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Mitochondrially mediated RNA interference, a retrograde signaling system affecting nuclear gene expression

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1 Mitochondrially-mediated RNA interference, a retrograde signaling  
2 system affecting nuclear gene expression

3

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6

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16 Abstract

17       Several functional classes of short noncoding RNAs are  
18 involved in manifold regulatory processes in eukaryotes, including,  
19 among the best characterized, miRNAs. One of the most intriguing  
20 regulatory networks in the eukaryotic cell is the mito-nuclear  
21 crosstalk: recently, miRNA-like elements of mitochondrial origin,  
22 called smithRNAs, were detected in a bivalve species, *Ruditapes*  
23 *philippinarum*. These RNA types originate in the organelle, but were  
24 shown *in vivo* to regulate nuclear genes. Since miRNA genes evolve  
25 easily *de novo* with respect to protein coding genes, in the present  
26 work we estimate the probability with which a newly arisen smithRNA  
27 finds a suitable target in the nuclear transcriptome. Simulations with  
28 transcriptomes of twelve bivalve species suggest that this probability  
29 is not species-specific and high: one in a hundred million ( $1 \times 10^{-8}$ ) if  
30 five mismatch between the smithRNA and the 3' mRNA are allowed,  
31 yet many more are allowed in animals. We propose that novel  
32 smithRNAs may easily evolve as exaptations of the pre-existing  
33 mitochondrial genome architecture, where suitable secondary  
34 structures are common and constitutive. In turn, the ability of evolving  
35 novel smithRNAs may have played a pivotal role in mito-nuclear  
36 interactions during animal evolution, including the intriguing  
37 possibility of acting as speciation triggers.

## 38 RNA-silencing pathways

39       Beside well-known ribosomal, messenger, and transfer RNAs,  
40 many short and long RNA type are known from the cell cytoplasm.  
41 Among short noncoding RNAs (sncRNAs), small interfering RNAs  
42 and microRNAs play a pivotal role in the regulation of eukaryotic  
43 cytoplasmic translation, and involve a DICER-related protein and an  
44 Argonaute-related protein (Shabalina and Koonin 2008; Ghildiyal and  
45 Zamore 2009; Auyeung et al. 2013; Fang and Bartel 2015;  
46 Michlewski and Cáceres 2019). DICER proteins are required to  
47 process the immature RNA transcript to its functional form (Bernstein  
48 et al. 2001; Bartel 2018), while Argonaute proteins load the mature  
49 sncRNA and take part in the repression of the target transcripts  
50 (Bartel 2009; O'Brien et al. 2018).

51       Primary small interfering RNAs (siRNAs) are generally  
52 produced from exogenous double stranded RNAs; conversely,  
53 primary microRNAs (miRNAs) are transcribed from specific genomic  
54 loci (for instance, Ghildiyal et al. 2008; O'Brien et al. 2018; and  
55 references therein). However, this distinction is blurred, since siRNAs  
56 have been documented arising from selfish elements integrated in  
57 the genome (Yang and Kazazian Jr 2006; Chen et al. 2012), hairpins  
58 or endogenous double stranded RNAs (Czech et al. 2008;  
59 Kawamura et al. 2008; Okamura et al. 2008; Tam et al. 2008;

60 Watanabe et al. 2008; Ghildiyal and Zamore 2009). Moreover,  
61 siRNAs involve a complete base pairing with the target mRNA,  
62 whereas miRNAs may show more flexible complementarity to their  
63 targets. This is the case of metazoans, where a short sequence at  
64 the 5' of the mature miRNA, called the "seed", is crucial in the  
65 interaction with mRNAs (Shabalina and Koonin 2008; Ghildiyal and  
66 Zamore 2009; Bofill-De Ros et al. 2020).

