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Paternal epigenetics: Mammalian sperm provide much more than DNA at fertilization

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21 Abstract

22 The spermatozoon is a highly differentiated cell with unique characteristics: it is mobile, thanks to its
23 flagellum, and is very compact. The sperm cytoplasm is extremely reduced, containing no ribosomes,
24 and therefore does not allow translation, and its nucleus contains very closed chromatin, preventing
25 transcription. This DNA compaction is linked to the loss of nucleosomes and the replacement of
26 histones by protamines. Based on these characteristics, sperm was considered to simply deliver
27 paternal DNA to the oocyte. However, some parts of the sperm DNA remain organized in a
28 nucleosomal format, and bear epigenetic information. In addition, the nucleus and the cytoplasm
29 contain a multitude of RNAs of different types, including non-coding RNAs (ncRNAs) which also carry
30 epigenetic information. For a long time, these RNAs were considered residues of spermatogenesis.
31 After briefly describing the mechanisms of compaction of sperm DNA, we focus this review on the
32 origin and function of the different ncRNAs. We present studies demonstrating the importance of
33 these RNAs in embryonic development and transgenerational adaptation to stress. We also look at
34 other epigenetic marks, such as DNA methylation or post-translational modifications of histones, and
35 show that they are sensitive to environmental stress and transmissible to offspring. The post-
36 fertilization role of certain sperm-borne proteins is also discussed.

37

38 Introduction

39 Our view of what sperm cells bring to the oocyte and its contribution to human reproduction has
40 dramatically changed since it was first understood, and remains the subject of intense research.
41 Sperm cells, simply called sperm, were identified in the 17th and 18th century, thanks to the
42 development of the microscope by Dutch scientist Antoni Van Leeuwenhoek (1632-1723). Van
43 Leeuwenhoek was the first to observe these strange moving objects within semen that he called
44 “animalcule” (1678, communication at the London Royal Society). At that time, it was hypothesized
45 that the head of the animalcules (sperm) contained a whole tiny human curled up inside
46 (homonculus at this time), whose development would be assured by the female, the egg was
47 considered to act as the placenta. Nicolaas Hartsoecker (1656-1725) was the first to propose an
48 illustration of this hypothesis in 1694 (Hartsoecker, 1694) (Figure 1).

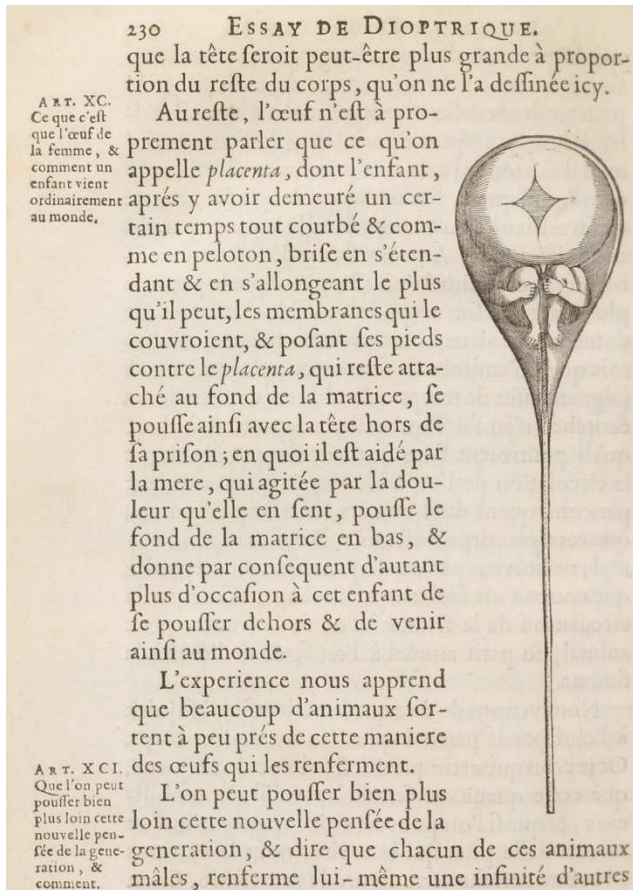


Figure 1. Illustration of the homunculus hypothesis proposed by Antoni Van Leeuwenhoek in 1678 and illustrated by Hartsoecker in 1694 (Hartsoecker, 1694). "Courtesy of The Linda Hall Library of Science, Engineering & Technology"

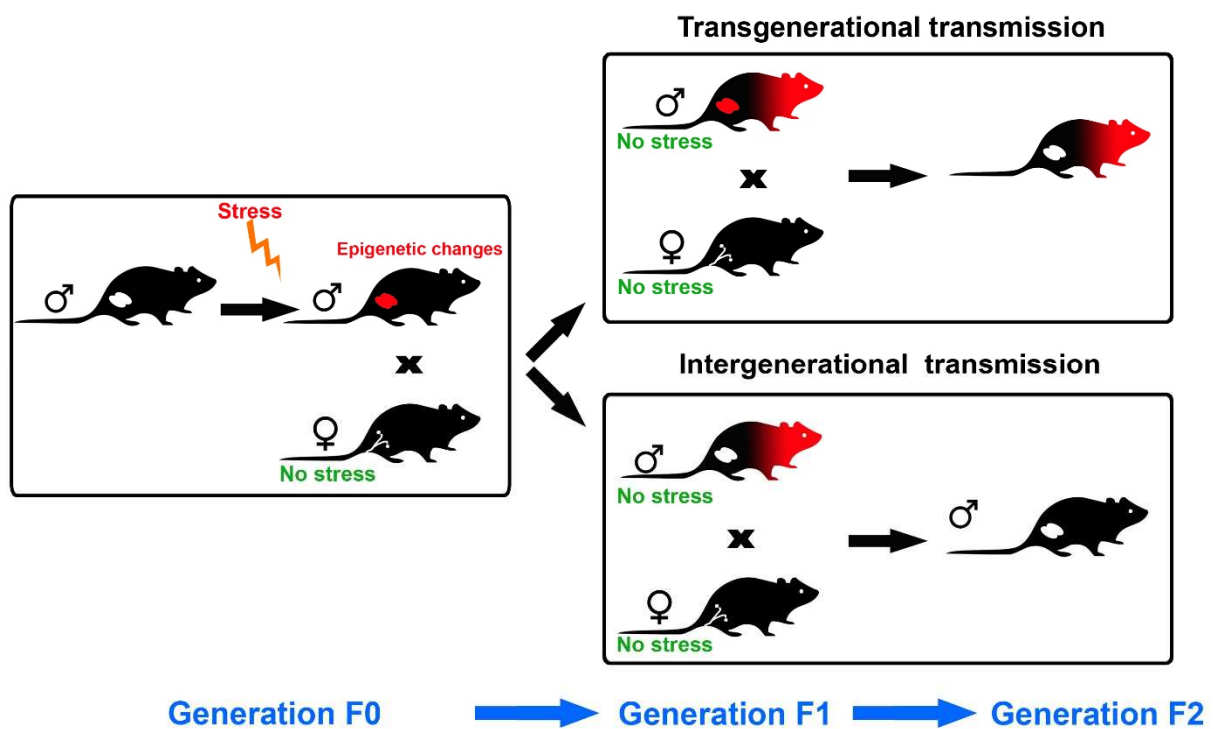
64

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66 We now know that this hypothesis was incorrect, but it took more than 200 years to start to decipher
 67 the contribution of sperm cells to the new living organism, and important discoveries are still being
 68 made. The fact that a spermatozoon provides half of the genetic material, and is at the origin of one
 69 of the pronuclei during fertilization was actually discovered two centuries later by several biologists
 70 between 1873 and 1883 using various animal models such as starfish (Fol, 1873), sea urchin (Hertwig,
 71 1876) and ascaris (van Beneden, 1883). The discovery that sperm contribute half of the heredity of
 72 the embryo was discovered simultaneously 20 years later by Theodor Boveri (1862-1915) and Walter
 73 Sutton (1877-1916). In 1902-1903, they proposed that chromosomes bear hereditary factors in
 74 accordance with Mendelian laws – first described 37 years previously by the monk Augustin Mendel
 75 (1822-1884) – and that sperm provide half of the chromosomes found in the zygote. Soon after this
 76 breakthrough, Jacques Loeb (Loeb, 1913) rapidly recognized that the male gamete not only transmits
 77 the paternal characteristics to the developing embryo, but also induces development by activating
 78 the egg. A second major function of the male gamete, essential for fertilization, was then identified.
 79 The characterization of the sperm-borne factor triggering egg activation was also an epic scientific
 80 journey, and culminated in the discovery of PLCzeta (*PLCZ1*) by teams led by Karl Swann and Antony

81 Lay in 2002 (Cox *et al*, 2002, Saunders *et al*, 2002). A description of this sperm factor is beyond the
 82 scope of this review, and for more information about this key protein, we refer readers to a recent
 83 review (Parrington *et al*, 2019).

84 For decades, the function of sperm was considered to be limited to these two roles: providing half of
 85 the hereditary material in the form of the paternal DNA, and activating the egg. In this review, we
 86 will discuss the sperm epigenome, the modifications to the genome that do not affect the DNA
 87 sequence but are capable of influencing gene expression, including non-coding RNA, DNA
 88 methylation and posttranscriptional modification of histones. We will first present the characteristics
 89 of sperm chromatin and the main elements necessary for its organization and compaction. Then, we
 90 will describe the epigenetic events occurring in the sperm, the different RNA molecules (mRNAs,
 91 regulatory non-coding RNAs) contained in sperm and their origins, and also significant sperm
 92 developmental factors including histones and proteins that are retained and transmitted. Finally, we
 93 highlight how environmental stress can alter epigenetic factors in sperm, such as DNA methylation
 94 and non-coding RNA, causing phenotypic changes in the offspring. These changes not only affect the
 95 phenotype of the F1 offspring, but also that of the following F2 generation, even though the F1
 96 generation was not exposed to the initial environmental stress. Nevertheless, this transgenerational
 97 transmission of a new phenotype is not permanent and vanishes in most cases after the F3
 98 generation. Readers should note that, “transgenerational transmission” is distinct from
 99 “intergenerational transmission”, in that the latter only affects the F1 generation (Figure 2).



101

102

103 *Figure 2. Stress (Nutritional, physical, ...) induces epigenetic changes in the male reproductive organs,*
104 *including testes and epididymides. These epigenetic modifications lead to an alteration of epigenetic*
105 *information carried by the sperm and the progeny obtained from stressed male crossed with a non-*
106 *stressed female exhibits phenotypic changes dependent of the nature of the stress. Epigenetic*
107 *alterations can be maintained in the male germ cells up to F3 generation, even though the*
108 *subsequent generations are not exposed to the initial (F0) environmental stress, a phenomena named*
109 *“transgenerational transmission”. If the epigenetic alterations of the germ cells are not transmitted*
110 *to the F2 generation, then the phenotypic change is named “intergenerational transmission”.*

111

112

113 1. Sperm DNA compaction

114

115 Sperm cells are produced during the spermatogenesis, a very complex physiological process which
116 can be split into three successive steps: mitosis, meiosis, and spermiogenesis and involves the
117 expression of several thousands of genes (Hess & Renato de Franca, 2008). The spermatozoon is
118 undoubtedly the most highly differentiated cell type in mammals, with the greatest number of
119 specific or enriched genes necessary for its biogenesis (Uhlén *et al*, 2016). Among the many
120 exceptional features of this cell, its DNA organization is unique, showing a high level of compaction
121 leading to an arrest of transcription. Sperm DNA compaction involves replacing nucleosomes by
122 donut-shaped DNA-protamine toroids, with each toroid containing 60 kbp of DNA. This
123 rearrangement results in a highly compact and transcriptionally inactive sperm chromatin (Steger,
124 1999). For a long time, the histone-to-protamine transition in mammals was considered to take place
125 in two successive stages, with a first stage involving the replacement of histones by transition
126 proteins, and a second stage during which transition proteins are replaced by protamines. However,
127 discoveries in this field over the years revealed the process to be much more complex, and not as
128 linear as it initially seemed. At the beginning of the transition, from spermatogonia to elongated
129 spermatids, the canonical histones (H2A, H2B, H3 and H4) and the H1-linker histone undergo post-
130 translational modifications to facilitate their progressive replacement in nucleosomes by somatic or
131 testicle-specific variants. Each histone variant has its own expression window that is directly related
132 to its specific role during the process of histone-to-protamine transition. The incorporation of histone

133 variants induces chromatin instability that favors nucleosome disassembly. Moreover, post-
134 translational modifications of the variants accentuate chromatin relaxation and allow DNA strand
135 breaks that facilitate the progressive elimination of histone variants and their replacement by
136 transition proteins and protamines (Rathke *et al*, 2014, Wang *et al*, 2019). Recently, it was proposed
137 that transition proteins and protamines could be added simultaneously, rather than successively,
138 leading to the formation of a nucleo-protamine structure (Barral *et al*, 2017).

