Identifying small RNAs derived from maternal- and somatic-type rRNAs in Zebrafish Development

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Abstract (200 words)

rRNAs are non-coding RNAs present in all prokaryotes and eukaryotes. In eukaryotes there are four rRNAs: 18S, 5.8S, 28S, originating from a common precursor (45S), and 5S. We have recently discovered the existence of two distinct developmental types of rRNA: a maternal-type, present in eggs and a somatic-type, expressed in adult tissues.

Lately, next-generation sequencing has allowed the discovery of new small-RNAs deriving from longer non-coding RNAs, including small-RNAs from rRNAs (srRNAs). Here, we systemically investigated srRNAs of maternal- or somatic-type 18S, 5.8S, 28S, with small-RNAseq from many zebrafish developmental stages.

We identified new srRNAs for each rRNA. For 5.8S, we found srRNA consisting of the 5' or 3' halves, with only the latter having different sequence for the maternal- and somatic-types. For 18S, we discovered 21nt srRNA from the 5' end of the 18S rRNA with a striking resemblance to microRNAs; as it is likely processed from a stem-loop precursor and present in human and mouse Argonaute-complexed small-RNA. For 28S, an abundant 80nt srRNA from the 3' end of the 28S rRNA was found. The expression levels during embryogenesis of these srRNA indicate they are not generated from rRNA degradation and might have a role in the zebrafish development.

Keywords: Ribosomal RNA, Small-rRNA derived, embryogenesis, zebrafish, development

Introduction

Several new classes of small non-coding RNAs have been discovered in the wake of the next-generation sequencing (NGS) revolution (Wittmann and Jäck 2010). This has fueled interest in small-RNAs derived from other non-coding RNAs, such as microRNA (miRNA) (Li et al. 2009), transfer RNA (tRNA) (Lee et al. 2009b), small nucleolar RNA (snoRNA) (Taft et al. 2009; Martens-Uzunova et al. 2013) and ribosomal RNA (rRNA) (Wei et al. 2013).

rRNAs are the predominant components of ribosomes. In eukaryotes there are four different rRNAs: 5S, 18S, 5.8S, and 28S. The genes coding for these rRNAs, often referred to as rDNA, are differently organized: 18S, 5.8S and 28S genes are in the same transcriptional unit, the 45S rDNA, which is present as tandem repeats in a genome (Prokopowich et al. 2003), whereas 5S genes are organized in clusters of tandem repeats separated by small non-transcribed spacers (NTS) (Ciganda and Williams 2011).

It has often been assumed that short reads mapping to rRNAs in whole-transcriptome sequencing experiments are a byproduct of RNA-degradation. Nevertheless, there is mounting evidence that small reads mapping to rRNAs represent stable and functional molecules. First, deep-sequencing studies have shown that small rRNA-derived RNAs (srRNAs) originate from a specific process that favors the formation of fragments from the 5' and/or 3' termini of the full-length rRNA (Li et al. 2012). Moreover, srRNAs seem to have a role during the response to DNA damage and stress (Lee et al. 2009a; Chen et al. 2013) and they resemble small interfering RNA (siRNA) and miRNA in structure and function, like binding to Argonaute (AGO) proteins (Castellano and Stebbing 2013; Zheng et al. 2014; Chak et al. 2015; Yoshikawa and Fujii 2016).

We have recently shown that in zebrafish, a well-studied and versatile model organisms (Nüsslein-Volhard and Dham 2002), all rRNAs (5S, 5.8S, 18S and 28S) have developmentally-regulated sequence variants, named maternal- and somatic-type (Locati et

al. 2017a, 2017b). Maternal-type rRNA, which makes up all the rRNA in mature oocytes, is replaced by somatic-type rRNA during embryogenesis, until exclusive somatic-type rRNA expression in adult tissue. These two rRNA types contain ample variations in their primary and secondary structures, which likely leads to different processing, diverse ribosomal protein binding and type-specific interactions with different mRNAs (Locati et al. 2017b). Given this particular developmental-specific expression of rRNA types in zebrafish, in this study we investigated the occurrence of associated 5.8S, 18S and 28S srRNAs during zebrafish development. We identified several new putative srRNAs and discuss their possible biological role.

