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non-protein-coding RNAs as regulators of development in tunicates

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Abstract Tunicates, or urochordates, are a group of small marine organisms that are found widely throughout the seas of the world. As most plausible sister group of the vertebrates they are of utmost importance for a comprehensive understanding of chordate evolution, hence they have served as model organisms for many aspects of the developmental biology. Current genomic analysis of tunicates indicates that their genomes evolved with a fast rate not only at the level of nucleotide substitutions but also in terms of genomic organization. The latter involves genome reduction, rearrangements, as well as the loss of some important coding and non-coding RNA (ncRNAs) elements and even entire genomic regions that are otherwise well conserved. These observations are largely based on evidence from comparative

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genomics resulting from the analysis of well-studied gene families such as the Hox genes and their non-coding elements. In this chapter the focus lies on the ncRNA complement of tunicates, with a particular emphasis on microRNAs, which have already been studied extensively for other animal clades. MicroRNAs are known as important regulators of key genes in animal development and they are intimately related to the increase morphological complexity in higher metazoans. Here we review the discovery, evolution, and genome organization of the miRNA repertoire, which has been drastically reduced and restructured in tunicates compared to the chordate ancestor. Known functions of microRNAs as regulators of development in tunicates are a central topic. For instance we consider the role of miRNAs as regulators of the muscle development and their importance in the regulation of the differential expression during the oral siphon regeneration. Beyond microRNAs, we touch upon the functions of some other ncRNAs such as Yellow Crescent RNA, mRNAs, RMST lncRNAs, or spliced-leader (SL) RNAs, which have diverse functions associated with the embryonic development, neurogenesis and mediation of mRNA stability in general.

1 Introduction

Tunicates are organisms characterized by a fast rate of genomic and developmental evolution. Some fast evolving evolutionary changes include loss of synteny, fast changes in cis-regulatory sequences, and loss of several key regulatory developmental genes (Satou et al., 2008; Denoeud et al., 2010), such as several central or posterior Hox genes involved in AP patterning of metazoans (Ikuta and Saiga, 2005) and Gbx involved in the establishment of the midbrain-hindbrain boundary in vertebrates (Yagi et al., 2003).

The role of noncoding RNAs in tunicate development has been studied since the 1990's, dating back to the seminal work by Swalla & Jeffry on the RNAs localized in the yellow crescent or myoplasm, a cytoskeletal domain in oocytes of the ascidian *Styela clava* (Swalla and Jeffery, 1995). The yellow crescent (YC) RNA identified to be present throughout embryonic development was the first example of what is now a large and rapidly growing class of ncRNAs with important roles in growth and development in tunicates. This asymmetrically distributed ascidian RNAs were part of the set of many other RNAs known as maternally synthesized cytoplasmically localized RNAs, discovered first in oocytes of *Xenopus* (Bashirullah et al., 1998).

The current state of knowledge on ncRNAs in tunicates is far from comprehensive and complete. Nevertheless, in particular microRNAs have been studied already in some detail in several tunicates species, such as, *Oikopleura dioica*, both *Ciona* species¹, *Didemnum vexillum*, and *Salpa thompsoni*. These studies have revealed many losses of miRNA families that are very well conserved outside the

¹ In accordance with the prevalent use in the ascidian community we use the term *Ciona robusta* reflecting that "Morphological evidence that the molecularly determined *C. intestinalis* type A and type B are different species: *Ciona robusta* and *C. intestinalis*" (Brunetti et al., 2015).

tunicates. At the same time, many gains of unique miRNAs among recently divergent lineages in the tunicates when compared to other groups of chordates (Fu et al., 2008; Velandia-Huerto et al., 2016). Relaxed constraints in the evolution of genomes and developmental trajectories in the tunicates may have been responsible for the plethora of reproductive strategies, morphologies, and life histories observed in the group (Holland, 2015).

2 miRNA families origin and evolutionary perspective

2.1 *Origins and Evolution of MicroRNAs*

MicroRNAs (miRNAs) have been described in almost all animals and plants as well as unicellular eukaryotes. They are important post-transcriptional regulators of gene expression affecting a sizable fraction of all mRNAs (Ameres and Zamore, 2013). Mechanistically, miRNAs depends on the presence of the evolutionarily even older RNA interference pathways (Cerutti and Casas-Mollano, 2006; Shabalina and Koonin, 2008) that leads to the suppression of double-stranded RNA molecules in a cell's cytoplasm.

Throughout animals, canonical miRNAs are processed through a well-characterized pathway. The primary precursor transcript (pri-miRNA) is transcribed by pol-II. While in most cases the pri-miRNA is a long noncoding RNA, some miRNAs are processed from protein-coding transcripts, where they are mostly derived from introns (Lin et al., 2006). In the next step, hairpin-shaped precursors, the pre-miRNAs, are excised while the RNA is still residing in the nucleus. These are exported into the cytoplasm (Lund et al., 2004) and then processed further into miRNA/miRNA* duplexes. In the final step the single-stranded mature miR or its complement, the miR*, is incorporated into the RISC complex. Sequence complementarity of miR and mRNA ensures the targeting specificity (Bartel, 2009). As a consequence, miRNAs share a set of structural characteristics, most importantly the extremely stable secondary structure of the precursor hairpin and the 2-bp overhang of miR and miR* generated by Dicer processing. These features make it possible to reliably identify miRNAs from short RNA-seq data, see e.g. (Langenberger et al., 2010; Friedländer et al., 2012; Langenberger et al., 2012).

Most animal microRNAs are among the most highly conserved genetic elements. The most stringent selection pressure acts on the mature miR sequence. This is a consequence of the fact that a single miR typically targets a large number of mRNAs. Mutations in the mature sequence thus simultaneously affect many interactions, and thus are almost always selected against. In conjunction with the stringent requirements on the secondary structure, the entire precursor is under strong stabilizing selection (Price et al., 2011), explaining the observed high levels of sequence conservation. As a consequence, even evolutionarily distant homologs of miRNAs can be readily detected despite the short sequence length. Most efficiently,

infernal (Nawrocki and Eddy, 2013a) is used for this purpose, since it makes use of both sequence and structure comparison. The evolution of miRNAs can thus be traced back in time with high accuracy (Hertel et al., 2006).

Like other gene families, miRNAs form paralogs (Tanzer and Stadler, 2004; Hertel et al., 2012) and hence often appear as families of homologous genes. This forms the basis of the *miRBase* nomenclature (Ambros et al., 2003). A series of investigations into the phylogenetic distribution of miRNA families showed that miRNAs are infrequently lost at family level and thus serve as excellent phylogenetic markers (Sempere et al., 2006; Heimberg et al., 2007, 2010; Wheeler et al., 2009), although the massive restructuring of the miRNA complement of tunicates is an important exception to this rule (Fu et al., 2008).

The innovation of new miRNA families is an on-going process. Experimental surveys of the miRNA repertoire thus have reported a large number of very young and even species-specific miRNAs (Bentwich et al., 2005; Berezikov et al., 2006). The process was studied quantitatively in fruit flies, where innovation rate of as many as 12 new miRNA genes per million years has been estimated (Lu et al., 2008). This is consistent with the fact that stable hairpins are abundant structural elements in random RNAs, which makes it not only possible but actually quite likely that miRNA precursors appear by chance in transcribed genomic regions (Tanzer and Stadler, 2004; Campo-Paysaa et al., 2011a; Marco et al., 2013). Of course, only a tiny fraction of these fortuitously processed hairpins have a function and hence are subject to selection, and an even smaller subset is conserved over long evolutionary time scales. Detailed studies showed that evolutionarily young miRNA have comparably low expression levels. Initially, they go through a phase of relatively fast sequence evolution (Liang and Li, 2009; Meunier et al., 2012), which slows down as the selective pressures from a gradual increase in the number of target site increases. A large, diverse set of targets then protects against miRNA loss (Lee et al., 2007). The rate of gain of miRNA families that are retained essentially permanently amounts to only 1 per several million years. This number is consistent with divergence of the miRNA complements between animal phyla.

