

Alma Mater Studiorum Università di Bologna  
Archivio istituzionale della ricerca

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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Plazzi F., Le Cras Y., Formaggioni A., Passamonti M. (2023). Mitochondrially mediated RNA interference, a retrograde signaling system affecting nuclear gene expression. *HEREDITY*, 2023, 1-6 [10.1038/s41437-023-00650-5].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/952489> since: 2024-05-21

*Published:*

DOI: <http://doi.org/10.1038/s41437-023-00650-5>

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).  
When citing, please refer to the published version.

(Article begins on next page)

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

3

4 Federico Plazzi<sup>1\*</sup>, Youn Le Cras<sup>1,2</sup>, Alessandro Formaggioni<sup>1</sup>, Marco  
5 Passamonti<sup>1</sup>

6

7 <sup>1</sup> Department of Biological, Geological and Environmental Sciences,  
8 University of Bologna, via Selmi, 3 – 40126 Bologna (BO)

9 <sup>2</sup> Present address: Magistère Européen de Génétique, Université  
10 Paris Cité, 85 Boulevard Saint Germain – 75006 Paris

11 \* Corresponding author: federico.plazzi@unibo.it

12

13 Word count for main text (excluding references, tables and figures):

14 3,683

15

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; Ovcariikova 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 Ca<sup>2+</sup> 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

405 Acknowledgements

406           This study was supported by Italian Ministry of University and  
407 Research PRIN 2020 (2020BE2BC3) funded to MP. YLC was  
408 supported by EUR G.E.N.E. (reference #ANR-17-EURE-0013) and is  
409 part of the Université Paris Cité (IdEx #ANR-18-IDEX-0001), funded  
410 by the French Government through its “Investments for the Future”  
411 program. We are grateful to people at the ESEB 2022 symposium  
412 “*Beyond transcription: the role of post-transcriptional gene regulation*  
413 *in adaptation and evolution*” for sharing ideas and suggestions. We  
414 also want to thank three anonymous reviewers, whose comments  
415 and criticism greatly improved the original manuscript.

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

429 References

- 430           Andersson DI, Jerlstrom-Hultqvist J, Nasvall J (2015)  
431 Evolution of New Functions De Novo and from Preexisting Genes.  
432 Cold Spring Harb Perspect Biol 7:a017996
- 433           Andrews S (2010) FastQC: A Quality Control Tool for High  
434 Throughput Sequence Data.  
435 <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>
- 436           Auyeung VC, Ulitsky I, McGeary SE, Bartel DP (2013) Beyond  
437 Secondary Structure: Primary-Sequence Determinants License Pri-  
438 miRNA Hairpins for Processing. Cell 152:844-858
- 439           Babiarz JE, Ruby JG, Wang Y, Bartel DP, Blelloch R (2008)  
440 Mouse ES cells express endogenous shRNAs, siRNAs, and other  
441 Microprocessor-independent, Dicer-dependent small RNAs. Genes  
442 Dev 22:2773-2785
- 443           Bartel DP (2009) MicroRNAs: target recognition and regulatory  
444 functions. Cell 136:215-233
- 445           Bartel DP (2018) Metazoan MicroRNAs. Cell 173:20-51
- 446           Berezikov E, Liu N, Flynt AS, Hodges E, Rooks M, Hannon  
447 GJ, et al. (2010) Evolutionary flux of canonical microRNAs and  
448 mirtrons in Drosophila. Nat Genet 42:6-9.

