

1 **Title:** Intergenerational and transgenerational epigenetic inheritance in animals

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12

13 **Abstract:**

14

15 Animals transmit not only DNA but also a diversity of other molecules, such as
16 RNA, proteins and metabolites, to their progeny via gametes. To what extent
17 do these molecules convey information between generations and does this
18 information change according to their physiological state and environment?
19 Here we review recent work on the molecular mechanisms by which 'epigenetic'
20 information is transmitted between generations over different timescales and
21 the importance of this information for development and physiology.

22

23 **Main text:**

24 Introduction

25

26 DNA is a reliable information transfer system because of the accuracy of
27 DNA replication. Humans, for example, copy 6 billion bits of information to their
28 offspring with an error rate of approximately 2 bits per 100 million¹. However,
29 eggs and sperm contain more than DNA, and it has become increasingly
30 apparent in recent years that other molecules beyond the genome sequence
31 transfer information between generations. Moreover, there are mounting
32 examples in which this information is altered depending upon the physiological
33 and environmental conditions of previous generations. Multiple mechanisms
34 have been proposed to underlie non-DNA sequence-based inheritance and
35 these can be either genome-associated (e.g. covalent modifications of DNA and
36 histones or transfer of small RNAs complementary to genomic sequences) or
37 genome-independent (e.g. microbiome transfer). They also vary in their
38 generational duration, with inheritance spanning one generation to a seemingly
39 indefinite number.

40 The terms 'intergenerational' and 'transgenerational' are often used to
41 describe such effects and require clarification. Transgenerational effects refer
42 exclusively to phenomena that could not be ascribed to direct effects of a
43 particular trigger on the affected organism. For instance, an environmental
44 stimulus can directly affect a gestating embryo (and the already-formed oocytes
45 within a female embryo in mammals^{2, 3}). As such, only altered phenotypes
46 occurring in the second or third generation after a trigger can truly be described
47 as transgenerational for male and female transmission, respectively. Effects
48 spanning shorter timescales are described as parental or intergenerational.
49 Nonetheless, many described intergenerational effects share established
50 mechanisms with transgenerational ones. Another term that warrants
51 discussion is epigenetic, whose once broader meanings⁴ have narrowed in
52 recent years, not without objections⁵, to most commonly refer only to genome-
53 associated mechanisms of non-DNA sequence-based inheritance - chiefly DNA
54 methylation, histone modifications and inherited RNAs⁶. These specific
55 'epigenetic mechanisms' underlie some, but not all, characterised examples of

56 intergenerational and transgenerational inheritance.

57 A key difference between DNA sequence-based and other mechanisms
58 of inheritance is the fidelity of information transfer. Whilst DNA-based
59 information transfer is extremely high-fidelity, other mechanisms are normally
60 far less robust. Consequently, the timescales of reliable information transfer by
61 DNA sequence-based and other mechanisms are usually very different⁷. One
62 point of confusion concerns two separate distinctions that are often conflated:
63 firstly, genetic (i.e. DNA-based) vs epigenetic mechanisms of inheritance, and
64 secondly, environmentally-responsive vs unresponsive phenomena. Inheritance
65 of environmentally-acquired traits can also be mediated through genetic
66 inheritance, as occurs in the CRISPR innate immunity system of prokaryotes⁸.
67 Conversely, stable long-term transcriptional repression is often achieved by an
68 inherited epigenetic memory, but one that is largely unresponsive to
69 environment and physiology. It is the question of whether epigenetic
70 mechanisms can provide a heritable (and potentially adaptive) memory of
71 ancestral environmental exposure that has proven most controversial³.

72 Numerous examples of intergenerational and transgenerational effects in
73 animals have now been described. Model organisms such as *Caenorhabditis*
74 *elegans* have emerged as powerful systems in which to study these
75 phenomena, owing to their short generation times and the ease with which
76 genomic variation can be controlled. However, before the spectre of Lamarck
77 rises anew, we would contest that few well-established transgenerational effects
78 are adaptive, in the sense of preparing future generations for enduring altered
79 environmental conditions. Indeed, such adaptive changes, conceivable for
80 rapidly reproducing species such as *C. elegans* with lifecycles that may be short
81 with respect to environmental fluctuations, would be unlikely for long-lived
82 animals such as humans. Our aim here will be to give examples of non-DNA
83 sequence-based inheritance in animals and an overview of how ancestral state
84 can affect future generations, by which mechanisms this can occur, both
85 genome-associated and genome-independent, and how the mechanisms
86 involved change as we look to increasing timescales. Our focus is on
87 inheritance of acquired information. However, we also discuss some examples
88 of non-environmentally responsive epigenetic inheritance because they are
89 often better characterised and, arguably, more important for animal physiology.

90

91 Parental effects

92

93 Examples of parental genotype or environment affecting progeny
94 phenotype independent of inherited DNA ('parental effects') are numerous.
95 However, with direct contact between the individuals exposed to a trigger and
96 their immediate progeny (or their mate), many potential mechanisms can be
97 involved. To confidently implicate specific mechanisms of inheritance, careful
98 experimental design and interpretation are required³. Particular research effort
99 has been directed at paternal effects⁶, with the expectation that limiting a male's
100 interactions with partner and progeny to the act of mating alone will narrow
101 potential mechanisms down to those transmitted via gametes. Even so,
102 genome-independent mechanisms may still affect progeny phenotypes (**Figure**
103 **1**). For example, microbiome transfer from father to mother can rescue the
104 intergenerational effects of maternal antibiotic use in *Drosophila melanogaster*⁹,
105 and apparent paternal effects may in fact be cryptic maternal effects, when
106 paternal condition, such as depression-like states in mice¹⁰, influences maternal
107 investment or care.