Many authors have observed that overall the miRNA repertoire has been expanding throughout animal evolution in a manner that at least roughly correlates with morphological complexity (Hertel et al., 2006; Sempere et al., 2006; Niwa and Slack, 2007; Prochnik et al., 2007; Lee et al., 2007; Heimberg et al., 2007; Peterson et al., 2009; Berezikov, 2011). Several bursts of miRNA innovation have been observed (Hertel et al., 2006; Heimberg et al., 2007; Tanzer et al., 2010; Hertel and Stadler, 2015), most notably at the root of the placental mammals, the ancestor of “free-living” nematodes, or the radiation of the drosophilids. Massive morphological simplification, on the other hand, is sometimes associated with a drastic loss of miRNA families. This has been observed most prominently for tunicates (Fu et al., 2008; Dai et al., 2009).

2.2 miRNA identification and validation

The first miRNA reported in any tunicate was let-7, which was first detected in *Ciona robusta* and *Herdmania curvata* (Pasquinelli et al., 2000). A previous study the same year in *C. elegans* had shown that small RNA let-7 (21 nt) was required for late larval to adult developmental transition (Reinhart et al., 2000). Small RNA let-7 was then shown to also be differentially expressed during the development of many distantly related animal taxa, but was not detected in Porifera, Ctenophora, Cnidaria, and Acoelomorpha, suggesting that let-7 was involved in the regulation of late temporal transitions during development or in the evolution of complex life histories in the Nephrozoa (Pasquinelli et al., 2000, 2003).

The first systematic computational screen of a tunicate genome in 2005 followed the first large animal sequencing projects. Beginning with *C. robusta* and *C. savignyi*, a profile-based strategy was implemented in the ERPIN program (Legendre et al., 2005). This work detected a set of new miRNAs candidates considered to be *C. robusta* specific, such as the members of the family miR-9 and miR-79 together with many other miRNA families that, as expected, were found to be homologous between both *Ciona* species. Among these were miR-124, miR-92, miR-98, miR-325, the miR310-313 group, and let-7. In the same year, a whole-genomic comparative approach in the urochordate lineage was performed on the species *C. robusta*, *C. savignyi*, and *O. dioica*. Using a computational screening of structured ncRNAs based upon homology between predicted precursor hairpin structures 41 miRNA candidates were detected including let-7 and other six known candidates in *C. robusta* (Missal et al., 2005). The same group in 2007 implemented a structure-based clustering approach in *C. robusta* predicted the presence of 58 miRNAs, of which only let-7, miR-7, miR-124, and miR-126 coincided with the previously annotated miRNAs (Will et al., 2007).

Up to this point, efforts to map the miRNAs in urochordate lineages were mainly focused on computational approaches. With improved sequence technologies, new hybrid strategies were employed that combined computational and experimental studies to validate candidate families previously detected. For instance, the first bona fide records for *C. robusta* miRNAs were registered in mirBase only in Release 11 based on the data reported in Norden-Krichmar et al. (2007). This study searched the genomes of *C. robusta* and *C. savignyi* for conservation of the seed regions of the known mature miRNA sequences compiled in the 2006 release of miRBase release. Those miRNAs were aligned locally using the FASTA/ssearch34 program, retaining only matches with at least 90% identity. The characteristic hairpin structure and the relative positioning of the mature sequences were used as additional filters. After manual curation a set of 18 miRNAs that appeared conserved in both *Cionas* was reported. The expression of 14 of these families, including let-7, miR-7, and miR-126, was then confirmed by Northern blot analyses in the adult tissue of *C. robusta*.

Until 2008, most of the miRNAs were annotated in *Cionas*, and few new miRNA families were discovered in other urochordates. The first repertory of miRNAs in tunicate beyond the *Ciona* species was published in 2008 for the larvacean *O. dioica*

(Fu et al., 2008). At that time the Fu *et al.* were studying the temporal-spatial expression patterns of conserved miRNAs in different developmental stages of oocytes, 1-cell zygote, 2-8 cell embryos, blastulas, gastrulas, tadpoles (in different stages) and 1-6 days old adults from *O. dioica*. Small RNAs were isolated, amplified by RT-PCR and rapid amplification of cDNA ends (RACE) of the developmental stages, cloned and sequenced. Blast searches using the sequences of cloned small RNA libraries were used to annotate small RNAs as miRNA candidates. In further steps the recovered genomic flanking sequences each side of those mapped candidates were used as input to predicted secondary structures by mfold v3.1. This step was used to detect candidates that folds like miRNA hairpins and aimed to decrease the set of false positive potential miRNAs in *O. dioica*. Finally, for this set of potential candidates a developmental miRNA array dot blot analyses were performed to detect miRNA expression. With this approach from 3066 sequenced small RNA clones, expression was detected for only 55 miRNAs. The authors suggested that these candidates were expressed throughout the short life cycle of *O. dioica* showing that some of them were stocked as maternal determinants prior to rapid embryonic development. The authors also identified a set of sex-specific miRNAs that appeared as male/female gonad differentiation became apparent and was maintained throughout spermatogenesis (Fu et al., 2008). Unexpectedly, the majority of the miRNAs loci in *O. dioica* were located in antisense orientations in the host genes, in contrast to the majority of the mammalian miRNAs known at the time.

Between the years 2009 and 2015 the majority of the studies of miRNAs in tunicates were focused on the validation of expression of computational predicted miRNAs in Cionas with the special focus on *C. robusta* as model organism of tunicates or on the test of new computational approaches as miRTRAP, miRDeep2 and miRRim2, which used next-generation sequencing libraries of small RNAs derived from *C. robusta* to validate their algorithms. Then by the year 2016 the first comparative homology based search strategy let us to identify the repertory on miRNAs and other ncRNAs in the carpet sea squirt *Didemnum vexillum* with a preliminary comparative analysis of gain and losses of miRNA families in chordates which included the *Cionas*, *O. dioica* and the colonial tunicate *Botryllus schlosseri* (Velanda-Huerto et al., 2016). By the same year, from the preliminary genome sequence assembled for the Southern Ocean salp, *Salpa thompsoni* (Urochordata, Thaliacea) a set of miRNAs families were detected (Jue et al., 2016) and in 2017 the prediction of miRNAs families were reported for *Halocynthia roretzi* species (Wang et al., 2017).