449           Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001)  
450    Role for a bidentate ribonuclease in the initiation step of RNA  
451    interference. *Nature* 409:363-366

452           Bettinazzi S, Plazzi F, Passamonti M (2016) The Complete  
453    Female- and Male-Transmitted Mitochondrial Genome of *Meretrix*  
454    *lamarckii*. *PLoS ONE* 11:e0153631

455           Bofill-De Ros X, Yang A, Gu S (2020) IsomiRs: Expanding the  
456    miRNA repression toolbox beyond the seed. *Biochim Biophys Acta*  
457    *Gene Regul Mech* 1863:194373

458           Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible  
459    trimmer for Illumina sequence data. *Bioinformatics* 30:2114-2120

460           Boore JL (1999) Animal mitochondrial genomes. *Nucleic Acids*  
461    *Res* 27:1767-1780

462           Bottje WG, Khatri B, Shouse SA, Seo D, Mallmann B,  
463    Orlowski SK, et al. (2017) Identification and differential abundance of  
464    mitochondrial genome encoding small RNAs (mitosRNA) in breast  
465    muscles of modern broilers and unselected chicken breed. *Front*  
466    *Physiol* 8:816

467           Bråte J, Neumann RS, Fromm B, Haraldsen AAB, Tarver JE,  
468    Suga H, et al. (2018) Unicellular Origin of the Animal MicroRNA  
469    Machinery. *Curr Biol* 28:3288-3295.e5

470 Breton S, Milani L, Ghiselli F, Guerra D, Stewart DT,  
471 Passamonti M (2014) A resourceful genome: updating the functional  
472 repertoire and evolutionary role of animal mitochondrial DNAs.  
473 Trends Genet 30:555-564

474 Burton RS, Barreto FS (2012) A disproportionate role for  
475 mtDNA in Dobzhansky-Muller incompatibilities? Mol Ecol 21:4942-  
476 4957

477 Cavalier-Smith, T (2010) Origin of the cell nucleus, mitosis and  
478 sex: roles of intracellular coevolution. Biol Direct 5:7

479 Cerutti H, Casas-Mollano JA (2006) On the origin and  
480 functions of RNA-mediated silencing: from protists to man. Curr  
481 Genet 50:81-99

482 Chen CZ, Schaffert S, Fragoso R, Loh C (2013) Regulation of  
483 immune responses and tolerance: the microRNA perspective.  
484 Immunol Rev 253:112-128

485 Chen L, Dahlstrom JE, Lee S-H, Rangesamy D (2012)  
486 Naturally occurring endo-siRNA silences LINE-1 retrotransposons in  
487 human cells through DNA methylation. Epigenetics 7:758-771

488 Clayton DA (2000) Transcription and replication of  
489 mitochondrial DNA. Human Reprod 15:11-17

490 Cohen P (2014) New role for the mitochondrial peptide  
491 humanin: protective agent against chemotherapy-induced side  
492 effects. *J Natl Cancer Inst* 106:dju006

493 Connelly SV, Manzella-Lapeira J, Levine ZC, Brzostowski J,  
494 Krymskaya L, Rahman RS, et al. (2021) Restructured Mitochondrial-  
495 Nuclear Interaction in *Plasmodium falciparum* Dormancy and  
496 Persister Survival after Artemisinin Exposure. *mBio* 12:e00753-21

497 Czech B, Malone CD, Zhou R, Stark A, Schlingeheyde C, Dus  
498 M, et al. (2008) An endogenous small interfering RNA pathway in  
499 *Drosophila*. *Nature* 453:798-802

500 D'Souza AR, Minczuck M (2018) Mitochondrial transcription  
501 and translation: overview. *Ess Biochem* 62:309-320

502 Desvignes T, Sydes J, Montfort J, Bobe J, Postlethwait JH  
503 (2021) Evolution after Whole-Genome Duplication: Teleost  
504 MicroRNAs. *Mol Biol Evol* 38:3308-3331

505 Dexheimer PJ, Cochella L (2020) MicroRNAs: From  
506 Mechanism to Organism. *Front Cell Dev Biol* 8:409

507 Dobzhansky T (1937) *Genetics and the Origin of Species*.  
508 Columbia University Press, New York

509 Dowling DK, Friberg U, Lindell J (2008) Evolutionary  
510 implication of non-neutral mitochondrial genetic variation. *Trends*  
511 *Ecol Evol* 23:546-554

512 Fang W, Bartel DP (2015) The Menu of Features that Define  
513 Primary MicroRNAs and Enable De Novo Design of MicroRNA  
514 Genes. *Molecular Cell* 60:131-145