108 The parental effects of diet and obesity is a well-studied paradigm
109 (reviewed in ¹¹), with obvious potential relevance to health given the rise in
110 obesity rates in Western countries in the past few decades¹². Intergenerational
111 effects of parental nutrition have been suggested in humans^{13, 14} and
112 demonstrated in rodents¹⁵⁻²², *D. melanogaster*²³ and *C. elegans*^{24, 25}. In
113 mammals, for example, under- or over-nutrition in either parent commonly
114 impacts offspring glucose metabolism¹¹. Counterintuitively, the effects of
115 maternal and paternal diet are often qualitatively and quantitatively similar^{21, 22,}
116 ^{26, 27}. However, such effects are often non-monotonic^{23, 24} and can be
117 dependent on the developmental context of parental or grandparental
118 exposure^{13, 15} and on progeny sex^{13-15, 17} and diet²³. For instance, both low- and
119 high-sugar paternal diets increased offspring adiposity in *D. melanogaster*, but
120 only when offspring were themselves challenged with a high-sugar diet²³.

121

122 *Maternal provisioning and metabolism*

123

124 Maternal provisioning to offspring may mediate effects of maternal diet²⁸,
125 ²⁹ or other physiological factors. For example, we recently found that increased
126 provisioning of a lipoprotein yolk complex to offspring with advancing maternal
127 age has a major impact on progeny growth rates and starvation resistance in *C.*
128 *elegans*³⁰. Offspring phenotypes may also be affected by provisioning of
129 specific regulatory products such as mRNAs^{31, 32} or essential micronutrients
130 such as zinc³³. Physiological alterations in maternally supplied organelles,
131 particularly mitochondria, could also underlie parental effects of diet, as a
132 maternal high-fat diet impairs fetal mitochondrial function in mice²¹. Perturbation
133 of maternal metabolism genetically³⁴ or by dietary intake of specific metabolites
134 can influence epigenomic regulation in progeny and even further generations
135 (reviewed in ³⁵). For instance, progeny DNA methylation can be influenced by
136 maternal dietary intake of methyl donors in mice³⁶ with striking heritable effects
137 on coat colour. Similar effects have also been suggested in humans, where
138 seasonal changes in dietary intake of methyl donors around conception in rural
139 mothers correlate with alterations in DNA methylation in children³⁷.

140

141 *Microbiome transfer*

142

143 Non-DNA-based inheritance may also act via transfer of an altered
144 parental microbiome⁹. Bacterial strains can be inherited maternally in humans³⁸,
145 although the mechanisms- whether by breast milk, birth canal or even placental
146 transfer - remain unclear³⁹. In mice, diet-induced microbiome changes,
147 specifically a progressive loss of taxonomic diversity due to a Western-style
148 low-fibre diet, are cumulative over generations and eventually irreversible via
149 extinction of specific microbiotic subpopulations⁴⁰. This suggests that
150 multigenerational environmental exposure could cause a stable
151 transgenerational alteration of progeny physiology via the microbiome.

152

153 *DNA methylation in sperm*

154

155 Methylation of DNA at cytosine residues has been suggested as
156 mediating parental dietary effects in mammals. Genomic imprinting – the
157 phenomenon whereby a gene’s expression depends upon whether it is

158 inherited from the male or female germline – is associated with differences in
159 DNA methylation and demonstrates that DNA methylation states *can* be
160 transmitted between generations in mammals⁴¹. The sperm methylome is
161 reportedly altered by various severe interventions which produce
162 intergenerational or transgenerational effects, such as *in utero* malnutrition^{42, 43},
163 early-life overnutrition⁴⁴ and diabetes⁴⁵ in mice and by obesity in humans⁴⁶.
164 However, the mechanisms by which sperm methylation could be modified at
165 specific sites are unclear. Moreover, methylation is largely erased upon
166 fertilisation⁴⁷ and it is not obvious how alterations could affect gene expression
167 in progeny with high penetrance¹¹. It was also reported that sperm methylation
168 was unaffected by several diets that induce phenotypic effects in progeny⁴⁸.

169 Although cytosine methylation is virtually absent from many organisms
170 such as *D. melanogaster*⁴⁹ and *C. elegans*⁵⁰, it is now apparent that DNA
171 methylation can also occur at adenosine residues, although the functional
172 significance of this mark, and whether it carries information across
173 generations⁵¹, is unclear⁵².

174

175 *Small noncoding RNAs in sperm*

176

177 Small noncoding RNAs (sncRNAs), particularly tRNA-derived small
178 RNAs (tsRNAs) and microRNAs (miRNAs), are emerging as possible mediators
179 of environmental information transmission through sperm in mammals
180 (reviewed by⁵³). Derived from precursor or mature tRNAs, tsRNAs are of
181 diverse size and biogenesis⁵⁴ and have in the last decade been implicated in a
182 range of cellular processes, including repression of transposable elements⁵⁴⁻⁵⁶.
183 Like miRNAs⁵⁷, tsRNAs can interact with small RNA-binding proteins of the
184 Argonaute family to induce post-transcriptional gene silencing^{54, 58} via sequence
185 complementarity to the 3'UTRs of target mRNAs^{58, 59}.