139 1.a Histone variants

140 The core canonical histones (H3, H4, H2A and H2B) can be replaced by histone variants, changing the
141 structural and functional features of the nucleosomes. Unlike canonical histones, which are
142 incorporated during the S-phase of the cell cycle, histone variants are synthesized and incorporated
143 throughout the cell cycle, in particular during spermiogenesis. Many histone variants have been
144 identified in mammals for H2A, H2B and H3 canonical histones, but none for H4. The H1-linker
145 histone, which influences the degree of compaction of the chromatin, and whose eviction leads to
146 decondensation, also has variants (Thoma *et al*, 1979). The variants are species-dependent, and
147 human and mouse histone variants only partially overlap.

148 Compaction of sperm DNA is associated with intense trafficking of histone and histone variants
149 within the nucleosome and its linker, a process that is not fully understood. Histone H1 has at least
150 11 variants, including the testis variants H1T, H1T2 and HILS1 (TS H1.6, TS H1.7 and TS H1.9,
151 according to the new nomenclature) (El Kennani *et al*, 2017). H1T is present from the spermatocyte I
152 stage to the elongating spermatid stage (Drabent *et al*, 1996). H1T maintains a relatively open
153 chromatin configuration, which is necessary for meiotic recombination and histone replacement
154 (Delucia *et al*, 1994). H1T2 is present from the round spermatid stage to the elongated spermatid
155 stage at the apical pole of the nucleus, under the acrosome. It is required for chromatin
156 condensation, protamine incorporation and cell restructuring (Martianov *et al*, 2005). HILS1, present
157 from the elongated to the condensed spermatid stages, has a higher DNA binding capacity than other
158 histones. It could be involved in transcriptional regulation and also contribute to nuclear
159 condensation through its interactions with transition proteins (Yan *et al*, 2003).

160 Several variants of the histone H2A (tH2A, H2A.L.1-3, H2A.B.1-3, H2AP) and the histone H2B (tH2B)
161 have been specifically identified in mouse testis, but their specific roles have not been fully
162 described. The histone variants tH2A and tH2B (TS H2A.1 and TS H2B.1, according to the new
163 nomenclature) are present from the spermatogonia stage and gradually disappear during
164 condensation of spermatid nuclei (El Kennani *et al*, 2017, Montellier *et al*, 2013, Trostle-Weige *et al*,
165 1982, van Roijen *et al*, 1998). They induce chromatin instability and could guide the placement of
166 transition proteins and protamines. These variants also seem to play a role in epigenetic

167 reprogramming (Padavattan *et al*, 2015). The class of histone variants H2A.L and H2B.L are present
168 from the round spermatid stage to the elongated spermatid stage, and H2A.L.1, H2A.L.2 and H2B.L.1
169 are still present in the mature sperm (Govin *et al*, 2007). H2A.L.2 allows the chromatin to open, and
170 could play a crucial role in the incorporation of transition proteins and protamines (Barral *et al*,
171 2017). H2A.B.3 is present from the spermatocyte I stage to the round spermatid stage (Soboleva *et*
172 *al*, 2012). H2A.B.3 could also promote the chromatin reorganization necessary for histone-to-
173 protamine transition, by modulating the incorporation and removal of H2A.L.2 and TP1 from
174 chromatin (Anuar *et al*, 2019). It could also play a role in pre-mRNA splicing (Soboleva *et al*, 2017).
175 Histone H3 (H3.1 and H3.2) has a testicle-specific histone variant (H3t), and a testicle-enriched
176 variant (H3.3) in mice. H3t (TS H3.4, according to the new nomenclature) is present from the
177 spermatogonia stage to the early spermatid stage (El Kennani *et al*, 2017, Trostle-Weige *et al*, 1984).
178 H3t could contribute to destabilizing chromatin, and play another as yet unknown role in later stages
179 of spermiogenesis that cannot be compensated for by other members of the H3 family (Tachiwana *et*
180 *al*, 2010, Ueda *et al*, 2017). H3.3 is a ubiquitous protein encoded by two genes, *H3f3a* and *H3f3b*.
181 These genes have different nucleotide sequences, but produce the same protein. *H3f3a* transcripts
182 are present in small amounts from the spermatogonia stage to the elongated spermatid stage,
183 whereas *H3f3b* transcripts are only detected during the spermatocyte I stage (Bramlage *et al*, 1997).
184 H3.3 allows destabilization of the chromatin, promotes transcription and plays an essential role in
185 the histone-to-protamine transition by modulating the incorporation of TP1 and P1 in chromatin
186 (Ahmad & Henikoff, 2002, Thakar *et al*, 2009, Yuen *et al*, 2014). Moreover, H3.3 is involved in Meiotic
187 Sex Chromosome Inactivation (MSCI) at the pachytene stage. Sex chromosomes are located at the
188 periphery of the nucleus in the sexual body and are inactivated during meiosis. Induction of MSCI is
189 associated with both removal of conventional H3 and accumulation of H3.3 and γ H2AX in the
190 sexual body, suggesting that these histone variants are needed for sex chromosome condensation
191 and silencing (Mahadevaiah *et al*, 2001, van der Heijden *et al*, 2007). The human-specific H3.5
192 variant is present from the spermatogonia to the spermatocyte I stages (Urahama *et al*, 2016). H3.5
193 could also be involved in chromatin destabilization. Moreover, another, as yet poorly characterized,
194 role related to DNA synthesis has been described (Shiraishi *et al*, 2018, Urahama *et al*, 2016).
195 Overall, this short description of the numerous histone variants involved in sperm DNA compaction
196 highlights the complexity of the process.

197 1.b Transition proteins

198 Transition proteins are basic nuclear proteins (more basic than histones and less basic than
199 protamines) that are synthesized during the advanced stages of spermiogenesis and disappear
200 before the final stage of spermiogenesis (Alfonso & Stephen Kistler, 1993). There are four types of

201 transition proteins: TP1, TP2, TP3 and TP4 (or TNP1, TNP2, TNP3 and TNP4). The first two are present
202 in all species, and are consequently the best characterized. TP1 is a 54-amino-acid protein that is
203 highly conserved in mammals. It is rich in serine, arginine, histidine and lysine residues (Kremling *et al*,
204 *al*, 1989). TP2 is a larger protein, with 117 to 138 amino acids depending on the species, it is rich in
205 the same basic amino acids as TP1, and also contains some cysteine residues (Kleene & Flynn, 1987,
206 Schlüter *et al*, 1992). Its N-terminal part contains two zinc finger domains allowing it to bind to
207 unmethylated CpG sites on DNA, whereas its C-terminal part is very basic (Baskaran & Rao, 1991,
208 Cole & Kistler, 1987, Meetei *et al*, 2000). Animals lacking TP1 or TP2 produce normal sperm but have
209 reduced fertility, demonstrating that there is some functional redundancy between TP1 and TP2. The
210 absence of both TP1 and TP2 causes male sterility, associated with protamine defects and major DNA
211 breaks. The cells produced are incompatible with fertilization, even by intracytoplasmic sperm
212 injection (ICSI) (Shirley *et al*, 2004, Yu *et al*, 2000, Zhao *et al*, 2004, Zhao *et al*, 2001). Although the
213 two proteins can partially compensate for each other, they play unique roles in the histone-to-
214 protamine transition. During the histone-to-protamine transition, TP1 could be involved in DNA
215 destabilization and thus promote histone removal; it could also contribute to DNA strand break
216 repair (Caron *et al*, 2001, Singh & Rao, 1987). TP2 could be necessary for stabilizing and initiating
217 DNA condensation (Baskaran & Rao, 1990). Finally, transition proteins also carry a significant number
218 of post-translational modifications (acetylation, phosphorylation and methylation) on their serine,
219 lysine, and arginine residues. These modifications are inserted at specific times during nuclear
220 condensation in spermatids and are crucial for the histone-to-protamine transition (Gupta *et al*,
221 2015).

222

223 1.c Protamines

224 Protamines were first isolated and discovered in salmon sperm at the end of the 19th century by
225 Friedrich Miescher (Miescher, 1874). There are three types of protamines: protamine 1 (P1),
226 protamine 2 (P2) and protamine 3 (P3), but only P1 and P2 are involved in sperm DNA compaction.
227 P1 is expressed in all vertebrates, whereas P2 is expressed only in certain mammalian species
228 (Primates, Rodents, Lagomorphs and equine and bovine species) (Balhorn, 2007, Corzett *et al*, 2002,
229 Hamilton *et al*, 2019b). Protamines, proteins of low molecular weight, are highly basic (more basic
230 than histones and TPs), and containing extensive proportions of arginine (47-70% of the amino acids)
231 and cysteine (8-16% of the amino acids) residues in placental mammals (Balhorn, 2007, Calvin, 1976,
232 McKay *et al*, 1985, MCKAY *et al*, 1986, Queralt *et al*, 1995). In placental mammals, P1 consists of
233 around 50 amino acids organized in three characteristic domains. A central arginine-rich DNA binding
234 domain, flanked on either side by short segments containing phosphorylation sites composed of

235 serine and threonine residues, and cysteine residues necessary for the formation of inter-protamine
236 disulfide bridges (Balhorn, 2007, McKay et al., 1985, Queralto et al., 1995). P2 contains 54 to 63 amino
237 acids depending on the species (McKAY et al., 1986, Yelick *et al*, 1987). Unlike P1, a precursor to P2 is
238 synthesized with about 100 amino acids. The mature form of P2 is obtained by cleavage, resulting in
239 the removal of about 40% of its N-terminus (Balhorn, 2007, Carré-Eusèbe *et al*, 1991, Yelick et al.,
240 1987). In humans, two mature forms of P2 have been described, P2a with 57 amino acids and P2b
241 with 54 amino acids (McKAY et al., 1986). Both bull and boar possess the *PRM2* gene, and P2 mRNA
242 transcripts have been detected, but no P2 protein has yet been identified in mature sperm from
243 these animals (Ganguly *et al*, 2013, Maier *et al*, 1990, Mazrimas *et al*, 1986, Tobita *et al*, 1982). They
244 are synthesized during the last stages of spermatogenesis to replace histones at the DNA, and highly
245 contribute to its compaction (Rathke et al., 2014).