Materials and Methods

Biological materials, RNA-isolation, small-RNA-seq

We used: i) Three pools of unfertilized eggs (oocytes); ii) one embryo at each of the 12 developmental stages: 64 cells (2 hours post-fertilization); high stage (3.3 hpf); 30% epiboly stage (4.7 hpf); 70% epiboly stage (7 hpf); 90% epiboly stage (9 hpf); 4-somite stage (11.3 hpf); 12-somite stage (15 hpf); 22-somite stage (20 hpf); prim-5 stage (24 hpf); prim-16 (31 hpf); long-pec stage (48 hpf); protruding-mouth stage (72 hpf), and iii) one whole–body male-adult zebrafish sample. The harvesting of the biological materials, RNA-isolation, and small-RNA sequencing have been described in detail previously (Locati et al. 2017a, 2017b)

Bioinformatics

Mapping

Reads <131 nt were mapped against the zebrafish 5.8S, 18S, 28S maternal- and somatic-type sequences with Bowtie2 (Langmead and Salzberg 2012) using default settings for reads between 20 nt and 131 nt, while for reads shorter than 20 nt the setting --score-min was set to L,-1,0.

RNA structures

Secondary RNA structures were predicted using the RNA-Folding Form in the mfold web-server (http://www.bioinfo.rpi.edu/applications/mfold, (Zuker 2003)) with standard settings.

AGO-complexed small-RNA pool analysis

The sequences of the miRNA- and miRNA*-like 18S srRNAs were searched through Fastq files of high-throughput sequencing of RNAs isolated by crosslinking-immunoprecipitation (HITS-CLIP), from mouse brains (Chi et al. 2009) and THP-1 cells (Burroughs et al. 2011).

Target Prediction and Ontology Analysis.

Putative targets of the 18S miRNA-like srRNA were predicted with miRanda using default settings (Enright et al. 2003). To limit identification of potential false positives we chose an arbitrary paring-score cutoff of ≥150 and an energy cutoff of ≤ -20. Categorization of putative target genes in Gene Ontology (GO) Biological Process (BP) terms was accomplished by using DAVID 6.8 web-service (https://david.ncifcrf.gov/home.jsp) (Huang et al. 2009) and discarding results with p-value >0.05.

Availability of data and material

All sequencing data are accessible through the BioProject database under the project accession number PRJNA347637 (www.ncbi.nlm.nih.gov/bioproject).

Results and Discussion

To systematically investigate srRNAs in zebrafish development, we applied an adapted small-RNA-seq approach to RNA from an egg pool and a whole-body adult-male sample.

With the knowledge that virtually all expressed rRNA in zebrafish eggs originates from maternal-type, whereas in adult tissues this is from somatic-type (Locati et al. 2017b), we mapped the reads from the egg pools (51 M reads) and three whole-body adult-male samples (40 M reads) to respectively maternal-type and somatic-type 5.8S, 18S and 28S rRNA. We focused on RNAs transcribed from the 45S rDNA, given the limitations to reliably sequence 5S rRNA with standard NGS protocols (Locati et al. 2017a). For RNA molecules to be considered potential srRNAs, we applied an arbitrary upper size limit of 131 nucleotides and assumed that, by absence of RNA-fragmentation in the small-RNA-seq protocol, every read represents an actual complete RNA molecule.

Small 5.8S rRNA-derived RNAs

The length distribution of the sequencing reads mapped to 5.8S rRNA showed two peaks at 75-76 nt and 83 nt for the maternal-type (= egg sample) and 74 nt and 81 nt for the somatic-type (= adult-male sample) (Figure 1A). Analysis of the 20 most abundant 5.8S srRNA sequences (Supplementary File A) shows that these peaks originate from two 5.8S fragments that roughly correspond to the 5.8S rRNA 5' and 3' halves, which are likely generated from a single cut in the 5.8S rRNA molecule (Figure 2A). The cutting-site lies in a loop and is exactly at the location where the maternal-type sequence has an AC insertion as compared to the somatic-type (Figure 2A). This is similar to the known tRNA halves, where a riboendonuclease cuts within the tRNA anticodon loop thus producing tRNA 5' and 3' halves (Anderson and Ivanov 2014; Dhahbi 2015).

The 5' and 3' halves resulting from the 5.8S rRNA cut display rather strong secondary structures, showing long stable stems (Figure 2B), which may explain their relative read abundance. While the sequence of the 5.8S rRNA 5' halves is the same between maternal- and somatic-type, the 3' halves contain some differences: these, however, do not alter their

secondary structure, since the differences are either in the loops or those in the stem regions seem compensated by coevolution (Figure 2B).