515 Formaggioni A, Luchetti A, Plazzi F (2021) Mitochondrial  
516 Genomic Landscape: A Portrait of the Mitochondrial Genome 40  
517 Years after the First Complete Sequence. *Life* 11:663

518 Furuse Y, Finethy R, Saka HA, Xet-Mull AM, Sisk DM, Smith  
519 KL, et al. (2014) Search for microRNAs expressed by intracellular  
520 bacterial pathogens in infected mammalian cells. *PLoS One*  
521 9:e106434

522 García-López J, Brieño-Enríquez MA, del Mazo J (2013)  
523 MicroRNA biogenesis and variability. *BioMol Concepts* 4:367-380

524 Gershoni M, Templeton AR, Mishmar D (2009) Mitochondrial  
525 bioenergetics as a major motive force of speciation. *Bioessays*  
526 31:642-650

527 Ghildiyal M, Seitz H, Horwich MD, Li C, Du T, Lee S, et al.  
528 (2008) Endogenous siRNAs derived from transposons and mRNAs in  
529 *Drosophila* somatic cells. *Science* 320:1077-1081

530 Ghildiyal M, Zamore PD (2009) Small silencing RNAs: an  
531 expanding universe. *Nat Rev Genet* 10:94-108

532 Ghiselli F, Gomes-dos-Santos A, Adema CM, Lopes-Lima M,  
533 Sharbrough J, Boore JL (2021) Molluscan mitochondrial genomes  
534 break the rules. *Phil Trans R Soc B* 376:20200159

535 Ghiselli F, Milani L, Guerra D, Chang PL, Breton S, Nuzhdin  
536 SV, et al. (2013) Structure, Transcription, and Variability of Metazoan  
537 Mitochondrial Genome: Perspectives from an Unusual Mitochondrial  
538 Inheritance System. *Genome Biol Evol* 5:1535-1554

539 Gouzy J, Carrere S, Schiex T (2009) FrameDP: sensitive  
540 peptide detection on noisy matured sequences. *Bioinformatics*  
541 25:670-671

542 Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA,  
543 Amit I, et al. (2011) Full-length transcriptome assembly from RNA-  
544 seq data without a reference genome. *Nat Biotechnol* 29:644-652

545 Ha M, Kim VN (2014) Regulation of microRNA biogenesis. *Nat*  
546 *Rev Mol Cell Biol* 15:509-524

547 Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD,  
548 Bowden J, et al. (2013) *De novo* transcript sequence reconstruction  
549 from RNA-seq using the Trinity platform for reference generation and  
550 analysis. *Nat Protoc* 8:1494-1512

551 Hemmi K, Kakehashi R, Kambayashi C, Du Preez L, Minter L,  
552 Furuno N, et al. (2020) Exceptional Enlargement of the Mitochondrial



553 Genome Results from Distinct Causes in Different Rain Frogs  
554 (Anura: Brevicipitidae: *Breviceps*). Int J Genomics 2020:6540343

555 Hertel J, Stadler PF (2015) The Expansion of Animal  
556 MicroRNA Families Revisited. Life (Basel) 5:905-920

557 Hill GE (2019) Mitonuclear ecology. Oxford Series in Ecology  
558 and Evolution. Oxford University Press, Oxford

559 Hillen HS, Temiakov D, Cramer P (2018) Structural basis of  
560 mitochondrial transcription. Nat Struct Mol Biol 25:754-765

561 Huang Z, Teeling EC (2017) ExUTR: a novel pipeline for  
562 large-scale prediction of 3'-UTR sequences from NGS data. BMC  
563 Genomics 18:847

564 Jiao Y, Zheng Z, Du X, Wang Q, Huang R, Deng Y, et al.  
565 (2014) Identification and Characterization of MicroRNAs in Pearl  
566 Oyster *Pinctada martensii* by Solexa Deep Sequencing. Mar  
567 Biotechnol 16:54-62

568 Kawamura Y, Saito K, Kin T, Ono Y, Asai K, Sunohara T, et al.  
569 (2008) *Drosophila* endogenous small RNAs bind to Argonaute 2 in  
570 somatic cells. Nature 453:793-797