67 Pathways for RNA interference (RNAi) have deep eukaryotic  
68 roots (Shabalina and Koonin 2008). The ancestral forms of RNAi  
69 most likely worked as defense mechanisms against viruses and  
70 transposons (Li and Ding 2005; Matzke and Birchler 2005). However,  
71 alternative hypotheses have been put forward. RNA-mediated gene  
72 silencing and suppression of exogenous or selfish elements may  
73 have been an exaptation after the evolution of an RNA machinery  
74 used for centromere assembly and proper formation of telomeres  
75 during eukaryogenesis (Cavalier-Smith 2010). Alternatively, a  
76 qualitative system drift has been proposed for RNAi, starting from the  
77 prokaryotic antisense RNA gene regulation mechanism (Torri et al.  
78 2022).

79 It is commonly accepted that the last eukaryotic common  
80 ancestor possessed a proto-RNAi mechanism (Cerutti and Casas-  
81 Mollano, 2006; Shabalina and Koonin 2008; Moran et al. 2017; Bråte

82 et al. 2018; Velandia-Huerto et al. 2022); moreover, it is increasingly  
83 clear that miRNAs arose multiple times among eukaryotes, exploiting  
84 the same ancient RNAi components (Moran et al. 2017; Yazbeck et  
85 al. 2017; Bråte et al. 2018; Velandia-Huerto et al. 2022; but see  
86 Poole et al. 2014). Conversely, miRNAs and their hairpin precursors  
87 have been shown to be highly conserved within eukaryotic  
88 supergroups (Hertel and Stadler 2015; Yazbeck et al. 2017;  
89 Velandia-Huerto et al. 2022).

90 In metazoans, hundreds of conserved miRNA families have  
91 been identified (for instance, Yazbeck et al. 2017; Velandia-Huerto et  
92 al. 2022). If confirmed by the growing knowledge about miRNAs in  
93 non-model species, this would mean that the expansion of miRNA  
94 families in the kingdom is coincidental with, if not associated to, the  
95 diversification of body plans and ultimately the evolution of bilaterians  
96 (Hertel and Stadler 2015; Dexheimer and Cochella 2020; Desvignes  
97 et al. 2021; Ma et al. 2021). However, multicellular organisms are  
98 particularly prone to the evolution of complex regulatory networks by  
99 neutral processes, and the evolution of miRNAs in animals may not  
100 be adaptive at its roots (Lynch 2007).

101 To date, there is virtually no eukaryotic cell phenomenon  
102 which has not been shown to be regulated by miRNAs, from stress  
103 response (Larriba and del Mazo 2016; Riggs et al. 2018) to

104 biomineralization (van Wijnen et al. 2013; Jiao et al. 2014), from  
105 immunity (Chen et al. 2013; Wang et al. 2018) to development and  
106 aging (Yekta et al. 2008; Kim and Lee 2019).  
107

108 Retrograde signaling through RNA-silencing: smithRNAs

109       The mitochondrion-to-nucleus communication is typically  
110 referred to as “retrograde signaling” or “Mitochondrial Retrograde  
111 Response” (MRR; Ovcariakova et al. 2022), because it was always  
112 clear that nucleus ought to regulate mitochondria in the eukaryotic  
113 cell, but the reverse regulatory function was not immediately  
114 understood. MRR may be mediated by cholesterol, reactive oxygen  
115 species and  $\text{Ca}^{2+}$  at nucleus-mitochondrion contact sites (Connelly et  
116 al. 2021). However, there are short RNAs (Maniataki and Mourelatos  
117 2005; Weber-Lofti and Dietrich 2018), long non-coding RNAs  
118 (Vendramin et al. 2017; Weber-Lofti and Dietrich 2018) and peptides  
119 (Lee et al. 2013; Cohen 2014) of mitochondrial origin that have been  
120 proposed to interact with the nucleus.