186 tsRNAs comprise most of the sncRNA pool in mature mammalian sperm
187⁶⁰, with miRNAs a distant second^{55, 61}. Sperm tsRNAs are reportedly altered by
188 diet⁶¹ or exposure to an endocrine disruptor⁶² in rodents and by obesity in
189 humans⁴⁶, while sperm miRNAs are altered by psychological stress in mice^{63, 64}
190 and men⁶⁵, and by parental genotype⁶⁶, diet⁶⁷⁻⁶⁹ and environmental
191 deprivation⁷⁰ in mice, all conditions associated with paternally-acquired

192 disorders. Crucially, in several cases zygotic injection of total sperm RNA^{64, 66,}
193 ^{69, 70}, sncRNA fractions^{61, 69} or specific sncRNAs^{55, 66, 68, 71} could partially or fully
194 recapitulate these paternally acquired phenotypes¹¹. In mice, inheritance of
195 sncRNA-mediated phenotypes has been reported to rely on the activity of the
196 RNA methyltransferase *Dnmt2*^{69, 72}, indicating that RNA modifications may
197 constitute an additional layer of regulation important for transmission of
198 acquired phenotypes through sperm⁶¹. In keeping with a role in repressing
199 transposons, which often use conserved tRNAs as primers for replication⁵⁶, a
200 specific sperm-borne tsRNA influenced by paternal diet was found to
201 specifically regulate genes governed by the pluripotency-promoting endogenous
202 retroviral element MERVL in the mouse zygote⁵⁵. Remarkably, it was shown
203 that sperm tsRNAs do not originate from sperm tRNAs but rather are acquired
204 via transfer of extracellular vesicles from the epididymis⁵⁵, offering a tantalising
205 hint of soma-to-germline transmission of information. Recent results indicate
206 that sperm miRNAs similarly acquired during epididymal transit could be
207 essential for embryonic development⁷³.

208

209 *Histone modifications*

210

211 There is some^{21, 23, 25}, but little, evidence for covalent modifications of histones
212 mediating parental effects. However, histone modifications are certainly
213 transmitted between generations at some loci in mammals⁷⁴, fish⁷⁵ and worms⁷⁶
214 and they have been implicated in longer-lasting transgenerational phenomena.
215 It is plausible, therefore, that they could also underlie some parental effects. In
216 *C. elegans* an epigenetic memory of germline transcription, mediated by
217 deposition of H3K36me3 on active genes^{77, 78} and H3K27me3 on repressed
218 genes⁷⁶, is passed from each generation to the next and is essential for
219 germline viability^{77, 78}, representing an example of non-environmentally-
220 responsive epigenetic inheritance that is critical for normal development and
221 physiology.

222

223 Multi-generation epigenetic inheritance

224

225 Documented examples of true transgenerational epigenetic inheritance

226 (TEI) induced by parental genotype, physiology or environment are becoming
227 increasingly numerous in model invertebrates. In most cases, however, the
228 effects described have a limited duration, for example typically spanning 3-4
229 generations in *C. elegans*, before reversion to the baseline phenotype⁷⁹⁻⁸².
230 Characterised mechanisms commonly involve inheritance via gametes of
231 genome-associated epigenetic information, such as histone modifications or
232 small RNAs. The likely reasons for the limited lifetime of many transgenerational
233 effects can be found in the passive and active mechanisms that underlie
234 changes in small RNA populations and histone modifications from generation to
235 generation⁸³.

236

237 *Inheritance of RNAi in C. elegans*

238

239 Although occurring in artificial laboratory conditions, the inheritance of
240 gene silencing induced by ancestral RNAi interference (RNAi) in *C. elegans* has
241 provided the most incontrovertible demonstration of TEI and has proven
242 invaluable in dissecting the mechanisms involved. Worms supplied with
243 exogenous double-stranded RNA (dsRNA), usually by feeding, employ an
244 amplification machinery which results in systemic silencing of complementary
245 genes in almost all tissues, including the germline. dsRNA is processed by
246 Dicer and accessory proteins to form primary short interfering RNAs (siRNAs).
247 Primary siRNAs bind to a member of the Argonaute family of small RNA-binding
248 proteins and guide them to complementary mRNA transcripts. RNA-dependent
249 RNA polymerases (RdRPs) are then recruited to produce abundant secondary
250 siRNAs (otherwise known as 22G RNAs for their length and 5' guanosine bias).
251 RdRP-associated silencing mechanisms are found in diverse taxa, although not
252 in vertebrates. In turn, these 22G RNAs engage a variety of Argonautes to
253 destroy complementary mRNAs, inhibit transcription⁸⁴ and deposit the
254 repressive chromatin marks H3K9me3 and H3K27me3 at the target locus⁸⁴⁻⁸⁶.

255 Gene silencing induced by dsRNA can be inherited^{87, 88}, typically for up
256 to 3 generations⁸⁰ but sometimes as long as 80 generations when selecting for
257 the resulting phenotype⁸⁸. The nuclear Argonaute *hrde-1* (heritable RNAi
258 defective) is dispensable for gene silencing in exposed worms but is necessary
259 for its inheritance in subsequent generations⁸⁹, demonstrating that *C. elegans*

260 possesses cellular machinery dedicated to the information transmission over
261 generations. The nuclear RNAi pathway, which shuttles 22G RNAs into the
262 nucleus⁹⁰, is required for the maintenance of inherited silencing in progeny⁹¹.
263 The limited typical duration of the silencing response may be due to dilution of
264 siRNAs over generations⁸⁵. Unlike primary siRNAs, secondary siRNAs rarely
265 serve as templates for further amplification of the gene silencing response
266 induced by dsRNA, which is therefore limited^{92, 93}. The repressive H3K9me3
267 and H3K27me3 footprints triggered by secondary siRNAs also persist in the
268 absence of the dsRNA trigger for at least 2 generations^{85, 86}, although H3K9me3
269 deposition is dispensable for heritable silencing at some loci^{94, 95}. Interestingly,
270 the H3K9 methylase *met-2*, responsible for H3K9me1/2, conversely limits the
271 generational duration of some dsRNA-induced silencing by altering siRNA
272 inheritance⁹⁶. Application of additional dsRNA triggers unrelated to the original
273 target in subsequent generations can extend the duration of inherited silencing,
274 suggesting that negative feedback by downregulation of the RNAi machinery
275 may act to limit the duration of a heritable response⁹⁷.