These conserved secondary structures of the 5.8 srRNAs may be useful in ribosome degradation to separate 5.8S rRNA from 28S rRNA. In mature ribosomes, 5.8S rRNA interacts with 28S rRNAs in at least three regions (Anger et al. 2013). Once the 5.8S rRNA is cut, the 5' srRNA only has two 28S rRNA binding regions and the 3' srRNA one. The self-binding secondary structure of both srRNA halves might enhance separation from the 28S rRNA. (Figure 2C). It is unclear if and what function these specific 5.8 srRNAs might have. Following the presence of 5.8S rRNA halves throughout embryogenesis, we observed that their relative presence is almost equal (Supplementary File Ba), whereas, in eggs and in adult tissues the 5.8S 5' half srRNA is over ~3 and 4 times more abundant than the 3' half srRNA, respectively, which may indicate that the 5' half srRNA is more stable. Moreover, it is worth noting that the somatic-type 3' half srRNA is detected only from the latest embryonic stage, even though the somatic-type 5.8S rRNA expression starts from the 90% epiboly stage (Supplementary File Ba). This means that although there is a lot of complete somatic-type 5.8S rRNA present, no processing via 5.8S srRNA seems to occur. Similarly, although maternal-type 5.8S rRNA is degraded during the late stages of embryogenesis, the level of 5.8S srRNA is relatively unaffected, suggesting these srRNAs are not a byproduct of normal 5.8S rRNA degradation.

Small 18S rRNA-derived RNAs

Both maternal- and somatic-type 18S srRNAs show a wide range of small fragments all present in a non-distinct distribution, with the exception of a miRNA-sized distribution peak (21 nt) in maternal-type srRNA (Figure 1B). In somatic-type srRNA this distribution peak is present at a markedly lower relative abundance. The most abundant (29%) potential maternal-type srRNA is indeed a 21 nt fragment (Supplementary File A), derived from the

utmost 5' end of the 18S rRNA (Supplementary File C). For somatic-type rRNA the most abundant (8%) 18S rRNA is the 130 nt fragment at the utmost 5' end of the 18S rRNA (Supplementary File A). We believe that the 130 nt fragment is the precursor of the 21 nt sequence because the 21 nt is a subsequence of the 130 nt sequence from the 5' of the mature 18S rRNA. Furthermore a relative high percentage 21 nt reads is present with a low percentage 130 nt in the egg sample, whereas in the adult sample a relatively low percentage 21 nt reads is present with a relatively high percentage of 130 nt reads (Figure 1B).

To substantiate this, we assessed the ability of both the maternal- and somatic-type (which differ only in 2 nucleotides) of this srRNA to form a stem-loop structure, similar to the ability of other non-coding RNAs, such as tRNAs and snoRNAs, to function as non-canonical precursor for the biogenesis of miRNAs (Scott et al. 2009; Scott and Ono 2011; Garcia-Silva et al. 2012; Martens-Uzunova et al. 2013; Abdelfattah et al. 2014). In one of the predicted structures from the *in silico* analysis, the 130 nt srRNA has a secondary structure consisting of a stem and a complex hinge with three smaller hairpins (Supplementary File Da) both for maternal- and somatic-type srRNA. The observed 21nt srRNA maps to 5' strand of the stem (Supplementary File Da and Figure 3), similar to where a miRNA originates from its precursor (Berezikov 2011). During miRNA-processing, one strand of the stem is preferentially selected for entry into a silencing complex (guide strand), whereas the other strand, known as the passenger strand or miRNA* strand, is usually degraded. As strand selection is not completely strict, miRNA* can also be present, albeit at a lower frequency, and be active in silencing (Ha and Kim 2014). We were able to detect the 3' strand of the stem in both samples, yet at a very low relative abundance (Supplementary File Db). In order to evaluate these miRNA-like srRNAs we analyzed whether they could bind to the Argonaute protein (AGO) as happens in the RNA interference (RNAi) silencing pathways. For this we analyzed the occurrence of identical rRNA sequences in the previously published AGO-

complexed small-RNA pool of other model organisms (Chi et al. 2009; Burroughs et al. 2011). Both the guide and passenger strand were detected in the small-RNA pool that co-immunoprecipitated with AGO in mouse and human samples, indicating that this sequence can bind to AGO, thus suggesting that this 21 nt srRNA may behave like a miRNA in gene regulation (Jonas and Izaurralde 2015) .

