

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

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27 **Abstract (200 words)**

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

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

36 We identified new srRNAs for each rRNA. For 5.8S, we found srRNA consisting of the 5' or
37 3' halves, with only the latter having different sequence for the maternal- and somatic-types.
38 For 18S, we discovered 21nt srRNA from the 5' end of the 18S rRNA with a striking
39 resemblance to microRNAs; as it is likely processed from a stem-loop precursor and present
40 in human and mouse Argonaute-complexed small-RNA. For 28S, an abundant 80nt srRNA
41 from the 3' end of the 28S rRNA was found. The expression levels during embryogenesis of
42 these srRNA indicate they are not generated from rRNA degradation and might have a role in
43 the zebrafish development.

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

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50 **Introduction**

51 Several new classes of small non-coding RNAs have been discovered in the wake of the next-
52 generation sequencing (NGS) revolution (Wittmann and Jäck 2010). This has fueled interest
53 in small-RNAs derived from other non-coding RNAs, such as microRNA (miRNA) (Li et al.
54 2009), transfer RNA (tRNA) (Lee et al. 2009b), small nucleolar RNA (snoRNA) (Taft et al.
55 2009; Martens-Uzunova et al. 2013) and ribosomal RNA (rRNA) (Wei et al. 2013).

56 rRNAs are the predominant components of ribosomes. In eukaryotes there are four different
57 rRNAs: 5S, 18S, 5.8S, and 28S. The genes coding for these rRNAs, often referred to as
58 rDNA, are differently organized: 18S, 5.8S and 28S genes are in the same transcriptional
59 unit, the 45S rDNA, which is present as tandem repeats in a genome (Prokopowich et al.
60 2003), whereas 5S genes are organized in clusters of tandem repeats separated by small non-
61 transcribed spacers (NTS) (Ciganda and Williams 2011).

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

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

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

84

85 **Materials and Methods**

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

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

94 **Bioinformatics**

95 *Mapping*

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

100 *RNA structures*

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

103 *AGO-complexed small-RNA pool analysis*

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

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108 *Target Prediction and Ontology Analysis.*

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

115 **Availability of data and material**

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

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120 **Results and Discussion**

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

123 With the knowledge that virtually all expressed rRNA in zebrafish eggs originates from
124 maternal-type, whereas in adult tissues this is from somatic-type (Locati et al. 2017b), we
125 mapped the reads from the egg pools (51 M reads) and three whole-body adult-male samples
126 (40 M reads) to respectively maternal-type and somatic-type 5.8S, 18S and 28S rRNA. We
127 focused on RNAs transcribed from the 45S rDNA, given the limitations to reliably sequence
128 5S rRNA with standard NGS protocols (Locati et al. 2017a). For RNA molecules to be
129 considered potential srRNAs, we applied an arbitrary upper size limit of 131 nucleotides and
130 assumed that, by absence of RNA-fragmentation in the small-RNA-seq protocol, every read
131 represents an actual complete RNA molecule.

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133 **Small 5.8S rRNA-derived RNAs**

134 The length distribution of the sequencing reads mapped to 5.8S rRNA showed two peaks at
135 75-76 nt and 83 nt for the maternal-type (= egg sample) and 74 nt and 81 nt for the somatic-
136 type (= adult-male sample) (Figure 1A). Analysis of the 20 most abundant 5.8S srRNA
137 sequences (Supplementary File A) shows that these peaks originate from two 5.8S fragments
138 that roughly correspond to the 5.8S rRNA 5' and 3' halves, which are likely generated from a
139 single cut in the 5.8S rRNA molecule (Figure 2A). The cutting-site lies in a loop and is
140 exactly at the location where the maternal-type sequence has an AC insertion as compared to
141 the somatic-type (Figure 2A). This is similar to the known tRNA halves, where a
142 riboendonuclease cuts within the tRNA anticodon loop thus producing tRNA 5' and 3' halves
143 (Anderson and Ivanov 2014; Dhahbi 2015).

