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# RNAi AND miRNA PATHWAYS IN ANNELIDS AND MOLLUSCS

**Keywords:** dsRNA, PKR, Dicer, TARBP2, PACT

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## ABSTRACT

RNA silencing denotes sequence-specific repression mediated by small RNAs. In metazoa, there are two mechanistically closely related pathways: RNA interference (RNAi) and microRNA (miRNA) pathway. The miRNA pathway regulates endogenous protein-coding gene expression and has been implicated in many biological processes. RNAi generally serves as a form of innate immunity targeting viruses and mobile elements. This review is summarizing information about RNAi and miRNA pathways in protostome phyla: *Annelida* and *Mollusca*. The molecular mechanisms of dsRNA and miRNA pathways in annelids remain largely unexplored. The available information points towards coexistence of miRNA and RNAi pathways, however, their integration or genetic separation remain unclear. Molluscs are an interesting taxon, which appears to have a unique setup of RNA silencing possibly employing a single Argonaute protein while it also employs interferon-like pathway elements.

## Introduction

The mechanistical principles of vertebrate miRNA and RNAi pathways were introduced in the first review of this series (Svoboda, 2019) and in further detail elsewhere (Bartel, 2018), I will focus here directly on features of these pathways reported from Annelid and Molluscs taxons, which do not have common model species, which would be investigated in depth like *C. elegans* or *Drosophila*. The review is divided into Annelid and Molluscs parts. In each of them, I'll review published data concerning components of miRNA and RNAi pathways and discuss biological roles of the two pathways.

## Annelids

Annelids are coelomate protostome worms whose body is organized into a series of ring-shaped repetitive segments. ~15 000 annelid species are grouped into three classes: Polychaeta (bristle worms, e.g. *Platynereis*), Oligochaeta (earthworms, e.g. *Lumbricus*), and *Hirudinea* (leeches, e.g. *Hirudo*). Literature on RNA silencing and dsRNA pathways is extremely limited. There were under twenty publications dealing with small RNAs, most of which was related to detection of miRNAs.

### **Dicer**

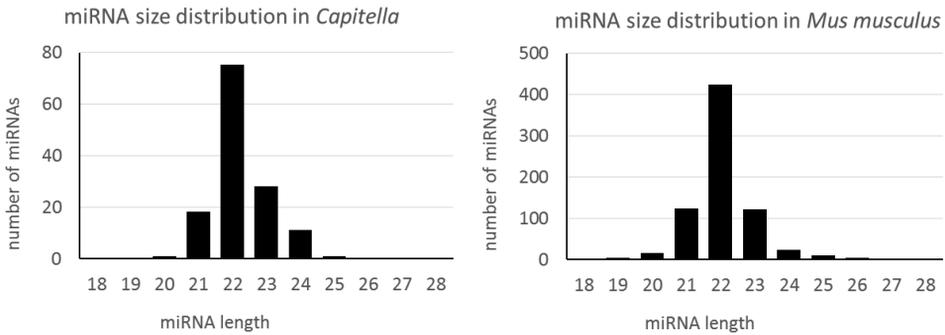
There was no publication concerning Dicer structure or function in annelids, thus all information provided here had to be extracted from genomic databases. Blast search of annelid entries in Genbank (query: murine Dicer protein, algorithm: tblastn, database: Nucleotide collection nr/nt, results restricted to *Annelida* (taxid:6340)) identified a single mRNA XM\_009031272 from the leech *Helobdella robusta* encoding a 1316 aa Dicer protein which lacked ~ 400 aminoacids at the N-terminus. The protein contained HELICc domain at the N-terminus but lacked the N-terminal DExD domain. Thus, this Dicer is structurally reminiscent of the N-terminally truncated Dicer capable of producing endo-siRNAs (Flemr et al., 2013). Importantly, analysis of *Helobdella robusta* genomic sequence identified a single Dicer gene on a contig ref|NW\_008705401.1|, which also carried the entire N-terminus, which was lacking in the identified mRNA. This would imply a similar scenario as observed in mouse oocytes – Dicer encodes two protein isoforms, where the longer one is adapted for the production of miRNAs, while the shorter can produce both, miRNAs and siRNAs. However, this information would need to be validated experimentally and it needs to be also tested how common would be this scenario for annelids in general. Finally, annelid Dicer also produces miRNAs with median length of 22 nucleotides as estimated from the miRBase data (Kozomara and Griffiths-Jones, 2014) (Fig. 1).