276 Why did *C. elegans* evolve the ability to respond to dsRNA with potent
277 and systemic targeted silencing? The RNAi machinery is required for some
278 antiviral responses in *C. elegans*⁹⁸⁻¹⁰⁰, and it has been suggested that
279 inheritance of parental antiviral small RNAs acts to block the transmission of
280 virus infection between generations^{81, 101}. However, a heritable response was
281 not observed for the only known natural virus of *C. elegans*¹⁰².

282

283 *Small RNAs and histone modifications in TEI*

284

285 The importance of small RNAs for the inheritance of RNAi-triggered
286 repression in *C. elegans* underscores mobile RNAs as an attractive candidate
287 for mediating transgenerational inheritance in multiple species (reviewed in¹⁰³).
288 dsRNA produced in somatic tissues, including neurons, can be inherited in *C.*
289 *elegans*¹⁰⁴ and reports indicate transfer of somatic RNAs to gametes in mice^{55,}
290 ^{73, 105}. The RNAi pathway in *C. elegans* was found to also target endogenous
291 genes, utilising a similar amplification mechanism as exogenous RNAi^{106, 107}.
292 Indeed, endogenous RNAi is necessary for transgenerationally-inherited gene
293 regulatory and physiological changes in response to ancestral starvation¹⁰⁸ and

294 heat stress⁸².

295 Histone modifications are important in the inheritance of RNAi in *C.*
296 *elegans*, and a variety of histone modifications have been implicated in other
297 cases of transgenerational inheritance, including methylation of H3K4 in mice¹⁰⁹
298 and *C. elegans*^{51, 79, 110, 111}, H3K9 in *C. elegans*^{51, 112-115} and H3K27 in *C.*
299 *elegans* and *D. melanogaster*^{114, 116, 117}. Stress-induced perturbations to histone
300 modifications may revert slowly over generations¹¹⁵, leaving a gradually fading
301 transgenerational memory. In some cases global levels of histone modifications
302 remain modified in later generations^{115, 116} although in others global levels are
303 unchanged^{25, 79}, implying differential regulation of specific loci¹¹⁸. In *C. elegans*,
304 transgenerational expression of longevity phenotypes caused by ancestral
305 mutations in the conserved COMPASS H3K4 methylases is dependent on the
306 corresponding demethylase⁷⁹, demonstrating that alterations in the antagonistic
307 activity of chromatin-modifying enzymes over generations can induce
308 transgenerational phenotypes⁵¹.

309

310 *TEI to pre-adapt progeny to environmental conditions*

311

312 Despite the increasing popularity of research into TEI, the evidence for
313 adaptive, environmentally-responsive transgenerational inheritance, whereby
314 ancestral experience equips progeny to better withstand environmental
315 challenges, remains scant. At the time of writing most documented cases of
316 inheritance of environmental experience occur in artificial contexts^{110, 115}, even
317 when those experiments attempt to mimic naturally occurring challenges⁸¹, and
318 the relationship of ancestral environment to alterations in progeny gene
319 regulation or physiology in terms of fitness is often far from clear^{81, 82, 108, 116}.
320 Nonetheless, a few reports suggest the possibility of adaptive TEI. A recent
321 study reports that exposure of *C. elegans* to a heavy metals leads to increased
322 resistance to the same stresses in future generations, what the authors call
323 transgenerational hormesis¹¹¹. Likewise, ancestral starvation in *C. elegans*
324 induces transgenerational resistance to starvation, by unknown mechanisms^{119,}
325 ¹²⁰. Despite most described TEI effects occurring in *C. elegans*, the most
326 striking case of potentially adaptive TEI involving soma-to-germline
327 communication is found in mice, where a conditioned fear response to a specific

328 odour in male mice can be inherited for two generations¹²¹. In this case, the
329 effect was associated with enlargement of neuroanatomical structures in
330 progeny, and with differential methylation of the locus encoding the
331 corresponding odour receptor in the sperm of exposed males (though not their
332 sons). Still, at present it seems that adaptive, environmentally-responsive TEI, if
333 it exists, is the exception rather than the rule. Nonetheless, it is clear that
334 epigenetic mechanisms can transfer information about ancestral state between
335 generations, and although the extent of this transfer is typically limited to a few
336 generations, some specific cases – arising from a loss of gene repression – can
337 lead to longer-lasting memories.

338

339 Long-lasting TEI

340

341 Despite the meagre evidence for adaptive memory of environmental
342 conditions, there undoubtedly exists an adaptive transgenerational memory that
343 serves to distinguish ‘self’ genetic elements from that of potentially harmful,
344 ‘foreign’ sequences. In many species repetitive genomic regions such as
345 transposons, are constitutively repressed by heterochromatin. Rather than
346 becoming re-established *de novo* each generation, it appears that the
347 heterochromatic state of repetitive regions is often inherited. Environmental
348 insults disrupting this repression can lead to a quantitative modulation of
349 expression from heterochromatic regions that takes many generations to
350 restore.