121       Recently, it has been shown that sncRNAs with some  
122 similarities with miRNAs are involved in MRR as well; they were  
123 termed small mitochondrial highly expressed RNAs (smithRNAs) and  
124 were originally found in the Manila clam *Ruditapes philippinarum*  
125 (Pozzi et al. 2017). Small RNAs were already known from animal  
126 mitochondria (e.g., Mercer et al. 2011; Ro et al. 2013; Bottje et al.  
127 2017; Riggs et al. 2018), but they had always been associated to  
128 mitochondrial targets (Mercer et al. 2011; Ro et al. 2013; Bottje et al.  
129 2017). Conversely, smithRNAs are transcribed from the

130 mitochondrial genome, but they regulate nuclear targets by definition.  
131 The complementarity of a small region of the sncRNA with the 3'  
132 UTR of target messengers was shown to be a good predictor of  
133 regulated target genes (Pozzi et al. 2017; Passamonti et al. 2020).

134 The original *in silico* prediction of smithRNAs was  
135 subsequently confirmed by *in vivo* experiments, which also showed  
136 that smithRNAs can affect the epigenetic status of the nuclear  
137 genome by regulating histone methylation/acetylation (Passamonti et  
138 al. 2020). Finally, far from being a bivalve oddity, smithRNAs were  
139 suggested to be present in distantly related bilaterians (Passamonti  
140 et al. 2020). Notably, putative mitochondrial noncoding RNAs have  
141 been also found in *Arabidopsis thaliana* (Marker et al. 2002), as well  
142 as in other plants (Weber-Lofti and Dietrich 2018).

143 As most sncRNAs, smithRNAs may well be genetic elements  
144 that commonly arise *de novo* during evolution (Velandia-Huerto et al.  
145 2022; and references therein). Duplication, reshuffling, transposition,  
146 retrotransposition, chimeric phenomena account for most new genes  
147 (Andersson et al. 2015; Schlotterer 2015; VanKuren and Long 2018;  
148 Zhao et al. 2021), but small noncoding loci like miRNAs may  
149 represent the most common source of *de novo* genes (Lu et al.  
150 2008b; Lyu et al. 2014; Zhao et al. 2021). Most miRNAs arising *de*  
151 *novo* are probably functionless (Lu et al. 2008b; Berezikov et al.

152 2010) or even dead-on-arrival (Petrov et al. 1996; Petrov and Hartl  
153 1998), but many may become adaptive miRNAs (Lu et al. 2008a;  
154 Mohammed et al. 2014; Lyu et al. 2014; Mohammed et al. 2018;  
155 Zhao et al. 2021).

156       Therefore, it can be stated that (i) at least some smithRNAs  
157 are miRNA-like molecules, structurally simple and requiring flexible  
158 base pairing to nuclear targets; (ii) at least some smithRNAs exert  
159 significant and broad-scope effects on the associated nuclear  
160 genome; (iii) smithRNAs may be widespread among animals and  
161 may have been present in the metazoan common ancestor; (iv)  
162 miRNA-like elements can easily evolve *de novo*, be conserved as  
163 adaptive traits, or be swept away by natural selection. Therefore, a  
164 fundamental evolutionary question arises: how common is the  
165 emergence of new smithRNAs and of novel smithRNA functions?

166

167 Target availability

168       As stated, at least some smithRNAs behave as animal  
169 miRNAs and require only partial pairing with 3' UTRs of target  
170 nuclear messengers. Namely, the extended seed region required to  
171 basepair and regulate the target encompasses nucleotides 1-8 of the  
172 mature miRNA molecule (Bartel 2009; McGeary et al. 2019).  
173 Although cases of alternative and noncanonical pairing sites are  
174 known (see Tan et al. 2014; Bartel 2018; McGeary et al. 2019; Bofill-  
175 De Ros et al. 2020; Rissland 2020; Komatsu et al. 2023; and  
176 reference therein), a handful of nucleotides are anyway involved in  
177 target regulation.