### **dsRBPs**

There is no literature concerning dsRBP proteins participating in RNA silencing, Rosani et al. suggest that annelids employ a single TARBP2 homolog (Rosani et al., 2016).

### **Argonaute proteins**

Likewise, there is essentially no information regarding the AGO subfamily of Argonaute proteins. There is a study of protein components in molluscs, which included two annelid species and found that annelids have three (*Capitella*) and four (*Helobdella*) Argonaute proteins from both, PIWI and AGO clades (Rosani et al., 2016). There are five papers concerning Argonaute, however of the PIWI clade, which is functioning in the germline (Giani et al., 2011; Kozin and Kostyuchenko, 2015; Ozpolat and Bely, 2015; Sugio et al., 2008; Weigert et al., 2013). Blast search of *Helobdella* genome identified two AGO paralogs (XM\_009021176.1 and XM\_009031816.1) and two possible PIWI proteins



**Figure 1** Annelid miRNA lengths

The left graph depicts size distribution of all 134 miRNAs of a polychaete worm *Capitella teleta* deposited in the miRBase (version 21). For comparison, the right graph shows size distribution of 721 high-confidence murine miRNAs.

(XM\_009015681.1 and XM\_009021522.1). The two paralogs of PIWI proteins would be consistent with the aforementioned analysis of *Myzostoma cirriferum* PIWI proteins (Weigert et al., 2013). Whether the two AGO paralogs are functionally dedicated to RNAi and miRNA pathways like AGO proteins in *Drosophila* is unknown. Furthermore, if annelids would have two PIWI proteins, it is possible that *Capitella* might have only one AGO protein serving in RNAi and miRNA pathways.

### **Other factors**

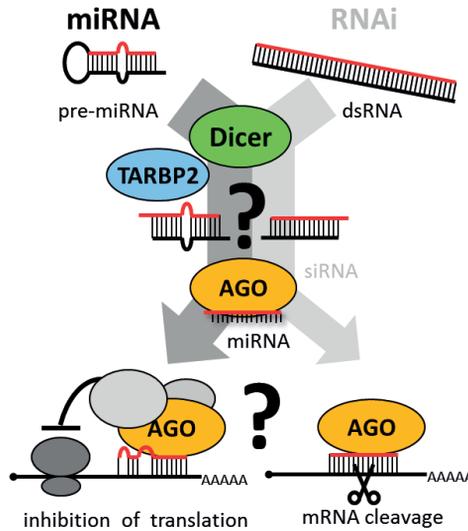
No other proteins factors from miRNA or RNAi pathway have been specifically reported. Data from Rosani et al. show that annelids employ microprocessor complex (Rosani et al., 2016)

### **miRNA**

There is one annelid species, which has annotated miRNAs in the miRBase: *Capitella teleta* -129 precursor miRNAs and 134 mature miRNAs. Literature search revealed seven publications reporting annelid miRNA identification and or expression (Christodoulou et al., 2010; Gong et al., 2010; Helm et al., 2012; Huang et al., 2012; Kenny et al., 2015; Sperling et al., 2009; Tessmar-Raible et al., 2007). As shown above, annelid miRNAs have an average length 22 nucleotides like other animal miRNAs.

### **RNAi**

The only information available concerning RNAi is that it is functional (Takeo et al., 2010; Yoshida-Noro and Tochikai, 2010). The only one published experimental paper reporting RNAi employed long dsRNA that was injected into the coelom (1  $\mu\text{g}/\mu\text{l}$ , 100 nl per worm, i.e. 100 ng of dsRNA/worm).