2.2.1 High throughput studies of *Ciona* miRNAs

At the end of the last decade the application of next generation sequencing technologies to sequence small RNA libraries changed the common way used to detect expression of miRNAs in many organisms including the tunicates. This technology became one of the most common approaches that supported methods like RT-PCR, microarrays or dot blotting which were previously used to validate miRNA expression in tunicates. In 2009 small RNA libraries prepared from various developmental

stages including unfertilized eggs, early embryos, late embryos and adults from *C. robusta* were assayed by high-throughput sequencing of cDNA with an Illumina 1G Genome Analyzer. These sequencing data revealed 80 miRNAs families in *C. robusta*. Unexpectedly, a distinct species of small RNAs processed from the distal parts of the miRNA precursor hairpins was found to be abundantly expressed. These were termed miRNA-offset RNAs (moRs) (Shi et al., 2009). Later on, after extracting non-coding conserved regions of whole genome alignments between *C. robusta* and *C. savigny* a set of 12 million sequences were computationally folded using RNAfold and mfold. Then after combining the following criteria: structure/sequence conservation, homology to known miRNAs, and phylogenetic footprinting the authors detected a set of 458 candidate sequences (Keshavan et al., 2010). Then in order to validate those candidates, RT-PCR and PAGE were conducted to design a custom microarray. 244 of the 458 miRNA predictions were represented either in their microarray data or in the Illumina sequences from (Shi et al., 2009), leading to an estimate of about 300 miRNA genes in *C. robusta*. It is worth noting that (Keshavan et al., 2010) did not recover 39 previously characterized miRNAs. A novel computational strategy for the systematic, whole-genome identification of microRNA from high throughput sequencing information was developed in 2010 in Hendrix et al. (2010) and applied to *C. robusta*. This method, miRTRAP, relies on the sequence patterns produced by mechanisms of microRNA biogenesis but also includes additional criteria regarding the prevalence and quality of small RNAs arising from the antisense strand and the neighboring loci. With that approach, nearly 400 putative microRNAs loci were detected. The miRTRAP approach depends crucially on the depth of the small RNAs mapped to a given locus and requires a very accurate assignment of small RNA sequences on their relative positions along the hairpin, that is, miR/miR*, moR/moR* and loop (Hendrix et al., 2010). A further improvement in the analysis of miRNA sequencing data became available with miRDeep2, a revised version miRDeep (Friedländer et al., 2012). It was reported to identify with an accuracy of 98.6% and 99.9% canonical and non-canonical miRNAs in different species. A reanalysis of the small RNA data from (Shi et al., 2009) with miRDeep2 reported 313 known and 127 novel miRNAs in *C. robusta*. In the same year the program miRRim2 (Terai et al., 2012) was applied to the *C. robusta* genome, in which some candidates identified from the work of Hendrix et al. (2010) and the several novel promising candidates were detected.

In 2013, (Kusakabe et al., 2013) investigated the expression patterns of the cluster miR-1 and miR-133 in *C. robusta* and in *C. savignyi*. RT-PCR amplification of miR-1/133 precursors were performed and PCR products were subcloned and sequenced. Whole-mount in situ hybridization to detect cin-miR-1/miR-133 primary transcript was performed and LNA Northern blotting was conducted on different developmental stages.

2.2.2 High throughput miRNA searches in other Urochordates

Since 2016 new approximations has increased our knowledge about new families in other tunicates thanks to the sequence of new urochordate genomes of the species *D. vexillum*, *S. thompsoni* and *H. roretzi*. A detailed homology-based computational survey of ncRNAs was performed for an early draft genome of *D. vexillum* (Velandia-Huerto et al., 2016). Blast and HMMer searches were performed with annotated small ncRNAs sequences from metazoans and hidden markov models from RFAM² to obtain the sort of candidates at sequence level. Structural alignments of those sequences were performed by infernal (Nawrocki and Eddy, 2013b), using metazoan-specific covariance models to annotate the small ncRNAs collection, which 57 families and 100 loci of miRNAs were found.

Small RNA libraries for the Southern Ocean salp *S. thompsoni* were sequenced with an Illumina Hiseq 2000 (Jue et al., 2016). After filtering data sets to 18-24 nt for miRNA and 28-32 nt for piRNA, the reads were aligned to *S. thompsoni* genome and miRNA gene folding predictions were performed using RNAfold. In this initial survey of small RNAs, were revealed the presence of known, conserved miRNAs, as well as novel miRNA genes and mature miRNA signatures for varying developmental stages. Then in 2017, the prediction of 319 miRNAs candidates in *H. roretzi* were obtained through three complementary methods. The experimental validation suggested that more than half of these candidate miRNAs are expressed during embryogenesis. The expression of some of the predicted miRNAs were validated by RT-PCR using embryonic RNA. In this approach *C. robusta* small RNA-Seq reads derived from *C. robusta* (Shi et al., 2009) (previously known as *C. intestinalis* today reclassified) was used to identify conserved miRNAs in *H. roretzi* (Wang et al., 2017).

Current repertoire of miRNAs (Figure 8) is based on the final matrix of miRNAs families from Hertel and Stadler (2015) and complemented by homology methods developed on Velandia-Huerto et al. (2016) and specifically for *S. thompsoni* and *H. roretzi* blast searches with structural alignments where applied on the reported candidates in Jue et al. (2016) and Wang et al. (2017).

2.3 miRNA in clusters

One of the most interesting aspects about the patterns of genomic locations of miRNAs is to know whether those loci are randomly distributed throughout the genome as single copies or if they are arranged on consecutive locations or in tandem copies clustered to be expressed from polycistronic primary precursors or to be transcribed independently (Tanzer et al., 2010). Interestingly in *O. dioica* miRNAs are located in the antisense orientations of protein-coding gene and immediately downstream of its corresponding 3'UTR region or even more frequently in the sense strand of introns

² <http://rfam.xfam.org/>

(Fu et al., 2008). Nevertheless, after those conspicuous distributions some clusters have been also identified in *O. dioica*. For instance four miRNAs, miR-1490a, miR-1493, miR-1497d, and miR-1504, are reported by Fu et al. (2008) to be present as two copies, and miR-1497d-1 and miR-1497d-2 are included in the large miR-1497 cluster. The current structure of this cluster is shown in Table 1 although only one copy for the miR-1497 has been reported for *C. robusta* located in an intergenic region (Fu et al., 2008; Hendrix et al., 2010) and one in *C. savigny* overlapped in an intron (Fu et al., 2008). By testing real time PCR co-expression of some miRNAs, their host and adjacent genes in *O. dioica* by Fu et al. (2008) it was discovered that in the case of the cluster miR-1487/miR-1488 there was no clear positive or negative correlation with the expression of its anti-sense hosting gene. The same authors showed that in males this cluster expression was not associated with the expression of its adjacent ABCA3 gene.

Table 1: Details of biggest miRNA cluster for chordate species.

Specie	Chr	Start	End	Size (Mb)	No.	miRNAs detail
<i>B. floridæ</i>	Bf_V2_118	216744	220351	3607	5	bfl-mir-4869, bfl-mir-4857, bfl-mir-4862, bfl-mir-4856b, bfl-mir-4856a
<i>O. dioica</i>	scaffold_3	2222857	2223714	857	6	odi-mir-1497e, odi-mir-1497d-2, odi-mir-1497d-1, odi-mir-1497c, odi-mir-1497b, odi-mir-1497a
<i>B. schlosseri</i>	chrUn	40003	41320	1317	2	mir-233, mir-10

<i>C. robusta</i>	7	4153284	4156782	3498	23	cin-mir-4006d, cin-mir-4006c, cin-mir-4001b- 2, cin-mir-4000i, cin-mir-4006g, cin-mir-4001e, cin-mir-4001d, cin-mir-4000g, cin-mir-4006f, cin-mir-4006b, cin-mir-4001b-1, cin-mir-4000c, cin-mir-4006e, cin-mir-4000b-2, cin-mir-4001a-1, cin-mir-4000b- 1, cin-mir-4002, cin-mir-4000d, cin-mir-4001h, cin-mir-4000a-2, cin-mir-4006a-2, cin-mir-4006a-3, cin-mir-4006a-1
<i>C. savignyi</i>	reftig_16	3924783	3925336	553	3	csa-mir-216b, csa- mir-216a, csa-mir- 217
<i>C. savignyi</i>	reftig_1	1335375	1336487	1112	3	csa-mir-92b, csa-mir- 92c, csa-mir-92a