571 Kim SS, Lee S-JV (2019) Non-coding RNAs in *Caenorhabditis*  
572 *elegans* aging. Mol Cells 42:379-385

573 Komatsu S, Kitai H, Suzuki HI (2023) Network Regulation of  
574 microRNA Biogenesis and Target Interaction. Cells 12:306

575 Kutyumov VA, Predeus AV, Starunov VV, Maltseva AL,  
576 Ostrovsky AN (2021) Mitochondrial gene order of the freshwater  
577 bryozoan *Cristatella mucedo* retains ancestral lophotrochozoan  
578 features. *Mitochondrion* 59:96-104

579 Landerer E, Villegas J, Burzio VA, Oliveira L, Villota C, Lopez  
580 C, et al. (2011) Nuclear localization of the mitochondrial ncRNAs in  
581 normal and cancer cells. *Cell Oncol (Dordr)* 34:297-305

582 Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast  
583 and memory-efficient alignment of short DNA sequences to the  
584 human genome. *Genome Biol* 10:R25

585 Larriba E, del Mazo J (2016) Role of non-coding RNAs in the  
586 transgenerational epigenetic transmission of the effects of  
587 reprotoxicants. *Int J Mol Sci* 17:452

588 Lavrov D, Pett W (2016) Animal Mitochondrial DNA as We Do  
589 Not Know It: mt-Genome Organization and Evolution in Nonbilaterian  
590 Lineages. *Genome Biol Evol* 8:2896-2913

591 Lavrov DV, Pett W, Voigt O, Wörheide G, Forget L, Lang BF,  
592 et al. (2013) Mitochondrial DNA of *Clathrina clathrus* (Calcarea,  
593 Calcinea): six linear chromosomes, fragmented rRNAs, tRNA editing,  
594 and a novel genetic code. *Mol Biol Evol* 30:865-880

595 Lee C, Yen K, Cohen P (2013) Humanin: a harbinger of  
596 mitochondrial-derived peptides? Trends Endocrinol Metab 24:222-  
597 228

598 Li HW, Ding SW (2005) Antiviral silencing in animals. FEBS  
599 Lett 579:5965-5973

600 Li Y, Kocot KM, Tassia MG, Cannon JT, Bernt M, Halanych  
601 KM (2018) Mitogenomics Reveals a Novel Genetic Code in  
602 Hemichordata. Genome Biol Evol 11:29-40

603 Lu J, Fu Y, Kumar S, Shen Y, Zeng K, Xu A, et al. (2008a)  
604 Adaptive evolution of newly emerged micro-RNA genes in  
605 *Drosophila*. Mol Biol Evol 25:929-938

606 Lu J, Shen Y, Wu Q, Kumar S, He B, Shi S, et al. (2008b) The  
607 birth and death of microRNA genes in *Drosophila*. Nat Genet 40:351-  
608 355

609 Lynch M (2007) The evolution of genetic networks by non-  
610 adaptive processes. Nat Rev Genet 8:803-813

611 Lyu Y, Shen Y, Li H, Chen Y, Guo L, Zhao Y, et al. (2014)  
612 New MicroRNAs in *Drosophila*—Birth, Death and Cycles of Adaptive  
613 Evolution. PLoS Genet 10:e1004096

614 Ma X, He K, Shi Z, Li M, Li F, Chen X-X (2021) Large-Scale  
615 Annotation and Evolution Analysis of MiRNA in Insects. Genome Biol  
616 Evol 13:evab083

617 Maniataki E, Mourelatos Z (2005) Human mitochondrial  
618 tRNAMet is exported to the cytoplasm and associates with the  
619 Argonaute 2 protein. RNA 11:849-852

620 Marker C, Zemann A, Terhörst T, Kiefmann M, Kastenmayer  
621 JP, Green P, et al. (2002) Experimental RNomics: identification of  
622 140 candidates for small non-messenger RNAs in the plant  
623 *Arabidopsis thaliana*. Curr Biol 12:2002-2013