**Figure 2** Overview of annelid pathways

dsRNA and miRNA pathways in annelids are poorly understood. Among the missing pieces of information are: 1) degree of separation of miRNA and RNAi pathways (dedicated AGO proteins, co-existence of Dicer isoforms). Proteins from other dsRNA pathways related to innate immunity include OAS, most likely RIG-I and MDA5 homologs but probably not PKR.

### **Other dsRNA responding pathways**

Only one reference mentioned another protein involved in dsRNA response – OAS (Kjaer et al., 2009). Blast search for murine PKR, RIG-I, and MDA5 (algorithm: tblastn, database: Nucleotide collection nr/nt, results restricted to *Annelida* (taxid:6340)) revealed many sequences of *Helobdella*, and six of *Platynereis*, which were similar only to the second half of PKR, suggesting they were not orthologs. RIG-I and MDA5 searches identified two hypothetical proteins in *Helobdella* (ref|XM\_009026626.1| and XM\_009014668.1) with ~35% identity and ~50% similarity, which could be orthologs.

### **Summary**

In terms of the molecular mechanism of dsRNA and miRNA pathways, annelids remain largely unexplored. While, the available information points towards coexistence of miRNA and RNAi pathways, their integration or genetic separation remain unclear.

### **Molluscs**

Molluscs are a large and extremely diverse group of coelomate protostomes, which have an unsegmented soft body, internal or external shell, and a muscular foot. There are ~ 50 000

described species, which makes molluscs the second largest phylum after *Arthropoda* (third if *Chelicerata* and *Hexapoda* would be considered separate phyla). Apart from the complex classification of molluscs into 7–10 classes, three groups of molluscs are commonly recognized: *Cephalopoda* (squid, octopus), *Gastropoda* (snails and slugs), and *Bivalvia* (clams, mussels, scallops, oysters). Literature on RNA silencing and dsRNA pathways is limited. There were 92 publications dealing with small RNAs, most of which was related to use of RNAi as an experimental tool for suppressing gene expression.

### **Dicer**

There was no specific functional analysis of Dicer in molluscs. There is one study from 2016, which identified and bioinformatically analyzed Dicer and other components of miRNA and RNAi pathways in marine bivalves with a focus on a mussel *Mytillus galloprovincialis* and oyster *Cassostrea gigas* (Rosani et al., 2016). Their results show that all examined molluscs (>30 species of cephalopods, gastropods, and bivalves) have a single Dicer protein, which participates in both, RNAi and miRNA pathways and that Dicer of *Mytillus galloprovincialis* and *Cassostrea gigas* has a common structure found in *Metazoa*.

Additional information regarding Dicer structure was extracted from genomic databases. Blast search of molluscs entries in Genbank (query: murine Dicer protein, algorithm: tblastn, database: Nucleotide collection nr/nt, results restricted to molluscs (taxid:6447)) identified transcripts from *Mytilus*, *Crasostrea*, *Lottia*, *Aplysia*, *Miomphalaria*, and *Octopus* that apparently encoded full-length Dicer orthologs. Molluscs Dicer also produces miRNAs with median length of 22 nucleotides as estimated from the miRBase data (Kozomara and Griffiths-Jones, 2014). Interestingly, the incidence of 23 nt long miRNAs seems to be higher in molluscs (Fig. 3). However, given the low number of miRNAs (64) and unexplored diversity of molluscs, it should not be considered a significant feature.