<i>D. rerio</i>	4	28738556	28754891	16335	60	dre-mir-430a-18, dre-mir-430c-18, dre-mir-430b-4, dre-mir-430a-15, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430a-15, dre-mir-430c-18, dre-mir-430b-3, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-8, dre-mir-430a-15, dre-mir-430c-18, dre- mir-430b-5, miR-430, dre-mir-430b-20, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430i-3, dre-mir-430c-18, dre-mir-430b-3, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-8, dre-mir-430a-11, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430i-3, dre-mir-430c-18, dre-mir-430b-19, dre-mir-430a-10, dre-mir-430c-18, dre- mir-430b-5, miR-430, dre-mir-430b-20, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430i-3, dre-mir-430c-18, dre-mir-430b-19, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430a-15, dre-mir-430c-18, dre-mir-430b-3, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-8, dre-mir-430a-15, dre-mir-430c-18, dre-mir-430b-5
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<i>L. chalumnae</i>	JH126646.1	1529355	1882777	353422	7	mir-233, mir-233, mir-672, MIR535, mir-233	mir-233, mir-598,
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In *C. robusta* some miRNAs are located in introns and a small class of miRNAs are derived from the exonic parts of mRNAs (Hendrix et al., 2010). In contrast, some miRNAs of in *O. dioica* are expressed from loci on the opposite strand on protein-coding genes. An example is miR-2246, which also produces antisense miRs derived from miRNA loci (moRs). Only 44 loci appeared to be expressed as antisense products from the 300 miRNA loci predicted in 2010 by Hendrix et al. (2010). In Cionas have been also detected miRNAs organized in clusters, for example in *C. robusta* a putative cluster was detected by Keshavan et al. (2010) using microarray analysis that shows a similar loci organization to the cluster let-7/miR-125/miR-100 observed in Drosophila. The miR-1473 was later classified as the orthologue of miR-100 in the analysis derived from the comparison of the evolution of this cluster conducted by Griffiths-Jones et al. (2011). The authors suggested that mir-100, mir125 and let7 are clustered in most of the bilaterian genomes including the 1473 that is an orthologue of mir-100.

Current analysis of this cluster shows that the distribution of miRNAs families on this let-7 cluster are present in all the studied chordate species. Vertebrate species such as (*D. rerio* and *L. chalumnae*) contain more than one let-7 cluster, extending the loci definition which is not restricted only to one element but for a cluster of many loci with different length distributions. It is important to see that let-7 is organized sometimes with another let-7 locus or with another miRNA's loci families. The distribution of this cluster reported in amphioxus is composed of 2 let-7 and 3 mir-10 (1 bfl-mir-100, 1 bfl-mir-125a and 1 bfl-mir-125b), this cluster architecture is conserved in vertebrates that apparently inverted the order and split the relation between let-7 and mir-10, creating two different cluster order groups: let-7 + mir-10 and let-7 + other families. In this way, tunicates belong the latter group, not including mir-10 on the cluster but including mir-233, mir-1473 or mir-125.

A second miRNA cluster consisting of the miR-182 and miR-183 was also detected in *C. robusta* in 2010 by Keshavan et al. (2010) which in the current predictions reported another member locus: the miR-96 organized in the middle of those loci, as is shown in the plot 7. Here the authors also found five additional paralogs of let-7 within a 1-kb stretch, but it is important to know that those elements had been identified on chromosome 4q on Ensembl release 54 version, in the current version only two of those elements have been identified by homology approaches (Figure 1).

The cluster miR-1/miR-133, expressed specifically in Cionas muscle tissues was also reported by Kusakabe et al. (2013). The authors reported that one copy of this cluster is presented in both Cionas. As is shown in the plot 2 a copy is also present in *L. chalumnae*. In 2012 a new cluster was proposed in *C. robusta* by Terai et al. (2012) located on the chromosome 10q and composed of the mir-4054 locus and

the mir-4091. In the current distribution of this cluster a new annotated family the mir-4008 with three paralogous is located on the middle of those loci. This current distribution is shown in Table 2 these loci were validated by Norden-Krichmar et al. (2007), Fu et al. (2008), Hendrix et al. (2010), and Terai et al. (2012). As was mentioned by Hendrix et al. (2010) it is not very common to find related miRNAs organized in clusters composed by closely related families that differ in just a single nucleotide in the seed sequence as was found on the cluster composed by nine Ci-mir-2200, seven Ci-mir-2201 and nine Ci-mir2203 which were previously reported under that putative names. The same authors also found a second large cluster composed of 11 miRNAs that gather into 4 paralogous families three of Ci-mir-2200, three Ci-mir-2201, four Ci-mir-2204 and two Ci-2217. Current distribution of miRNAs families in *C. robusta* and curated annotations indicate than in other regions of the chromosome 7q the miRNAs are organized in tandem copies of families. An example is the big cluster composed of the families miR4000, miR-4001, miR4002, and miR4006 located on chromosome 7q (Table 2). Another cluster is also located on the same chromosome composed by the families miR-4003, miR4005 and miR4077 in (Table 2). Some other clusters are also found on the chromosome 1a, 10q and 3p. See this structure in Table 2, most of them were validated by Hendrix et al. (2010).

Some other clusters shared between both Cionas are the cluster 92, 124 and 200 validated by Norden-Krichmar et al. (2007), Fu et al. (2008), and Hendrix et al. (2010), which structure is shown in Table 2.

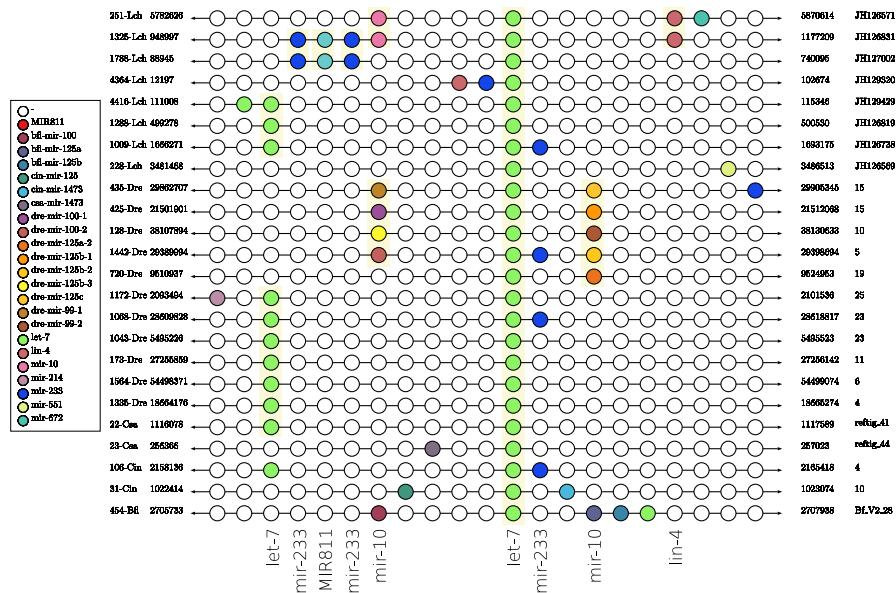


Fig. 1 Multiple alignment of let-7 clusters. Specific names from annotations and homology predictions are described in the legend. Names from miRBase families are reported at the bottom of the aligned elements.