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

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

150 These conserved secondary structures of the 5.8 srRNAs may be useful in ribosome
151 degradation to separate 5.8S rRNA from 28S rRNA. In mature ribosomes, 5.8S rRNA
152 interacts with 28S rRNAs in at least three regions (Anger et al. 2013). Once the 5.8S rRNA is
153 cut, the 5' srRNA only has two 28S rRNA binding regions and the 3' srRNA one. The self-
154 binding secondary structure of both srRNA halves might enhance separation from the 28S
155 rRNA. (Figure 2C). It is unclear if and what function these specific 5.8 srRNAs might have.

156 Following the presence of 5.8S rRNA halves throughout embryogenesis, we observed that
157 their relative presence is almost equal (Supplementary File Ba), whereas, in eggs and in adult
158 tissues the 5.8S 5' half srRNA is over ~3 and 4 times more abundant than the 3' half srRNA,
159 respectively, which may indicate that the 5' half srRNA is more stable. Moreover, it is worth
160 noting that the somatic-type 3' half srRNA is detected only from the latest embryonic stage,
161 even though the somatic-type 5.8S rRNA expression starts from the 90% epiboly stage
162 (Supplementary File Ba). This means that although there is a lot of complete somatic-type
163 5.8S rRNA present, no processing via 5.8S srRNA seems to occur. Similarly, although
164 maternal-type 5.8S rRNA is degraded during the late stages of embryogenesis, the level of
165 5.8S srRNA is relatively unaffected, suggesting these srRNAs are not a byproduct of normal
166 5.8S rRNA degradation.

167 **Small 18S rRNA-derived RNAs**

168 Both maternal- and somatic-type 18S srRNAs show a wide range of small fragments all
169 present in a non-distinct distribution, with the exception of a miRNA-sized distribution peak
170 (21 nt) in maternal-type srRNA (Figure 1B). In somatic-type srRNA this distribution peak is
171 present at a markedly lower relative abundance. The most abundant (29%) potential
172 maternal-type srRNA is indeed a 21 nt fragment (Supplementary File A), derived from the

173 utmost 5' end of the 18S rRNA (Supplementary File C). For somatic-type rRNA the most
174 abundant (8%) 18S rRNA is the 130 nt fragment at the utmost 5' end of the 18S rRNA
175 (Supplementary File A). We believe that the 130 nt fragment is the precursor of the 21 nt
176 sequence because the 21 nt is a subsequence of the 130 nt sequence from the 5' of the mature
177 18S rRNA. Furthermore a relative high percentage 21 nt reads is present with a low
178 percentage 130 nt in the egg sample, whereas in the adult sample a relatively low percentage
179 21 nt reads is present with a relatively high percentage of 130 nt reads (Figure 1B).

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

198 complexed small-RNA pool of other model organisms (Chi et al. 2009; Burroughs et al.
199 2011). Both the guide and passenger strand were detected in the small-RNA pool that co-
200 immunoprecipitated with AGO in mouse and human samples, indicating that this sequence
201 can bind to AGO, thus suggesting that this 21 nt srRNA may behave like a miRNA in gene
202 regulation (Jonas and Izaurralde 2015) .

203 Through zebrafish development, this miRNA-like srRNA shows higher presence in egg and
204 the 64-cell stage (2 hpf) and from then on is relatively low (Supplementary File B).

205 Interestingly the relatively high presence of the non-canonical precursor in adult is not
206 associated with higher miRNA-like srRNA presence.