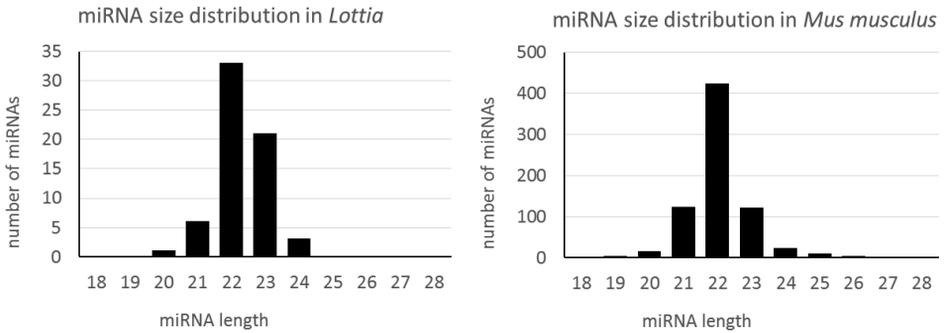
### **dsRBPs**

There is no literature concerning dsRBP proteins participating in RNA silencing in molluscs. The above-mentioned analysis of miRNA and RNAi pathway components identified only a single dsRBP (TARBP2) homolog (Rosani et al., 2016).

### **Argonaute proteins**

Likewise, there is essentially no information regarding the AGO subfamily of Argonaute proteins. The above-mentioned analysis of miRNA and RNAi pathway components identified one to four Argonaute proteins from both, AGO and PIWI clades (Rosani et al., 2016). However, general and derived roles of AGO proteins in molluscs remain unknown at the moment. Data from *Mytillus galloprovincialis* indicate presence of one AGO and two PIWI proteins (Rosani et al., 2016). This is remarkable because this AGO protein would act in both, RNAi and miRNA pathways like AGO2 in mammals.

In addition, there were two articles concerning the PIWI clade, which is acting in the piRNA pathway in the germline. In one of them, authors reported differential proteomic



**Figure 3** Molluscs miRNA lengths

The left graph depicts size distribution of all 64 miRNAs of sea snail *Lottia gigantea* deposited in the miRBase (version 21). For comparison, the right graph shows size distribution of 721 high-confidence murine miRNAs.

responses to generic dsRNA (poly I:C and poly A:U) in two oyster species (*Saccostrea glomerata* and *Crassostrea gigas*), which have differential susceptibility to ostreid herpesvirus infection. Interestingly, *Saccostrea glomerata*, which is not susceptible, showed production of proteins implicated in the TLR signalling pathway and PIWI protein was also found in *Saccostrea glomerata* but not in *Crassostrea gigas* when challenged with dsRNA (Masood et al., 2016). Although it is unclear whether PIWI could be mistaken for AGO, it is possible that piRNAs might have acquired additional roles in molluscs, perhaps including also immunity. This notion would be supported by the second report, which identified 28nt piRNAs in brain (while piRNAs are generally restricted to gonads if not into germline cells only). These piRNAs had unique biogenesis patterns, nuclear localization, sensitivity to serotonin, and were implicated in stable long-term changes in neurons associated with memory (Rajasethupathy et al., 2012).

### Other factors

According to the genome analysis of *Mytilus galloprovincialis*, Precambrian molluscs/mammalian ancestors must have shared all ancestral proteins in the miRNA pathway, including DROSHA, DGCR8 and GW182 (Rosani et al., 2016). There are no published mechanistic data suggesting that there would be any difference in activity of any of these factors in molluscs.

Importantly, we examined if molluscs genomes also contain an RdRP, which is found in RNA silencing in plants and Nematodes but not in insects or mammals: query: *C. elegans* RRF-1 NP\_001250555, algorithm: tblastn, database: Nucleotide collection nr/nt, results restricted to molluscs (taxid:6447) identified transcripts six different transcripts from *Crassostrea gigas* (XM\_011450789, XM\_011427600, XR\_900019, XR\_902698, XM\_011450791, XR\_900018) suggesting that molluscs might indeed employ RdRPs in RNAi. This would be a significant observation, making molluscs RNA silencing an intermediate type between those found in nematodes, arthropods, and mammals.