Through zebrafish development, this miRNA-like srRNA shows higher presence in egg and the 64-cell stage (2 hpf) and from then on is relatively low (Supplementary File B). Interestingly the relatively high presence of the non-canonical precursor in adult is not associated with higher miRNA-like srRNA presence.

To investigate targets of this miRNA-like srRNA, we used the miRanda algorithm (Enright et al. 2003) and obtained 532 putative target transcripts (Supplementary File Ea). After their classification in Gene Ontology (GO) Biological Process, it is worth noting that amongst the most statistically significant over-represented GO Biological Process terms there are several involved in embryogenesis, such as: embryonic morphogenesis, gastrulation, heart development and embryonic organ development (Supplementary File Eb).

Small 28S rRNA-derived RNAs

There is a clear peak at 80 nt in the length distribution of the sequencing reads mapped to 28S rRNA in both maternal- (35%) and somatic-type (7%) RNA (Figure 1C). This peak is essentially composed of srRNA that corresponds to the most 3' part of the 28S rRNA molecule (Supplementary File A and Supplementary File C). Five nucleotides differ between the maternal- and somatic-type 3' 28S srRNA (Figure 4).

As part of 28S rRNA, this sequence can form a stem-loop structure (Figure 4). Thus, this 3' srRNA can also reverse-complement bind to the 3' end of another complete 28S rRNA molecule (Figure 4 and Supplementary File F). As such, it may provide a protective hairpin, which could be part of a (short) feedback loop for 28S rRNA-degradation.

Relative presence of this 80 nt sRNA is substantially higher in egg and adult tissue compared to other embryonic stages (Supplementary File Bc). The somatic-type 28S 3' srRNA is detected only in adult tissues (Supplementary File Bc), similarly to the somatic-type 5.8S 3' half srRNA.

Conclusion

Taken together, our results show that 5.8S, 18S, and 28S rRNA genes each produce one or more srRNAs. These srRNAs are present during zebrafish development and most appear not to be generated during degradation of the associated complete rRNAs. Besides, the degradation rate of mature cytoplasmic rRNAs is generally undetectable in normal condition (Houseley and Tollervey 2009), as the rRNA is first fragmented by endoribonucleases and then the resulting fragments are rapidly degraded to mononucleotides by exoribonucleases (Basturea et al. 2011; Sulthana et al. 2016); this implies that the srRNAs we observe are likely stable products and not the result of the regular cellular ribosome turnover. Moreover, although their biological significance remains obscure, some srRNA could have a role in rRNA processing/degradation and in miRNA-like pathways.

Funding

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Competing interests

The authors declare that they have no competing interests

List of abbreviations

NGS: next-generation sequencing

srRNA: small rRNA-derived RNA
miRNA: microRNA
tRNA: transfer RNA
snoRNA: small nucleolar RNAs
rRNA: ribosomal RNA
rDNA: genes coding for rRNAs
NTS: non-transcribed spacers
tRFs: tRNA fragments
siRNA: small interfering RNA
hpf: hours post fertilization
GO: Gene ontology
BP: Biological Process
AGO: Argonaute protein
RNAi: RNA interference

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368 **Figure legends**

369

370 **Figure 1. sRNA-seq read length distribution in zebrafish.**

371 Bar plots showing the relative abundance of sRNA-seq read lengths (A: 5.8S rRNA; B: 18S

372 rRNA; C: 28S rRNA) in zebrafish eggs (blue) and adult-male whole-body (red).

373

374 **Figure 2. Structure and function of the 5.8S "half" srRNAs.**

375 A. Putative secondary structure for maternal-type 5.8S rRNA (Petrov et al. 2014) with the

376 associated srRNAs halves highlighted in yellow (5' half srRNA) and green (3' half srRNA).

377 The sequence differences from somatic-type 5.8S rRNA are shown as coloured circles (red =

378 insertion; blue = substitution).

