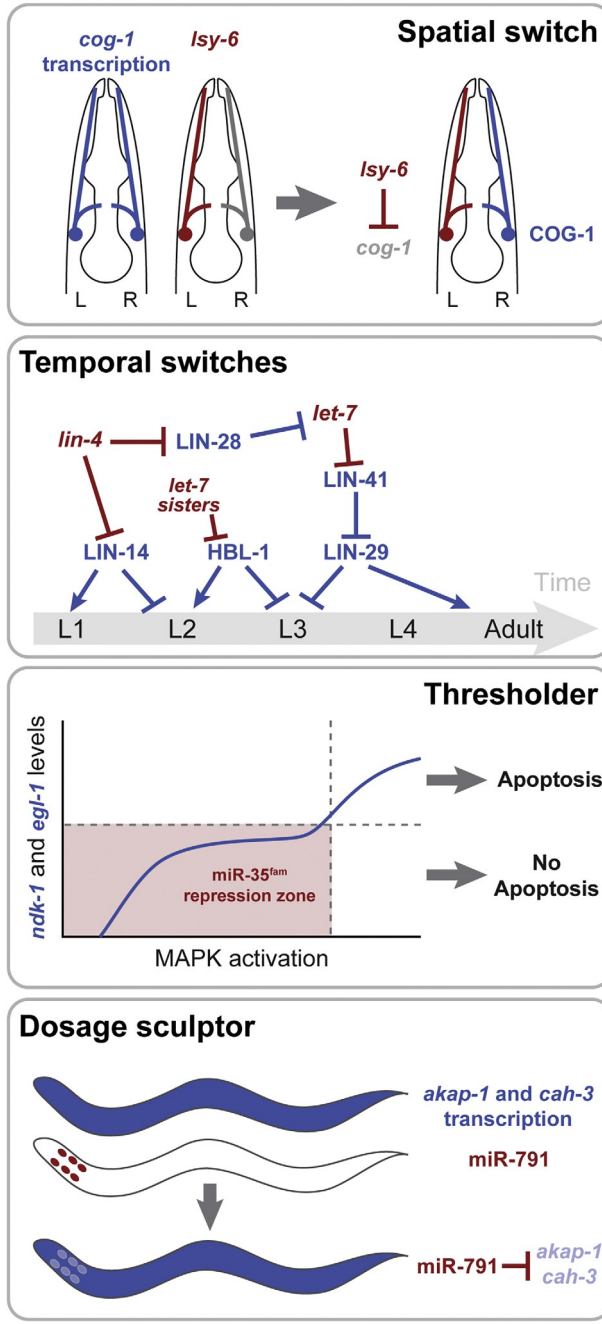

Fig. 5 See legend on next page.

introducing a left/right neuronal asymmetry (Johnston & Hobert, 2003) and (ii) miR-791, that acts in the CO_2-sensing neurons by repressing two house-keeping genes, providing a means to carve out specificity out of otherwise ubiquitously-transcribed genes (Drexel et al., 2016) (Fig. 5).

3. *Multiple miRNAs affect neuronal development and physiology*: While this might still be biased due to the small number of different miRNAs that have been studied in depth, the best characterized cell-specific miRNAs to date affect neuronal differentiation, migration and physiology: *lsy-6* (Johnston & Hobert, 2003), miR-79 (Pedersen et al., 2013), miR-2 (O'Hern et al., 2017), miR-791 (Drexel et al., 2016), miR-71 (Finger et al., 2019; Hsieh, Chang, & Chuang, 2012). This is consistent with the observation that miRNAs are enriched in the mammalian brain (Cao, Yeo, Muotri, Kuwabara, & Gage, 2006). Nevertheless, other cell-specific miRNAs have also been implicated in the development and function of other tissues and organs such as the intestine (Kemp et al., 2012), muscle (Nehammer et al., 2019; Simon et al., 2008), and hypodermis (discussed below in the context of larval development).

Fig. 5 Consequences of miRNA-mediated repression. Examples of non-mutually exclusive roles for miRNAs in development. The different functions are determined by the specificity of expression of the miRNA, the magnitude of repression on its target/s and the way the targets are embedded within a Gene Regulatory Network (GRN). MiRNAs are shown in red and their targets in blue. *Spatial switch*: a spatially restricted miRNA (*lsy-6*) fully represses a more broadly expressed transcription factor (COG-1) in the left ASE neuron. COG-1 is high in the GRN hierarchy of these neurons (Fig. 2) and its repression causes a switch in cell identity, introducing an asymmetry between the left and right ASE neurons. *Temporal switches*: the heterochronic miRNAs provide the best example for this. A series of repressive interactions determines the timing of expression of key transcription factors (LIN-14, HBL-1, LIN-29) across multiple tissues, to coordinate developmental events throughout the animal. *Thresholder*: the repressive action of miRNAs can buffer target expression within a certain concentration range effectively establishing a binary output depending on whether target concentration is kept below, or crosses a given threshold. This is the proposed mode of action for the miR-35 family as a repressor of apoptosis in the germline (where it regulates *ndk-1* and *egl-1* levels) and in the embryo (where it acts through *egl-1* repression). *Dosage sculptor*: ubiquitously expressed genes, which typically carry out house-keeping functions, were thought to escape miRNA-mediated repression as this would likely be detrimental. However, miR-791 is specific to the CO_2-sensing neurons and represses to otherwise ubiquitous genes in these cells, modulating the physiological properties of these neurons and having an impact on the animal's behavior.