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

213 **Small 28S rRNA-derived RNAs**

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

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

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

227 **Conclusion**

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

238

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242

243 **Competing interests**

244 The authors declare that they have no competing interests

245 **List of abbreviations**

246 NGS: next-generation sequencing

247 srRNA: small rRNA-derived RNA
248 miRNA: microRNA
249 tRNA: transfer RNA
250 snoRNA: small nucleolar RNAs
251 rRNA: ribosomal RNA
252 rDNA: genes coding for rRNAs
253 NTS: non-transcribed spacers
254 tRFs: tRNA fragments
255 siRNA: small interfering RNA
256 hpf: hours post fertilization
257 GO: Gene ontology
258 BP: Biological Process
259 AGO: Argonaute protein
260 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

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

394

395 **Figure 4. Structure of the interactions between the 80 nt 28S srRNA and the mature**
396 **28S rRNA.**

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

400 **Supplementary Files**

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

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

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

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

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

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

408

409

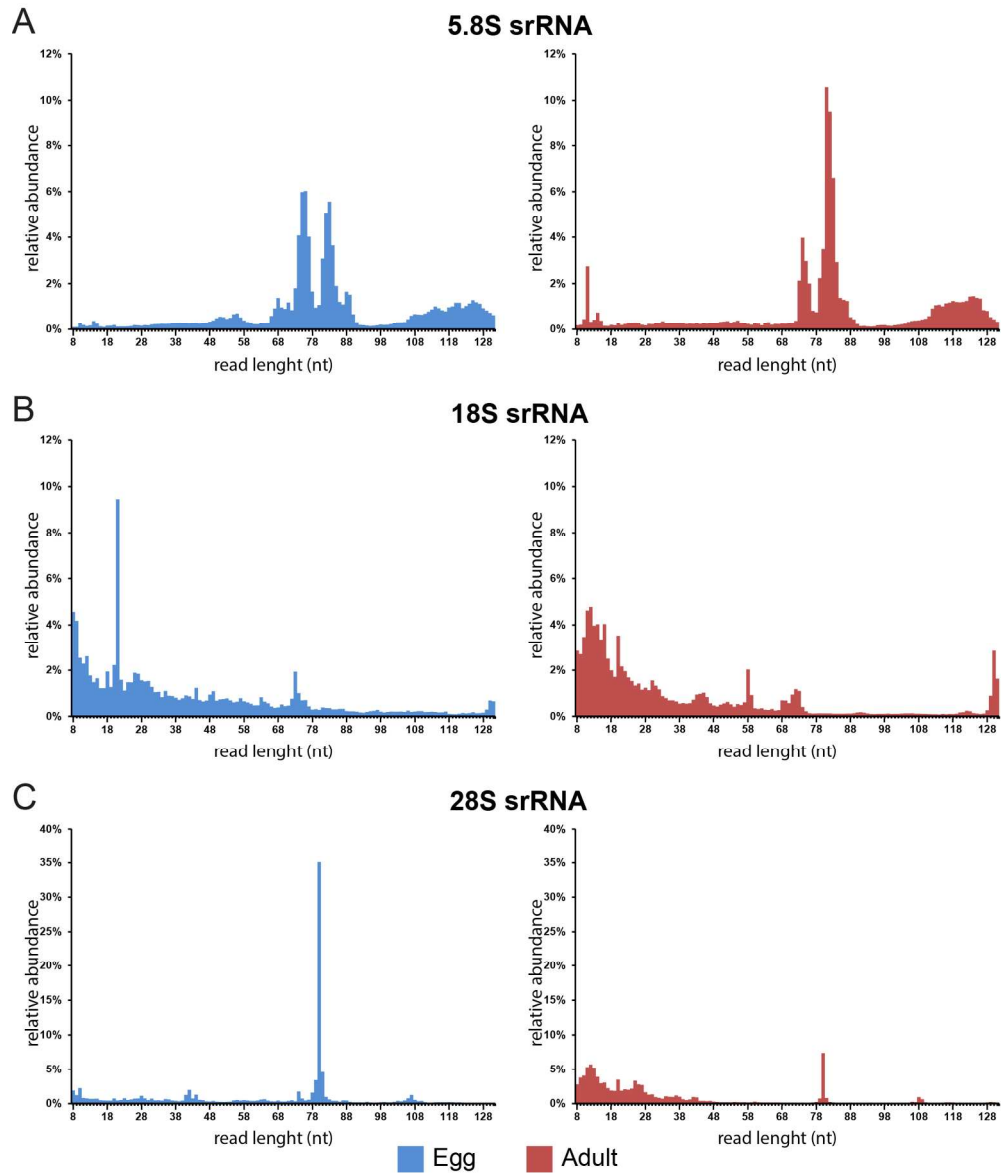


Figure 1. sRNA-seq read length distribution in zebrafish.