379 B. Putative secondary structure of maternal- and somatic-type 5' half srRNA (5.8S srRNA

380 5'), maternal-type 3' half srRNA (5.8S srRNA M 3'), and somatic-type 3' half srRNA (5.8S

381 srRNA S 3'). Sequence differences between maternal- and somatic-type 3' half srRNAs are

382 highlighted in blue (5.8S srRNA M 3') or red (5.8S srRNA S 3').

383 C. Proposed processing of the 5.8S half srRNAs: a putative riboendonuclease cuts 5.8S rRNA

384 in the loop, leading to the release of the 5.8S half srRNAs, which cannot interact with 28S

385 rRNA anymore, due to their secondary structures.

386 The thick black segments in the 28S rRNA lines indicate the interaction sites with 5.8S rRNA

387 (Petrov et al. 2014).

388

389 **Figure 3. Proposed 18S miRNA-like srRNA biogenesis.**

390 A fragment of ~130 nt at the utmost 5' end of the 18S rRNA is cut and it folds into a stem-

391 loop structure. As a potential non-canonical miRNA precursor it may be further processed

and the stem can be loaded into an Argonaute protein. Only one strand is preferentially selected (purple) to behave like a miRNA, while the other is usually degraded (grey).

Figure 4. Structure of the interactions between the 80 nt 28S srRNA and the mature 28S rRNA.

The 80 nt srRNA (green) originates from the utmost 3' part of the 28S rRNA (grey). It can interact with the 3' region of the 28S rRNA forming a strong stem structure (Supplementary File E).

Supplementary Files

gen-2017-0202Suppla.xlsx: 20 most abundant 5.8S, 18S and 28S srRNA sequences.

gen-2017-0202Supplb.pdf: Presence of srRNAs during zebrafish development.

gen-2017-0202Supplc.pdf: srRNAs read abundance over the length of mature rRNAs.

gen-2017-0202Suppld.pdf: Structure and presence of examined 18S srRNAs.

gen-2017-0202Supple.xlsx: Analysis of the putative 18S miRNA-like srRNA targets

gen-2017-0202Supplf.pdf: Structure of the interactions between mature 28S and the examined 28 srRNA.