5.6 Larval development

Post-embryonic development in *C. elegans* is a dynamic process with critical decision points (e.g. to enter the dauer stage or not) and requiring coordinated changes across multiple body parts. Specifically, during larval development the animal undergoes controlled growth and remodeling of different structures, coordinating changes across the soma and germline to reach sexual maturity and acquire adult physiology and behaviors. A number of miRNAs are involved in these processes. Given the history of the miRNA field a large part of the focus on miRNA functions in larval development has been on *lin-4*, *let-7* and the *let-7 sisters*, uncovered for their roles in establishing the timing and coordination of larval events across multiple tissues (Abbott et al., 2005; Ambros, 1989; Reinhart et al., 2000). The basis for control of temporal progression is an intricate network of predominantly repressive interactions that determines the temporal specificity of different transcription factors necessary for the different larval stages (Fig. 5). We refer the interested reader to other reviews that cover these networks more extensively (Faunes & Larrain, 2016; Rougvie & Moss, 2013). The heterochronic pathway has been instrumental to uncover most of what we know about miRNAs in *C. elegans* and other animals, from the initial discovery of *lin-4* and *let-7* and beyond (reviewed in Ambros & Ruvkun, 2018). Given the elaborate interplay of transcriptional and post-transcriptional regulation (by miRNAs but also RNA binding proteins) in this pathway, it will likely continue to provide fertile ground for the discovery of exciting biology.

The developmental timing effects of *lin-4* and *let-7* have been predominantly studied in the context of hypodermis, seam cell and vulva development, where their main targets are *lin-14* and *lin-41*, respectively (Ambros & Ruvkun, 2018). However, these miRNAs are broadly expressed, and play roles in the context of other tissues and organs. In the nervous system, *lin-4* contributes to the timing of events required for maturation of neuronal circuits during the L1 stage, such as axon growth and synaptogenesis (Zou, Chiu, Domenger, Chuang, & Chang, 2012) and motorneuron rewiring along the body (Hallam & Jin, 1998; Howell, White, & Hobert, 2015). In both cases, *lin-4* acts by repressing *lin-14*, as it does in the hypodermis and seam cells. *Let-7* also times neuronal events that are important for the transition to adulthood, such as the changes in neuronal properties and circuit rewiring necessary for sexual maturation (Pereira et al., 2019), and impacts on the synaptic polarity of the DA motorneurons (Armakola & Ruvkun, 2019). The decline in axonal regeneration potential observed in

later larval stages is also determined by *let-7* and other heterochronic genes (Zou et al., 2013). In these cases, *let-7* also acts through the repression of *lin-41* and is embedded in the same gene regulatory network across different tissues, ultimately resulting in increased activity of the transcription factor LIN-29 (Aeschimann et al., 2017; Azzi, Aeschimann, Neagu, & Grosshans, 2020). This is consistent with the deep conservation of these regulatory modules, which have been implicated in developmental transitions, including sexual maturation, in diverse animals (Faunes & Larrain, 2016). It remains possible that additional targets may have been co-opted for certain *let-7*-mediated functions, e.g. it has been proposed that *ced-7* is a target in the context of axon regeneration by self-fusion (Basu et al., 2017).

Other miRNAs have also been implicated in larval development. Specifically, miR-34 and miR-83 act together in providing robustness to the migration of the distal tip cells, in particular under stress caused by temperature oscillations (Burke, Hammell, & Ambros, 2015). It has been proposed that these miRNAs act at least in part by inhibiting noisy gene expression caused by temperature fluctuations (Burke et al., 2015). miR-34 has also been shown to contribute to resistance to diverse stressors (Isik, Blackwell, & Berezikov, 2016). It has been more generally argued that miRNA-mediated repression can help buffer influence from the environment on gene expression (Ebert & Sharp, 2012). Another miRNA whose function becomes evident under conditions of environmental hardship, although with a conceptually different mechanism, is miR-235. Normally, a number of blast cells divide, migrate and differentiate to initiate post-embryonic development in the L1 stage. These events, however, need to be suspended upon starvation as animals enter the L1 diapause. Instead, in starved L1s that lack miR-235, blast cells fail to enter quiescence and some animals even molt (Kasuga, Fukuyama, Kitazawa, Kontani, & Katada, 2013). The function of miR-235 is mediated, at least in part, by repression of the transcription factor *nhr-91*.