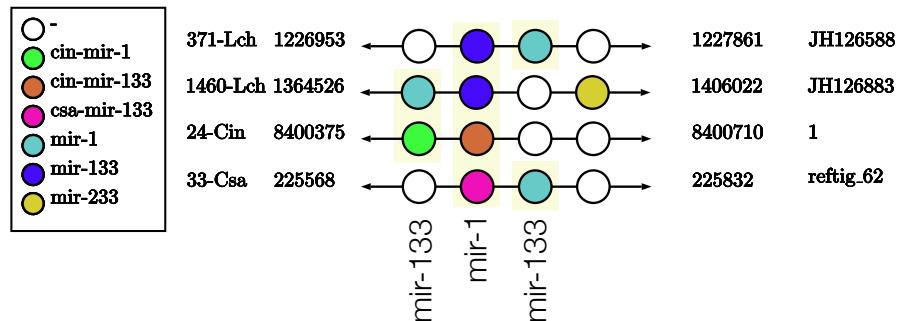
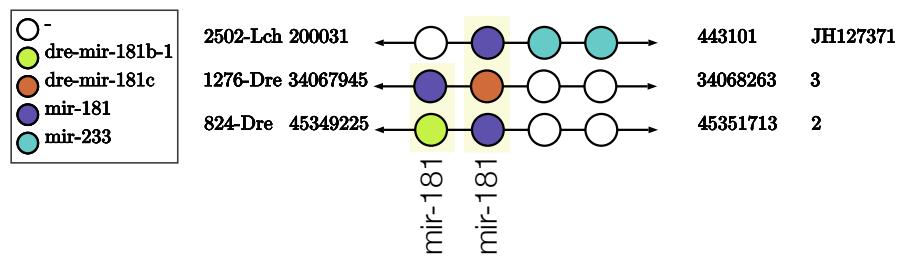
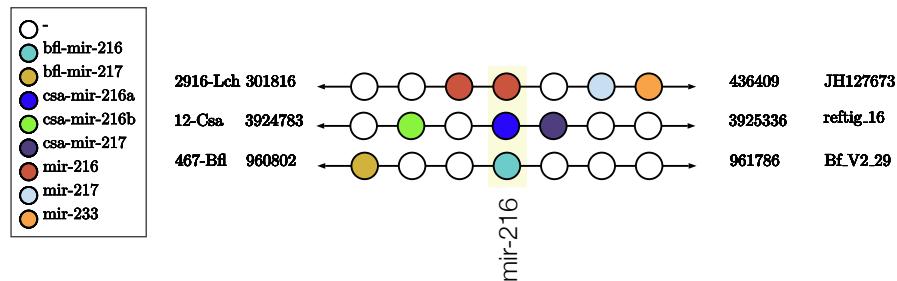
**Fig. 2** mir-1/mir-133**Fig. 3** mir-181**Fig. 4** mir-216/mir-217

Table 2: Reported clusters in the literature. Bold text represent those miRNAs elements that are currently annotated and validated, but could not be detected by homology strategies. **Ciro**: *C. robusta*, **Cisa**: *C. savignyi* and **Oidi**: *O. dioica*

Species	Chr	Start	End	miRNAs	Comments	Source DB	Ref.
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Ciro	4q	2082260	2083286	cin-let-7a-1, cin-let-7f, cin-let-7b, cin-let-7c, cin-let-7a-2	Reported on miRBAse and annotated on Ensembl	miRBase (Hendrix et al., 2010), (Fu et al., 2008)
Cisa	refrig_41	1114139	1117597	csa-let-7c-1, csa-let-7b, csa-let-7c-2	Reported on miRBase and does not detected by homology strategies.	miRBase (Fu et al., 2008)
Ciro	10q	3226200	3228884	cin-mir-34, mir-4091, 4008a, 4008c, 4008b, cin-mir-4054	NA	miRBase, Homology (Norden-Krichmar et al., 2007), (Fu et al., 2008), (Hendrix et al., 2010), (Terai et al., 2012)
Ciro	7q	4828431	4835967	cin-mir-4077b, cin-mir-4003b, cin-mir-4005b, cin-mir-4077d, cin-mir-4003a-1, cin-mir-4003c, cin-mir-4077a, cin-mir-4003a-4, cin-mir-4003d	NA	miRBase, Homology (Hendrix et al., 2010)
Ciro	3q	567478	571031	cin-mir-4001f, cin-mir-4000e, cin-mir-4001c, cin-mir-1502d, cin-mir-4018a, cin-mir-4019, cin-mir-1502b, cin-mir-1502a, cin-mir-4007, cin-mir-4000f, cin-mir-4001i, cin-mir-4018b, cin-mir-1502c	Inclusion of 4019 and cin-mir-4007	miRBase, Homology (Hendrix et al., 2010)
Ciro	HT000037.1	4884	5250	cin-mir-367, cin-mir-4009c, cin-mir-4009b, cin-mir-367, cin-mir-4009c, cin-mir-4009a, cin-mir-4009b	Non-highlighted names could be found in the current genome at HT000037.1 scaffold	miRBase, Homology (Hendrix et al., 2010)

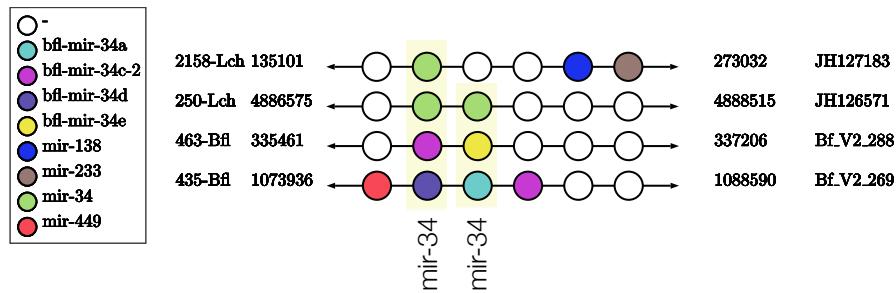
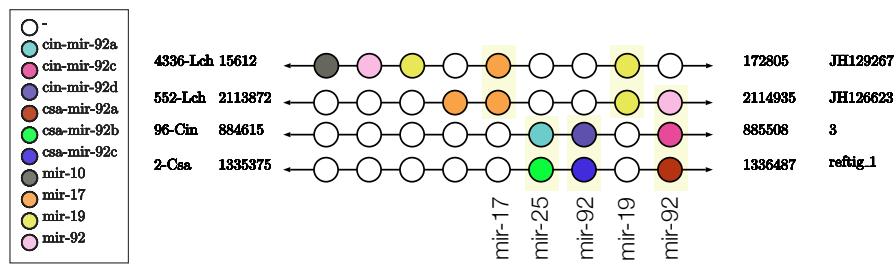
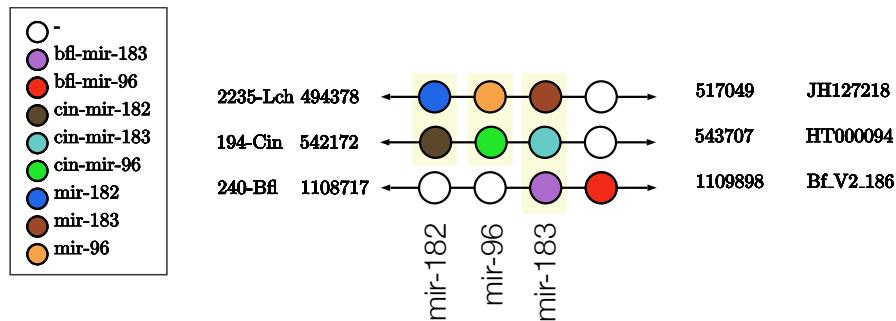
Ciro	10q	3226200	3228884	cin-mir-34, mir-4091, 4008a, 4008c, 4008b, cin-mir-4054	cin- NA	miRBase, Homology	(Hendrix et al., 2010)
Ciro	3q	884615	885508	cin-mir-92a, mir-92d, cin-mir-92c	cin- NA	miRBase, Homology	(Hendrix et al., 2010)
Cisa	reftig_1	1335375	1336487	csa-mir-92b, mir-92c, csa-mir-92a	csa- NA	miRBase, Homology	(Fu et al., 2008)
Oidi	scaffold_1	3086369	3086586	odi-mir-92b, mir-92a	odi- NA	Homology	(Fu et al., 2008)
Ciro	7q	4969691	4969912	cin-mir-124-1, cin-mir-124-2	NA	miRBase, Homology	(Hendrix et al., 2010), (Fu et al., 2008)
Cisa	reftig_262	49392	49620	csa-mir-124-1, mir-124-2	csa- NA	miRBase, Homology	(Fu et al., 2008)
Ciro	HT000325.1	8331	8778	cin-mir-200, mir-3575, 141, cin-mir-5611	cin- NA	miRBase, Homology	(Norden- Krichmar et al., 2007), (Fu et al., 2008), (Hen- drix et al., 2010), (Friedländer et al., 2012)
Cisa	reftig_613	31353	31949	csa-mir-200, mir-141	csa- NA	miRBase, Homology	(Fu et al., 2008)