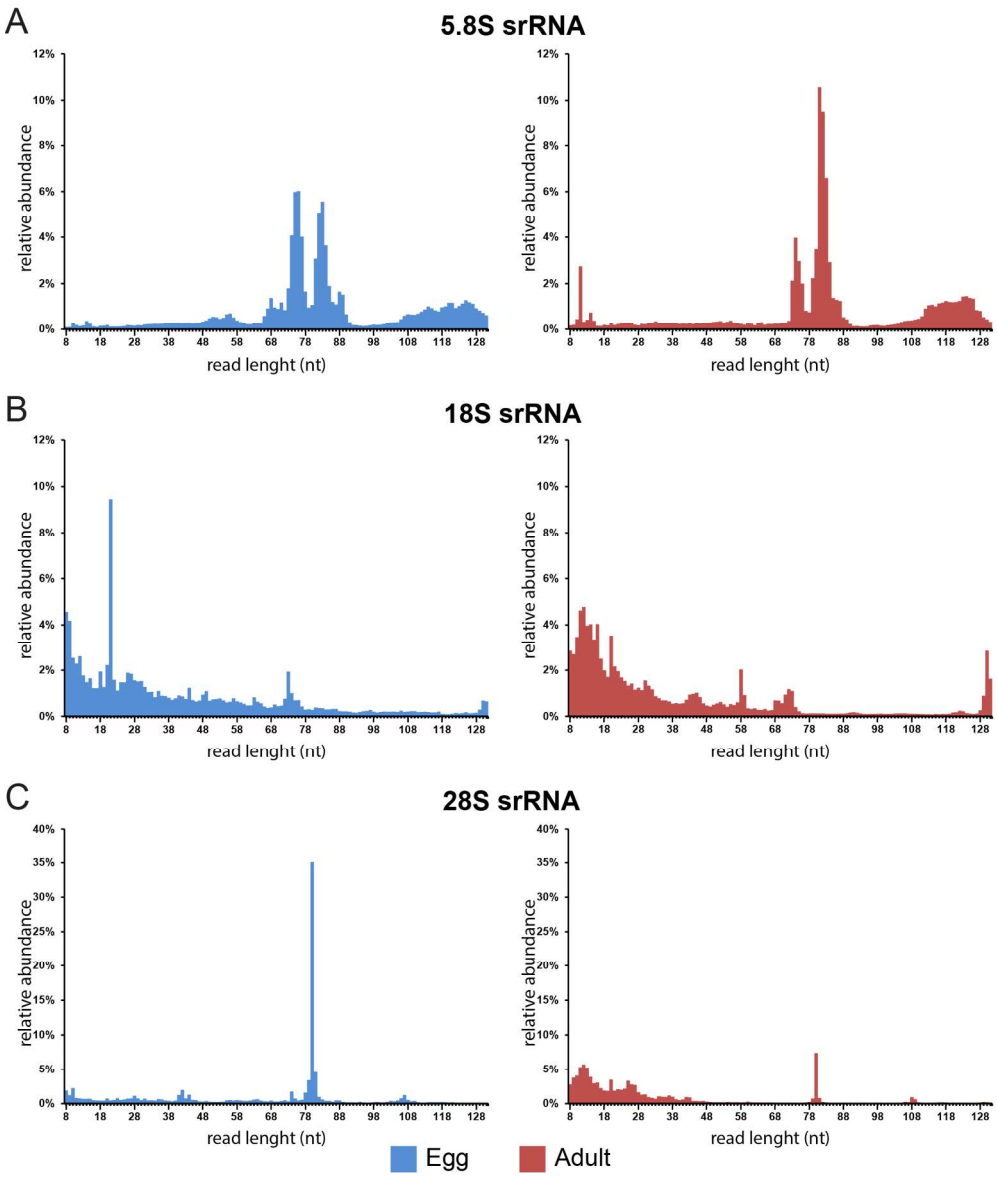

Figure 1. sRNA-seq read length distribution in zebrafish.

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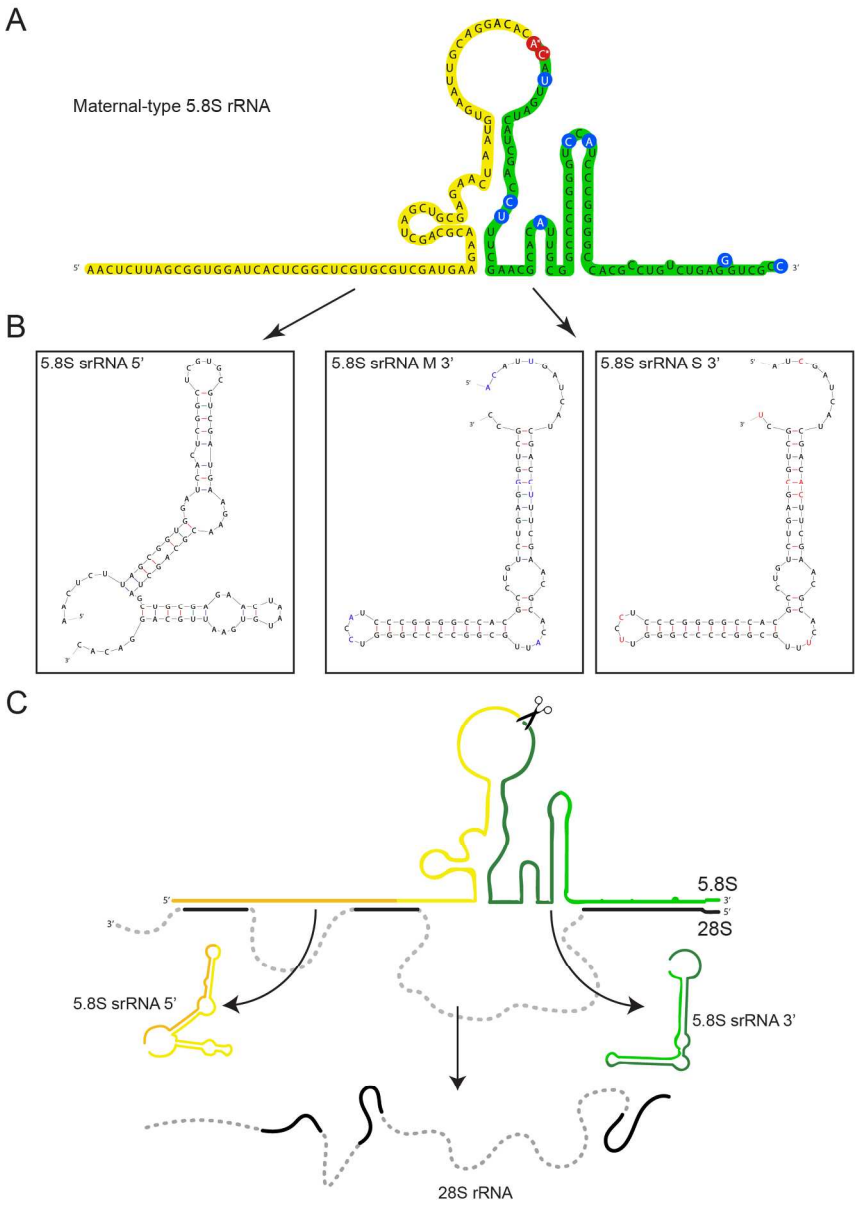