246

247 1.d Organization of sperm chromatin

248

249 The presence of a large number of positively-charged amino acids in protamines, and in particular
250 the arginine clusters, constitute zones for DNA tethering, thanks to the electrostatic bonds created
251 by their opposing charges. These proteins therefore form very stable complexes with DNA (Balhorn,
252 2007, Brewer *et al*, 2003, DeRouchey & Rau, 2011). Protamine binding will thus neutralize the charge
253 on the DNA molecules and allow them to pack more closely. The structural organization of the
254 protamine-DNA interaction is still unclear, but it seems that protamines wrap around the double-
255 stranded DNA by positioning themselves in one of the grooves of the double helix (Balhorn, 2007,
256 Fita *et al*, 1983, Hud *et al*, 1994). P1 can bind about 11 bp of DNA, whereas the larger P2 can bind up
257 to 15 bp, at a rate of one protamine per turn of the helix (Bench *et al*, 1996). Protamine binding in
258 the DNA grooves induces curvature, resulting in the formation of a toroidal loop-like structure or
259 "donut loop", with each toroidal loop connecting the DNA to the nuclear matrix (Allen *et al*, 1997,
260 Allen *et al*, 1993, Balhorn, 2007, Björndahl & Kvist, 2010, Hud *et al*, 1993, Ward, 2010). Each toroidal
261 loop can pack up to 60 kb of DNA, and the sperm nucleus can contain up to 50,000 toroidal loops by
262 the end of spermiogenesis (Hud et al., 1993).

263

264 Protamines also have cysteine residues, the thiol (-SH) groups of which are oxidized to disulfide (-SS-)
265 by nuclear glutathione peroxidase 4 (nGPx4) to form a three-dimensional network of disulfide
266 bridges (Puglisi *et al*, 2012). The intra-protamine disulfide bridges stabilize the folding of different
267 protamine domains and are essential for DNA condensation. The inter-protamine disulfide bridges, in
268 complement, serve to increase the structural stability of the nucleoprotamines and to maintain the
269 strained conformation of the structure, which further enhances the level of DNA compaction

270 (Hutchison *et al*, 2017). The chromatin stability is proportional to the number of disulfide bridges
271 formed, and this stabilization process is progressive, beginning in the testis and continuing as sperm
272 transit through the epididymis (Balhorn *et al*, 1992, Gosálvez *et al*, 2011, Saowaros & Panyim, 1979,
273 Yossefi *et al*, 1994).

274 In P2, zinc finger domains composed of cysteine and histidine residues and stabilized by zinc salt
275 bridges contribute to the formation of P2-DNA complexes (Bench *et al*, 2000, Bianchi *et al*, 1992). In
276 addition to its role in stabilizing the nucleoprotamine structure as effectively as disulfide bridges, zinc
277 also prevents superstabilization by reducing the availability of free thiol groups to form disulfide
278 bridges (Björndahl & Kvist, 2010). Zinc salt bridges are also easier to disrupt than disulfide bridges,
279 and could allow faster decondensation of the chromatin upon oocyte fertilization (Björndahl & Kvist,
280 2010). This setup probably explains why species expressing only P1 have the most extensively
281 compacted chromatin, and why species expressing more P2 are more sensitive to DNA damage
282 (Gosálvez *et al*., 2011).

283 In addition to their structural and binding residues, protamines have serine and threonine residues
284 which are phosphorylated immediately after protamine synthesis by SRPK1 (serine/arginine-rich
285 protein-specific kinase-1) for P1 and Camk4 (Calcium/calmodulin-dependent protein kinase type IV)
286 for P2 (Papoutsopoulou, 1999, Wu *et al*, 2000). These phosphorylation events are necessary to
287 initially establish correct binding between the protamines and the DNA. Phosphorylation reduces the
288 affinity of the protamines for DNA (by increasing the negative charge of protamines), to give the
289 protamines time to position themselves optimally with respect to the DNA, thus reducing
290 mismatching. Subsequently, the progressive dephosphorylation of protamines gradually increases
291 the affinity of protamines for DNA and induces chromatin condensation (Green *et al*, 1994). Optimal
292 positioning of protamines is essential for the appropriate formation of inter-protamine disulfide
293 bridges (Marushige & Marushige, 1978). Other post-translational modifications of protamines have
294 been identified and could contribute to the protamine-DNA binding or protamine removal process
295 after fertilization, suggesting the existence of a protamine code equivalent to the histone code
296 (Brunner *et al*, 2014).

297 During spermatogenesis, not all histones are replaced by protamines, and variable fractions of
298 histones remain associated with DNA in the mature sperm (1-8% for mouse (Balhorn *et al*, 1977, Jung
299 *et al*, 2017), 13% for bull (Samans *et al*, 2014), 3-15% for human (Bench *et al*., 1996, Samans *et al*.,
300 2014, Tanphaichitr *et al*, 1978), and 25% in some marsupial species (Soon *et al*, 1997)). These
301 residual histones were first considered a defect in the replacement of histones by protamines, but it
302 is now clear that persistent histones (canonical or variant) in mature sperm are not randomly
303 distributed and have a role to play. Histone-enriched regions appear to be distributed either in large
304 10-100-kb regions corresponding to the solenoids present between toroidal loops, or in short

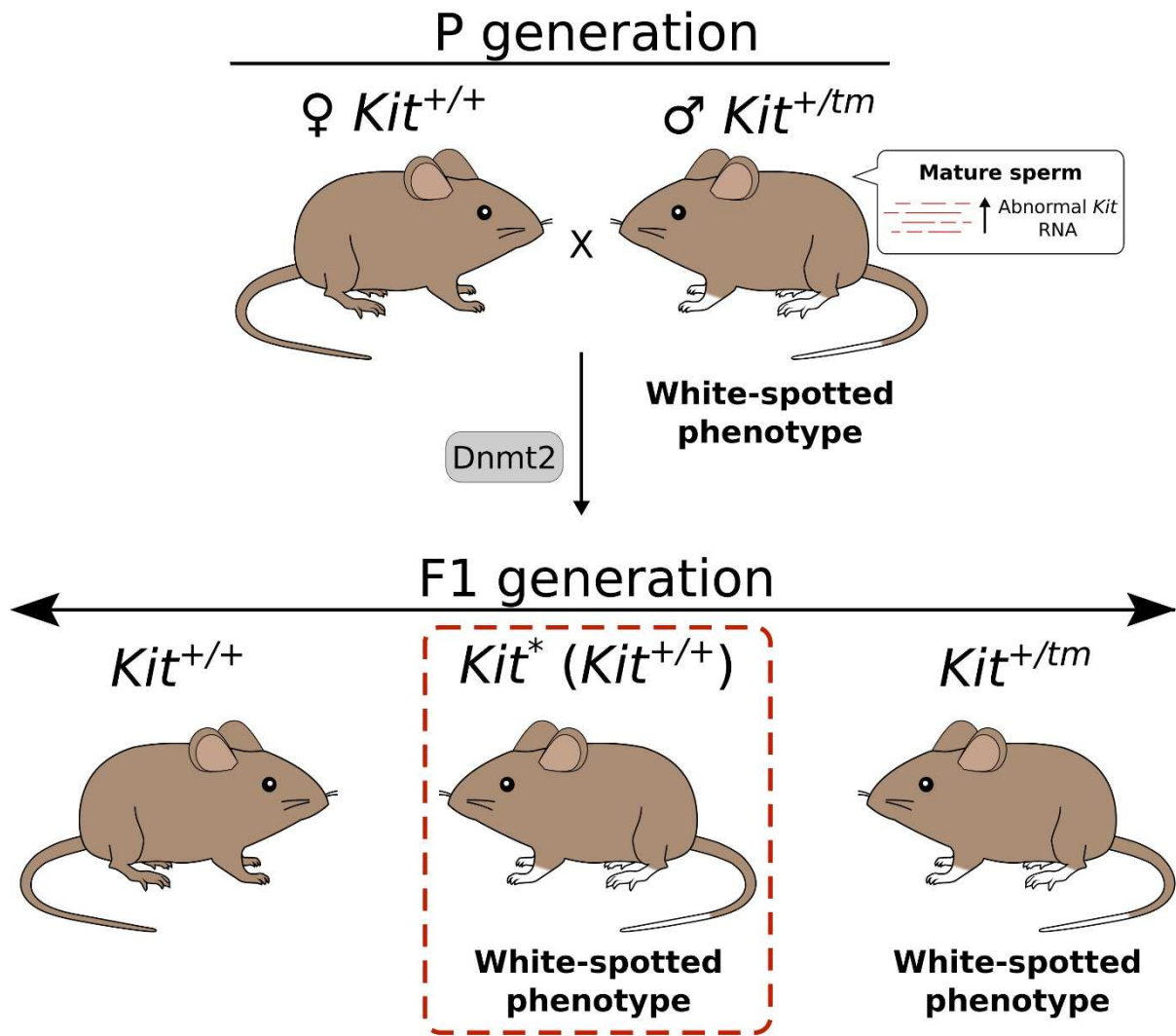
305 domains dispersed throughout the genome, corresponding to stretches of DNA anchoring the
306 toroidal loops to the nuclear matrix (Arpanahi *et al*, 2009, Hammoud *et al*, 2009, Ward, 2010). The
307 precise location of persistent histones in the genome remains a subject of debate. Some studies
308 found that nucleosomes are enriched at the level of hypomethylated CpG sites corresponding to
309 promoters and genes that are important for embryogenesis (transcription factors, signaling factors,
310 Homeobox gene clusters, globin genes, imprinted gene clusters, genes encoding microRNAs, etc.)
311 (Arpanahi *et al.*, 2009, Erkek *et al*, 2013, Gardiner-Garden *et al*, 1998, Hammoud *et al.*, 2009, Royo *et*
312 *al*, 2016, Yoshida *et al*, 2018). Nevertheless, other authors have reported that the nucleosomes
313 persist at intergenic regions, regions rich in repeated sequences (telomeres, subtelomeres,
314 centromeres, pericentromeres, LINE1 and SINE) and rRNA and SRP RNA (RNA component of Signal
315 Recognition Particle) gene clusters (Carone *et al*, 2014, Houghoughi *et al*, 2020, Meyer-Ficca *et al*,
316 2013, Samans *et al.*, 2014, Sillaste *et al*, 2017, Zalenskaya *et al*, 2000). These divergent results could
317 be due to the fact that different protocols were used to isolate histones, but could ultimately be
318 complementary: even if the majority of persistent histones are localized in gene deserts, some of the
319 histones are also deposited in specific regions that are important for embryonic development
320 (Yamaguchi *et al*, 2018). Importantly, histones retained in sperm carry epigenetic marks that are
321 transferred to the embryo, and they could be involved in embryonic development (Bui *et al*, 2011,
322 Ihara *et al*, 2014, van der Heijden *et al*, 2008).

323 In summary, sperm DNA compaction is highly complex and involves a multitude of steps and
324 molecules. Interestingly, the transition from nucleosome to nucleoprotamine does not result in total
325 erasure of epigenetic markers, as evidenced by the presence of histones and histone variants in
326 mature sperm. Histones are only one facet of epigenetic control over transcription/translation, and
327 the question of the significance of other markers in paternal inheritance remains to be evaluated.