351 For example, growth at elevated temperature¹¹⁵ or impaired DNA
352 replication during embryogenesis¹²² can result in a loss of repression of
353 heterochromatic transgene arrays in *C. elegans* that can take more than 10
354 generations to fully re-establish (**Figure 1b**). Importantly, expression of a subset
355 of endogenous repetitive elements repressed by H3K9me3 also heritably
356 increased at elevated temperature, albeit for fewer generations¹¹⁵. Heat can
357 also derepress pericentromeric heterochromatin in *D. melanogaster*¹²³, leading
358 to a long transgenerational epigenetic memory of ancestral environment. In both
359 *C. elegans* and *D. melanogaster*, multiple generations of heat exposure and
360 consequent de-repression were required to maximise the generational duration
361 of the resulting memory^{115, 123}. These results are consistent with the gradual

362 restoration of heterochromatic regions perturbed by stress, the ‘healing’ of an
363 ‘epigenetic wound’⁸³. This memory may therefore result from a limited capacity
364 to restore disturbed heterochromatin within a single generation, although it is
365 unclear why this would be so. It is also not clear whether this potential for long-
366 term memory of environmental information has ever been co-opted for an
367 adaptive purpose.

368

369 *The mortal germline of C. elegans*

370

371 A reciprocal phenomenon to this slow recovery following chromatin
372 perturbation is found in the mortal germline (Mrt) phenotypes of *C. elegans*
373 mutants (and some naturally-occurring strains¹²⁴), which display a progressive
374 reduction in fertility, often temperature-sensitive, that accumulates over
375 generations and ultimately results in sterility (**Figure 1c**). While Mrt phenotypes
376 of some mutations result from genetic changes such as telomere loss^{125, 126},
377 many genes with a mutant Mrt phenotype are involved in histone
378 modifications^{51, 89, 96, 127-130} or small RNA pathways^{89, 129, 131, 132} and the
379 phenotype can be rapidly reverted by returning animals to the permissive
380 temperature^{118, 124, 129}, altering diet¹³³, re-introducing functional gene copies¹²⁷
381 or introducing downstream mutations⁹⁶, demonstrating that these
382 transgenerational phenotypes are epigenetic in nature. Interestingly, a recent
383 study found that the Mrt phenotype of *C. elegans* Piwi mutants results not from
384 a profound loss of germline totipotency but rather from the aberrant (and
385 reversible) induction of reproductive quiescence, normally induced under stress,
386 as a consequence of transcriptional dysregulation in the germline¹³³. If this
387 finding is generally applicable it suggests why the reversion of accumulated Mrt
388 phenotypes can be achieved so rapidly.

389

390 Stable TEI: enjoy the silence

391

392 *Small-RNA-triggered stable silencing*

393

394 The inherited repression of transposons and foreign DNA is essential for
395 maintaining the fitness of a lineage. How are these elements recognised and

396 silenced? Single-copy germline-expressed GFP transgenes in *C. elegans*, a
397 clear example of 'foreign' DNA, can undergo spontaneous silencing, resulting in
398 fully penetrant, stably inherited silencing for more than 20 generations with no
399 evidence of reversion^{112, 113, 134, 135}. This indefinite silencing is triggered by
400 endogenous small RNAs called piRNAs and so was christened RNAe (RNA-
401 induced epigenetic silencing). piRNAs are sncRNAs expressed from genomic
402 clusters ranging from tens to thousands of individual piRNA sequences¹³⁶.
403 Although their length and biochemical characteristics vary across species,
404 piRNAs interact with widely conserved Piwi proteins, part of the Argonaute
405 family, to effect silencing (reviewed in¹³⁷). Broadly, genomically-encoded
406 primary piRNAs guide Piwi proteins to complementary transcripts and initiate
407 amplification of secondary small RNAs, resulting in gene silencing. In zebrafish,
408 mice and *D. melanogaster*, the destruction of transposon mRNA guided by Piwi-
409 bound primary piRNAs can be coupled to the production of secondary piRNAs
410 from the targeted transcript, leading to a feed-forward amplification response
411 christened the Ping-Pong cycle¹³⁷. In *C. elegans*, transcript targeting by piRNAs
412 instead leads to the RdRP-catalysed production of 22G RNAs, which effect
413 heritable silencing through the nuclear RNAi pathway in conjunction with *hrde*-
414 ^{112, 113, 134, 135}, a machinery shared with heritable dsRNA-induced silencing.
415 piRNA-mediated silencing not only represses transposons but also targets
416 many endogenous transcripts, which can potentially be subject to
417 transgenerational epigenetic memory⁸².
418 Recent work in *C. elegans* has elucidated how primary piRNAs provide
419 surveillance over germline transcription¹³⁸⁻¹⁴¹. While piRNAs in mammals and *D.*
420 *melanogaster* exhibit near-perfect complementary base pairing with targets¹³⁷,
421 *C. elegans* piRNAs, like miRNAs⁵⁷, tolerate significant mismatches outside of a
422 5' seed region¹⁴¹. In this way, thousands of piRNAs can engage the entire
423 germline mRNA transcriptome¹³⁸. How do the genes necessary for germline
424 function escape this promiscuous silencing? In *C. elegans*, recognition of 'self'
425 has been associated with at least three potential mechanisms. Periodic
426 sequence elements called PATCs, largely intronic, are associated with
427 germline-expressed genes¹⁴² and protect foreign sequences from becoming
428 silenced via an unknown mechanism^{141, 143}. Another mechanism may involve
429 as-yet-uncharacterised features intrinsic to the coding sequence which prevent

430 silencing¹³⁹. A third mechanism is associated with the Argonaute CSR-1, whose
431 bound 22G RNAs display complementarity to almost all germline-expressed
432 genes¹⁴⁴ and which has been proposed to license gene expression^{145, 146} by
433 protecting mRNAs from piRNA targeting and subsequent siRNA generation¹³⁸.
434 Interestingly, both CSR-1 and the *C. elegans* Piwi orthologue PRG-1, along with
435 newly discovered proteins that seem to have a role in transgenerational
436 epigenetic inheritance^{147, 148}, reside in perinuclear phase-separated liquid-like
437 granules^{144, 149} with a defined spatial organisation¹⁴⁷, suggesting that the
438 temporal order of transit through this system of granules of mRNAs exiting the
439 nucleus may be important for RNA-directed silencing and licensing
440 mechanisms^{147, 148}. However, this hypothesis awaits experimental verification.