538x629mm (96 x 96 DPI)

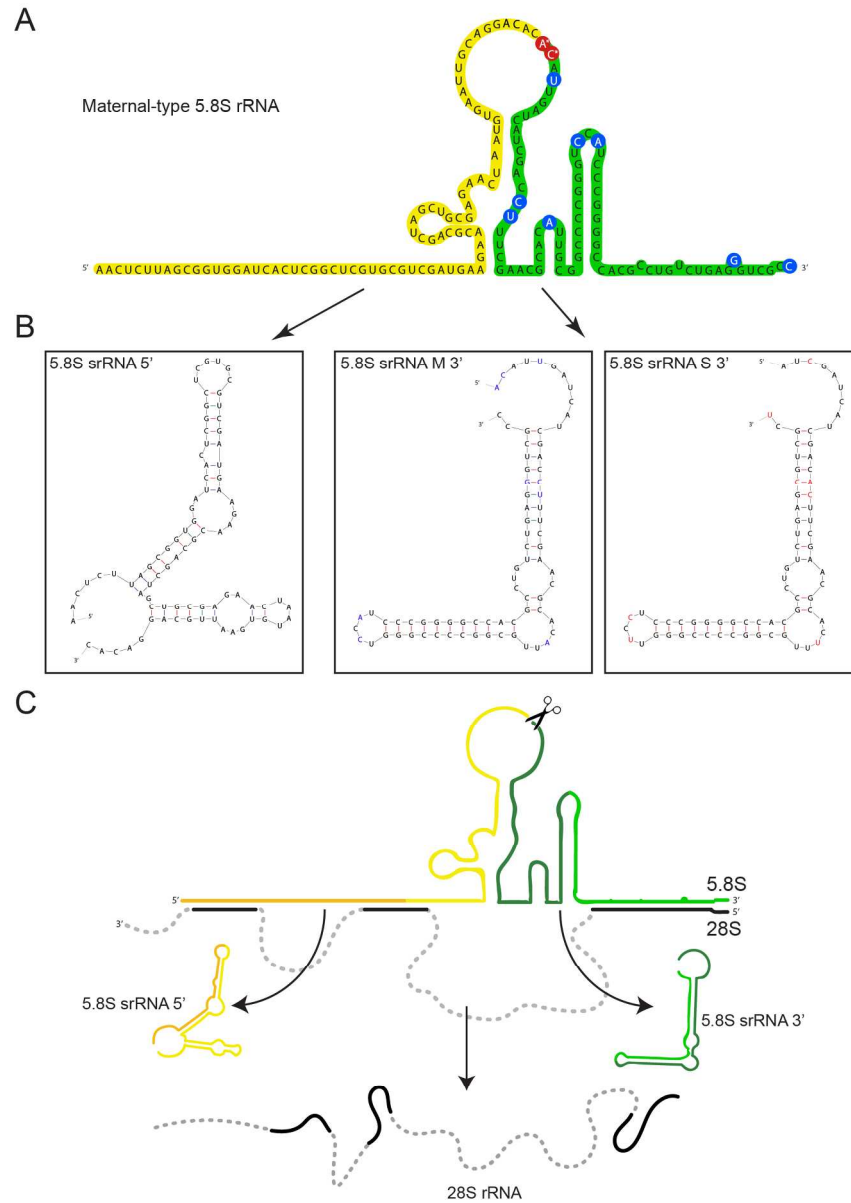


Figure 2. Structure and function of the 5.8S "half" srRNAs.