Ciro 7q	4153284 4156782	cin-mir-4006d, cin-mir-4006c, cin-mir-4001b- 2, cin-mir-4000i, cluster, but in another cin-mir-4006g, cin-mir-4001e, cin-mir-4001d, cin-mir-4000g, cin-mir-4006f, cin-mir-4000h*, cin-mir-4006b, cin-mir-4001b-1, cin-mir-4006e, cin-mir-4001a-1, cin-mir-4001a-2, cin-mir-4002, cin-mir-4001h, cin-mir-4000a-2, cin-mir-4006a-2, cin-mir-4006a-3, cin-mir-4006a-1, cin-mir-4006e, cin-mir-4001a-1, cin-mir-4006b, cin-mir-4000c, cin-mir-4006e, cin-mir-4000b-2*, cin-mir-4001a-1, cin-mir-4000b-1*, cin-mir-4001a-2, cin-mir-4002, cin-mir-4000d*, cin-mir-4001h, cin-mir-4006a-3, cin-mir-4006a-1, cin-mir-4000a-1	Elements marked with * miRBase, are identified by homology strategies at the same order reported by miR-Base. Homology et al., 2010)
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3 miRNAs and its role in development

3.1 miRNAs discovery and its role in development

Both MicroRNAs (miRNAs or miRs) as well as MicroRNA offset RNA (moRNAs or moRs) are developmentally regulated as shown during *C. robusta* development (Shi et al., 2009). In spite of the considerably higher abundance of miRs and miRs* in cells than their corresponding abundance of moRs, all three small RNA types have been shown to have regulatory roles in gene expression. Although a vast ma-

**Fig. 5** mir-34**Fig. 6** mir-92**Fig. 7** mir-182/mir-96/mir-183

jority of miRNAs remain to be studied, there are already many cases of well-studied miRNAs (including many that are mentioned in this chapter that have been studied in tunicates) that are known to target mRNAs, modulate their levels of expression, and affect developmental processes both in plants and animals (Zhao et al., 2018). Only recently two studies demonstrated for the first time that two moRs (viral moR-R1-3-5p and moR-21) could also modulate gene expression, and were not merely the byproduct of miRNA biogenesis (Umbach et al., 2010; Zhao et al., 2016).

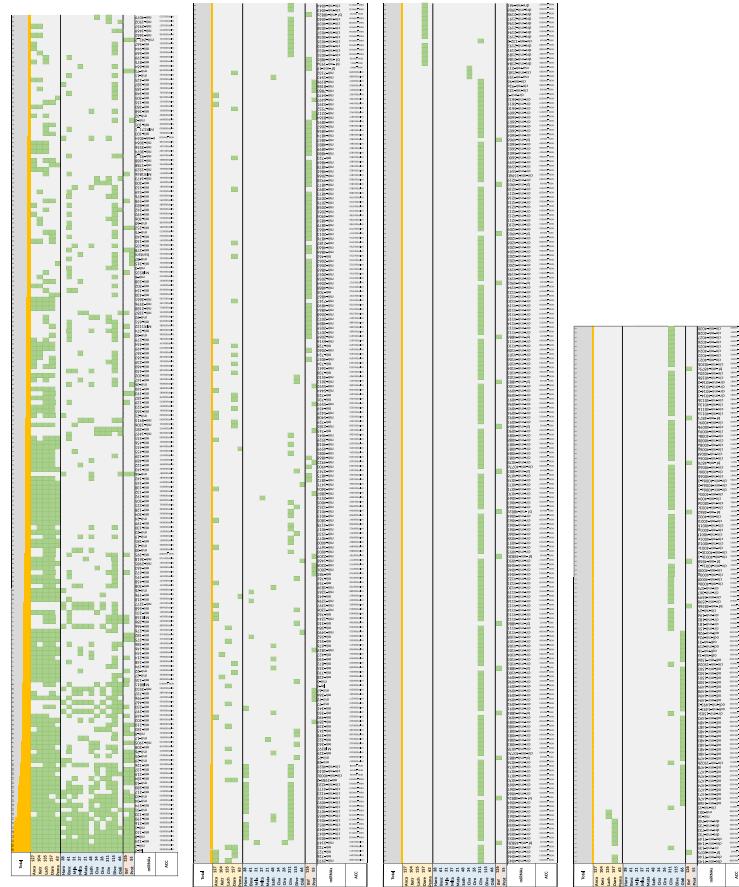


Fig. 8 Absence/Presence Matrix of miRNAs families along Bilaterian species. **Prot:** Protostomata, **Brfl:** *B. floridae*, **Oidi:** *O. dioica*, **Dvex:** *D. vexillum*, **Ciin:** *C. robusta*, **Cisa:** *C. savignyi*, **Ciro:** *C. robusta*, **Sath:** *S. thompsoni*, **Mata:** *M. oculata*, **Mlta:** *M. occulta*, **Mlis:** *M. occidentalis*, **Bosc:** *B. schlosseri*, **Haro:** *H. roretzi*, **Pema:** *P. marinus*, **Dare:** *D. rerio*, **Lach:** *L. chalumnae*, **Xetr:** *X. tropicalis* and **Anca:** *A. carolinensis*.