178       To provide a rough estimate of the probability of a random  
179 sequence to behave as a miRNA-like regulatory element for a  
180 transcript within the same organism, we generated 189,339,429  
181 random pri-miRNA-like sequences using custom-tailored Python  
182 scripts. The pri-miRNA is the canonical primary transcript of a miRNA  
183 element: it will be cleaved by the protein DROSHA within the nucleus  
184 at specific sites associated to its secondary structure, producing the  
185 pre-miRNA. As described above, the pre-miRNA will be cleaved by  
186 DICER in the cytoplasm to produce the functional molecule (Ghildiyal  
187 and Zamore 2009; García-López et al. 2013; Ha and Kim 2014;  
188 Bartel 2018; and reference therein). Sequences were randomly

189 generated following the canonical pri-miRNA structure detailed in  
190 Bartel (2018): all sequences were then matured *in silico*, respecting  
191 the sites of DROSHA and DICER cleavage (see Ha and Kim 2014;  
192 Bartel 2018).

193         Since functional smithRNAs have been demonstrated *in vivo*  
194 in the Manila clam only (Passamonti et al. 2020), we assembled  
195 transcriptomes from 12 bivalve species for which transcriptome data  
196 are available on GenBank: *Ruditapes decussatus* (SRR527757);  
197 *Arctica islandica* (SRR1559269); *Galeomma turtoni* (SRR1560274);  
198 *Sphaerium nucleus* (SRR1561723); *Laternula elliptica*  
199 (SRR1687084); *Lyonsia floridana* (SRR1560310); *Margaritifera*  
200 *margaritifera* (SRR1560312); *Arca noae* (SRR1559268); *Mytilus*  
201 *edulis* (SRR1560431); *Placopecten magellanicus* (SRR1560445);  
202 *Solemya velum* (SRR330465); *Yoldia eightsii* (SRR3205073).

203         Transcriptomes were curated using the software FastQC  
204 (Andrews 2010), Trimmomatic (Bolger et al. 2014), BUSCO (Simão  
205 et al. 2015), and Trinity (Grabherr et al. 2011; Haas et al. 2013). The  
206 software Kraken2 (Wood et al. 2019) was used to classify potential  
207 contaminants of human and prokaryotic origin, using a custom-  
208 assembled database of prokaryotic sequences updated to June  
209 2019. Peptide detection on noisy matured sequences was carried out  
210 with FrameDP (Gouzy et al. 2009), and 3' UTRs were predicted

211 using ExUTR (Huang and Teeling 2017) and the invertebrate dataset  
212 of 3' UTRs.

213 *In silico*-matured RNAs were mapped onto assembled  
214 transcriptomes using Bowtie (Langmead et al. 2009), using the minus  
215 strand of the Bowtie index and requiring at least a perfect match  
216 between the 3' UTR and nucleotides 2-8 of the simulated miRNA-like  
217 element, thus conservatively restricting the analysis to “canonical”  
218 targeting only. Scripts, commands, and settings are available by YLC  
219 and AF upon request.

220 The number of simulated miRNA-like elements able to find  
221 targets in the transcriptome were normalized over the number of *k*-  
222 mers ( $k = 22$  nucleotides) available in the 3' UTRs of the focal  
223 transcriptome: the result was divided by 189,339,429 (the number of  
224 random pri-miRNAs) to get an estimate of the probability for a single  
225 miRNA-like element to find a suitable target in a given *k*-mer.

226 The probability for a random pri-miRNA-like sequence to result  
227 in a mature miRNA having a target on a transcriptome is  
228 exponentially linked to the number of mismatches outside the seed  
229 region, irrespective of the species the transcriptome is obtained from  
230 (Fig. 1). Specifically, this probability is approximately one in a  
231 hundred million ( $1 \times 10^{-8}$ ) if exactly five mismatches between the

232 mature miRNA-like molecule and a 3' UTR are considered (provided  
233 that the seed basepairs perfectly).

234 Recall the large amount of replicating mitochondrial genomes  
235 in the germline, and the huge number of individuals and populations  
236 of these species, one in a hundred million should be regarded as a  
237 high chance for a *de novo*-arisen mitochondrial miRNA-like element  
238 to find a regulative target in the nuclear transcriptome of the same  
239 cell. Notably, this probability does not change across species, which  
240 means that it is independent from nuclear transcriptome features.