328 2. Paramutations in sperm and the discovery of the importance of 329 RNA in paternal heritage

330 Paramutations were first described in plants as an epigenetic modification of one allele induced by
331 crosstalk between the two alleles of the same gene, giving rise to a new phenotype for the modified
332 allele (Chandler, 2007). Importantly, despite its name, paramutation causes changes in the
333 expression of the affected allele without any DNA mutation. Nevertheless, the modified locus can
334 induce a similar change in homologous sequences, and consequently this change of allelic expression
335 is transmitted to future generations and is thus heritable. In fact, paramutation allows the
336 transmission of heritable characteristics through epigenetic mechanisms. Paramutation was reported
337 in mice for the Kit gene (Rassoulzadegan *et al*, 2006). The Kit gene, coding for a tyrosine kinase

338 receptor, is essential for the maintenance of primordial germ cells (PGCs) in both sexes in the
339 embryo, but it is also expressed in adult testis where it is required for DNA synthesis in differentiating
340 spermatogonia (Rossi *et al*, 2000). In addition, Kit is expressed in melanocytes and is necessary for
341 melanin expression. Kit-null animals (insertion of a lacZ cassette within the gene, Kittm) are not viable,
342 in contrast to heterozygous animals Kit^{+tm}, which have a white tail tip and white fingers
343 (Rassoulzadegan *et al.*, 2006) . Interestingly, the authors reported that WT animals (Kit^{+/+}) obtained
344 from crosses between two heterozygote animals (Kit^{+tm}) or a heterozygote animal and a WT animal
345 also had a white tail tip and white fingers, despite their full WT genotype (Figure 3). This observation
346 suggests that the Kit⁺ allele from the heterozygous animal has been modified (called Kit*). The white-
347 spotted phenotype was transmitted to the F2 generation, even though no Kittm allele was present,
348 but vanished in subsequent generations. Levels of the polyadenylated messenger RNA for Kit were
349 generally decreased in animals with white-spotted fur and a full WT genotype (paramutated
350 animals), but spliced fragments of the full Kit transcript with multiple sizes were strongly expressed in
351 the testis. More surprisingly, extensive deregulation of the temporal window of Kit mRNA expression
352 was observed, with strong expression in postmeiotic cells in heterozygous males compared to WT
353 animals, where Kit mRNA was barely detectable. Moreover, Kit mRNA is not eliminated in the
354 residual bodies during the sperm cytoplasmic reduction, and remains present in mature epididymal
355 sperm, suggesting a role for Kit mRNA fragments in the transmission of the white-spotted phenotype.
356 This role for fragmented Kit mRNA in the paramutation was demonstrated by injecting sperm mRNA
357 extracts from heterozygous males into WT oocytes. The offspring expressed the white-spotted
358 phenotype, in contrast to offspring from WT males. The role of fragmented Kit mRNA was also
359 supported by the fact that an injection of 2 microRNA species (miR-221 and miR-222) targeting Kit
360 mRNA mimicked the effect of sperm RNA injection. Importantly, animals generated from WT sperm
361 and miRNA-injected WT oocytes could transmit the white-spotted phenotype to the F2 generation.
362 These results allowed the authors to propose that the modified phenotype is induced by the transfer
363 of cleaved Kit RNA molecules to embryos. These findings demonstrated that microRNA injected into
364 the zygote can induce paramutation by altering gene expression, and thus confirmed that sperm
365 miRNA can alter the phenotype of the progeny. This idea was supported by Grandjean and coll.
366 (Grandjean *et al*, 2009), who showed that injection of miR-124 induced a large body size as a result of
367 modification of Sox9 RNA, and Wagner and coll. (Wagner *et al*, 2008), who demonstrated that
368 microinjection of miR-1 led to deregulation of the transcript Cdk9, a transcription factor, and cardiac
369 hypertrophy in mice. In both cases, the phenotypes were transmitted to subsequent generations.



370

371 **Figure 3: Model for transgenerational inheritance of paramutated *Kit*.**

372 Heterozygous mice carry a wild-type (+) and a modified/inactivated *Kit* allele (*tm*), and bear a white-
 373 spotted phenotype with white fingers and white tail tips, while wild-type homozygotes have solid
 374 color. The heterozygote males produce abnormal *Kit* RNA from the Kit^{tm} allele, which are packaged in
 375 the sperm and delivered to the egg upon fertilization. Some resulting progeny with two wild-type
 376 alleles develop the heterozygote white-spotted phenotype (Kit^*). The aberrant RNAs are transmitted
 377 to the progeny and thought to regulate expression of the wild-type allele in the zygote, thus
 378 propagating the paramutation to the progeny. The establishment and transgenerational inheritance
 379 of the paramutation also requires the expression of an RNA methyltransferase *Dnmt2* (Kiani et al,
 380 2013), but its role remains unclear. The paramutation might gradually disappear over successive
 381 generations due to the reduction in aberrant *Kit* RNAs.

382 **3. Sperm contains numerous messenger RNA (mRNA) and non-**
 383 **coding RNA (ncRNA)**

384 The coding region of DNA represents only 2.5% of the genome. Nevertheless, most of the genome
 385 (~85%) can be transcribed as non-coding RNA (ncRNA). There are two types of ncRNAs, housekeeping
 386 RNA such as transferRNA (tRNA) and ribosomalRNA, and regulatory ncRNAs, which have many

387 functions, including post-transcriptional regulation of mRNA. For a detailed review of the numerous
388 classes of regulatory ncRNA and more detail on their structure and functions, we refer readers to
389 (Hammond, 2015, Hombach & Kretz, 2016). Here, we will focus only on short ncRNAs, including Piwi-
390 interacting RNA (piRNA), microRNA (miRNA), small interfering RNAs (endo-siRNAs), and transferRNA-
391 derived fragments (tRFs).

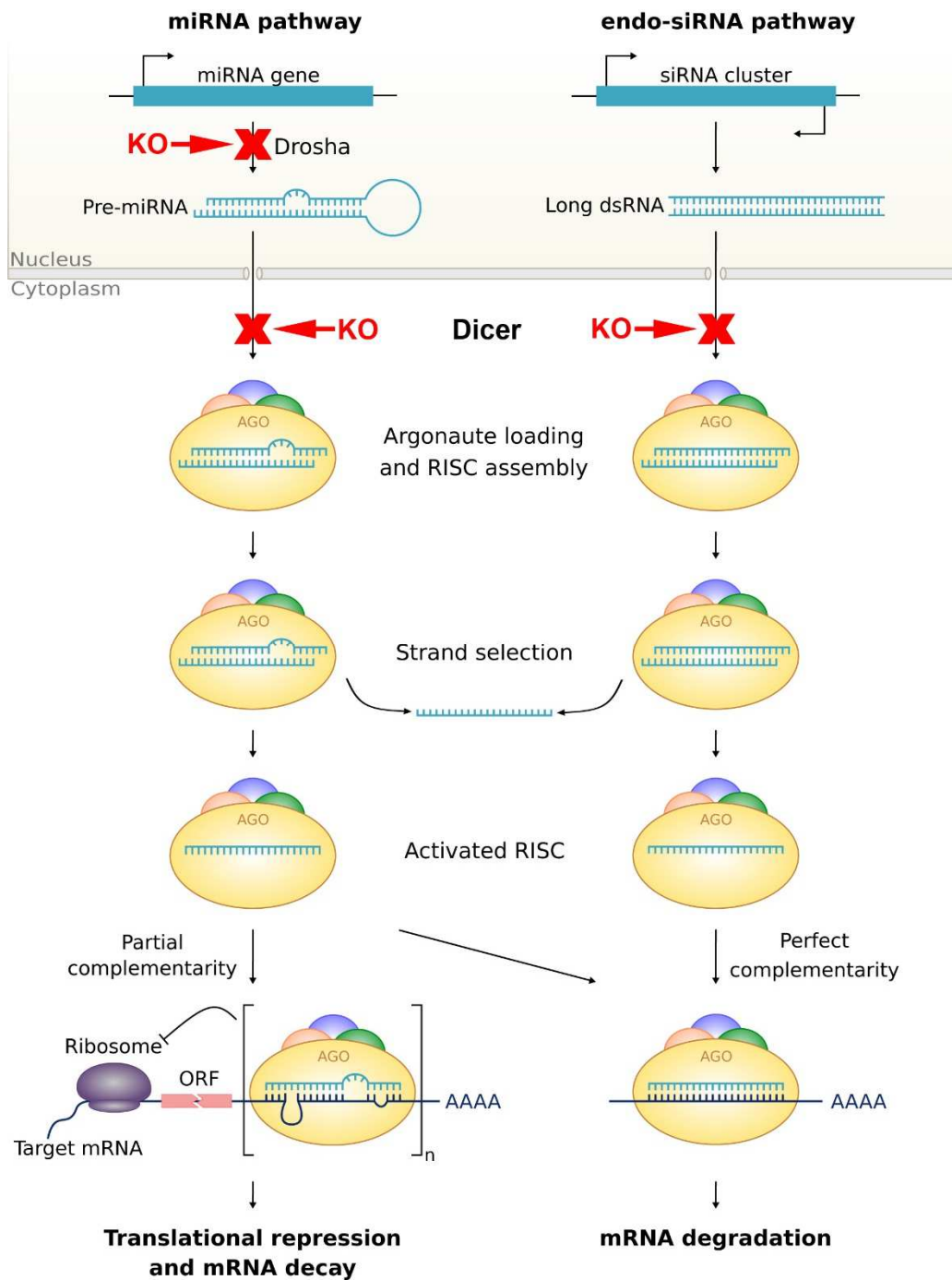
392 Short ncRNAs can be split into two classes: those coded at specific loci in the genome, and those
393 resulting from endonucleolytic cleavage of other types of RNA. The first class corresponds to piRNA,
394 miRNA, and endo-siRNA. piRNA and miRNA genes, located throughout the genome, are non-coding
395 genes which exclusively generate ncRNA. Some miRNAs are located within introns or untranslated
396 regions (UTR) of protein coding genes. Endo-siRNAs have only been mapped to genomic areas that
397 produce transcripts capable of forming double-stranded RNA (dsRNA) structures, such as
398 retrotransposons or bidirectional transcription sites. tRFs, in contrast, are generated through the
399 cleavage of transfer RNA by endonucleases such as Angiogenin, DICER, and probably some
400 uncharacterized proteins.

401 During the cytoplasmic sperm compaction occurring at the end of spermiogenesis, most of the
402 cytoplasm, including ribosomes, the endoplasmic reticulum, and the Golgi apparatus, are discarded
403 in the residual body. Moreover, as explained above, the sperm DNA becomes highly compacted as
404 nucleosomes are replaced by protamines, eventually resulting in closed chromatin. As a result of
405 these changes, transcription and translation are considered to be arrested in sperm. Nevertheless,
406 RNA was first detected in sperm over 30 years ago (Pessot *et al*, 1989). The more recent
407 development of high-throughput sequencing technologies has revealed the vast diversity of RNA
408 present, with more than 10,000 distinct transcripts identified (El Fekih *et al*, 2017, Selvaraju *et al*,
409 2017, Sandler *et al*, 2013). Various types of RNA were identified, including messenger RNAs and
410 several classes of ncRNAs, including short and long ncRNAs (Dadoune, 2009). Moreover, RNA-seq
411 technology identified both full and truncated RNAs, which is an interesting result when considering
412 Kit-induced paramutation results. A multispecies sperm-borne RNA database to allow comparative
413 annotation of all sperm RNA has been established (Schuster *et al*, 2016). When sperm RNAs were
414 discovered, they were at first considered to be remnants of spermatogenesis. However, the studies
415 showing that microinjections of RNAs into the zygote alter the heredity of the progeny over several
416 generations (Grandjean *et al.*, 2009, Wagner *et al.*, 2008) stimulated a reevaluation of the role of the
417 various endogenous sperm RNAs in embryo development and epigenetic inheritance, as explained
418 below.