441

442 *Mechanisms of stable silencing*

443

444 In *C. elegans*, once silencing has been initiated by piRNAs, target
445 sequences can remain stably repressed for many generations even in the
446 absence of the triggering piRNA-Piwi complex^{112, 113, 150}, although in some
447 cases Piwi may still act to maintain silencing¹³⁹. The maternal transmission of
448 tertiary 22G RNAs, downstream of secondary 22G RNAs and the germline
449 nuclear RNAi pathway including *hrde-1*, is sufficient for inherited piRNA-initiated
450 silencing, indicating that a feed-forward amplification loop maintains high levels
451 of siRNAs in the absence of both the trigger and the initially silenced locus⁹³.
452 Mutually reinforcing feedback between small RNAi pathways and repressive
453 chromatin, such as those demonstrated in *Schizosaccharomyces pombe* and
454 *Arabidopsis thaliana* (reviewed in¹⁵¹), would explain the extraordinary stability
455 of this silencing⁸³. An analogous mechanism has been proposed in *D.*
456 *melanogaster* (reviewed in¹⁵²), although to date such a feedback has not been
457 convincingly demonstrated in animals. Nonetheless, it is clear that stable gene
458 silencing generally involves multiple epigenetic pathways. In *C. elegans*, the
459 multigenerational stability of piRNA-initiated silencing requires both the RNAi
460 pathway and chromatin modifiers, especially H3K9 methyltransferases^{113, 135}.
461 Secondary piRNAs also guide DNA methylation at the targeted locus in mice¹⁵³,
462¹⁵⁴ and the formation of heterochromatin at the targeted locus in *D.*
463 *melanogaster*¹⁵⁵⁻¹⁵⁸ (Figure 2).

464

465 Conclusions and outlook

466

467 Non-DNA sequence-based inheritance of information occurs in multiple animals
468 and is important for development and physiology. One of the main purposes of
469 epigenetic inheritance is the perpetuation of repression of repetitive elements.
470 However, it may also serve to transmit information about particular gene
471 expression programs, e.g. the germline program in *C. elegans*. What is more
472 controversial is the extent to which transmitted epigenetic information is
473 modulated by the environment and physiology, and whether this is ever
474 adaptive.

475 We have shown that non-DNA sequence-based inheritance of acquired
476 information can occur over different timescales, with the set of mechanisms
477 changing and narrowing as we look to further generations. Parental effects over
478 a single generation can act via many mechanisms and can have large
479 phenotypic consequences. However, there is still little evidence for
480 physiologically consequential multi-generation memory of environmental
481 change, even though the potential for longer-lasting memories has now been
482 repeatedly demonstrated and the underlying mechanisms dissected. Epigenetic
483 inheritance of transcriptional repression can, for example, sometimes be
484 perturbed by environmental insults, with a gradual restoration over generations
485 of perturbed repression leading to a transgenerational transfer of information
486 about ancestral environmental experience. Similarly, on shorter timescales,
487 inheritance of small RNAs can occur. However, evidence is still lacking for
488 either of these capacities for information transfer ever being employed to alter
489 progeny physiology adaptively in the light of ancestral experience. Due to the
490 long generation time of humans, adaptive epigenetic inheritance seems unlikely
491 over any generational timescale, although instances of environmental insults
492 leading to intergenerationally-inherited disorders, as demonstrated in rodents,
493 could have a medically relevant impact on individual physiology.

494 Regardless of the species, parental experiences are more likely to predict
495 environmental conditions than those of more distant ancestors. As such,
496 adaptive effects seem more plausible in the context of intergenerational, rather
497 than transgenerational, paradigms. The more numerous and often more

498 tractable cases of inheritance over a single generation therefore offer fertile
499 ground for researchers who wish to probe the mechanisms and adaptive
500 significance of environmentally-responsive non-DNA sequence-based
501 inheritance, despite the hype surrounding transgenerational inheritance. For
502 example, the details of how soma-to-germline information transfer could occur
503 are still elusive and may be better understood by studying experimentally
504 tractable intergenerational systems. Indeed, research effort may be better
505 directed at confirming and expanding the often-scant mechanistic details of
506 previously described cases of intergenerational and transgenerational
507 inheritance rather than seeking out novel phenomena. Much work remains to
508 establish how epigenetic information survives and is propagated between
509 tissues and across generations, how widespread intergenerational and
510 transgenerational phenomena are in natural contexts and what the physiological
511 relevance of naturally-occurring intergenerational and transgenerational
512 inheritance may be.

513

514 **Conflict of interest statement**

515

516 The authors report no conflict of interest.

517

518 **Figure/Table Legends**

519

520 **Table 1. Examples of intergenerational or transgenerational inheritance**
521 **over different timescales.** Here we provide illustrative examples of some of
522 the more compelling and better-characterised reports of inter- and
523 transgenerational inheritance. These examples are chosen with a view to
524 providing a diversity of mechanisms and demonstrating which mechanisms are
525 more typical over different generational timescales. Many other examples are
526 discussed in the main text.