163x230mm (300 x 300 DPI)

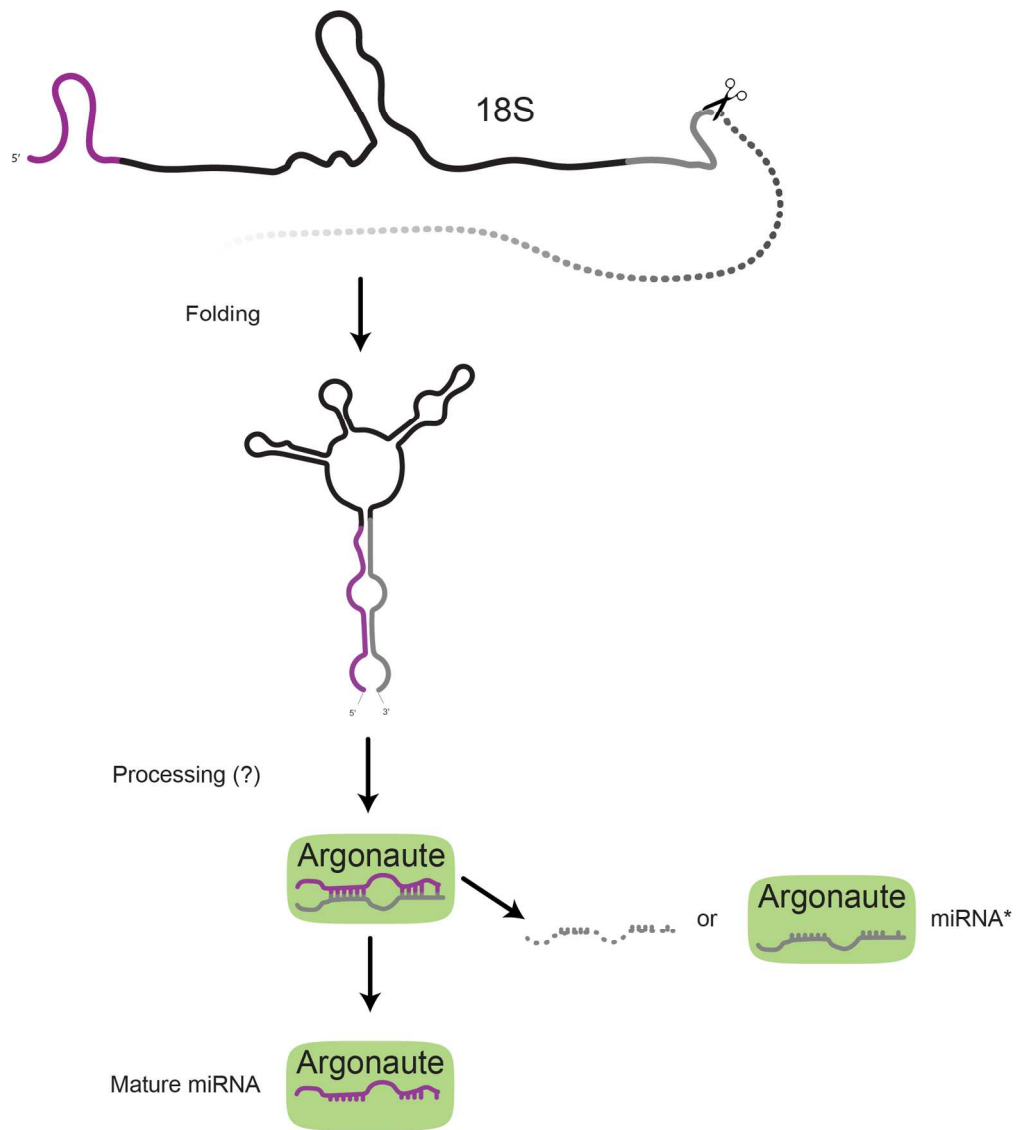


Figure 3. Proposed 18S miRNA-like srRNA biogenesis.