241 It is worth noting that we conservatively focused on the 2-8  
242 eptamer seed pairing, but other types of seed pairing are  
243 conceivable, and, thus, this probability is largely underestimated.  
244 Moreover, more than five mismatches are normally allowed in  
245 miRNA-driven regulation in animals (Shabalina and Koonin 2008;  
246 Ghildiyal and Zamore 2009; Bofill-De Ros et al. 2020), thus again  
247 increasing the chances for a *de novo* mitochondrial miRNA-like  
248 element, since the decimal logarithm of probability is positively  
249 correlated with mismatches outside the seed ( $r = +0.9858$ ; Fig. 1).

250 If this trend will be confirmed outside bivalves, it will be  
251 tempting to conclude that the DNA chemistry and nucleotide  
252 composition of eukaryotes, as well as constraints on pri-miRNA  
253 structures, do result in a significant probability that a miRNA-like

254 element finds a suitable nuclear target, after having originated merely  
255 by chance and random mutations on a mitochondrial genome.  
256

257 Mitochondrial secondary structures are easily co-opted to deliver new  
258 functions

259 Obviously, the probability of a simulated sequence to match a  
260 3' UTR is not enough to state that smithRNA commonly arise *de*  
261 *novo*. A smithRNA is a sncRNA associated to a specific biogenesis  
262 pathway, which requires molecular signals for processing enzymes,  
263 such as secondary structures.

264 In the traditional view, the animal mitochondrial genome is  
265 believed to be small and compact, containing a conserved set of  
266 protein-coding genes associated with the mitochondrial oxidative  
267 phosphorylation (OXPHOS) pathway (Boore 1999). However, recent  
268 research has shown that this may not always be the case,  
269 challenging the notion of ubiquitous features in metazoan  
270 mitochondrial genomics (Lavrov et al. 2013; Breton et al. 2014;  
271 Formaggioni et al. 2021). Actually, animal mitochondrial genomes  
272 are highly variable for what concerns genome architecture (Lavrov  
273 and Pett 2016); genome size (Pu et al. 2019; Hemmi et al. 2020);  
274 use of different genetic codes (Lavrov et al. 2013; Li et al. 2018);  
275 gene arrangement (Trindade Rosa et al. 2017; Pu et al. 2019;  
276 Hemmi et al. 2020; Monnens et al. 2020; Ghiselli et al. 2021;  
277 Kutyumov et al. 2021); Doubly Uniparental Inheritance (DUI;  
278 Passamonti and Ghiselli 2009; Zouros and Rodakis 2019;

279 Passamonti and Plazzi 2020); and post-transcriptional regulation  
280 (Osigus et al. 2017; Schuster et al. 2017).

281         The finetuning of some of these mechanisms (for instance,  
282 DUI, post-transcriptional regulation) and the origin of these features  
283 involves a complex crosstalk with nuclear genomes, as well as the  
284 availability of regulatory sequences and signals along the  
285 mitochondrial genome (e.g., Ghiselli et al. 2013, 2021). For example,  
286 since mitochondrial DNA is normally transcribed as a single  
287 polycistron (e.g., Hillen et al. 2018), structural signals ought to be  
288 present to cleave single transcripts, which are normally found  
289 between protein coding genes as tRNA genes or short noncoding  
290 regions with stem-and-loop secondary structures (e.g., Plazzi et al.  
291 2013; Bettinazzi et al. 2016).

292         Therefore, mitochondrial genomics itself requires multiple  
293 secondary structures to regulate the organellar functions. Moreover,  
294 many of these structural sites are processing and cleavage signals,  
295 as is the case for protein coding gene spacers, that are excised to  
296 separate single transcripts. These RNA hairpins are normally  
297 processed and degraded as part of the normal cellular turnover of  
298 macromolecules.