Figure 2. Structure and function of the 5.8S "half" srRNAs.

163x230mm (300 x 300 DPI)

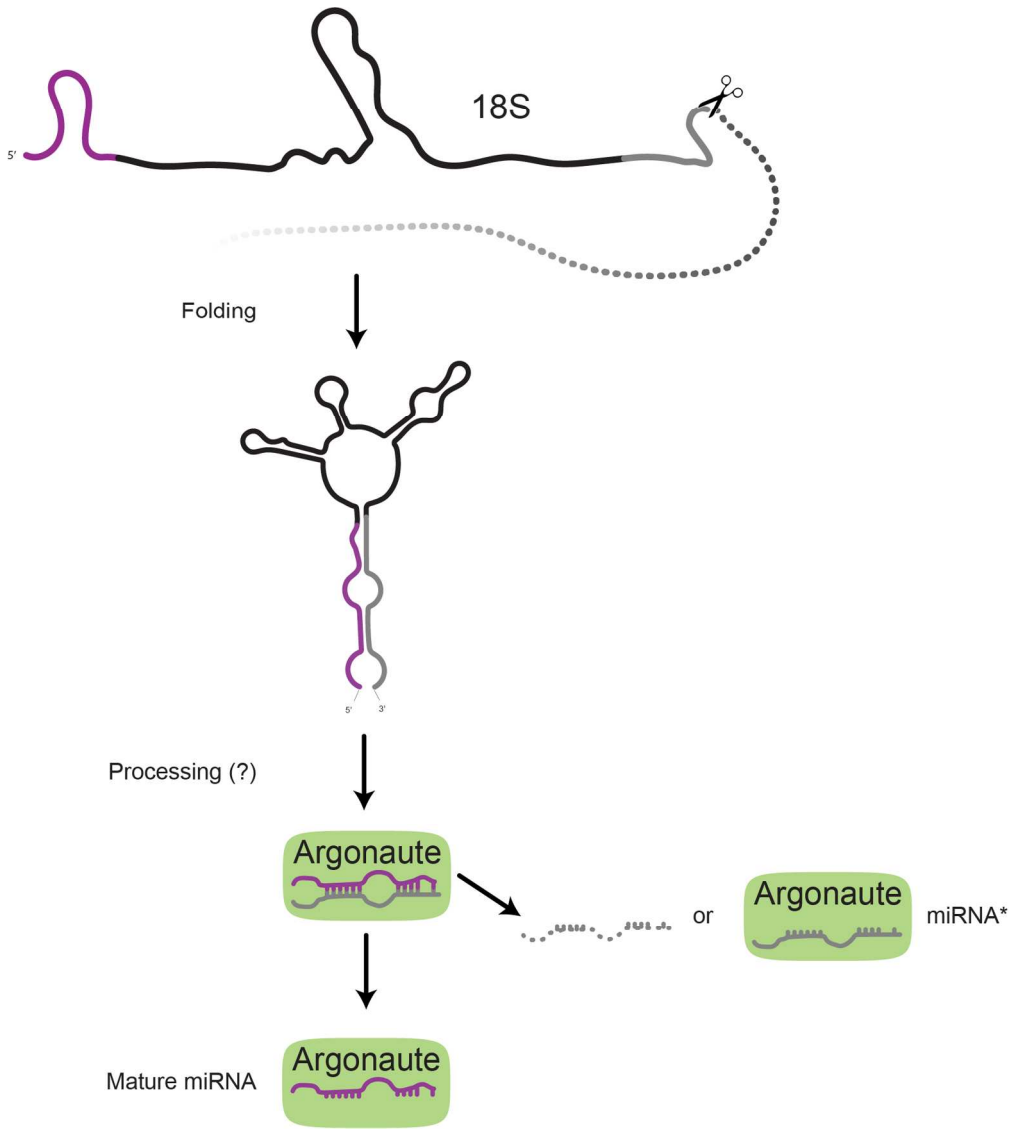

Figure 3. Proposed 18S miRNA-like srRNA biogenesis.