527 **Figure 1. Mechanisms of transfer of information about ancestral**
528 **environment or physiology over generations. a)** Many mechanisms of
529 transmission of information about environmental experience or physiological
530 state can underlie inheritance over a single generation, from parents to

531 progeny, both genome-associated (e.g. covalent modifications of histones) and
532 genome-independent (e.g. microbiome transfer). Apparent paternal effects are
533 not always mediated by gametes but may act via the mother. **b)** Gradual
534 changes in epigenetic marks might underlie transgenerational memory. A loss
535 of gene repression caused by an environmental or physiological insult, for
536 example by perturbation of heterochromatin-mediated transcriptional
537 repression, can reset gradually over generations, providing a transgenerational
538 memory of ancestral experience. **c)** Mutations or natural variation in various
539 epigenetic pathways can lead to mortal germline (Mrt) phenotypes in *C.*
540 *elegans*, where fertility is lost gradually over generations but can be rapidly
541 restored by changing conditions. The prevalence of this phenotype in mutants
542 affecting chromatin modifications and small RNA pathways indicates the
543 importance of epigenetic pathways in the maintenance of normal development
544 and physiology.

545 **Figure 2. Small RNA pathways can direct histone/DNA methylation to**
546 **repress specific loci.** Small RNAs guide proteins of the Argonaute family to
547 destroy target mRNA transcripts and deposit repressive marks on
548 corresponding genomic loci. These marks are often heritable and cross-talk
549 between small RNA and chromatin pathways may be essential for stable gene
550 silencing.

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552 **References:**

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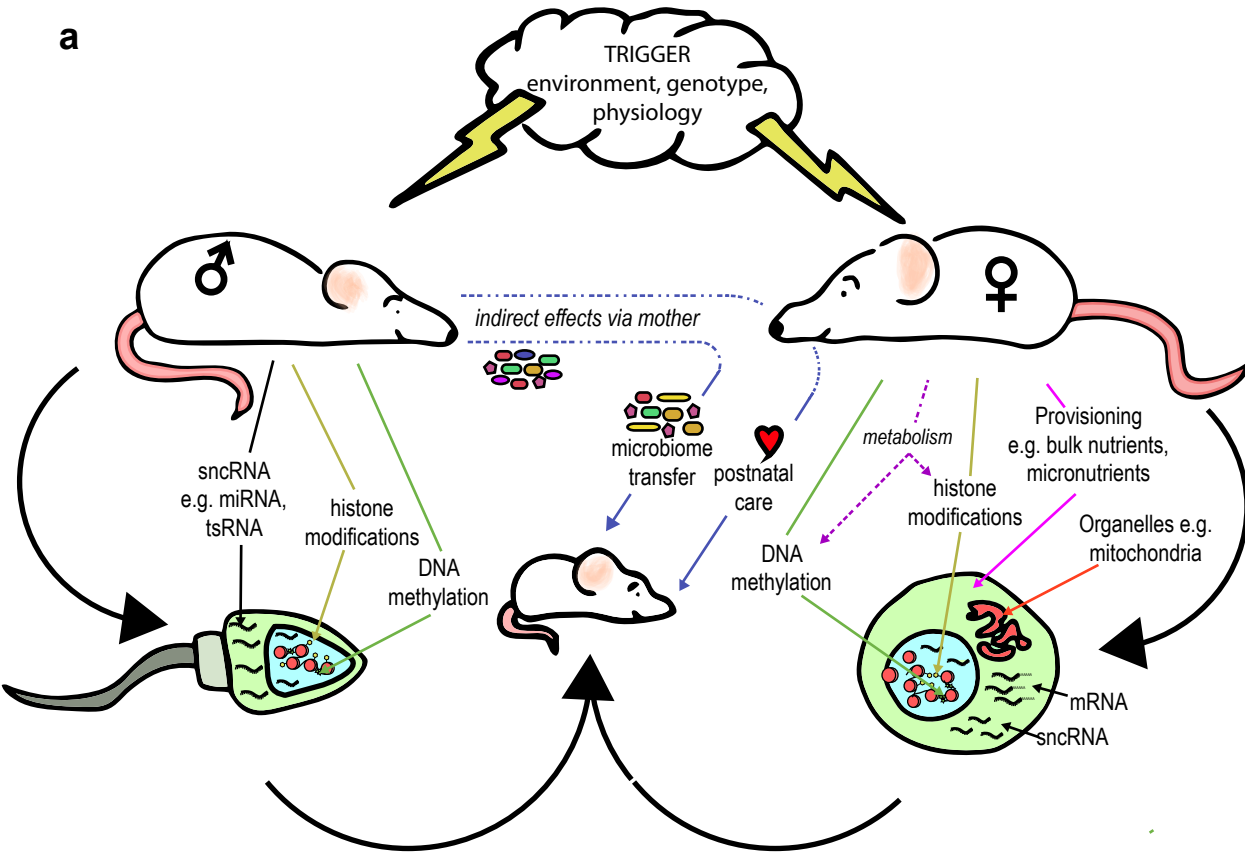
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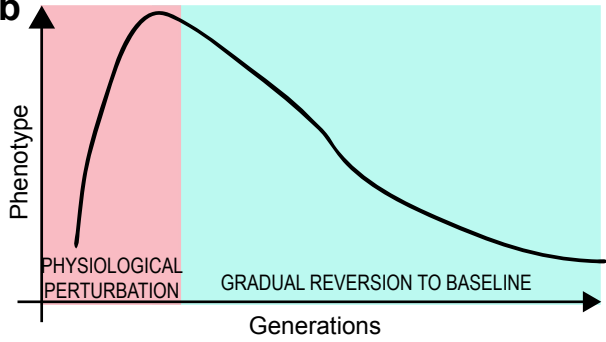
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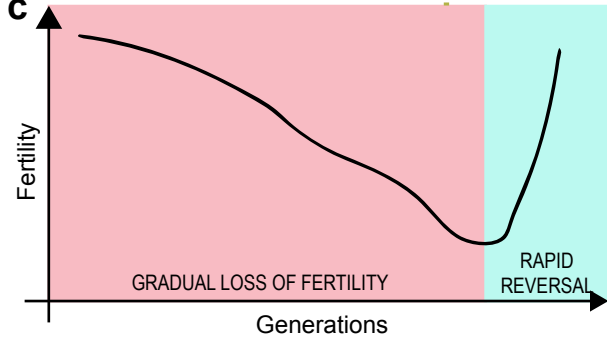
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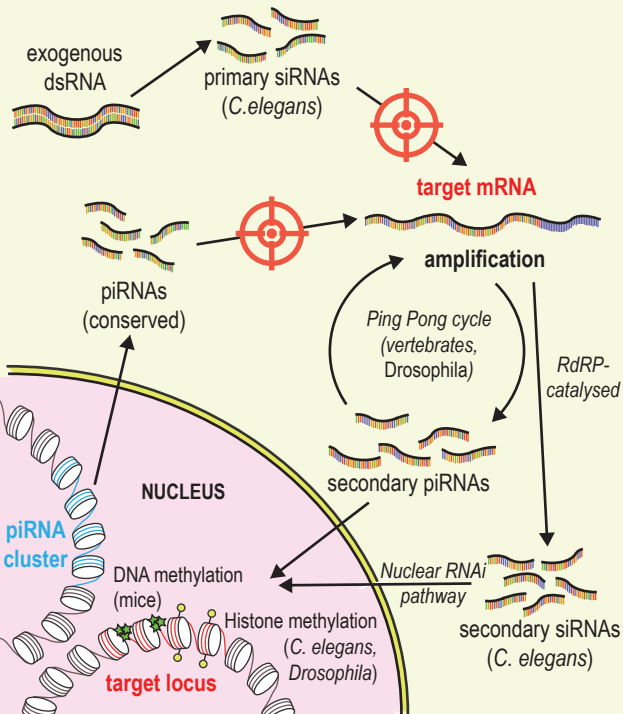