624 Matzke MA, Birchler JA (2005) RNAi-mediated pathways in  
625 the nucleus. Nat Rev Genet 6:24-35

626 McGeary SE, Lin KS, Shi CY, Pham TM, Bisaria N, Kelley  
627 GM, et al. (2019) The biochemical basis of microRNA targeting  
628 efficacy. Science 366:eaav1741

629 Mercer TR, Neph S, Dinger ME, Crawford J, Smith MA,  
630 Shearwood A-MJ, et al. (2011) The human mitochondrial  
631 transcriptome. Cell 146:645-658

632 Michlewski G, Cáceres JF (2019) Post-transcriptional control  
633 of miRNA biogenesis. RNA 25:1-16

634 Milani L, Ghiselli F, Maurizii MG, Passamonti M (2011) Doubly  
635 uniparental inheritance of mitochondria as a model system for  
636 studying germ line formation. PLoS One 6:e28194

637 Mohammed J, Bortolamiol-Becet D, Flynt AS, Gronau I, Siepel  
638 A, Lai EC (2014) Adaptive evolution of testis-specific, recently  
639 evolved, clustered miRNAs in *Drosophila*. RNA 20:1195-1209

640 Mohammed J, Flynt AS, Panzarino AM, Mondal MMH, DeCruz  
641 M, Siepel A, et al. (2018) Deep experimental profiling of microRNA  
642 diversity, deployment, and evolution across the *Drosophila* genus.  
643 Genome Res 28:52-65

644 Monnens M, Thijs S, Briscoe AG, Clark M, Frost EJ,  
645 Littlewood DTJ, et al. (2020) The first mitochondrial genomes of  
646 endosymbiotic rhabdocoels illustrate evolutionary relaxation of *atp8*  
647 and genome plasticity in flatworms. Int J Biol Macromol 162:454-469

648 Moran Y, Agron M, Praher D, Technau U (2017) The  
649 evolutionary origin of plant and animal microRNAs. Nat Ecol Evol  
650 1:27

651 Muller HJ (1942) Isolating mechanisms, evolution, and  
652 temperature. Biol Symp 6:71-125

653 O'Brien J, Hayder H, Zayed Y, Peng C (2018) Overview of  
654 MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Front  
655 Endocrinol 9:402

656 Okamura K, Chung W-J, Ruby JG, Guo H, Bartel DP, Lai EC  
657 (2008) The *Drosophila* hairpin RNA pathway generates endogenous  
658 short interfering RNAs. Nature 453:803-806

659 Osigus H-J, Eitel M, Schierwater B (2017) Deep RNA  
660 sequencing reveals the smallest known mitochondrial micro exon in  
661 animals: The placozoan *cox1* single base pair exon. PLoS ONE  
662 12:e0177959

663 Ovciarikova J, Shikha S, Sheiner L (2022) Nuclear  
664 Interactions: A Spotlight on Nuclear Mitochondrial Membrane Contact  
665 Sites. Contact 5:1-7

666 Passamonti M, Calderone M, Delpero M, Plazzi F (2020)  
667 Clues of *in vivo* nuclear gene regulation by mitochondrial short non-  
668 coding RNAs. Sci Rep 10:8219

669 Passamonti M, Ghiselli F (2009) Doubly Uniparental  
670 Inheritance: two mitochondrial genomes, one precious model for  
671 organelle DNA inheritance and evolution. DNA Cell Biol 28:79-89

672 Passamonti M, Plazzi F (2020) Doubly Uniparental Inheritance  
673 and beyond: The contribution of the Manila clam *Ruditapes*  
674 *philippinarum*. J Zool Syst Evol Res 58:529-540

675 Petrov DA, Hartl DL (1998) High Rate of DNA Loss in the  
676 *Drosophila melanogaster* and *Drosophila virilis* Species Groups. Mol  
677 Biol Evol 15:293-302