153x172mm (300 x 300 DPI)

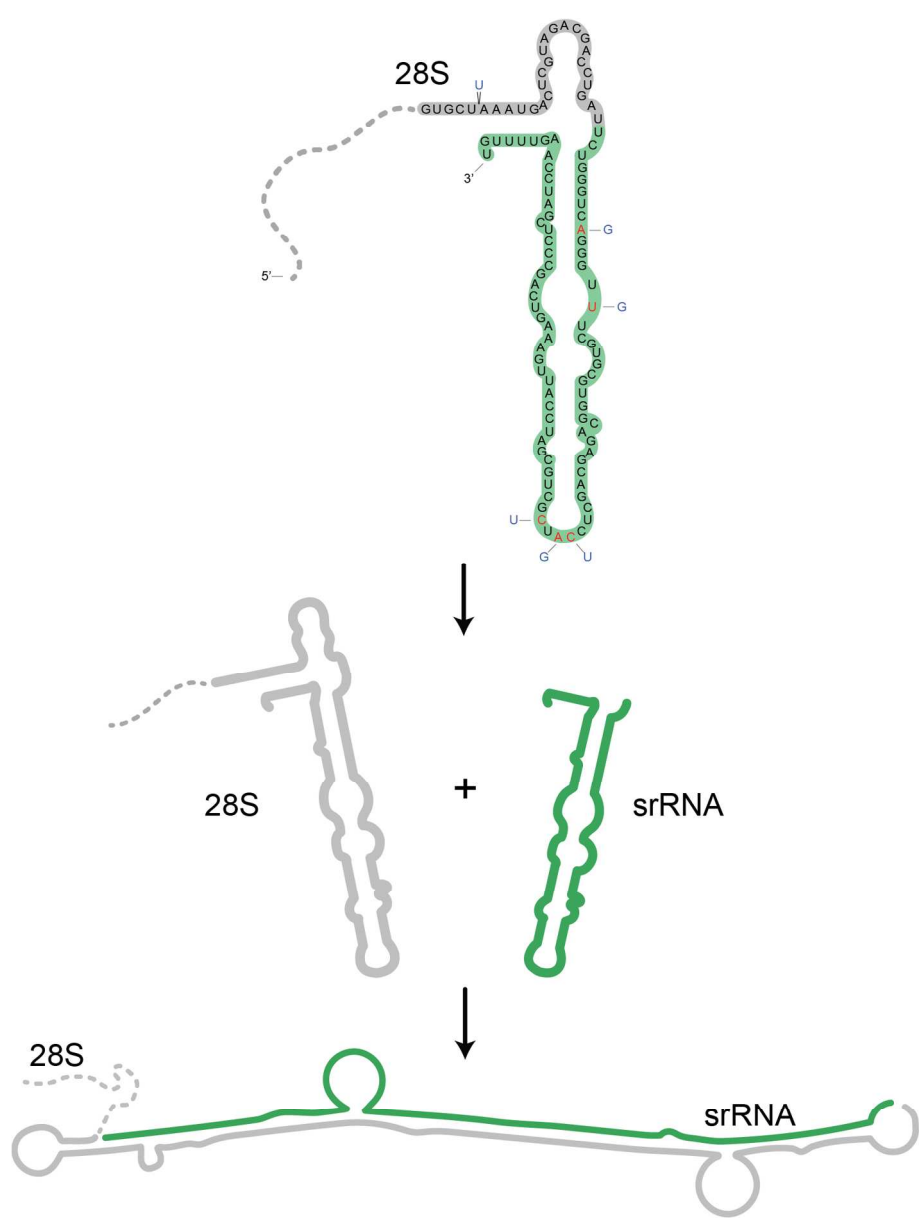


Figure 4. Structure of the interactions between the 80 nt 28S srRNA and the mature 28S rRNA.

144x190mm (300 x 300 DPI)