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3.a MicroRNA (miRNA) and small interfering RNAs (endo-siRNAs)

MicroRNA (miRNA) and small endogenous interfering RNAs (endo-siRNAs) are small ncRNAs characterized by transcript sizes smaller than 200 nt. Single-stranded miRNAs are generally the most abundant class of small ncRNAs, mediating gene silencing at the post-transcriptional level. Double-stranded siRNAs are key players in the RNA interference (RNAi) pathway, an alternative pathway for post-transcriptional gene silencing. Considerable light into the molecular processes governing small RNA biogenesis pathways has been shed by several teams: for miRNA, the canonical pathway involves two endoribonucleases, DROSHA in the nucleus and DICER in the cytoplasm, which sequentially generate the active, ~22-nucleotide mature miRNA from the hairpin shaped primary miRNA (Hammond, 2015) (Figure 4). siRNA are produced from double-stranded RNA by DICER, without input from DROSHA. Both ncRNA are loaded onto the RNA-induced silencing complex (RISC), which cleaves the target complementary mRNA to induce RNA decay and gene silencing (Figure 3). As they target the 3' untranslated regions of mRNAs, miRNA are not very specific, and a single miRNA is predicted to induce the cleavage of hundreds of mRNAs. In contrast, siRNAs are more specific. More than 2000 distinct miRNAs have been characterized so far, targeting around 1/3 of all mRNAs.



436

437 **Figure 4: The mechanism of miRNA and endo-siRNA biosynthesis and function.**

438 *miRNA and endo-siRNA are involved in post-transcriptional gene silencing through translational*
 439 *inhibition and mRNA decay, or mRNA cleavage by Argonaute and subsequent degradation. (Adapted*
 440 *from (Chu & Rana, 2007, Lam et al, 2015), Chu CY, Rana TM (2007) and Clancy, S (2008)). AGO:*
 441 *Argonaute; endo-siRNA: endogenous small interfering RNA; miRNA: micro-RNA; pre-miRNA: precursor*
 442 *miRNAs; ORF: open reading frame; RISC: RNA-induced silencing complex*

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444 RNA interference pathways, and particularly those involving miRNAs, are emerging as important
 445 regulators of spermatogenesis and epididymal maturation. Complete and ubiquitous deletions of

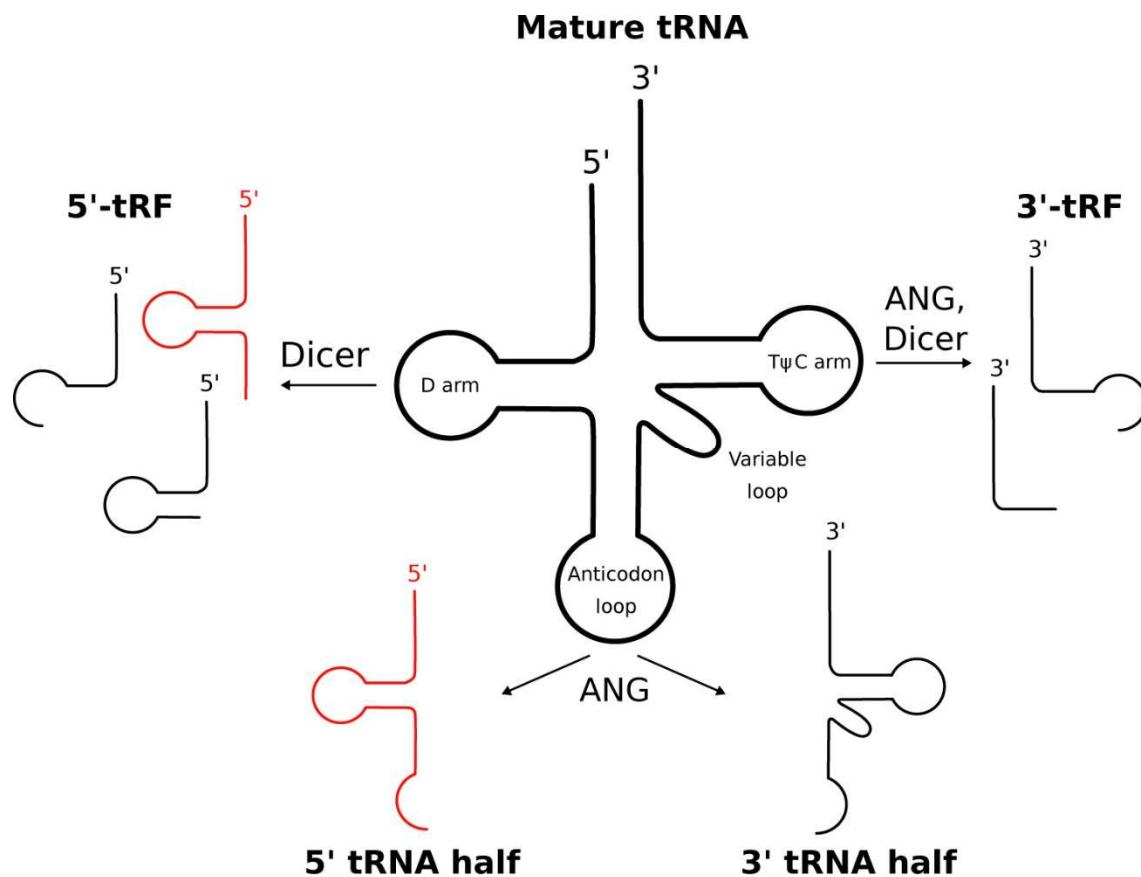
446 DICER or DROSHA are lethal, but their deletion in germ cells only causes male and female infertility,
447 demonstrating the importance of ncRNAs during gametogenesis. Around 600 distinct miRNAs have
448 been described in the mouse testis (Schuster et al., 2016), and both miRNAs and siRNAs are
449 abundantly expressed during spermatogenesis (Song *et al*, 2011). The nature and copy number of
450 miRNAs changes during sperm maturation (Nixon *et al*, 2015); their origins and importance will be
451 discussed below.

452

453 3.b Transfer RNA-derived fragments (tRFs)

454 Transfer RNAs, key elements of the transcriptional / translational machinery, have a characteristic
455 shape, with several domains, organized from 3' to 5': the T ψ C arm, variable loop, anticodon loop and
456 the D arm. Endonucleases can generate two types of transfer RNA-derived fragments (tRFs). In
457 response to stress, Angiogenin cuts the anticodon loop and produces halved fragments. Other
458 cleavage events, orchestrated by DICER, Angiogenin, and other uncharacterized enzymes near the D-
459 or T ψ C-arms produce small RNA known as 5' tRFs and 3' tRFs, respectively (Keam & Hutvagner, 2015)
460 (Figure 5). Numerous functions have been attributed to tRFs. The first to be identified was a function
461 similar to miRNA (see above), silencing gene expression by binding to the 3' UTR of mRNAs. Other
462 functions were since identified, such as direct regulation of translation by tRFs binding to ribosomes.
463 Finally, tRFs can also form complexes with RNA-binding proteins such as Ybx1 to regulate RNA
464 degradation and stability. Mature sperm from the cauda epididymis contain numerous ncRNA, and
465 80% of them are tRFs, mostly 5' tRFs (Peng *et al*, 2012, Sharma *et al*, 2016).

466



467

468 **Figure 5: tRNA fragments derived from mature tRNAs.**

469 Processing of mature tRNA can give rise to small RNA fragments. tRNA-derived fragments are shorter
 470 than tRNA halves and are produced by the action of Angiogenin and Dicer. In mammalian cells under
 471 stress, a cleavage in the anticodon loop produces tRNA halves by the action of the nuclease
 472 Angiogenin (ANG). tRNA fragments identified in mouse cauda sperm are predominantly enriched in
 473 fragments derived from the 5' ends of tRNAs (in red), as shown by (Sharma et al., 2016)

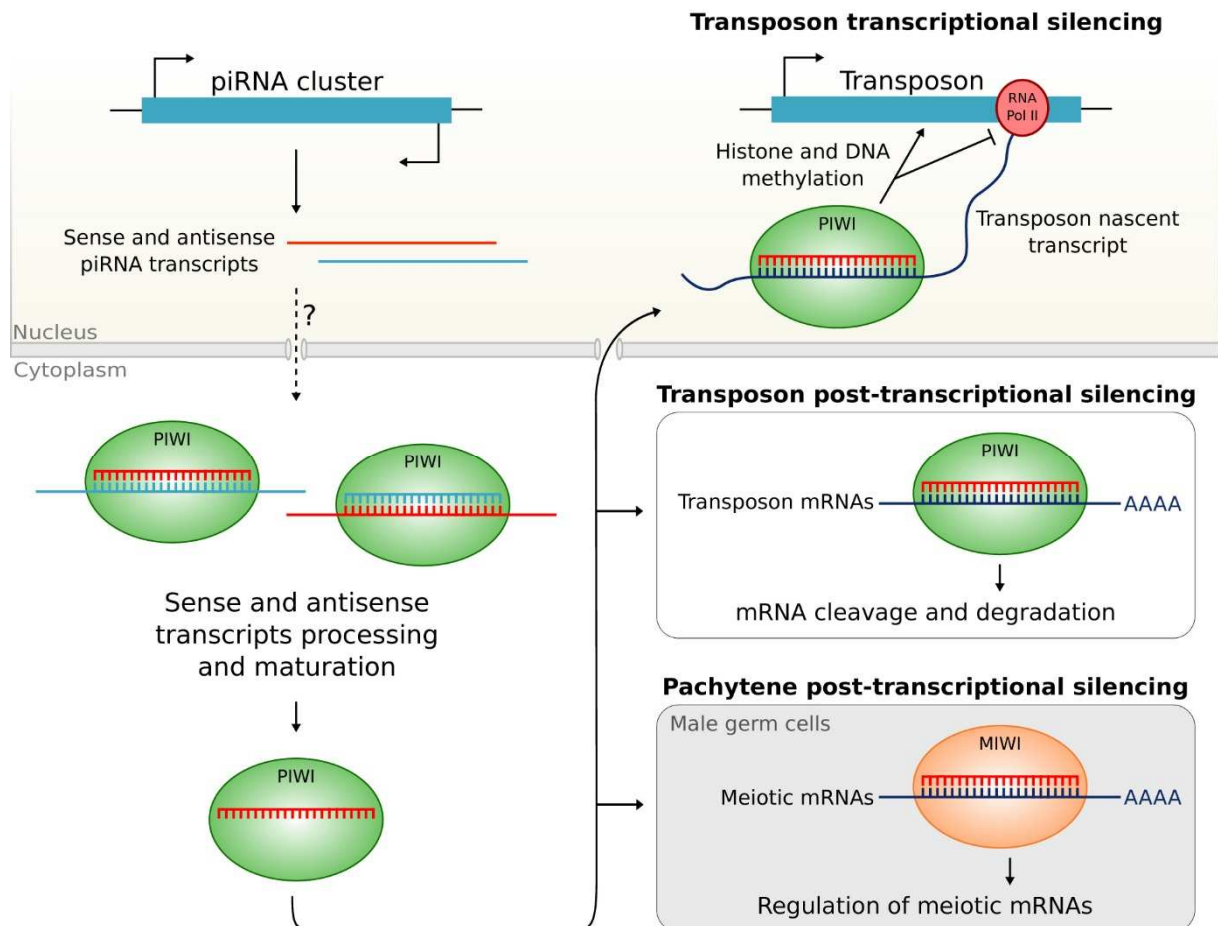
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476 3.c Piwi-interacting RNA (piRNA)

477 Numerous transposable elements, likely derived from viruses, are found in mammalian genomes.
 478 Their movement can disrupt gene expression when transposed into exons, introns, or into 5' or 3' -
 479 UTRs. It is thus crucial to inhibit their dissemination to preserve the integrity of the genome (Parhad
 480 & Theurkauf, 2019). Dissemination is prevented in germ cells by the expression of 22-27-nt small-
 481 ncRNA, known as piRNA, the primary function of which is transposon silencing. piRNA, located in
 482 clusters, are expressed from the pre-pachytene stage, and their production is DICER-independent
 483 (Figure 6). The piRNA silencing pathway involves several proteins from the PIWI clade of Argonaute
 484 proteins, such as MILI, MIWI2, and MIWI. Mutations that disrupt this pathway cause transposon
 485 overexpression, leading to sterility. Interestingly, a second wave of piRNA synthesis occurs over the
 486 course of the pachytene stage, during which several hundred piRNAs with unique sequences are

487 expressed from piRNA clusters containing few transposons and repeat sequences. The piRNAs
 488 expressed during this second wave control the expression of genes coding for proteins involved in
 489 meiosis (Goh *et al*, 2015), and massively eliminate the corresponding mRNAs at the elongated
 490 spermatid stage (Gou *et al*, 2015).