678 Petrov DA, Lozovskaya ER, Hartl DL (1996) High intrinsic rate  
679 of DNA loss in *Drosophila*. Nature 384:346-349

680 Phillips D, Reilley MJ, Aponte AM, Wang G, Boja E, Gucek M,  
681 et al. (2010) Stoichiometry of STAT3 and mitochondrial proteins:  
682 implications for the regulation of oxidative phosphorylation by protein-  
683 protein interactions. J Biol Chem 285:23532-23536

684 Plazzi F, Ribani A, Passamonti M (2013) The complete  
685 mitochondrial genome of *Solemya velum* (Mollusca: Bivalvia) and its  
686 relationships with Conchifera. BMC Genomics 14:409

687 Poole CB, Gu W, Kumar S, Jin J, Davis PJ, Bauche D, et al.  
688 (2014) Diversity and expression of microRNAs in the filarial parasite,  
689 *Brugia malayi*. PLoS One 9:e96498

690 Pozzi A, Dowling DK (2022) New Insights into Mitochondrial-  
691 Nuclear Interactions Revealed through Analysis of Small RNAs.  
692 Genome Biol Evol 14:evac023

693 Pozzi A, Plazzi F, Milani L, Ghiselli F, Passamonti M (2017)  
694 SmithRNAs: could mitochondria “bend” nuclear regulation? Mol Biol  
695 Evol 34:1960-1973

696 Pu L, Liu H, Wang G, Li B, Xia G, Shen M, et al. (2019)  
697 Complete mitochondrial genome of the cockle *Anadara antiquata*  
698 (Linnaeus, 1758). Mitochondrial DNA Part B 4:2293-2294

699 Rackham O, Shearwood AM, Mercer TR, Davies SM, Mattick  
700 JS, Filipovska A (2011) Long noncoding RNAs are generated from

701 the mitochondrial genome and regulated by nuclear-encoded  
702 proteins. RNA 17:2085-2093

703         Rand DM, Hanley RA, Fry AJ (2004) Cytonuclear coevolution:  
704 the genomics of cooperation. Trends Ecol Evol 19:645-653

705         Riggs CL, Summers A, Warren DE, Nilsson GE, Lefevre S,  
706 Dowd WW, et al. (2018) Small non-coding RNA expression and  
707 vertebrate anoxia tolerance. Front Genet 9:230

708         Rissland OS (2020) Big Insights into Small RNAs.  
709 Biochemistry 59:1551-1552

710         Ro S, Ma H-Y, Park C, Ortogero N, Song R, Hennig GW, et al.  
711 (2013) The mitochondrial genome encodes abundant small  
712 noncoding RNAs. Cell Res 23:759-774

713         Ruby JG, Jan CH, Bartel DP (2007) Intronic microRNA  
714 precursors that bypass Drosha processing. Nature 448:83-86

715         Schlotterer C (2015) Genes from scratch – the evolutionary  
716 fate of *de novo* genes. Trends Genet 31:215-219

717         Schuster A, Lopez JV, Becking LE, Kelly M, Pomponi SA,  
718 Wörheide G, et al. (2017) Evolution of group I introns in Porifera: new  
719 evidence for intron mobility and implications for DNA barcoding. BMC  
720 Evol Biol 17:82

721         Shabalina SA, Koonin EV (2008) Origins and evolution of  
722 eukaryotic RNA interference. Trends Ecol Evol 23:P578-P587



723 Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV,  
724 Zdobnov EM (2015) BUSCO: assessing genome assembly and  
725 annotation completeness with single-copy orthologs. *Bioinformatics*  
726 31:3210-3212

727 Taanmann JW (1999) The mitochondrial genome: structure,  
728 transcription, translation and replication. *Biochim Biophys Acta (BBA)*  
729 – *Bioenergetics* 1410:103-123

730 Tam OH, Aravin AA, Stein P, Girard A, Murchison EP,  
731 Cheloufi S, et al. (2008) Pseudogene-derived small interfering RNAs  
732 regulate gene expression in mouse oocytes. *Nature* 453:534-538