3.2 Neuronal fate determination and regulation by miR-124

The miRNA miR-124 is expressed in the nervous system of many animals, including *Drosophila* (Aboobaker et al., 2005), *C. elegans* (Clark et al., 2010) and humans (Sempere et al., 2004). As was first observed in *in vitro* studies of mouse brain cells, low expression of miR-124 was related to neural stem cell maintenance, whereas high expression of miR-124 induced the differentiation of neuronal cell types (Cheng et al., 2009). A regulative role of miR-124 in non-neural vs. neural fate decisions was further investigated by embryonic experiments *in vivo* (Chen et al., 2011) and by theoretical and *in silico* modeling analyses in *C. robusta* (Chen et al.,

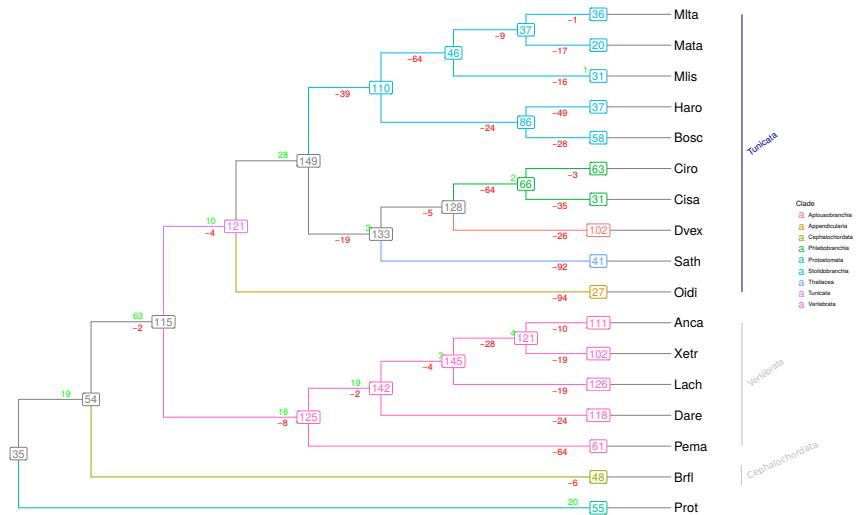


Fig. 9 Dollo parsimony of miRNAs families distribution in some chordates genomes. **Prot:** Protostomata, **Brfl:** *B. floridae*, **Oidi:** *O. dioica*, **Dvex:** *D. vexillum*, **Ciro:** *C. robusta*, **Cisa:** *C. savignyi*, **Sath:** *S. thompsoni*, **Mata:** *M. oculata*, **Mltia:** *M. occulta*, **Mlis:** *M. occidentalis*, **Bosc:** *B. schlosseri*, **Haro:** *H. roretzi*, **Pema:** *P. marinus*, **Dare:** *D. rerio*, **Lach:** *L. chalumnae*, **Xetr:** *X. tropicalis* and **Anca:** *A. carolinensis*. The phylogenetic distribution of this species was obtained from (Delsuc et al., 2017; Kocot et al., 2018).

2014). These studies showed that miR-124 promotes nervous system development by feedback interactions with Notch signaling. During nervous system development of *C. robusta*, cells in the dorsal and ventral midline epidermis of the tailbud embryo either take an epidermal sensory neuron (ESN) or peripheral nervous system (PNS) fate, a decision mediated by lateral inhibition using a classical model of feedback loop regulation Notch-Delta signaling in neighboring cells (Collier et al., 1996; Chen et al., 2014). Cells that take an ESN fate showed low expression of miR-124 presumably by Notch inhibition, whereas cells that take a PNS fate expressed high levels of miR-124, which in the latter case it was shown to target and repress non-neuronal genes (e.g. neuronal repressors SCP1 and PTBP1) downstream of Notch signaling (Chen et al., 2011). In addition, expression of miR-124 in larval epidermal cells was sufficient for ectopic neural specification, which resembled mis-expression experiments using Pou4, an important transcription factor for sensory neuron specification (Chen et al., 2011; Joyce Tang et al., 2013). Whereas miR-124 targeting to SCP1 is thought to have evolved in the vertebrates+tunicates, miR targeting to PTBP1 may be conserved among bilaterians except for ecdysozoans (Chen et al., 2011) suggesting that the miRNA regulatory logic in lateral inhibition models of Notch-Delta signaling may have broader implications in other organisms yet to be studied (Chen et al., 2014). The research team also showed that miR-124 acted at the gastrula stage and targeted other non-neural genes such as muscle determinant

Macho-1 and notochord determinant Brachyury to allow for ectodermal fate specification (Chen et al., 2011).

3.2.1 Muscle development and the polycistronic miR-1/miR-133 cluster

A well-studied case of miRNA regulation in muscle development is the miR-1/miR-133 polycistronic cluster. Whereas miR-1 promotes differentiation of muscle, miR-133 promotes proliferation of muscle precursors (Chen et al., 2005). In the chordates, these two miRNAs are encoded in an antisense direction in a relatively close localization (3-11 kb apart) within the gene *mind bomb 1* (MIB1), and transcribed as a single primary (i.e. polycistronic) transcript. Except for Drosophila and ambulacrarians (i.e. echinoderms and hemichordates), a close proximity of these two miRNAs has been documented in most animal taxa suggesting some form of functional regulatory constraint of a condensed miR-1/miR-133 cluster for the bilaterians (Campo-Paysaa et al., 2011b). During *C. robusta* development, the polycistronic transcription can be detected in the nuclei of presumptive tail muscle cells from the gastrula stage onward, and its transcription is regulated by an 850 bp sequence upstream of the transcript start site (Kusakabe et al., 2013). Differential expression of the two miRNAs in muscle tissues was only detected in the adult, where body wall muscle expressed similar levels of miR-1 and miR-133 and heart muscle expressed significantly higher levels of miR-1 (Kusakabe et al., 2013).

3.2.2 miRNA expression during oral siphon (OS) regeneration

Three stages of regeneration have been proposed that reconstruct main events of regeneration that match expected miRNAs expression profiles in the corresponding timeframes (Knapp et al., 2013). The three phases correspond to: i. wound Healing, ii. transition, and iii. re-development. The miRNA-mRNA transcriptional profiling using a correlation network, correlated differential expression of mRNAs to miRNA profiles during the three regeneration windows mentioned above in *C. robusta* oral siphon regeneration (Spina et al., 2017). In the first phase, i.e. wound healing, miRNA target clusters of miR 4178b-5p and miR 4_20211 were found to be correlated to the differential expression of genes involved in the following Gene Ontology (GO) term functional classifications: immune response, stress response and apoptosis. In the second phase, i.e. transition, miR 4008c-5p, miR 4123-5p, miR 4178-5p, miR 2_15911, miR 4_20211, and miR 11_7539 were correlated and known to target Wnt, TGF β and MAPK pathway genes that may be regulating the proliferative state characteristic of this particular timeframe. In the third phase, i.e. re-development, miR4008c-5p, miR 10_4533, and miR 11_6940 known to target ECM peptidase inhibitors are correlated with the characteristic extracellular matrix remodeling that occurs at the final phase of regeneration and which resembles the original developmental processes. In contrast other miRs were found expressed throughout the regenerative process. MiRNA miR 10_4533 known to target IGF and

IGFb was found expressed presumably regulating the proliferation of progenitor cells. Also miR-9 was found expressed throughout regeneration and is known to be essential for neural development and function, presumably by targeting and regulating genes involved in cytoskeleton and cell cycle functions (Galderisi et al., 2003; McBeath et al., 2004), instead of targeting Notch or Hes-1 (Spina et al., 2017).

3.2.3 miRNA expression during *O. dioica* development

A most thorough study of the miRNA repertoire expressed during development has been published for the larvacean *O. dioica* (Fu et al., 2008). Using a miRNA array approach with 55 candidate miRNAs and 10 developmental stages for analyses, some general patterns of miRNA occurrence emerged. MicroRNAs were expressed throughout the life cycle of the animal, and were deposited in eggs as maternal determinants for early zygotes. Expression of zygotic miRNAs, such as miR-1487 and miR-1488, was observed starting in the blastula stage (1.5h post fertilization). Most miRNAs analyzed showed developmental regulation (for specific miRNAs that were differentially expressed at each stage see (Fu et al., 2008)), except for some such as miR-1497 that was expressed throughout all stages (Fu et al., 2008). From this study, the first sex specific miRNAs were revealed: miR-1478 was expressed in 6 days old females in the oocytes, whereas miR-1487/88 were expressed in 6 days old males. Interestingly, the compact genomes of *O. dioica* showed one single copy of most miRNA loci, except for miR-1490a, miR-1493, miR-1497d, and miR-1504 that were in two copies (Fu et al., 2008).