299         However, it is easy to speculate that a hairpin might survive  
300 being directly co-opted as pre-miRNA. It is sufficient that its

301 secondary structure can be recognized by some DICER ortholog:  
302 hairpin structure that are normally found in cleavage signals are  
303 indeed very similar to hairpin structure normally shown by pre-  
304 miRNAs. In that case, the RNA would be cleaved and a miRNA  
305 would be produced skipping the pri-miRNA/DROSHA stage – and will  
306 find a suitable nuclear target one in a hundred million times, and  
307 probably more (as per our simulation above). Other examples of  
308 DROSHA-independent biogenesis of miRNAs are indeed known  
309 (Ruby et al. 2007; Babiarz et al. 2008; O'Brien et al. 2018).

310 Obviously, a hairpin excised within the mitochondrion must be  
311 delivered to the cytoplasm prior to the final, and in this case only,  
312 maturation step driven by DICER. In fact, many studies found  
313 mitochondrial RNA outside the source organelle, which accounts for  
314 the possibility for RNA molecules to be exported. For example,  
315 several tRNAs of mitochondrial origin were found in the cytoplasm of  
316 human cells, even in association with Ago2, an Argonaute protein  
317 included in the formation of the functional complex involved in RNA  
318 silencing (Maniataki and Mourelatos 2005). Mitochondrially-encoded  
319 RNAs can bind Ago2 as well (Pozzi and Dowling 2022), and long  
320 non-coding RNAs from the mitochondrion were also reported within  
321 the nucleus (Landerer et al. 2011; Rackham et al. 2011; Vendramin  
322 et al. 2017). Interestingly, mitochondria of *R. philippinarum* have

323 been observed while releasing their content in the cytoplasm (Milani  
324 et al. 2011), which would be a straightforward mechanism for  
325 smithRNAs to enter cytoplasm, at least in this species.

326         RNAi driven by mitochondria might be a remnant of their origin  
327 as free-living, aerobic prokaryotes. Notably, the intracellular  
328 pathogen *Mycobacterium marinum* synthesize small, antisense  
329 regulatory RNAs which are exported to the host cell and processed  
330 as if they were miRNAs (Furuse et al. 2014) and, generally speaking,  
331 many bacterial small RNAs show complex secondary structures  
332 (Wagner and Simons 1994). Indeed, a connection between small  
333 antisense regulatory RNAs in prokaryotes and the cytoplasmic proto-  
334 RNAi system in eukaryotes has been suggested (Torri et al. 2022). In  
335 sum, we propose that smithRNAs arise as an exaptation at the  
336 molecular level of secondary structures that were always present in  
337 mitochondrial genomes, possibly since their origin as endosymbionts.  
338 Moreover, we also predict that this phenomenon might be more  
339 common than thought, given the similar selective constraints on  
340 hairpins.

341

342 Retrograde RNAi and mitonuclear co-adaptation

343 Mitochondrial and nuclear genomes must coevolve to provide  
344 an efficient energy production (Hill 2019). The electron transport  
345 system of mitochondria (ETS), to which the efficiency of energy  
346 production through OXPHOS is strictly linked, is delivered by a  
347 complex assembly of nuclear and mitochondrial subunits that are  
348 forced to function together (Rand et al. 2004). An effective OXPHOS  
349 is achieved by three different mechanisms: (i) protein-protein  
350 interaction forming the ETS complexes (Phillips et al. 2010); (ii)  
351 protein-RNA/DNA interactions during transcription and translation of  
352 mitochondrial genes (Taanmann 1999; D'Souza and Minczuck 2018);  
353 and (iii) protein-DNA interaction in the replication of the mitochondrial  
354 genome (Clayton 2000).

355 In fact, speciation soon started to be discussed in the context  
356 of mito-nuclear coadaptation, as a mechanism that may easily evolve  
357 mito-nuclear incompatibilities (Dowling et al 2008; Gershoni et al.  
358 2009; Burton and Barreto 2012). Examples of these mitonuclear  
359 incompatibilities are for instance available for *Drosophila* and  
360 *Tigriopus* copepods (see Hill 2019; and references therein).