491 **Figure 6: A simplified mechanism of piRNA biosynthesis and function. OR The mechanism of piRNA**
 492 **biosynthesis and function.**
 493

494 Mammalian piRNAs derive from distinct transposon sequences known as piRNA clusters. They can be
 495 transcribed in the sense or antisense direction, and give rise to single-stranded long non-coding RNA
 496 transcripts. The precursors are transported into the cytoplasm by an unknown mechanism and
 497 processed in a Dicer-independent manner. Sense and antisense transcripts are in turn loaded onto
 498 PIWI clade proteins and trimmed to the length of mature piRNA, their 5' and 3' ends are modified,
 499 and they are used in an amplification loop to generate other complementary mature sense and
 500 antisense piRNAs. Mature piRNAs are mainly involved in transposon silencing by repressing their
 501 transcription or by cleaving their mRNAs. Transposon transcriptional silencing is thought to occur
 502 when a piRNA-guided PIWI protein recognizes a nascent transposon transcript in the nucleus and
 503 recruits a DNA and histone methylation machinery to the transposon locus, which silences
 504 transcription. In the cytoplasm, piRNA induces post-transcriptional silencing of target transposon
 505 transcripts in an RNAi-like manner. In mouse spermatocytes, pachytene piRNAs are depleted of
 506 transposon sequences, and these piRNAs are thought to regulate gene expression during meiosis and
 507 late spermiogenesis, but the exact mechanism is unknown. (Adapted from (Meister, 2013, Ozata *et al*,
 508 2019)).

509

510 4. Origin of sperm ncRNA

511 Some of these RNAs appear to be deeply embedded in the sperm nucleus, since harshly purified
512 nuclei devoid of acrosomes, plasma membrane, perinuclear theca, and nuclear envelope still contain
513 ncRNA and mRNA (Yan *et al*, 2008). Due to their deep localization, they are probably produced during
514 spermatogenesis and not discarded during sperm cytoplasm compaction. Interestingly, when these
515 purified sperm heads were injected into artificially activated oocytes, 2-cell embryos developed, 17%
516 of which produced live pups when re-implanted in foster mothers. Furthermore, the purification of
517 epididymal sperm heads and flagella indicate that ncRNAs are present in both subcellular
518 compartments, but that the composition of these ncRNAs differs between the two compartments.
519 Whereas the heads contain mostly tRFs, the flagella are comparatively enriched in piRNA and
520 miRNAs (Sharma *et al*, 2018).

521 Sperm released into the lumen of the seminiferous tubules transit through the epididymis and are
522 then stored in the cauda epididymis until ejaculation. The epididymis is separated into several
523 functional areas. This very long epithelial tubule is composed of several cell types, including secretory
524 cells. It is well known that during epididymal transit the composition of sperm changes dramatically,
525 and that these changes are necessary for sperm to become competent. For instance, the lipid
526 composition of the plasma membrane is extensively changed as a result of phospholipase A2
527 secretion, in addition group III sPLA2 (Sato *et al*, 2010) and proteins important for sperm/egg binding
528 are inserted into the plasma membrane of the sperm as it travels (Caballero *et al*, 2013, Da Ros *et al*,
529 2015). Epididymal proteins are transferred to the sperm by the fusion of small membrane vesicles
530 known as epididymosomes. Interestingly, Belleannée and coll. (Belleannée *et al*, 2013) showed that
531 these vesicles can transport miRNA, and hypothesized that via this route miRNA synthesized and
532 secreted in one epididymal region could modulate the function of epididymal cells in another region.
533 Since these epididymosomes can fuse with sperm, they could also provide new ncRNAs to sperm. To
534 verify this hypothesis, several types of experiments were performed.

535 First, the composition of ncRNA in sperm was measured using RNA sequencing on immature sperm
536 (testicular sperm) and maturing sperm located in different regions of the epididymis. These analyses
537 showed that the ncRNA composition of sperm changes dramatically, with loss and acquisition of
538 ncRNA, mostly in the distal part of the epididymis (Nixon *et al*, 2015). A study tracking the
539 composition of the ncRNA in the testis from pachytene spermatocytes to immature sperm cells, and
540 in the epididymis from caput sperm to cauda sperm indicated that the predominant ncRNA in testis
541 are piRNA, and that transit through the epididymis is associated with a loss of almost all piRNAs and a

542 remarkable increase in tRFs (Sharma et al., 2018). The results of this study also confirmed a notable
543 qualitative and quantitative increase in miRNAs during transit through the caput epididymis (Figure
544 7A).

545 Second, to demonstrate that these changes were due to uptake of epididymosomes, the
546 concentration of ncRNA was measured in immature sperm before and after co-incubation with caput
547 epididymosomes. Reilly and coll. (Reilly *et al*, 2016) showed that this co-incubation actually increased
548 the RNA copy numbers for miR-191, miR-375, miR-467a, miR-467d, miR-467e demonstrating that the
549 sperm RNA payload is strongly influenced by secretions in the epididymis. Similarly, another
550 comparison of ncRNA content between testicular sperm and testicular sperm incubated with caput
551 epididymosomes indicated that incubation with caput epididymosomes changes the composition of
552 ncRNA, with notable increases in miRNA, tRFs and a decrease in piRNA (Sharma et al., 2016, Sharma
553 et al., 2018).

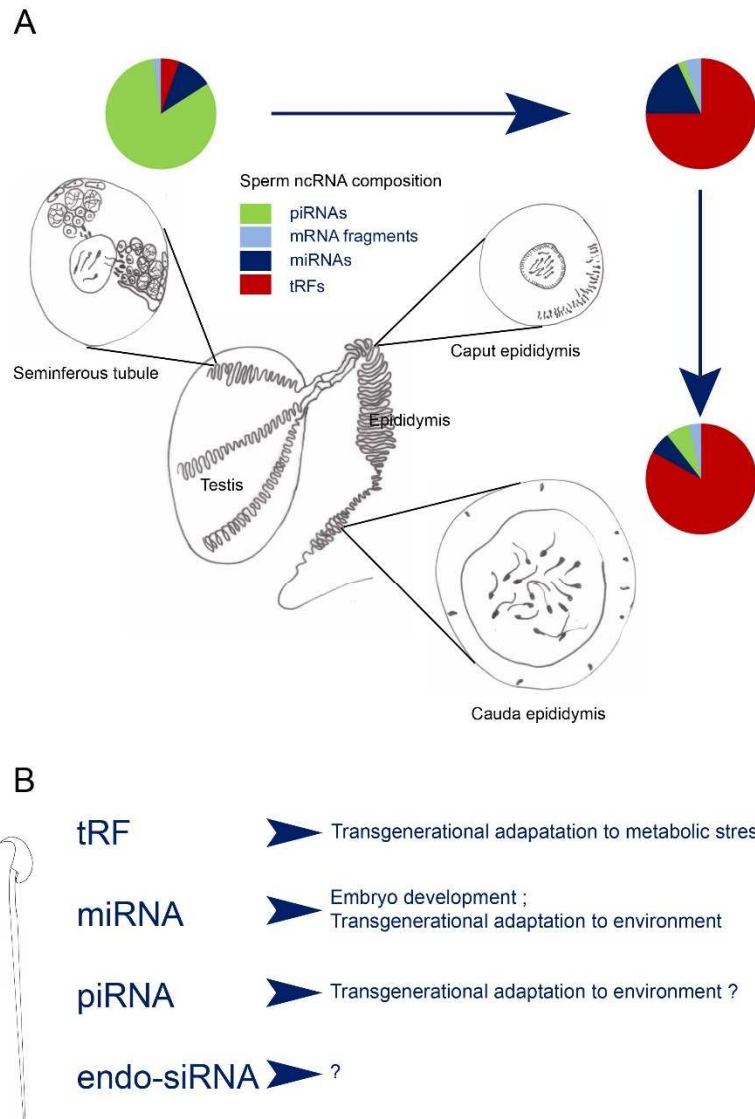
554 Epididymosomes not only provide new epididymal ncRNA to sperm but also increase the copy
555 number of ncRNA in sperm. These observations reinforce the overall potential epigenetic influence of
556 sperm after fertilization. The composition of sperm ncRNA and their evolution from the testis to the
557 cauda epididymis is shown in figure 7A.

558

559 5. Functions of sperm-borne ncRNA

560 5.a Role of sperm-borne ncRNA in embryonic development

561 Although the presence of several types of ncRNA, including tRFs, miRNA, siRNA, and piRNA was
562 clearly demonstrated in mature cauda epididymis sperm, the function of the sperm ncRNA upon
563 fertilization and early embryo development may not be so clear-cut because (i) the quantity of
564 sperm-borne ncRNA delivered at fertilization into the oocyte remains very low compared to the
565 amount already present within the oocyte, (ii) the most abundant sperm miRNA are also present in
566 the oocyte before fertilization (Amanai *et al*, 2006), (iii) oocyte maturation and preimplantation
567 embryonic development are not affected by complete depletion of maternal miRNA, as
568 demonstrated by *Dgcr8* oocyte-deficient females, (of note, this observation suggests that the miRNA-
569 dependent gene silencing pathway is not activated at these stages (Suh *et al*, 2010)) and (iv) In
570 several species including mouse and Human, injection of round spermatids and testicular sperm, with
571 a very different miRNA contents than fully mature sperm, permits to obtain live healthy offspring
572 (Tanaka *et al*, 2015) Altogether, these results raise questions about any role of sperm-borne ncRNA,
573 and in particular miRNA function in the development of mammalian zygotes and embryos.



575

576 **Figure 7. Origin and functions of sperm ncRNA.**

577 *A. The composition of sperm ncRNA changes dramatically as cells travel from the testis to the*
 578 *cauda epididymis. In the testis, the most abundant type of ncRNA is piRNA, which plays a*
 579 *crucial role in transposon control. After spermiation, sperm transit through the caput*
 580 *epididymis, where tRFs and miRNAs are gained. piRNAs are no longer present, but how they*
 581 *are eliminated remains unknown. In the cauda epididymis, the miRNA composition is also*
 582 *strongly modified.*

583 *B. In the absence of miRNAs and tRFs, embryo development is abnormal. The miRNA are also*
 584 *key nucleic acids for transgenerational adaptation in response to paternal environmental*
 585 *stress. Adapted in part from (Sharma et al., 2016, Sharma et al., 2018)*

586

587 To address these questions, several approaches were used to investigate the role of sperm ncRNAs in
 588 fertilization and embryo development:

589 Although tissue-specific inactivation of DICER or DROSHA in male germ cells leads to infertility, some
590 non-motile and abnormal sperm are produced that can be used for ICSI. Interestingly, embryos
591 generated with sperm lacking DICER or DROSHA and WT oocytes developed poorly in comparison to
592 those generated with WT sperm and oocytes, but their development was improved when KO sperm
593 were co-injected with small amounts of ncRNA from WT sperm (Yuan *et al*, 2016). The absence of
594 DICER affected around 47% of sperm miRNAs and 8% of sperm siRNAs, and the absence of DROSHA
595 had an impact on around 52% of sperm miRNAs (DROSHA is not involved in siRNA biogenesis),
596 suggesting that some ncRNAs produced during spermatogenesis play a crucial role in embryonic
597 development. Interestingly, DICER and DROSHA-deficient sperm did not perform equally. Embryos
598 generated with DICER-deficient sperm developed poorly at all stages (from 2-cell to blastocyst),
599 whereas those generated with DROSHA-deficient sperm developed almost normally up to the 4-cell
600 stage, from which point they showed marked developmental defects. These results suggest that
601 sperm-borne siRNA plays a role in the early stages of embryonic development, whereas sperm-borne
602 miRNA are more important at later stages, beyond the 4-cell stage. These results also highlight a
603 long-term effect of these sperm-borne ncRNAs, after embryonic gene activation, which occurs at the
604 2-cell stage in mice. It is interesting to note that the improvement was greater when total WT RNA
605 was injected than with small RNA only, suggesting that sperm not only deliver regulatory RNA
606 molecules but also mRNA.