## miRNA

There is three molluscs species, which have annotated miRNAs in the miRBase (Kozomara and Griffiths-Jones, 2014): *Haliotis rufescens* (red abalone sea snail), *Lottia gigantea* (owl limpet sea snail), and *Melibe leonine* (lion nudibranch sea slug). Unfortunately, there are no other representatives of other main mollusk groups such as Cephalopodes or Bivalves. Annelid miRNAs have are ~ 22 nt long like other animal miRNAs (Fig. 4). There is a small number of reports concerning miRNA identification/annotation and/or analysis of expression/function in molluscs (Biggar et al., 2012; Bitel et al., 2012; Chen et al., 2014; Jiao et al., 2014; Jiao et al., 2015; Kenny et al., 2015; Martin-Gomez et al., 2014; Millan, 2011; Rajasethupathy et al., 2009; Tian et al., 2015; Xu et al., 2014; Zhao et al., 2016; Zheng et al., 2016a; Zheng et al., 2016b; Zhou et al., 2014).

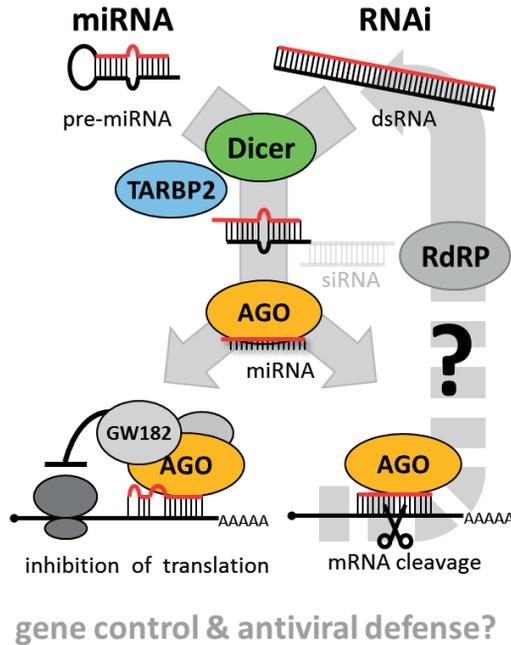
## RNAi

RNAi is functional in molluscs as evidenced by 18 reports, which employed RNAi in different molluscs species (see the table below). The canonical RNAi (i.e. using long dsRNA) has been observed in the following species upon various forms of delivery including larva soaking, animal injection (adductor muscle, gonad, brain, larva, post-renal sinus etc.), polyethyleneimine-mediated delivery, or cell injection. Collectively, these data imply that different molluscs have an intact machinery to execute RNAi. Effects of injection into body cavity would suggest that molluscs might have some cellular uptake mechanism for dsRNA or systemic RNAi but the direct evidence for any of that is lacking at the moment. Other dsRNA responding pathways

Molluscs seem to have a complex dsRNA response, which includes interferon-like response functioning in antiviral response (reviewed in Green et al., 2015a; Wang et al., 2015b). 21 articles dealt with dsRNA-induced interferon-like response. Several studies in Oyster have reported that dsRNA mimic poly(I:C) can strongly induce non-specific antiviral immune responses (De Zoysa et al., 2007; Green and Barnes, 2009; Green and Montagnani, 2013; Green et al., 2015b; Masood et al., 2016; Wang et al., 2016b; Wang et al., 2016c).

Molluscs have 2'5'- oligoadenylate synthetases (Kjaer et al., 2009; Pari et al., 2014), RIG-I-like protein (Zhang et al., 2014), MDA5 (Green et al., 2014), and PKR (Green et al., 2014; Green and Montagnani, 2013; Green et al., 2015b). Poly I:C can be also bound by Leucine-rich repeat (LRR)-only protein found in scallop *Chlamys farreri* (Wang et al., 2016b; Wang et al., 2016c). Another gene, which is induced by poly I:C or sodium alginate is Myxovirus resistance (Mx) protein, which has been found in Abalone (Cheng et al., 2012; De Zoysa et al., 2007). Mx is one of intensely studied antiviral proteins, which is induced by the type I interferon system (IFN alpha/beta).

However, it is important to recognize that molluscs are a heterogeneous group with distinct antiviral adaptations. For example, proteomic profiling of two oyster species with differential susceptibility to ostreid herpesviruses showed that the resistant species has a stronger manifestation of the interferon-like response in the proteome upon induction with poly I:C (Masood et al., 2016).