4 Other ncRNAs associated with development

4.1 Yellow Crescent RNA

Yellow crescent RNA, i.e. YC RNA, consists of an about 1.2 kb long polyadenylated RNA, which can be present throughout the embryonic development of ascidians (Swalla and Jeffery, 1995). Its name refers to the fact that *in situ* hybridization YC RNA is localized in the yellow crescent region of one-cell zygotes. The YC transcripts are actually already found in the cortex of unfertilized eggs, segregating with the myoplasm to the yellow crescent after fertilization (Swalla and Jeffery, 1995). Subsequently, most YC transcripts enter the primary muscle cell lineage after cleavage and are also present in the secondary muscle cell lineage (Swalla and Jeffery, 1995). YC RNA was first discovered in the club tunicate *Styela clava* (Swalla and Jeffery, 1995). As the presence of the 1.2-kb RNA in oocytes and early cleaving embryos indicates that it is a maternal transcript, YC RNA is considered to be a maternal RNA (Swalla and Jeffery, 1995). It is associated with the cytoskeleton and segregates to the muscle cells during ascidian embryogenesis. Although the YC

ORF encodes for a putative polypeptide of 49 amino acids, this protein is relatively small and does not show any significant homology to any known proteins. As the YC RNA shows various features indicating that it actually functions as an RNA rather than as a protein coding molecule, it is considered to be a noncoding RNA that may play an important role in growth and development (Swalla and Jeffery, 1995).

4.2 MicroRNA-offset RNAs

MicroRNA-offset RNAs, i.e. moRNAs, are about 20 nucleotides long RNAs that lie adjacent to pre-miRNAs. They can originate from both ends of these pre-miRNAs, although prevalently they are derived from the 5' arm (Bortoluzzi et al., 2011). During a study focused on identifying miRNAs in the simple chordate *C. robusta* moRNAs were first discovered (Shi et al., 2009). Unexpectedly, half of the *C. robusta* miRNA loci that were detected in this study turned out to encode the previously uncharacterized small RNAs, in addition to conventional miRNA and miRNA* products. This new class of RNAs was hereafter referred to as ‘moRNAs’, for miRNA-offset RNAs. It became clear that these moRNAs are probably produced by RNase II-like processing and are observed, like miRNAs, at specific developmental stages (Shi et al., 2009). These results and subsequent studies gave rise to the hypothesis that moRNAs represent a new class of functional regulators whose qualitative alteration and/or expression dysregulation might even impact human diseases (Bortoluzzi et al., 2011). Evidence supporting this hypothesis is still fragmentary however. After the discovery in *Ciona*, moRNAs were also found in human cells by deep sequencing analysis. Hereby it was reported that moRNAs from 78 genomic loci were weakly expressed in the prefrontal cortex (Langenberger et al., 2009). Additional indications that moRNA have a distinct function include the fact that some moRNAs are as conserved as miRNAs and are in fact conserved across species to the extent that correlated with expression level (Shi et al., 2009). The expression level of certain moRNAs can even be greater than for their corresponding miRNA (Umbach and Cullen, 2010). Finally, it can be argued (Bortoluzzi et al., 2011) that it is likely that moRNAs might represent a functional class of miRNA-related agents as moRNAs are prevalently produced by the 5' arm of the precursor, independent of which arm produces the most expressed mature miRNA (Langenberger et al., 2009; Umbach and Cullen, 2010). What functions moRNAs may have, varies. For example, moRNA expression was recorded in solid tumours, together with other small RNAs (Meiri et al., 2010). In addition the fact that an 18-fold enrichment of moRNAs was observed in the nucleus (Taft et al., 2010) indicates that at least some moRNAs may have functions related to nuclear processes (Bortoluzzi et al., 2011). Recently, a specific class of moRNAs (moRNA-21) has been associated with post-transcriptional gene regulation, proliferation of vascular smooth muscle cells (VSMC) and mediated gene down-regulation in a process mediated by Ago2 (Zhao et al., 2016). Although these studies do provide good indications, the potential functional roles that moRNAs can play, remain still largely unknown.

4.3 Long Noncoding RNA RMST

Long noncoding RNAs, i.e. lncRNAs, are abundantly found within mammalian transcriptomes. One of the known groups of lncRNAs, includes the rhabdomyosarcoma 2-associated transcript (RMST), which is indispensable for neurogenesis (Ng et al., 2013). Human RMST was shown to be responsible for the modulation of neurogenesis as its expression is regulated by the transcriptional repressor REST while it increases during neuronal differentiation (Ng et al., 2013). Hereby it was found that RMST is actually necessary for the binding of SOX2 to promoter regions of neurogenic transcription factors. SOX2, a transcription factor known to regulate neural fate, in combination with RMST were actually found to coregulate a large pool of downstream genes implicated in neurogenesis, i.e. more than 1 000 genes were differentially expressed upon RMST knockdown (Ng et al., 2013). These results illustrated the role of RMST as a transcriptional coregulator of SOX2 and a key player in the regulation of neural stem cell fate (Ng et al., 2013). A further confirmation of the importance of RMST came with the discovery of a homologue of this lncRNA in the simple chordate *D. vexillum*, i.e. the carpet sea-squirt (Velandia-Huerto et al., 2016). While homologues of “human” lncRNAs are rarely found across all chordates due to their low levels of sequence conservation, a plausible homolog of RMST 9, the conserved region 9 of the Rhabdomyosarcoma 2 associated transcript known for its interaction with SOX2, was found in *D. vexillum*. Subsequently putative homologs were also found in the genomes of the ascidians *C. robusta*, *C. savignyi* and *B. schlosseri* and the Florida lancelet *B. floridae*, illustrating that RMST lncRNA are thus conserved across chordates, making them one of the best conserved lncRNAs known to date (Velandia-Huerto et al., 2016).

4.4 Splices-leader RNA

mRNA 5’leader trans-splicing is a mode of gene expression in which the 5’ end of a pre-mRNA is discarded and replaced by the 5’ segment of a spliced leader (SL) RNA (Vandenbergh et al., 2001). Spliced-Leader RNAs, i.e. SL RNAs, hereby consist of a 5’ exon and a 3’ intron with a conserved consensus 5’ splice donor site at the exon-intron boundary (Ganot et al., 2004). SL RNA trans splicing has not only been described for euglenoids, kinetoplastids, cnidarians, nematodes, and Platyhelminthes (Ganot et al., 2004), but also for deuterostomes like the simple chordate *C. robusta* (Vandenbergh et al., 2001) and the appendicularian *O. dioica* (Ganot et al., 2004). Hereby *O. dioica* was shown to not only trans-splice SL RNAs to mRNAs, as does *C. robusta*, but also to use trans splicing in resolving polycistronic transcripts (Ganot et al., 2004). During trans splicing, the capped SL RNA exon moiety is covalently linked to the 5’ ends of mRNAs, forming a leader sequence ranging from 16 nt in *C. robusta* to 41 nt in trypanosomatids (Ganot et al., 2004). The role of SL trans-splicing is still unknown in many cases. SL trans-splicing may potentially having functions varying from the mediation of mRNA stability or translatability (Maroney

et al., 1995) and the resolution of polycistronic pre-mRNAs (Agabian, 1990; Blumenthal, 1995), to the production of functional mRNAs from RNA polymerase I transcripts (Lee and der Ploeg, 1997).

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