361 Although the abovementioned system may suggest a strict  
362 need of mito-nuclear coadaptation, other systems point in the  
363 opposite direction. In bivalves with DUI, two mitochondrial genomes

364 are transmitted to offspring in a sex-linked way (Passamonti and  
365 Ghiselli 2009; Zouros and Rodakis 2019; Passamonti and Plazzi  
366 2020) and there is evidence of a functional assembly of the ETS with  
367 two, highly divergent sets of mitochondrial proteins. Therefore, the  
368 correct protein-protein interaction forming the ETS complexes is less  
369 strict than previously thought, at least in these bivalve mollusks.

370         The existence of mitochondrially mediated RNAi provides a  
371 fourth mechanism for the evolution of mito-nuclear incompatibilities,  
372 which can arise much faster than the other three. When a set of  
373 smithRNAs is adapted to regulate nuclear gene expression in a  
374 species, the system could easily produce genetic barriers with other  
375 species having a differently adapted smithRNA subset. To our  
376 knowledge, there is currently no study on this issue, but we strongly  
377 suggest that the cases of mito-nuclear incompatibilities may be  
378 reconsidered in light of the role of the mitochondrial genome in  
379 regulating nuclear gene expression. In this conception, smithRNAs  
380 (and maybe other MRR mechanisms) may represent classical  
381 Dobzhansky-Muller speciation triggers (Dobzhansky 1937; Muller  
382 1942), which lead to the evolution of postzygotic genetic barriers.

383

#### 384 Concluding remarks

385         Notwithstanding their recent discovery (Pozzi et al. 2017), it is  
386 likely that smithRNAs are not a peculiar feature of a single bivalve  
387 species: they are probably widespread among metazoans  
388 (Passamonti et al. 2020). This does not necessarily imply that they  
389 are phylogenetically related, nor that the origin of smithRNAs is a  
390 single event in evolutionary history. The peculiar features of  
391 mitochondrial genomes involve the possibility that smithRNAs  
392 spontaneously arose multiple times from the secondary structure  
393 repertoire that is normally available along the mitochondrial genome.

394         Therefore, it is important to characterize the smithRNA toolbox  
395 in as many animal species as possible, and functional studies are  
396 required to prove that smithRNAs are regulatory elements *in vivo*.  
397 This will increase the list of functions smithRNAs can exert in the cell;  
398 moreover, light will be shed on the evolutionary conservation of  
399 smithRNAs and on their multiple origin through molecular exaptation,  
400 being the two things not mutually exclusive. Finally, if smithRNA  
401 precursors (or at least some of them) arise as exaptation of ancient  
402 legacies from free living bacteria, smithRNAs might be strictly  
403 connected with early eukaryogenesis.

404

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416

417 Author contribution statement

418       FP and MP conceived and supervised the study; YLC and AF  
419 analyzed data; FP and MP drafted the original manuscript; all authors  
420 read and approved the final manuscript.

421

422    Conflict of Interest

423            The authors declare no conflict of interest.

424

425 Data archiving

426 All data used for the present study are publicly available in

427 GenBank.

428

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788           Figure Legends

789           Figure 1. Frequency of miRNA-like simulated molecules that  
790 found at least one suitable target on 3' UTRs of the same species.  
791 The seed was conservatively defined as nucleotides 2-8 of the  
792 miRNA; a match was accepted if it was perfect at the seed and if it  
793 included a maximum of 5 mismatches outside. An example of an  
794 alignment with three mismatches is included in the insert. The  
795 number of elements with an acceptable match was normalized on the  
796 number of 22-mers in the relative 3' UTR set and divided by the  
797 number of simulated pri-miRNAs. The y axis is Log-transformed for  
798 the sake of readability. Regression line details:  $y = 1.0757x -$   
799  $12.8616$ ;  $R^2 = 0.9719$ ;  $P < 2 \times 10^{-16***}$ .