**Figure 4** Overview of mollusc pathways

dsRNA and miRNA pathways in molluscs are poorly understood. Among the missing pieces of information are: 1) degree of separation of miRNA and RNAi pathways (dedicated AGO proteins, co-existence of Dicer isoforms). 2) existence and functionality of RdRPs in molluscs, 3) derived roles of piRNAs in gene regulation and antiviral defense. Proteins from other dsRNA pathways related to innate immunity include PKR, OAS, RIG-I and MDA5 homolog, Mx protein and others.

**Summary**

Taken together, molluscs are an interesting taxon, which appears to have a unique setup of RNA silencing (Fig. 4) and its nexus with antiviral responses, which warrant further investigation.

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**Table 1** RNAi in molluscs induced with short dsRNA molecules

species	siRNA amount	delivery method & effect	reference
<i>Chlamys farreri</i>	1 µg/g	adductor muscle siRNA injection 70% KD at 72h	(Miao et al., 2016)
<i>Biomphalaria glabrata</i>	772 ng/250 µl	polyethyleneimine-mediated delivery >50–90% KD at 72h by ELISA	(Knight et al., 2011)
<i>Lymnaea stagnalis</i>	5 µl of 200 ng/µl	gonad injection, 27-mer 30–50% KD	(Fei et al., 2007)
<i>Lymnaea stagnalis</i>	2 µl of 20 µM	head injection above central ganglia, 27-mer,	(Hui et al., 2007)

**Table 2** RNAi in molluscs induced with long dsRNA molecules

species	long dsRNA		delivery method	reference
	concentration	length		
<i>Haliotis diversicolor</i>	5 µg/ml	136–819 bp	larva soaking	(Wang et al., 2016a)
<i>Haliotis diversicolor</i>	5 µg/ml	136–819 bp	larva soaking	(Wang et al., 2015a)
<i>Crassostrea gigas</i>	150–750 µg/ml 10 µg/g	652 bp	2x 100 µl injection ~50% KD at 48h	(Huvet et al., 2015)
<i>Crassostrea gigas</i>	50 µg/oyster	723 bp	adductor muscle inject. 1–7 days, good effect	(Choi et al., 2013)
<i>Crassostrea gigas</i>	530 µg/ml 53 µg/oyster	425 bp	gonad injection 77.52% KD	(Huvet et al., 2012)
<i>Nipponacmea fuscoviridis</i>	5 µg/ml	947 bp 667 bp	larva injection	(Hashimoto et al., 2012)
<i>Aplysia</i>	500 ng/µl	316 bp	sensory cell injection protein not decreased	(Lyles et al., 2006)
<i>Lymnaea stagnalis</i>	500 ng/µl 2 µg/oyster	~300 bp	central ring ganglia 60% KD at 24 h	(Guo et al., 2010)
<i>Lymnaea stagnalis</i>	5 µl of 200 ng/µl	321 bp	snail ganglia injection 30–50% KD	(Fei et al., 2007)
<i>Crassostrea gigas</i>	20 or 100 µg/oyster	525 bp 877 bp	gonad injection 39% & 87% KD	(Fabioux et al., 2009)
<i>Biomphalaria glabrata</i>	0.1, 1.0 and 5.0 µg/snail	537 bp 541 bp	post-renal sinus inj. 70–80% KD	(Jiang et al., 2006)
<i>Biomphalaria glabrata</i>	120 ng/250 µl	397 bp	polyethyleneimine-mediated delivery >50% KD at 72h by ELISA.	(Knight et al., 2011)
<i>Aplysia</i>	500 ng/µl	316 bp	sensory cell injection protein not decreased	(Lyles et al., 2006)
<i>Aplysia</i>	up to 700 µg/mL	N.A.	~20 giant neurons of the abdominal ganglion inj., 80–95% KD	(Lee et al., 2001)
<i>Aplysia</i>	500 µg/mL	800 bp	sensory neuron injection decrease by microscopy	(Ormond et al., 2004)

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