733 Tan GC, Chan E, Molnar A, Sarkar R, Alexieva D, Isa IM, et  
734 al. (2014) 5' isomiR variation is of functional and evolutionary  
735 importance. *Nucleic Acids Res* 42:9424-9435

736 Torri A, Jaeger J, Pradeu T, Saleh M-C (2022) The origin of  
737 RNA interference: Adaptive or neutral evolution? *PLoS Biol*  
738 20:e3001715

739 Trindade Rosa M, Oliveira DS, Loreto ELS (2017)  
740 Characterization of the first mitochondrial genome of a catenulid  
741 flatworm: *Stenostomum leucops* (Platyhelminthes). *J Zool Syst Evol*  
742 *Res* 55:98-105

743 van Wijnen AJ, van de Peppel J, van Leeuwen JP, Lian JB,  
744 Stein GS, Westendorf JJ, et al. (2013) MicroRNA functions in

745 osteogenesis and dysfunctions in osteoporosis. *Curr Osteoporos*  
746 *Rep* 11:72-82

747 VanKuren NW, Long M (2018) Gene duplicates resolving  
748 sexual conflict rapidly evolved essential gametogenesis functions.  
749 *Nat Ecol Evol* 2:705-712

750 Velandia-Huerto CA, Yazbeck AM, Schor J, Stadler PF (2022)  
751 Evolution and Phylogeny of MicroRNAs - Protocols, Pitfalls, and  
752 Problems. *Methods Mol Biol* 2257:211-233

753 Vendramin R, Marine JC, Leucci E (2017) Non-coding RNAs:  
754 the dark side of nuclear-mitochondrial communication. *EMBO J*  
755 36:1123-1133

756 Wagner EG, Simons RW (1994) Antisense RNA control in  
757 bacteria, phages, and plasmids. *Annu Rev Microbiol* 48:713-742

758 Wang M, Jiang S, Wu W, Yu F, Chang W, Li P, et al. (2018)  
759 Non-coding RNAs function as immune regulators in teleost fish. *Front*  
760 *Immunol* 9:2801

761 Watanabe T, Totoki Y, Toyoda A, Kaneda M, Kuramochi-  
762 Miyagawa S, Obata Y, et al. (2008) Endogenous siRNAs from  
763 naturally formed dsRNAs regulate transcripts in mouse oocytes.  
764 *Nature* 453:539-543

765 Weber-Lotfi F, Dietrich A (2018) Intercompartment RNA  
766 Trafficking in Mitochondrial Function and Communication. In: Cruz-

767 Reyes J, Gray M (eds) RNA Metabolism in Mitochondria. Nucleic  
768 Acids and Molecular Biology 34. Springer, Cham, pp 73-124

769 Wood DE, Lu J, Langmead B (2019) Improved metagenomic  
770 analysis with Kraken 2. Genome Biol 20:257

771 Yang N, Kazazian HH Jr (2006) L1 retrotransposition is  
772 suppressed by endogenously encoded small interfering RNAs in  
773 human cultured cells. Nat Struct Mol Biol 13:763-771

774 Yazbeck AM, Tout KR, Stadler PF, Hertel J (2017) Towards a  
775 Consistent, Quantitative Evaluation of MicroRNA Evolution. J Integr  
776 Bioinform 14:20160013

777 Yekta S, Tabin CJ, Bartel DP (2008) MicroRNAs in the Hox  
778 network: an apparent link to posterior prevalence. Nat Rev Genet  
779 9:789-796

780 Zhao Y, Lu G-A, Yang H, Lin P, Liufu Z, Tang T, et al. (2021)  
781 Run or Die in the Evolution of New MicroRNAs—Testing the Red  
782 Queen Hypothesis on De Novo New Genes. Mol Biol Evol 38:1544-  
783 1553

784 Zouros E, Rodakis GC (2019) Doubly Uniparental Inheritance  
785 of mtDNA: An Unappreciated Defiance of a General Rule. Adv Anat  
786 Embryol Cell Biol 231:25-49

787

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  $12.8616$ ;  $R^2 = 0.9719$ ;  $P < 2 \times 10^{-16***}$ .