5.7 Adult physiology and lifespan/aging

miRNAs play various roles during adult life, with most studied functions being related to sensing of the environment and responses to different stressors (Bezler et al., 2019; Dai, Gao, Zou, Ma, & Zhang, 2015; Jiang et al., 2018; Nehammer et al., 2019; O'Hern et al., 2017). The connection to stress is not unique to *C. elegans* and might reflect for example the advantage of post-transcriptional regulation to elicit fast responses upon different

stimuli, or a common need to buffer noisy gene expression caused by different stressors (Emde & Hornstein, 2014).

The ability to cope with stress is intimately linked to lifespan and healthy aging (Kenyon, 2010), and the presence of miRNAs has been implicated in these processes (Aalto et al., 2018; Finger et al., 2019; Kogure, Uno, Ikeda, & Nishida, 2017; Lehrbach et al., 2012). miRNAs can contribute to lifespan both positively and negatively, depending on the miRNA and the processes each regulates (Boehm & Slack, 2005; Boulias & Horvitz, 2012; de Lencastre et al., 2010; Finger et al., 2019; Kato, Kashem, & Cheng, 2016; Shen, Wollam, Magner, Karalay, & Antebi, 2012; Vora et al., 2013). This dichotomy is represented also at the level of the two miRNA-dedicated Argonautes ALG-1 and ALG-2: in adults, these two proteins are differentially expressed and regulate distinct sets of genes, resulting in the surprising observation that ALG-1 promotes longevity while ALG-2 restricts it (Aalto et al., 2018). Ultimately, the effects of different miRNAs on lifespan will depend on how they are embedded in the gene regulatory networks that impact on the aging process, most prominently the insulin/IGF- and the mTOR-signaling pathways (reviewed in Dall & Faergeman, 2019; Kinser & Pincus, 2020).

One of the miRNAs studied in greatest depth in the context of aging and stress is miR-71: loss of miR-71 causes a significant decrease in lifespan, while its overexpression can modestly extend it (Boulias & Horvitz, 2012; de Lencastre et al., 2010). The decrease in lifespan in the absence of miR-71 is accompanied by increased sensitivity to heat and oxidative stress (Boulias & Horvitz, 2012; de Lencastre et al., 2010) as well as proteotoxic stress (Finger et al., 2019). Interestingly, while miR-71 is broadly expressed, it is required in the AWC sensory neurons for correct neuronal activity (Finger et al., 2019; Hsieh et al., 2012), which has a non-autonomous impact on proteostasis and DAF-16 activity in the intestine and ultimately the animal's lifespan (Boulias & Horvitz, 2012; Finger et al., 2019).

PRG-1 and associated proteins appear to be very specifically expressed in the gonad of *C. elegans*. Yet, in other animals, most notably in some arthropods, somatic piRNA activity has been convincingly demonstrated (Lewis et al., 2018). This raises the question whether somatic functions of PRG-1 may exist in *C. elegans*, even if driven by thus far undetectable expression levels of the involved proteins in the soma. Intriguingly, PRG-1 has been linked to a repressive effect on axon regeneration (Kim et al., 2018), and NRDE-3 (acting downstream of the germline Argonaute ERGO-1) has

been implicated in neuronal adaptation to odor in adults (Juang et al., 2013). Given the described transgenerational effects of these small RNA pathways, and impact of germline functions on the soma, it is possible that such effects still find their origin in germ cells. More work on this front is required to clarify how precisely 21U- and other assumed-germline-small RNAs can affect adult physiology.

6. Closing remarks

Small RNA pathways have a big impact on different aspects of development. However, mechanistic explanations for many of the observed effects are still lacking. Efforts in two broad areas should help close this knowledge gap: (i) better assignment of biologically relevant targets and the impact of small RNA-mediated regulation on these and (ii) deeper understanding of the molecular mechanisms of the different pathways, their variations over developmental time and the cross-talk with other branches of RNA metabolism. Related to the latter, while small RNAs provide the target specificity, it is the Argonautes and their specific partners that determine the mechanism of action. As discussed for ALG-1 and PRG-1, their mode of action differs in the germline versus the embryo. Given the diversity of Argonautes within *C. elegans*, it seems plausible that additional variations in the activity of individual Argonautes exist, either across different cell types or different signaling contexts. The contribution of the small RNA pathways must also be considered within the tightly intertwined gene regulatory program of a cell, and keeping in mind that proteins acting in small RNA pathways may also affect other processes in RNA metabolism. Only such a comprehensive understanding of small RNA function within different contexts will unveil a deconvolved view of the roles of small RNA in development.

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