b



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Duration	Trigger	Species	Effects on progeny	Proposed mechanism of inheritance	Ref
1 generation	Paternal high-sugar diet	<i>D. melanogaster</i>	High triglyceride levels (on high-sugar diet)	Chromatin modifications in sperm (H3K9me3, H3K27me3)	23
1 generation	Young mother	<i>C. elegans</i>	Slow development, reduced resistance to starvation, reduced fecundity	Reduced maternal provisioning of yolk to embryos (for starvation resistance and development)	30
1 generation	Paternal low-protein or high-fat diet	<i>Mus musculus</i> , <i>Rattus norvegicus</i>	Differential gene regulation during embryogenesis, metabolic disorders	Somatic tsRNAs acquired by sperm during epididymal transit	18, 20, 55, 61
1 generation (for developmental phenotype)	Maternal antibiotic exposure	<i>D. melanogaster</i>	Delayed development	Heritable depletion of riboflavin-producing commensal bacteria	9
1-2 generations	Ancestral high glucose diet	<i>C. elegans</i>	Reduced fecundity, resistance to oxidative stress	COMPASS H3K4 methylases required for inheritance of stress resistance	25
2 generations	Maternal dietary supplementation with methyl donors	<i>M. musculus</i>	Alterations in coat colour	Increased DNA methylation at the agouti locus caused by retrotransposon insertion	36
2 generations	Undernourishment during pregnancy	<i>M. musculus</i>	Metabolic alterations	Hypomethylation of specific loci in F1 males	17, 43
2 generations	Paternal odour-conditioned fear response	<i>M. musculus</i>	Inherited fear response to specific odour	Neuroanatomical changes in progeny, locus-specific hypomethylation in sperm	121
2-3 generations	Exposure to various mild stresses	<i>C. elegans</i>	Increased stress resistance and proteostasis	Somatic insulin signaling, COMPASS H3K4 methylases in germline	111
3 generations	Ancestral mutation in COMPASS H3K4 methyltransferases	<i>C. elegans</i>	Increased longevity	Altered histone methylation, longevity phenotypes due to possible alteration in lipid metabolism	79; 159
3 generations	Overexpression of H3K4 demethylase in sperm	<i>M. musculus</i>	Reduced survival, developmental abnormalities	Alterations in sperm-borne RNA	109
3 generations	Ancestral development at elevated temperature	<i>C. elegans</i>	Alterations in gene expression	Disruption of piRNA-initiated repression of endogenous transcripts by the RNAi pathway	82
Up to 3-4 generations (typically)	RNAi triggered by exogenous dsRNA	<i>C. elegans</i>	Inherited gene repression	Secondary siRNAs; histone methylation	80, 88, 89
3 generations	Ancestral starvation during larval stage in wildtype worms	<i>C. elegans</i>	Alterations in gene expression and plasticity; increased stress resistance and lifespan	Inheritance of siRNAs bound to the nuclear Argonaute HRDE-1 (for expression differences)	108, 119, 120

3 generations	Heat shock during embryogenesis (multiple generations)	<i>D. melanogaster</i>	Alterations in eye colour	Disruption of heterochromatin by phosphorylation of ATF-2	123
3-9 generations	Ancestral starvation during larval stage in AMPK mutants	<i>C. elegans</i>	Reduced fecundity	Abnormal methylation of H3K4 by COMPASS histone methylases	110
14 generations	Growth at elevated temperature (multiple generations)	<i>C. elegans</i>	Increased expression from repetitive transgene array	Loss of H3K9me3-mediated repression	115
Indefinite	Spontaneous transgene silencing in the germline	<i>C. elegans</i>	Stable gene silencing with no reversion	piRNA-targeting induced nuclear RNAi guided by secondary siRNAs; histone methylation	112, 113, 134, 135

Table 1.