607 To assess the importance of the different types of ncRNA in embryo development, and in particular
608 ncRNAs provided by epididymosomes, embryos were generated by ICSI with testicular, caput and
609 cauda sperm, and embryo development was studied. mRNA from embryos were sequenced at
610 various stages (4-cell, 8-cell, morula, and blastocyst). Overexpression of transcripts coding for RNA-
611 binding proteins (Hnrnpab, Hnrnpu, Pcbp1, Eif3b, Yrdc, Ythdf1, Srsf2, and Ybx1) and chromatin-
612 associated factors (Smarcd2, Smarca5, Smarcc1, Trim28, and Ezh2) was observed only in embryos
613 generated with caput sperm and not those generated with cauda sperm (Conine *et al*, 2018). These
614 changes affected embryo development: 50% of embryos were absent at day 7.5 of gestation,
615 suggesting that their post-implantation development was abnormal, and finally no pups were
616 obtained at term. This result suggests that the ncRNA gained as sperm pass through the caput
617 transiently make sperm unable to sustain embryo development and that the ncRNA payload is
618 drastically modified in the cauda epididymis. Interestingly, co-incubation of caput sperm with
619 epididymosomes purified from the cauda epididymis rescued the level of expression of transcripts for
620 RNA-binding and chromatin-associated factors in embryos, allowing the embryos to develop to term
621 (Conine *et al.*, 2018). Remarkably, only the injection of miRNAs, but not tRFs, purified from cauda
622 sperm restored normal embryo development following fertilization with caput sperm. This

623 observation suggests that miRNAs gained during epididymal transit are involved in embryo
624 development, and that the tRFs acquired play a distinct role. Several sperm-borne miRNAs were
625 identified as crucial for early embryo development, such as miR-34c, which is involved in DNA
626 synthesis and inhibition of Bcl-2 expression in the zygote. Inhibition of miR-34c leads to failure of the
627 first mitosis (Liu *et al*, 2012).

628 5.b Roles of ncRNAs in the environmental stress-response

629 Environmental injuries suffered by the parents and their impact on phenotypes in the next
630 generation were first discovered by a study monitoring the health of adults whose mothers lived
631 through the Dutch famine during the Second World War. The results showed that the adults exposed
632 to famine in utero during the first trimester of pregnancy had a higher risk of obesity and those
633 exposed in the third trimester had a lower risk (Ravelli *et al*, 1976). Most early studies on
634 transgenerational effects of parental stress focused on the influence of metabolic stress over the
635 course of pregnancy. The effects of preconceptional stress on female gametes were more difficult to
636 demonstrate since any preconceptional stress that affects oogenesis may also have an impact on the
637 subsequent pregnancy. Nevertheless, a recent study showed that corticotropin releasing factor type
638 1 (CRF1) mRNA, a key component in the stress response, is increased in eggs harvested from stressed
639 female rats, and that offspring obtained after mating stressed females with control males exhibited
640 higher levels of CRF1 in the brain and behavioral defects (Zaidan *et al*, 2013). Thus, female germ cells
641 appear to be able to transmit stress-induced traits to their progeny. Similar experiments aiming to
642 demonstrate transgenerational transmission of environmental stress by sperm were easier to carry
643 out since stress suffered by the male impacts neither full embryo development nor the raising of
644 pups, which are mother-dependent.

645 Obesity is a significant health issue, and several epidemiologists have compared the increase in the
646 rate of obesity in Western countries to an epidemic-like phenomenon, suggesting possible
647 transmission of this condition. The first observations that a father's diet can affect his daughters'
648 health, leading to obesity and diabetes, were reported quite recently (Ng *et al*, 2010). This paternal
649 diet-induced phenotype is associated with reprogramming of metabolic gene expression in liver due
650 to alteration of epigenetic marks (Carone *et al*, 2010). These first reports were followed by numerous
651 studies investigating the transgenerational impact of paternal diet on the progeny's susceptibility to
652 metabolic disorders, and seeking to identify the molecular basis for its transgenerational
653 transmission. The transmission of the phenotype was observed up to the third generation. A high-fat
654 diet (HFD) leads to extensive modifications to the ncRNA content in both testis and sperm cells
655 (Fullston *et al*, 2013) and miRNAs, piRNAs, and tRFs were found to be differentially expressed in
656 sperm from animals consuming the HFD, piRNA being the most likely (de Castro Barbosa *et al*, 2016).

657 Interestingly, Let-7c, one miRNA that is upregulated in sperm from males fed with HFD , was also
658 upregulated in adipose tissues of their F1 offspring. This miRNA is known to regulate glucose
659 homeostasis and insulin sensitivity, and has been linked to a predisposition for type-2 diabetes. The
660 importance of ncRNA in paternal transmission of obesity and diabetes was also supported by an
661 experiment where ncRNA were injected into zygotes: the injection of spermiatic ncRNA from a male
662 fed with the HFD into a control zygote led to metabolic disorders in the progeny, whereas injection of
663 spermiatic ncRNA from a male fed with the control diet had no effect (Grandjean *et al*, 2015).
664 Interestingly, the injection of miR19b into a control zygote mimicked the phenotype observed after
665 injection of ncRNA from the HFD male. It is worth noting that the various reports on miRNAs involved
666 in the paternal transmission of metabolic disorders fail to agree. Even more surprisingly, the change
667 in miRNA content induced by HFD is not observed in sperm from male progeny, despite the fact that
668 they can transmit the metabolic phenotype to their own offspring, the grand-children of HFD males
669 (Fullston *et al*, 2016). This unexpected result strongly suggests that components other than miRNA
670 are also important for transgenerational paternal effects. This hypothesis was recently confirmed by
671 Sharma and coll. (Sharma *et al.*, 2016), who showed that sperm from males consuming a low-protein
672 diet contains higher levels of tRF-Gly-GCC, that regulates the endogenous retroelement MERVL.
673 Importantly, the long terminal repeat (LTR) in MERVL regulates a subset of genes expressed at high
674 levels in preimplantation embryos. The impact of the low-protein diet on gene expression in
675 preimplantation embryos was reproduced by microinjection of a synthetic tRF-Gly-GCC oligo (Sharma
676 *et al.*, 2016). A HFD also deregulates several thousand long ncRNAs in sperm, and it was thus
677 suggested that these long ncRNA could be involved in paternal heredity (An *et al*, 2017). Taken
678 together, these results suggest that stress activates the production of numerous ncRNA types in the
679 testis and/or epididymis, and that it consequently affects gene expression in post-fertilization
680 embryos at multiple levels.

681 It is worth noting the plasticity of ncRNA production. The transgenerational metabolic phenotype can
682 easily be reversed by exercise. Progeny produced from males fed a HFD exhibit metabolic disorders
683 than can be restored when obese fathers engage in low- to moderate-intensity exercise for 2 months
684 (McPherson *et al*, 2017, McPherson *et al*, 2015). This phenotype reversion following exercise is
685 supported by the fact that physical training modifies sperm ncRNA, and in particular a subset of
686 piRNAs in humans (Ingerslev *et al*, 2018) as well as a subset of miRNA (miR-19b, miR-455 and miR-
687 133a) and two species of tRF derived from tRNA-Gly and tRNA-Pro in mice (Short *et al*, 2017). As with
688 diet, there is no consensus on the type and identity of the ncRNA induced in sperm by physical
689 training.

690 Transgenerational stress transmission by sperm is not restricted to metabolic stress, and several
691 reports suggest that physical stress (loud noises, predator odor, physical constraints, etc.) or
692 hormonal stress (cortisone injection) experienced by the father alters behavioral responses to stress
693 in the progeny, partly due to deregulation of the hypothalamic-pituitary-adrenal axis (Rodgers *et al*,
694 2013, Short *et al*, 2016). Importantly, the stress experienced by the father modifies a specific subset
695 of spermatic miRNAs: nine miRNA (miR-193-5p, miR-204, miR-29c, miR-30a, miR-30c, miR-32, miR-
696 375, miR-532-3p, and miR-698) were determined to be over-expressed by (Rodgers *et al.*, 2013), and
697 three (miR-98, miR-144 and miR-190b) were reported by (Short *et al.*, 2016). Another observation of
698 the transgenerational impact of stress applied early during life reported effects on 73 miRNAs,
699 including miR-375-3p and -5p, miR-200b-3p, miR-672-5p, and miR-466-5p, which were upregulated
700 (Gapp *et al*, 2014). Yet another report indicated that more than 100 miRNAs were modified in
701 response to hormonal stress (Chan *et al*, 2020). It is important to highlight that there was no overlap
702 in deregulated miRNA expression in sperm between these reports, although all linked the effects to
703 the hypothalamic-pituitary-adrenal axis. Furthermore, injection of the nine miRNA reported by
704 Rodgers *et al.* (Rodgers *et al.*, 2013) into a control zygote fully reproduced the disruption of the
705 hypothalamic-pituitary-adrenal axis observed with sperm from stressed fathers, confirming the
706 importance of these miRNAs (Rodgers *et al*, 2015). Interestingly, injection of just one miRNA partly
707 reproduced the stress phenotype, suggesting that the combination of several miRNAs is important
708 for transgenerational stress adaptation. The injection of the nine miRNAs modified the expression of
709 75 mRNAs present in oocytes, including genes playing roles in chromatin remodeling. This epigenetic
710 control induced long-term modifications of gene expression in the target tissue, with 298 genes
711 found to have altered expression in the paraventricular nucleus of the hypothalamus (Rodgers *et al.*,
712 2015). Interestingly, changes to miRNA levels were observed up to 12 weeks after a short initial
713 stress, suggesting that stress has a long-term effect on the transcriptomic program of epididymal
714 cells (Chan *et al.*, 2020). Thus, physical stress experienced by the father induces altered stress
715 responsivity in offspring, but physical exercise produces a compensatory anxiolytic effect in the
716 progeny (Short *et al.*, 2017).

717 Finally, stress-induced ncRNA modifications of the father's sperm modify the cancer susceptibility of
718 the progeny, in particular for breast cancer following metabolic stress (da Cruz *et al*, 2018, Fontelles
719 *et al*, 2016), and medulloblastoma following irradiation (Paris *et al*, 2015).

720 In conclusion, the composition of sperm ncRNA is very sensitive to environmental stress, and these
721 changes can have long-term effects on the expression of genes in various tissues, allowing rapid
722 adaptability of the progeny without the need for DNA mutations, which are rare and random and
723 therefore require a large population and many generations to achieve efficient adaptability.