153x172mm (300 x 300 DPI)

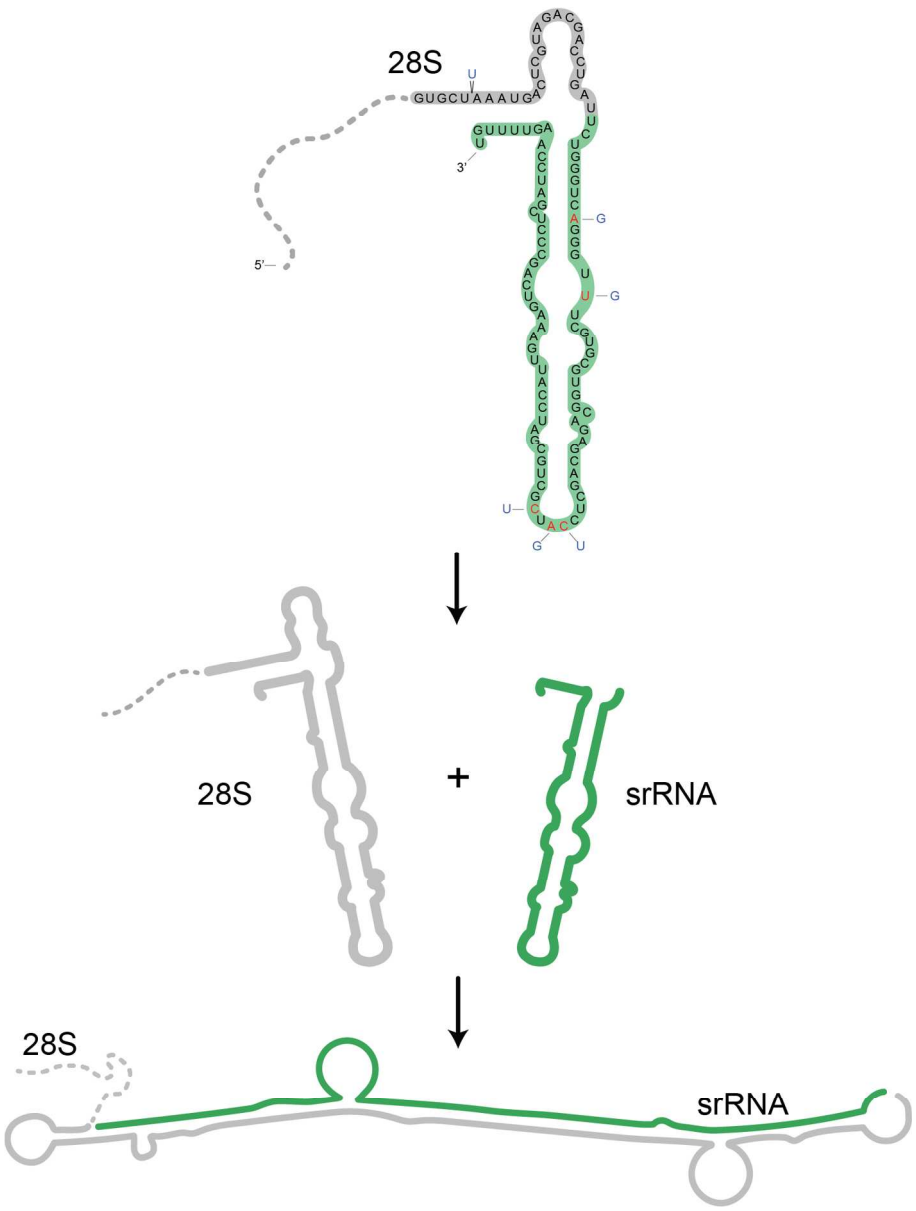

Figure 4. Structure of the interactions between the 80 nt 28S srRNA and the mature 28S rRNA.

144x190mm (300 x 300 DPI)