724 The functions of the different ncRNAs are summarized in figure 7B.

725 6. Importance of sperm DNA methylation in the environmental 726 stress-response

727 DNA methylation, which consists in the methylation of cytosine residues in CpG dinucleotides to form
728 5-methylcytosine, is a well-characterized mechanism driving gene expression. DNA methylation of a
729 gene promotor is known to inhibit transcription of the corresponding gene. Consequently, DNA
730 methylation is an important regulator of spermatogenesis, and its deregulation is associated with
731 multiple forms of male infertility (Gunes *et al*, 2016). DNA methylation of male germ cells is mostly
732 acquired in utero, and is completed by the end of the pachytene spermatocyte stage (Oakes *et al*,
733 2007).

734 There is now some evidence that the sperm methylation profile influences the phenotype of the
735 progeny. First, several researchers modified the level of folate in the paternal diet (Lambrot *et al*,
736 2013, Ly *et al*, 2017). Folate is a precursor of co-enzymes necessary for several synthetic pathways,
737 including those producing purine bases or thymidine monophosphate. These co-enzymes are also
738 methyl donors and are involved in DNA methylation among other things. When the paternal diet
739 lacks folate, a significant change in sperm DNA methylation is observed, with 57 genomic regions
740 showing altered methylation profiles (Lambrot *et al*, 2013). Notably, these different methylated sites
741 are close to the TSS of genes involved in development. These changes were associated with
742 developmental abnormalities in the progeny, supporting the importance of sperm DNA methylation
743 in the phenotype of the progeny. Second, reproduction in dairy cattle mainly relies on artificial
744 insemination, and the best bull sires can have several thousand offspring. It is thus possible to study
745 any relationship between sperm methylation and complex phenotypes in the offspring. In the study
746 by Liu *et al*. (Liu *et al*, 2019), the levels of methylation of variably methylated regions (VMRs) in
747 sperm from 19 sires correlated with complex traits in their daughters including reproduction, milk
748 production, and health. A high level of correlation was observed between methylation levels of VMR
749 and reproductive traits, clearly demonstrating the intergenerational transmission of traits through
750 DNA methylation (Liu *et al*, 2019).

751 We showed above that ncRNA are involved in the paternal epigenetic legacy, and in particular in the
752 transgenerational transmission of environmental insults. Is there any evidence showing that
753 methylation of sperm DNA also contributes to the paternal legacy? First, the possible role of sperm
754 DNA methylation in the transgenerational transmission of obesity was observed in several studies.
755 Obesity in rats induces a change in the methylation profile of epididymal sperm, and 18 regions were

756 identified as differentially methylated between control rats and rats fed a HFD. Interestingly, the
757 same regions were also differentially methylated in sperm from F1 offspring, but not in control
758 progeny. Some regions were found to be hyper- or hypo-methylated (de Castro Barbosa et al., 2016).
759 Similarly, physical training modified the methylation profile of sperm in humans, and around 300
760 differentially-methylated regions were identified following a 6-week exercise regimen (Ingerslev et
761 al., 2018). Second, paternal stress can also modify the behavior of offspring in rodents. For instance,
762 males separated from their mothers at an early age showed anxiety in adulthood. This separation
763 anxiety induced a change in the methylation of genes, including *MeCP2*, cannabinoid receptor 1
764 (*CB1*), and corticotropin releasing factor receptor 2 (*CRFR2*) in sperm. The offspring and grand-
765 offspring of these males, reared normally, also showed anxious behavior and similar alterations of
766 the methylation of these genes in neural tissues (Franklin et al, 2010).

767 The transgenerational transmission of methylation profiles and their contribution to the phenotype
768 of progeny remain a mystery when considering the two waves of general erasing of methylation
769 marks. The first wave occurs just after fertilization and preserves only imprinted marks (Abdalla et al,
770 2009, Jenkins & Carrell, 2012)), and the second wave of DNA demethylation occurs before meiosis in
771 PGCs in the developing embryo, corresponding, respectively, to embryonic days E11.5 and E12.5 in
772 mice (Yamaguchi et al, 2012); for review, see (Cantone & Fisher, 2013). It is worth highlighting that in
773 most studies relating to transgenerational transmission of environmental stress through paternal
774 germ cells, simultaneous changes were observed in both methylation profiles and ncRNA content in
775 sperm. It is therefore difficult to weigh up the respective contributions of DNA methylation and
776 ncRNA in the paternal legacy. Surprisingly, the changes in ncRNA observed in the father are generally
777 not observed in the subsequent generation even though the phenotype is transmitted to the grand-
778 offspring (Gapp et al., 2014). It is therefore possible that the paternal stress-induced ncRNA signal is
779 converted into DNA methylation or histone post-translational modifications. Are ncRNA and DNA
780 methylation two sides of the same coin? If DNA methylation is involved in such a process, it suggests
781 that specific methylation marks made in response to environmental events are protected from the
782 first wave of demethylation occurring within the zygote, in a similar way to imprinted marks, or that
783 the transmitted ncRNA could contribute to apposing these marks after the demethylation wave. In
784 contrast to most of the genome, which is compacted with protamines, imprinted genes remain
785 associated with histones in nucleosomes. The presence of nucleosomes at these loci allows them to
786 escape from the general erasing of marks. The localization of stress-dependent methylation marks in
787 regions of compacted DNA in sperm with nucleosomes, and their escape from the first
788 demethylation wave in zygotes remain to be formally demonstrated. Moreover, it is expected that
789 species such as humans containing a larger proportion of sperm chromatin packaged in nucleosomes

790 are more sensitive to environmental stress than species with a more limited proportion of their DNA
791 compacted in nucleosomes, such as murine or bovine species. This hypothesis deserves further
792 exploration.

793

794 7. Histones and other proteins as sperm-borne factors

795 As indicated in the first paragraph above, nucleosomes retained in sperm are important for paternal
796 genome activation, and subsequent embryo development. Histones play key roles in epigenetics, and
797 their post-translational modifications (PTM) such as methylation, acetylation, crotonylation, ..., allow
798 transcription to be adapted by changing the relaxation state of DNA. The histones retained in sperm
799 carry several types of PTM (La Spina *et al*, 2014, Steilmann *et al*, 2011), and these histone marks have
800 been described as repressive or active (Brykczynska *et al*, 2010).

801 As expected from the localization of the histones retained in the promoters of genes important for
802 development (see above), changing the histone marks in sperm has a negative impact on embryo
803 development. For instance, reducing H3K4 and H3K9 methylation in sperm by overexpression of
804 histone H3 lysine 4 demethylase (*KDM1A*) increased embryo resorption during pregnancy and the
805 rate of morphological defects in live offspring (Perez-Cerezales *et al*, 2017, Siklenka *et al*, 2015).
806 More surprisingly, developmental and morphological defects were trans-generationally transmitted
807 from the F1 to the F2 generation, even though germ cells in F1 males lacked *KDM1A* overexpression
808 (Siklenka *et al.*, 2015). This finding suggests that epigenetic marks at the histone level also support
809 transgenerational adaptation to stress. Modification of histone marks was also observed with
810 Vinclozolin, an agricultural fungicide used as a model to study transgenerational inheritance of
811 chemical stress. Vinclozolin is an endocrine disruptor that decreases sperm count and viability,
812 causing decreased male fertility (Anway *et al*, 2005). In this model, the F1 generation is treated at
813 embryonic stages, and thus the F2 is produced from treated sperm. Studying transgenerational
814 inheritance in this model revealed extra areas of histone H3 retention in sperm from the F3
815 generation of Vinclozolin-exposed lineages compared to a control lineage (Ben Maamar *et al*, 2018).
816 Unexpectedly, the number of histone retention sites was normal in sperm from the F2 generation
817 but increased in sperm from the F3 generation and beyond (Skinner *et al*, 2018), suggesting that
818 histone modifications may be induced by other epigenetic factors. In addition to histone
819 modifications, Vinclozolin treatment induces DNA methylation and alters the ncRNA payload (Skinner
820 *et al.*, 2018), and the various contributions and interactions of these epigenetic modifications to the
821 infertility phenotype remain to be clarified.

822 Finally, apart from the sperm factor PLCzeta, which triggers oocyte activation and the absence of
823 which leads to human infertility (Escoffier *et al*, 2016, Parrington *et al*, 2019), other sperm-borne
824 proteins have been shown to play a role in embryo development. For instance, glutathione-S-
825 transferase omega 2 (GSTO2), localized in the perinuclear theca of the sperm head, in conjunction
826 with GSTO from the oocyte speeds up sperm DNA decondensation. Selective inhibition of GSTO2 in
827 sperm before ICSI delays sperm DNA decondensation, leading to abnormal cleavage timings, and
828 ultimately to a decreased rate of blastocyst formation (Hamilton *et al*, 2019a).

829

830 8. Perspectives and discussion

831 The discovery of the role of ncRNA in embryo development and adaptation to environmental stress
832 has potentially significant medical consequences and technical applications.

833 8.a Medical implications

834 Several reports indicated that analysis of the ncRNA composition of semen may be predictive of the
835 fertilization competence of sperm (Cui *et al*, 2015, Munoz *et al*, 2015, Salas-Huetos *et al*, 2015).
836 ncRNA profiling of infertile males may therefore be of interest to identify possible epigenetic causes
837 for couples presenting with embryonic development failures after several cycles of ovarian
838 stimulation. Nevertheless, all sperm-borne miRNAs necessary for optimal embryo development in
839 humans remain to be characterized. Because of extensive variability in ncRNA between species, the
840 short list identified so far in mice may not be relevant for human fertility. This identification step is
841 required before any attempt to rescue or optimize embryonic development using synthetic ncRNA
842 can be made.

843 We have also shown above that environmental insults can modify the ncRNA load in sperm and have
844 dramatic effects on the physiopathology of the progeny, including the emergence of metabolic
845 disorders and stress responses. A transient change in lifestyle, with a balanced diet, physical exercise
846 and stress management approaches will likely modify the sperm ncRNA payload and could be
847 recommended to couples consulting for infertility, to optimize the chances of fertilization, embryo
848 development, and the health of any child born.

849 Finally, it would be interesting, for ICSI with testicular sperm to co-inject the most important and
850 specific epididymal ncRNAs to improve embryo development. Again, this necessitates to characterize
851 them in the human species because of species variability.

852 8.b Technical applications

853 The most promising application of the discovery of the role of ncRNA in embryo development is in
854 embryo cloning from somatic cells. It is well known that embryos obtained through somatic cell
855 nuclear transfer (SCNT) show abnormal development, with disrupted kinetics of the first cleavage
856 and defects in histone methylation, leading to low live birth rates and overweight offspring.
857 Interestingly, co-injection of purified sperm ncRNAs along with a somatic cell nucleus decreases
858 histone H3 methylation (H3K9me3), and increases K40 acetylation of tubulin, both of which are
859 associated with increased SCNT yields (Qu *et al*, 2019). Among the classes of miRNAs involved in
860 embryo development, miR-449a seems to be relevant since, when SCNT is performed with co-
861 injection of miR-449a, the cleavage rate increases, epigenetic reprogramming of the embryo is
862 improved, and the apoptotic index of blastocysts decreases (Wang *et al*, 2017). Sperm ncRNA is also
863 a marker of the quality of sperm and could be used in animal reproduction to select bulls with a high
864 fertility index as, for example, the level of expression of miR-216b in sperm is inversely correlated
865 with the fertility index for bulls (Alves *et al*, 2019).

866 Conclusion

867 The data presented in this review show that the spermatozoon not only provides the paternal DNA
868 component, but also a large number of epigenetic regulators, which influence embryonic
869 development and allow rapid adaptation of the offspring to environmental stress. Much remains to
870 be discovered, such as the identity and function of all the ncRNAs involved in embryonic
871 development and stress adaptation. The targets of these ncRNAs and the signaling pathways
872 activated have yet to be characterized, and also need to be investigated.

873

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875

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